BIOREACTOR SYSTEM DESIGN

BIOPROCESS TECHNOLOGY

Series Editor

W. Courtney McGregor

XOMA Corporation
Berkeley, California

1. Membrane Separations in Biotechnology, *edited by W. Courtney McGregor*
2. Commercial Production of Monoclonal Antibodies: A Guide for Scale-Up, *edited by Sally S. Seaver*
3. Handbook on Anaerobic Fermentations, *edited by Larry E. Erickson and Daniel Yee-Chak Fung*
4. Fermentation Process Development of Industrial Organisms, *edited by Justin O. Neway*
5. Yeast: Biotechnology and Biocatalysis, *edited by Hubert Verachtert and René De Mot*
6. Sensors in Bioprocess Control, *edited by John V. Twork and Alexander M. Yacynych*
7. Fundamentals of Protein Biotechnology, *edited by Stanley Stein*
8. Yeast Strain Selection, *edited by Chandra J. Panchal*
9. Separation Processes in Biotechnology, *edited by Juan A. Asenjo*
10. Large-Scale Mammalian Cell Culture Technology, *edited by Anthony S. Lubiniecki*
11. Extractive Bioconversions, *edited by Bo Mattiasson and Olle Holst*
12. Purification and Analysis of Recombinant Proteins, *edited by Ramnath Seetharam and Satish K. Sharma*
13. Drug Biotechnology Regulation: Scientific Basis and Practices, *edited by Yuan-yuan H. Chiu and John L. Gueriguian*
14. Protein Immobilization: Fundamentals and Applications, *edited by Richard F. Taylor*
15. Biosensor Principles and Applications, *edited by Loïc J. Blum and Pierre R. Coulet*
16. Industrial Application of Immobilized Biocatalysts, *edited by Atsuo Tanaka, Tetsuya Tosa, and Takeshi Kobayashi*
17. Insect Cell Culture Engineering, *edited by Mattheus F. A. Goosen, Andrew J. Daugulis, and Peter Faulkner*
18. Protein Purification Process Engineering, *edited by Roger G. Harrison*
19. Recombinant Microbes for Industrial and Agricultural Applications, *edited by Yoshikatsu Murooka and Tadayuki Imanaka*
20. Cell Adhesion: Fundamentals and Biotechnological Applications, *edited by Martin A. Hjortso and Joseph W. Roos*
21. Bioreactor System Design, *edited by Juan A. Asenjo and José C. Merchuk*
22. Gene Expression in Recombinant Microorganisms, *edited by Alan Smith*

ADDITIONAL VOLUMES IN PREPARATION

BIOREACTOR SYSTEM DESIGN

edited by

Juan A. Asenjo
University of Chile
Santiago, Chile

University of Reading
Reading, England

José C. Merchuk
Ben Gurion University of the Negev
Beersheva, Israel

CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an informa business

Reprinted 2010 by CRC Press
CRC Press
6000 Broken Sound Parkway, NW
Suite 300, Boca Raton, FL 33487
270 Madison Avenue
New York, NY 10016
2 Park Square, Milton Park
Abingdon, Oxon OX14 4RN, UK

Library of Congress Cataloging-in-Publication Data

Asenjo, Juan A.
Bioreactor system design / edited by Juan A. Asenjo, José C. Merchuk.
p. cm. — (Bioprocess technology; v. 21)
Includes bibliographical references and index.
ISBN 0-8247-9002-2 (acid-free paper)
1. Bioreactors. I. Merchuk, José C. II. Title. III. Series.
TP248.25.B55A84 1994
660'.6—dc20
94-35424
CIP

The publisher offers discounts on this book when ordered in bulk quantities. For more information, write to Special Sales/Professional Marketing at the address below.

Copyright © 1995 by MARCEL DEKKER, INC. All Rights Reserved.

Neither this book nor any part may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, microfilming, and recording, or by any information storage and retrieval system, without permission in writing from the publisher.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

Series Introduction

Bioprocess technology encompasses all the basic and applied sciences as well as the engineering required to fully exploit living systems and bring their products to the marketplace. The technology that develops is eventually expressed in various methodologies and types of equipment and instruments built up along a bioprocess stream. Typically in commercial production, the stream begins at the bioreactor, which can be a classical fermentor, a cell culture perfusion system, or an enzyme bioreactor. Then comes separation of the product from the living systems and/or their components followed by an appropriate number of purification steps. The stream ends with bioproduct finishing, formulation, and packaging. A given bioprocess stream may have some tributaries or outlets and may be overlaid with a variety of monitoring devices and control systems. As with any stream, it will both shape and be shaped with time. Documenting the evolutionary shaping of bioprocess technology is the purpose of this series.

Now that several products from recombinant DNA and cell fusion techniques are on the market, the new era of bioprocess technology is well established and validated. Books of this series represent developments in various segments of bioprocessing and have paralleled progress in the life sciences. For obvious proprietary reasons, some developments in industry, although validated, may be published only later, if at all. Therefore, our continuing series will follow the growth of this field as it is available from both academia and industry.

W. Courtney McGregor

Foreword

In rapidly evolving fields such as biochemical engineering and, more broadly, biotechnology, it is healthy (if not imperative) to reassess core subjects of the discipline every few years. The bioreactor is physically at the core of each and every bioprocess, and bioreactor design remains firmly a central subject of biochemical engineering. A serious effort, such as the one represented by this book, is good news and a resource companion for biotechnology practitioners, teachers, and students alike.

Mixing and oxygen transfer defined and dominated not only bioreactor design but biochemical engineering itself in the formative years after World War II. These processes became intimately associated with the successes of large-scale antibiotics production, an activity that dominated fermentation technology in size and importance until the arrival of the recombinant-DNA biotechnology era.

Recombinant-DNA fermentations, animal-cell and plant-cell culture, photobioreactors, immobilized cell technologies, and advanced wastewater and biore-

mediation processes are imposing new and frequently urgent demands on bioreactor design and control. New products and the need to reach commercialization within the shortest possible time are forcing biochemical engineers to design and operate bioreactors under suboptimal, if not marginal, conditions. Frequently, the organism(s) involved is not well studied, especially with respect to how it responds to key parameters of the bioreactor environment. The territory is often uncharted as several (albeit anecdotal) industrial processes in animal-cell technology have demonstrated in the last few years. Practicing biotechnologists can use all the help they can get. Another common feature of all these newer biotechnological processes is that cells are sensitive to small changes in the cultural environment and, thus, monitoring and control—largely an academic pursuit in the past—has now become an industrial necessity.

The chapters of this book address several established and newer aspects of bioreactor design, operation, and preparation. Organism selection, cellular stoichiometry, and medium design are treated as integral parts of the subject, as they should be. Transport processes and mixing also remain central to the coverage of the subject. The special characteristics of various types of bioreactors are critically analyzed and discussed. Sterilization is an established unit operation but containment is still evolving as new processes and products pose fresh challenges. The holistic and global approach taken here by the editors is quite different from past efforts. The coverage is as complete and creative as one can hope to see in these fast-changing times of ours. They have organized and presented simple and complex, fundamental and applied, and general and specific aspects of this ever-expanding and ever-fascinating subject. There is plenty of material here to satisfy a large spectrum of needs, from those of the practical-oriented biotechnologist and applied biologist to the educational needs of the quantitative- and fundamentals-oriented graduate student. The experiences and efforts of so many are now available to all of us, thoughtfully collected and concisely presented, in this compact volume. For this, the editors and authors have earned our gratitude.

Eleftherios Terry Papoutsakis
Department of Chemical Engineering
Northwestern University
Evanston, Illinois

Preface

Industrial bioprocesses are the integration of many, sometimes very complex, operations; the bioreactor is essentially at the core of these processes. Thus the bioreactor is the natural point of reference, and operations before and after are called upstream and downstream operations. Some controversy exists about whether bioreactor design or downstream processing is the main area in which research efforts should be concentrated as well as about the relative importance of upstream processing. It is clear, however, that the most important ingredient of a successful biochemical process is a careful consideration of the effect that the main variables in each stage have on the other stages and on the overall system performance.

The preceding statement can be clearly illustrated using a classical problem in chemical reactor optimal design. When a reaction proceeds to a high extent of conversion, a range of low rates is used and, therefore, it makes sense to stop the reaction at an optimal point that depends not only on the cost of operation of the bioreactor and of the feed, but also on the influence of the final product concen-

tration on the separation and purification costs. In this way the parameters that define the separation processes are variables to be taken into account in the design of the reactor itself.

In biochemical engineering processes this becomes even more critical because of the high sensitivity of biological cells, and also of enzymes, to the composition of substrate, and the extremely narrow range of the final product purity required, especially when dealing with products to be used for therapeutic purposes. Reason and experience indicate that small changes in the feed to a bioreactor may affect both reactor design and operation as well as downstream processing strategy. Typical examples are the penicillin and citric acid fermentations, enzyme synthesis subject to induction and catabolite repression, and mammalian cell culture with and without serum in the medium.

The aim of this book is to be a comprehensive volume for bioreactor system design, where the influence of variables associated with upstream and downstream stages is taken into account, considering the bioreactor as part of the system as a whole. Particular consideration has been given to the very first steps in the development of a bioprocess, such as strain and media selection, which can have an overwhelming influence on all further operations. It is intended to serve as a comprehensive guide for selection and design of bioreactor systems, starting from first principles. It includes both the large variety of cell systems in use today and those that show potential for application in the near future as well as their scale-up and operation modes and the bioreactor support systems, including sterilization and containment and system supplies.

Juan A. Asenjo
José C. Merchuk

Contents

Series Introduction W. Courtney McGregor iii
Foreword Eleftherios Terry Papoutsakis v
Preface vii
Contributors xi

1. Design of a Bioreactor System: Overview 1
José C. Merchuk and Juan A. Asenjo

Part I: Biological Systems and Media Design

2. Organism Selection 13
Francisco Javier Castillo

3. Bacterial, Yeast, and Fungal Cultures: Effect of Microorganism Type and Culture Characteristics on Bioreactor Design and Operation 47
Moshe D. White, Bernard R. Glick, and Campbell W. Robinson

4. Design, Formulation, and Optimization of Media 89
Rodolfo J. Ertola, Ana Maria Giulietti, and Francisco Javier Castillo

Part II: Bioreactor Design

5. Fundamentals of Bioreactor Design — 139
José C. Merchuk and Juan A. Asenjo

6. Stirred Tank Bioreactors — 207
Matthias Reuss

7. Pneumatically Agitated Bioreactors — 257
Karl Schügerl and Andreas Lübbert

8. Membrane Reactors — 305
Peter M. Salmon and Channing R. Robertson

9. Immobilized Microorganism Bioreactors — 339
Hideki Fukuda

10. Immobilized Animal Cell Bioreactors — 377
Matthew S. Croughan, Tzyy-Wen Chiou, and Daniel I. C. Wang

11. Plant Cell Bioreactors — 413
Peter D. G. Wilson and Martin G. Hilton

12. Photobioreactors — 441
Aleš Prokop and Larry E. Erickson

13. Bioreactor Operation Modes — 479
Tsuneo Yamane

14. Scale-Up — 511
Carles Solà and Francesc Gòdia

Part III: Bioreactor Support Systems

15. Sterilization and Containment — 553
Andrew Sinclair and Michael H. J. Ashley

16. Bioreactor System Supplies — 589
T. M. Roberts, M. J. Kearns, and T. J. Latham

Index — *615*

Contributors

Juan A. Asenjo Advanced Centre for Biochemical Engineering and Biotechnology, University of Chile, Santiago, Chile, and Biochemical Engineering Laboratory, University of Reading, Reading, England

Michael H. J. Ashley John Brown Engineers and Constructors Limited, Portsmouth, England

Francisco Javier Castillo Cell Culture Development and Fermentation, XOMA Corporation, Berkeley, California

Tzyy-Wen Chiou Institute of Chemistry, Academia Sinica, Taipei, Taiwan

Matthew S. Croughan Manufacturing Sciences, Genentech, Inc., South San Francisco, California

Larry E. Erickson Center for Hazardous Substance Research, and Department of Chemical Engineering, Kansas State University, Manhattan, Kansas

Rodolfo J. Ertola Centro de Investigacion y Desarrollo en Fermentaciones Industriales (CINDEFI), Facultad de Ciencias Exactas, University of La Plata, La Plata, Argentina

Hideki Fukuda* Kaneka Corporation, Takasago-shi, Hyogo, Japan

Ana Maria Giulietti Catedra de Microbiología, Inmunología, y Biotecnología, Facultad de Farmacia y Bioquímica, University of Buenos Aires, Buenos Aires, Argentina

Bernard R. Glick Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

Francesc Gòdia Unit of Chemical Engineering, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain

Martin G. Hilton Department of Nutrition, Diet, and Health, Institute of Food Research, Norwich Research Park, Colney, Norwich, England

M. J. Kearns Foster Wheeler Energy Limited, Reading, England

T. J. Latham Foster Wheeler Energy Limited, Reading, England

Andreas Lübbert Institut für Technische Chemie, Universität Hannover, Hannover, Germany

José C. Merchuk† Biotechnology Program and Department of Chemical Engineering, Ben Gurion University of the Negev, Beersheva, Israel

Aleš Prokop Department of Chemical Engineering, Vanderbilt University, Nashville, Tennessee

Matthias Reuss Institut für Bioverfahrenstechnik, Universität Stuttgart, Stuttgart, Germany

T. M. Roberts Foster Wheeler Energy Limited, Reading, England

Channing R. Robertson Department of Chemical Engineering, Stanford University, Stanford, California

Campbell W. Robinson Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada

Peter M. Salmon Fermentation Pilot Plant, Merck & Company, Inc., Rahway, New Jersey

Current affiliation: Department of Chemical Science and Engineering, Kobe University, Kobe, Hyogo, Japan

†*Current affiliation*: Visiting Professor, Biochemical Engineering Laboratory, University of Reading, Reading, England

Karl Schügerl Institut für Technische Chemie, Universität Hannover, Hannover, Germany

Andrew Sinclair John Brown Engineers and Constructors Limited, Portsmouth, England

Carles Solà Unit of Chemical Engineering, Universitat Autònoma de Barcelona, Bellaterra, Catalonia, Spain

Daniel I. C. Wang Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts

Moshe D. White Department of Biotechnology, Israel Institute for Biological Research, Ness-Ziona, Israel

Peter D. G. Wilson Department of Nutrition, Diet, and Health, Institute of Food Research, Norwich Research Park, Colney, Norwich, England

Tsuneo Yamane Department of Applied Biological Sciences, Faculty of Agriculture, Nagoya University, Nagoya, Japan

BIOREACTOR SYSTEM DESIGN

1

Design of a Bioreactor System

Overview

José C. Merchuk*

Ben Gurion University of the Negev
Beersheva, Israel

Juan A. Asenjo

University of Chile
Santiago, Chile
and University of Reading
Reading, England

I. INTRODUCTION

The selection and specific design of a bioreactor system include a series of decisions on matters ranging from basic microbiology and biochemistry to process engineering and marketing [1–4]. All those disciplines must contribute in a harmonic way to make possible the proper determination of the basic type of bioreactor system and its correct dimensions to provide an optimal environment for the biological system, operating at minimal cost in order to maximize the

**Current affiliation:* Visiting professor, University of Reading, Reading, England.

productivity and benefits of the process. The key issues for bioreactor system design are related to a series of concepts which must be clarified for each specific case, among which optimal cell concentration, specific rates of product synthesis, specific rate of oxygen uptake, and specific rates of heat evolution are of the utmost importance. In the present chapter the main steps of the procedure to follow in the design are described, and references are made to the chapters in this book which show in detail the treatment of the specific issues related to particular systems.

II. PRODUCTION REQUIREMENT

The final design of a system for industrial production of biotechnological products is related in many ways to the definition of the commercial objectives. These are determined by market needs of a certain product. The potential products cover an extremely wide range, from inexpensive, large-quantity products like ethanol, clean water, air, or soil to the most sophisticated and complex biochemical molecules, which are expensive and are required in small amounts. The former include conventional wastewater treatment and less conventional processes such as fly-ash treatment to reduce environmental contamination and reclaim valuable metals, soil decontamination, and biohydrometallurgy. In these processes the initial investment in hardware and the energy consumption during operation are key elements. Equipment amortization and operation costs chiefly determine the price of the product, and therefore the objective function for optimization is centered around these elements. At the other extreme, for the sophisticated, high-price speciality products, the price is usually related closely not to the production costs and investment in equipment but rather to costs associated with legal approval by regulatory authorities and the uniqueness of the product (e.g., hepatitis B vaccine or TPA). The design is therefore aimed at improving quality and purity regardless of the cost. In between these two extremes a wide range of processes exist, and in most of them the bioreactor design has an impact on both quality and cost-efficiency.

The first step, as shown in Fig. 1, is the specification of the chemicals to be produced. Once the target product has been specified and it is clear that the process is a biochemical one, the system to be used must be chosen.

III. BIOLOGICAL SYSTEM IDENTIFICATION

The identification of the system for synthesizing a given product starts with the selection of the biological system: microorganism, plant, or animal cells may be considered. For production of proteins or enzymes, recombinant microorganisms such as bacteria (*Escherichia coli* or *Bacillus*), mammalian cells (CHO, hybridoma), or insect or plant cells are usually chosen.

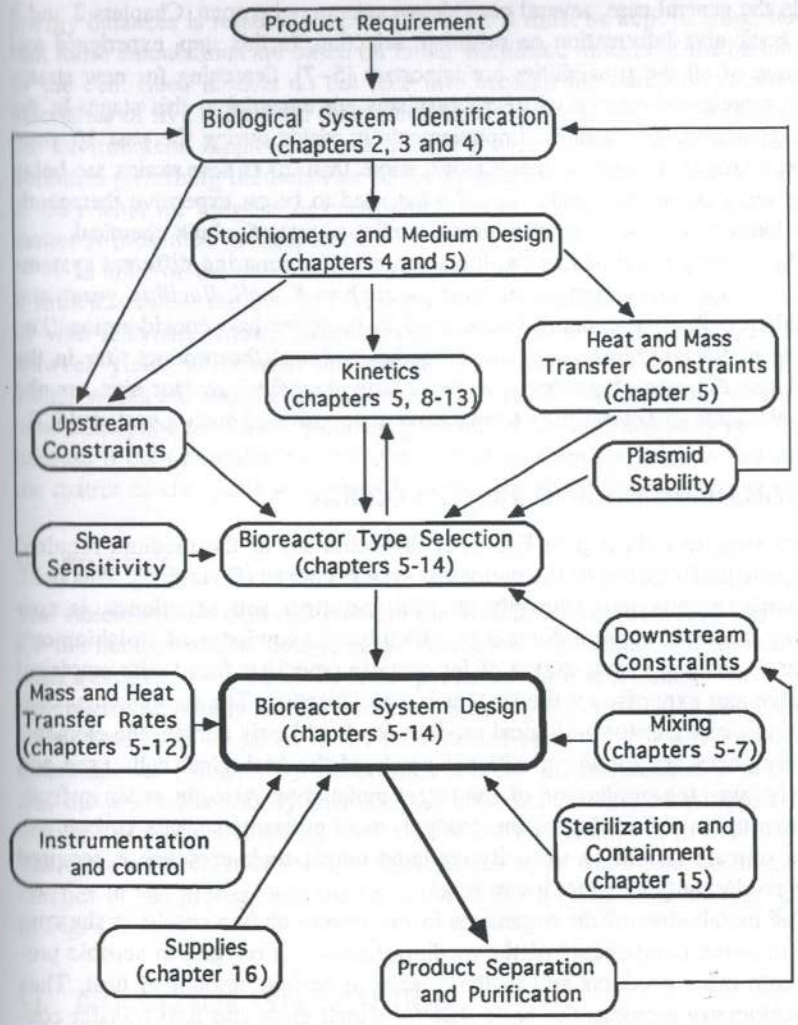


Figure 1 Schematic representation of the process of bioreactor system design.

The last two possibilities, plant organelles and cells (Chapter 11) or animal cells (Chapter 10), are sensitive and costly systems. This is indeed the case for many very valuable products. In those cases the sensitivity of the cells makes it necessary to consider the shear imposed on them as one of the important issues in the selection of the reactor type and in reactor design.

In the general case, several possible avenues may be open. Chapters 2 and 3 in this book give information on organism selection. In this step, experience and knowledge of all the possibilities are important [5–7]. Searching for new strains and the genetic engineering of microorganisms are essential at this stage. In the case of penicillin, for example, improvements in yields during the past 10 years have been crucial. In one company alone, more than 2000 new strains are being tested every year for the production of what used to be an expensive therapeutic but has today a worldwide market almost similar to that of a bulk chemical.

As recently discussed in the literature, when comparing different systems for the production of recombinant proteins, such as *E. coli*, *Bacillus*, yeast, and mammalian cells, where many issues need to be taken into consideration (i.e., refolding of the protein or secretion into the medium) the product titer in the reactor, specific rate of product synthesis, and thus final reactor size are the crucial elements in determining the process economics of such a system [5,7].

IV. STOICHIOMETRY AND MEDIUM DESIGN

The next step, as indicated in Fig. 1, is the definition of the medium required for adequate performance of the biological system chosen (Chapter 4). This field, which until recently relied heavily on trial and error and experience, is now becoming more and more a domain in which basic knowledge of stoichiometry (Chapter 5) and of rational design of fermentation media is fused with empirical knowledge and expertise on the system in consideration. The fundamental concept is that a medium for biological production should only contain the elements absolutely necessary for the nutritional needs of the biological cells used and for maximizing the production of the target metabolite. Also, in order to facilitate downstream processing of the product, most nutrients such as carbon and nitrogen sources should be virtually depleted unless their presence is required to keep productivity at a maximum level.

The metabolism of the organisms in the system chosen results in the conversion of some components of the medium (including oxygen in aerobic processes) into other products and in the release of certain amount of heat. Thus the stoichiometry provides the basic data on which mass and heat transfer considerations are based. This is indicated in Fig. 1. In the case of photosynthetic microorganisms, light transfer is to be included together with mass and heat transfer at this stage (Chapter 12).

The quantitative relationship between reactant consumption, formation of products, and heat evolution is the first element in reactor design in general (stoichiometry); bioreactors are not an exception in this. But, although yields were a main objective in the first stages in the development of fermentation as a technology, as the history of the development of penicillin production shows most dramatically, the in-depth consideration of the regularities in mass and

energy balances is relatively recent [2,8–10]. It must be kept in mind, however, that those calculations are based on rather simplified models of the biochemistry of the cell. Such models do not take into account the complex dynamic characteristics of live cells, with their amazing capacity for adaptation to changes in the environment. Approximations are valid only within a certain range of the variables governing the behavior of the system (and sometimes it is not known exactly what the range is or even which are the variables). More detail on this matter is presented in Chapter 5.

In spite of the reservations formulated here, when the system is known to a sufficient extent the information obtained from stoichiometric considerations is of vital relevance. Stoichiometric calculations provide quantitative relationships between yields of biomass and product synthesis, maintenance, and energetic yields, which are extremely important for process design. Although product yield is obviously the most basic parameter, the rest of the information is very valuable, because it both provides the basis for correct medium formulation and describes the matrix of chemicals which will influence the downstream processing.

V. KINETICS

The stoichiometric data complement the kinetic information, and together they are the nucleus for the design of the bioreactor. The kinetics of the bioreaction itself (Chapter 5) is obtained from published data if available, or from data generated specifically for the process being developed. At this point consideration should also be given to the influence of mass transfer and mixing on the macrokinetics (Chapter 5). Those elements are all interrelated in such a way that the process is usually iterative: the observed kinetics of a bioreaction will usually differ for cells if they are immobilized on a matrix or in free suspension. Therefore an interactive relation exists between the step of the definition of the kinetics of the process and the selection of the type of bioreactor, as indicated in Fig. 1.

VI. BIOREACTOR TYPE SELECTION

Once the stoichiometric and kinetic calculations are completed, a set of interrelated values regarding the concentrations of substrates and products, including off-gas, and of heat evolution should be available.

The bioreactor conception starts with the choice of the state of the biocatalyst: bacterial, yeast, animal, or insect cells, suspended cells versus the many forms of immobilization, including membrane bioreactors and cell recycle (Chapters 9 and 10). Each of these possibilities must be evaluated not only in the light of the time courses of the bioreaction provided by stoichiometry and kinetics but also considering mixing and gas exchange requirements and heat

removal. This will enable the designer to decide on a reactor type (Chapters 6, 7, and 8) and on operation mode (Chapter 13). The main basic types of bioreactor choice are shown in Figs. 2, 3, and 4. It is quite common to operate some of those at high cell concentrations by recycling part or all of the biomass. The recycled biomass is separated by settling, centrifugation, or filtration (Fig. 5).

At this stage, consideration has to be given to the possible existence of constraints in those concentrations. Such constraints are quite common upstream, due to substrate inhibition, and may be avoided by choice of a lower feed concentration or by manipulation of the operation mode. One of the most important reasons for the use of fed-batch operation (Chapter 13) is to avoid high concentrations of nutrients which may inhibit growth. Another reason for the use of fed-batch operation, in the specific case of baker's yeast, is the apparent presence of pockets of sufficient dissolved oxygen to produce ethanol if an excess of sugar is present in solution (Crabtree effect), which lowers the yeast yield.

Downstream concentration constraints are also important. High conversion may lead to a very viscous broth, generating problems of mixing and mass transfer and later on of separation. The quality of the product may change during the time course of the fermentation, creating a dilemma between quality and quantity. Such is the case in the production of polysaccharides like xanthan [11]. The maximal molecular weight and viscosity are obtained in those cases before the maximal product concentration, and again the optimal reactor concept and design depend on the objective set for the process.

In the case of biochemicals for therapeutic use, the purity of the product is of paramount importance [12,13]. The separation and purification stages downstream of the bioreactor in this case dictate constraints that influence the selection of the cells used for production and/or the type of bioreactor and reach farther upstream to the design of an optimal medium to make downstream product purification easier.

When using recombinant strains a large number of issues need to be taken into consideration, such as plasmid stability, copy number, and/or the use of a strong promoter. As the population of cells with the appropriate plasmid and thus expressing the target protein would decrease with time, fed-batch cultures would usually be the choice as opposed to continuous culture. On the other hand, the use of selection by antibiotics partially eliminates this constraint, and a two-stage continuous culture in which growth is optimized in the first stage and product synthesis in the second one appears particularly attractive [14].

VII. SYSTEM DESIGN

The next stage, as indicated in Fig. 1, is the actual design of the bioreactor system, based on the kinetic expressions for the process, the heat and mass transfer rates required, and the mixing conditions. Since this design is usually

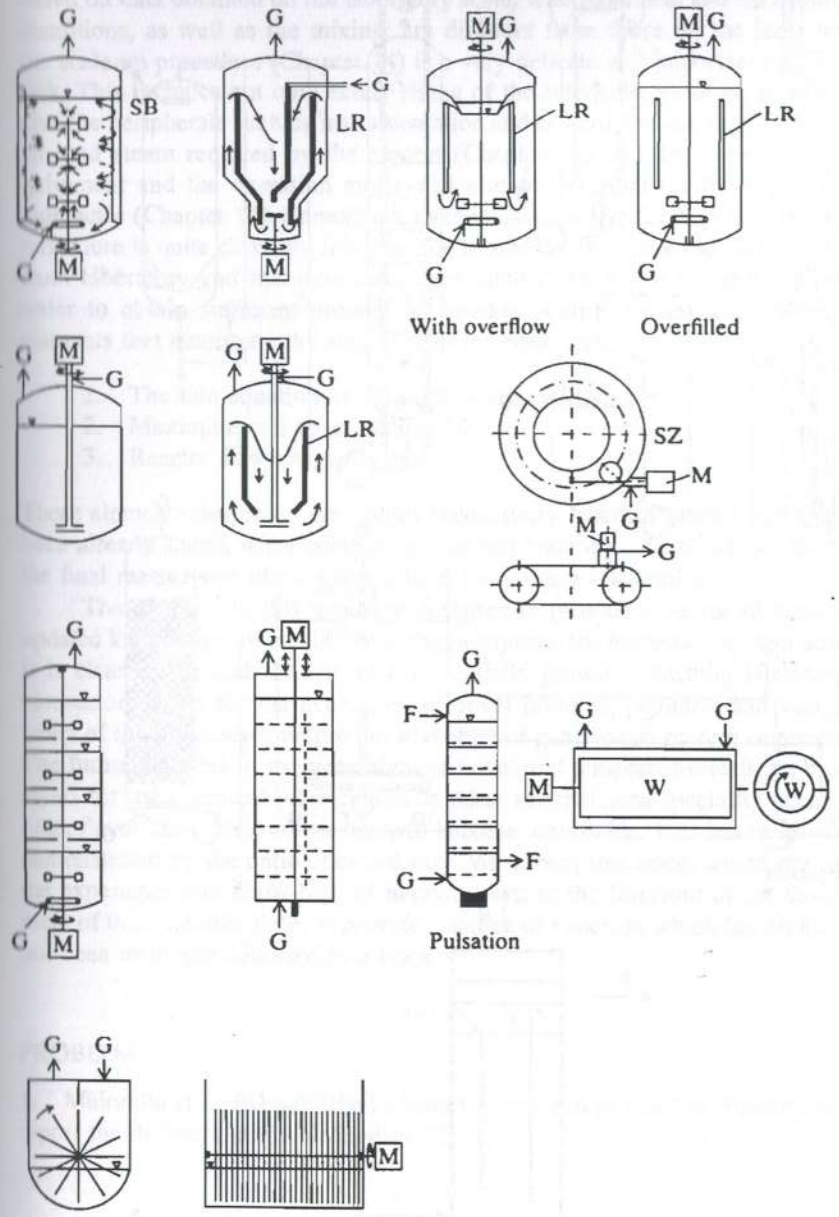
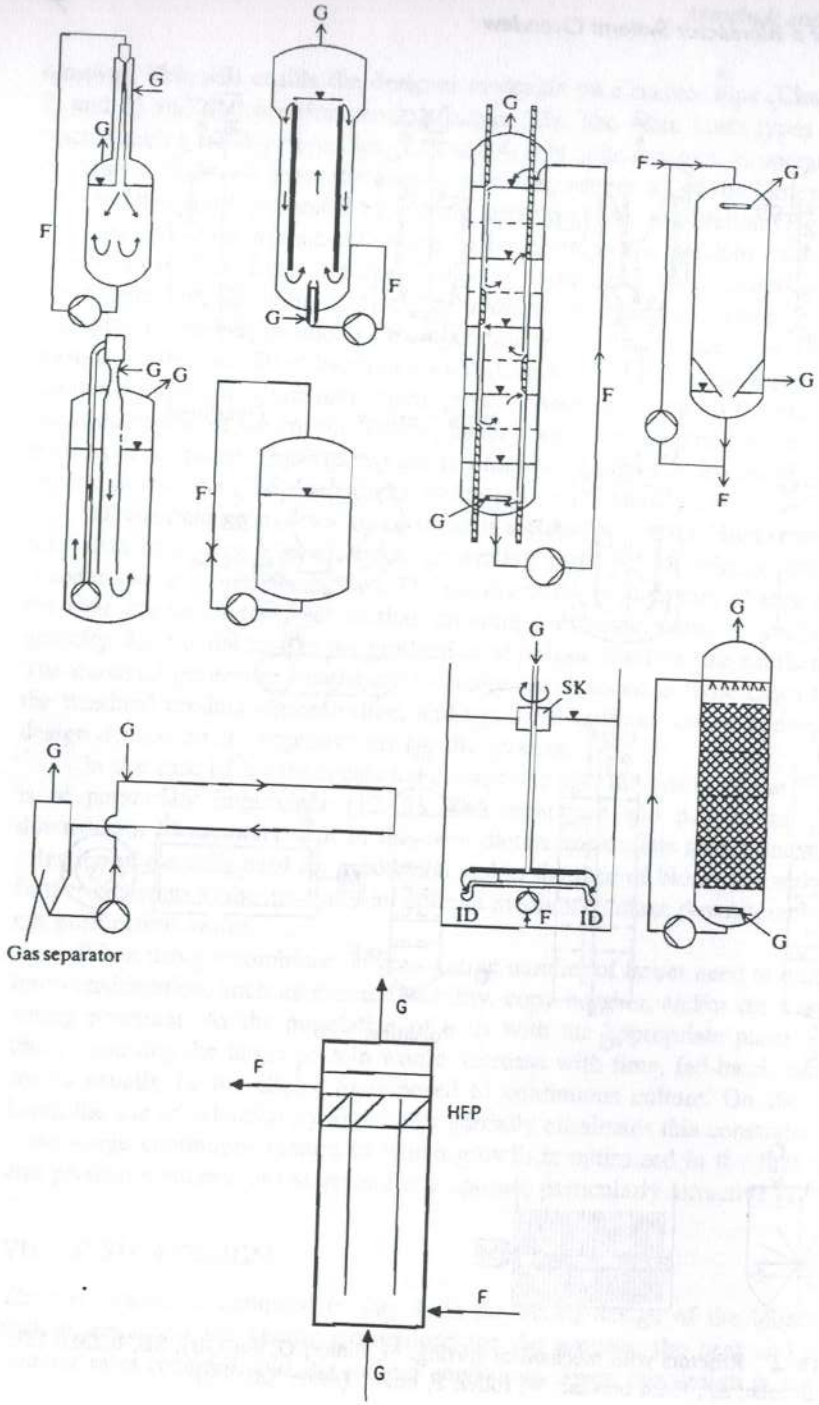


Figure 2 Reactors with mechanical stirring. M, motor; G, gas (air); SB, baffles; LR, conduit tube; SZ, foam breaker; W, roller; F, liquid. (From Ref. 15.)



based on data obtained on the laboratory scale, where the heat and mass transfer conditions, as well as the mixing, are different from those on the large scale, the scale-up procedure (Chapter 14) is a very delicate and important part of the task. This includes not only actual sizing of the selected type of equipment but also the peripherals such as instrumentation and control and the supply of water, air, and steam required by the system (Chapters 15 and 16). The market requirement and the operation mode will also be essential for the scale of the bioreactor (Chapter 14). Sometimes, even in cases where the engineering design procedure is quite clear and it is possible to size the full-scale bioreactor directly from laboratory and literature data, pilot plant experiments are still needed in order to obtain sufficient product for market studies. In any case, the basic elements that determine the size of the bioreactor are

1. The rate equation, usually including yield values
2. Maximum cell concentration
3. Reactor flow characteristics

These elements determine the reactor productivity and thus reactor size. As has been already stated, when comparing different reactor/production systems [5,7], the final reactor size plays a key role in the process economics.

The chapters in this book are designed to provide a source of basic and updated knowledge on the different stages required for bioreactor system design. It is clear to the authors that an exponentially growing potential is arising in bioreactor design for the generation of novel proteins, peptides, and vaccines, many of them unknown before the adventure of genetic and protein engineering. The future also lies in the generation of novel and temperature-resistant biocatalysts for the chemical industry and in other sophisticated speciality chemicals (e.g., flavor enhancers) whose use will become worldwide. This has clearly been demonstrated by the antibiotics industry. We expect this book, which draws on the experience and know-how of many experts at the forefront of the development of this scientific field, to provide a source of expertise which has previously not been available collected in a book.

PROBLEM

1. Maiorella et al. [6] published a report on ethanol production. Identify in the report the different stages defined in Fig. 1.

Figure 3 Reactors with pneumatic stirring. ID, injector nozzle; SK, float; HFP, helical flow promoter, see Fig. 2 for other abbreviations. (Adapted from Ref. 15.)

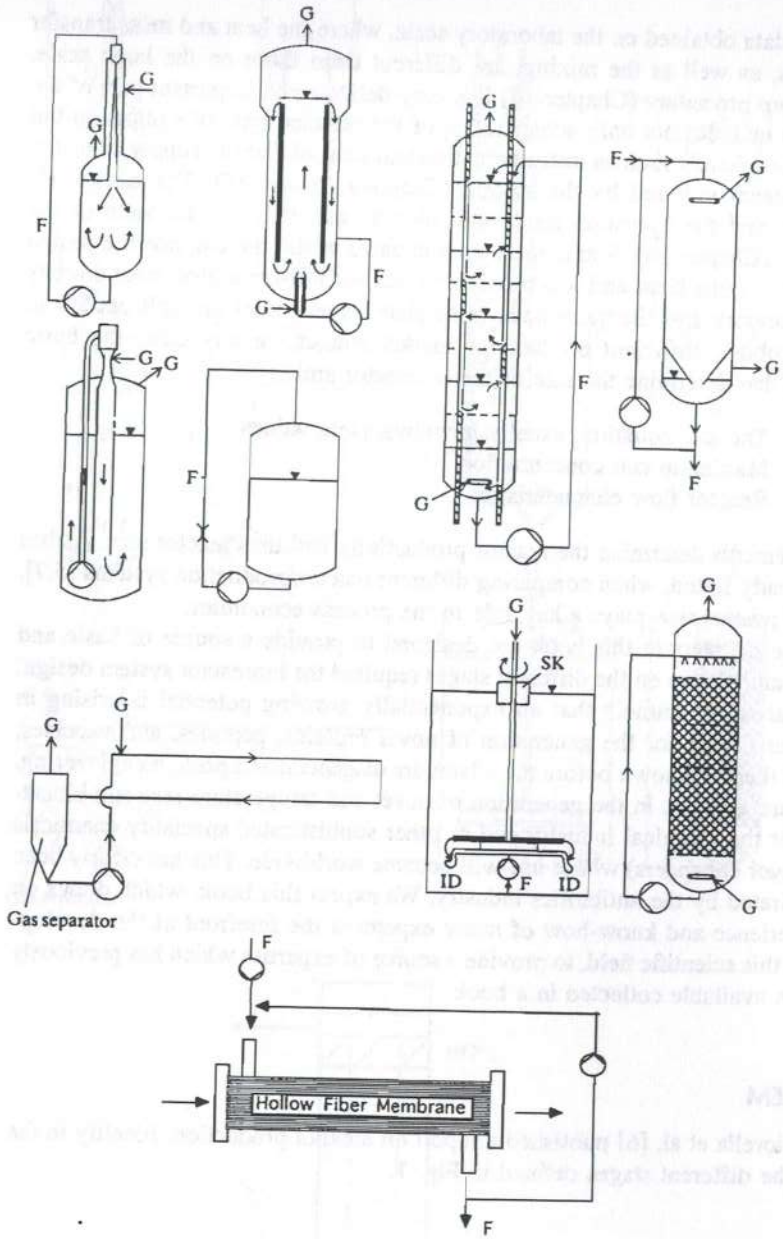


Figure 4 Reactors stirred by pumped fluid. See Figs. 2 and 3 for abbreviations. (Adapted from Ref. 15.)

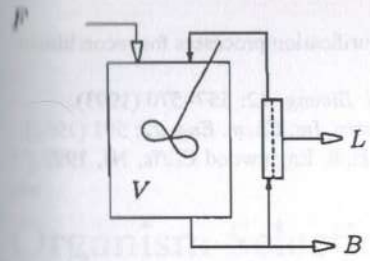


Figure 5 Schematic diagram of a continuous stirred tank with cell recycling. F, feed; L, product; B, purge. (From Ref. 16.)

REFERENCES

1. C. L. Cooney, Bioreactors: design and operation, *Science 219*: 728 (1983); B. L. Maiorella, H. W. Blanch, and C. R. Wilke, Biotechnology report. Economic evaluation of alternative fermentation processes, *Biotechnol. Bioeng. 26*: 1003–1025 (1984).
2. J. A. Roels, *Energetics and Kinetics in Biotechnology*, Elsevier, Amsterdam, 1983.
3. van't Riet and J. Tramper, *Basic Bioreactor Design*, Marcel Dekker, New York, 1991.
4. R. Pollard and C. J. Seharer, The application of chemical engineering concepts to the design of fermentors, *The Chemical Engineer*, February (1977).
5. R. V. Datar, T. Cartwright, and C. G. Rosen, Process economics of animal cell and bacterial fermentations: a case study analysis of tissue plasminogen activator, *Bio/Technology 11*: 349–355 (1993).
6. B. L. Maiorella, H. W. Blanch, and C. R. Wilke, Biotechnology report. Economic evaluation of alternative ethanol fermentation processes, *Biotechnol. Bioeng. 26*: 1003–1025 (1984).
7. R. W. van Leen, J. G. Backhuis, R. F. W. C. van Beckhoven, H. Burger, L. C. J. Dorssers, R. W. J. Hommes, P. J. Lemson, L. Noordam, N. L. M. Persoon, and G. Wagemaker, Production of human interleukin-3 using industrial microorganisms, *Bio/Technology 9*: 47–52 (1991).
8. L. E. Erickson, I. G. Minkevich, and V. K. Eroshin, Application of mass and energy balance regularities in fermentation, *Biotechnol. Bioeng. 20*: 1595–1621 (1978a).
9. J. G. Minkevich and V. K. Eroshin, Productivity and heat generation of fermentation and oxygen limitation, *Folia Microbiol. 18*: 376–386 (1973).
10. J. A. Roels and N. W. F. Kossen, On the modelling of microbial metabolism, *Prog. Ind. Microbiol. 14*: 95 (1978).
11. A. Schumpe and W. D. Deckwer, Viscous media in tower bioreactors: hydrodynamic characteristics and mass transfer properties, *Bioproc. Eng. 2*: 79 (1987).

12. J. A. Asenjo, *Separation Processes in Biotechnology*, Marcel Dekker, New York, 1990.
13. E. Leser and J. A. Asenjo, Rational design of purification processes for recombinant proteins, *J. Chromatogr. 584*: 43–57 (1992).
14. A. Togna, P. Fu, and M. L. Shuler, *Biotechnol. Bioeng. 42*: 557–570 (1993).
15. K. Schugerl, New bioreactors for aerobic processes, *Int. Chem. Eng. 22*: 591 (1982).
16. J. M. Lee, *Biochemical Engineering*, Prentice Hall, Englewood Cliffs, NJ, 1992.

2
Organism Selection

Francisco Javier Castillo

XOMA Corporation
Berkeley, California

I. INTRODUCTION

The great majority of products from biotechnology continue to be of microbial origin and hundreds are manufactured worldwide by a steadily growing, multibillion dollar industry. Microbial products include primary and secondary metabolites, polymers, enzymes, and the cells themselves. In addition, microbes are also used in biotransformations, preparation of fermented foods, feeds, and beverages, metal leaching, biodegradations, and waste treatment (Table 1). Most of these products and microbial uses involve processes that traditionally rely on the isolation, screening, selection, and improvement of microorganisms capable of producing high product titers or activities.

The rapid advances in recombinant DNA technology and cell fusion continue to open new ways for the production of biochemicals using microbes and the production of heterologous proteins in different expression systems that include bacteria, yeasts, filamentous fungi, insect cells, mammalian cells, and transgenic plants and animals. These technologies have been applied successfully in the production of high-value proteins for diagnostics, vaccines, and therapeutics that have reached combined sales in the billions of dollars projected to grow exponentially [152–153]. For the production of heterologous proteins the choice of expression system is a critical one; in addition to product titer, it has

Table 1 Microbial Products and Processes

Product/process	References
Ethanol	1–11
Acetone/butanol	7, 8, 12–15
Glycerol and other polyols	8, 16
Organic acids	4–8, 17–24
Amino acids	8, 25–30
Nucleosides, nucleotides	8, 31–35
Enzymes	8, 17, 36–50
Vitamins	8, 51–56
Antibiotics	8, 57–70
Ergot alkaloids	8, 17, 56, 71, 72
Biotransformation, bioconversions	8, 17, 73–80
Single cell protein	4, 8, 81–89
Polysaccharides	4, 90–93
Edible mushrooms	4, 17, 94, 95
Fermented foods and drinks	17, 96–102
Baker's yeasts	103–107
Metal leaching	8, 108–111
Biosurfactants	112–117
Biodegradation, bioremediation, and waste treatment	8, 17, 118–137
Agroactive/bioactive compounds	138, 139
Insecticides	4, 140–145
Nitrogen fixation	4, 146–148
Vaccines	149
Plastic	150
Chiral epoxides	151

to consider issues that include appropriate protein characteristics such as correct primary structure and posttranslational modifications, the complexity and yield of the required purification steps in each case, and potential regulatory hurdles associated with endogenous retroviruses and other contaminants from the cells and culture media. Thus, as with traditional microbial processes, the identification, preparation, isolation, and screening of several potential expression alternatives are crucial for the selection of an appropriate system capable of generating in a stable manner not only the correct molecule but also an overall cost-effective process.

II. TRADITIONAL PROCESSES

A. Selection of Microorganisms

The steps involved in traditional microbial selection include definition of product or microbial activity, literature searches, obtention of microorganisms from culture collections, development of screen procedures, isolation of microorganisms from natural samples, primary and secondary screening, and maintenance of microbial stocks.

The definition of product or microbial activity of interest precedes the initiation of a selection process. This definition may be dictated by specific needs, such as for the treatment of a specific waste or contaminant or to improve the economics of current fermentation processes. Other reasons may include the desire to manufacture new microbial products or to use a microbial process as a substitute for a chemical one.

Once the product or activity is defined, the next step may involve a literature search to identify microorganisms known to possess the desired properties or with the potential to have them. This is followed by obtaining the specific microorganisms from culture collections and/or by isolating them from natural samples by primary screening.

There are hundreds of culture collections throughout the world, and lists with addresses can be found in several sources [75,154–163]. A most comprehensive list of the major ones has been published by Kirsop and Doyle [164], and a directory of over 300 culture collections worldwide can be obtained from the World Data Center for Collections of Cultures of Microorganisms RIKEN (Table 2). Culture collections often provide information on media formulations and procedures for the recovery, growth, and maintenance of cultures obtained from them.

The isolation of microorganisms from nature by primary screening represents the second alternative. Primary screening is a predominantly qualitative effort [165] defined by Casida [2] as "the use of highly selective procedures to allow the detection and isolation of only those microorganisms of interest among a large microbial population." Although no single isolation method will reveal all the microorganisms present in a sample [166], it is possible to isolate many microorganisms by using general and specific enrichment techniques, providing a suitable environment for the growth of desired microorganisms while inhibiting or killing others. Steele and Stowers [165] emphasize the importance of understanding the physiology of the microorganisms of interest in the design of successful enrichment techniques. To develop an appropriate isolation strategy, it is advisable to consider the characteristics of the product or activity desired and to obtain samples from sources naturally enriched by existing selective pressures, applying for instance the steps delineated in the "ecological approach" [167,168], selecting environments that may yield the microorganisms of interest [50,131,134,169–175]. Techniques for the enrichment and isolation of many kinds of microorganisms from natural samples, including description of habitats, sample collection, selective media formulations, culture, maintenance, and storage, have previously been compiled [167,176–180].

Table 2 Institutions and Companies that Provide Services

Service	Address
Directory of culture collections	World Data Center for Collections of Cultures of Microorganisms RIKEN, 2-1 Hirosawa, Wako-Shi, Saitama 351-01, Japan. Telephone: 81-484-645651 and 81-484-621111 Fax: 81-484-645651
Recombinant protein expression systems	
Baculovirus expression kits	BacPak6, Clontech Laboratories, Inc., 4030 Fabian Way, Palo Alto, California 94303-4607, USA. Telephone: 800-662-2566 and 415-424-8188 Fax: 800-424-1350 and 415-424-1064
	MaxMBac, Invitrogen Corporation, 3985 B Sorrento Valley Road, San Diego, California 92121, USA. Telephone: 800-955-6288 Fax: 619-597-6201
	BaculoGold, PharMingen, 11555 Sorrento Valley Road, San Diego, California, 92121, USA. Telephone: 800-848-6227 Fax: 619-792-5238
Expression in CHO cells and murine myelomas	GS (glutamine synthetase system), Celltech Group, 216 Bath Road, Slough, Berkshire, SL1 4 EN, UK. Telephone: 0753-34655 Fax: 0753-36632
Expression in yeast *Pichia pastoris*	Pichia GEN, SIBIA, 505 Coast Boulevard South, La Jolla, California 92037-4641, USA. Telephone: 619-452-5892 Fax: 619-452-9279
Expression in yeast *Hansenula polymorpha*	Rhein Biotech GmbH, Eichsfelder Strase 11, D-40595 Dusseldorf 13, Germany. Telephone: 211-709010 Fax: 211-7090130
Expression in *Bacillus licheniformis*, *Kluyveromyces fragilis*, and *Aspergillus niger*	PLUGBUG, Royal Gist-brocades nv. Wateringseweg 1, P.O. Box 1, 2600 MA Delft, The Netherlands. Telephone: 015-799111 and 015-972108 Fax: 015-793480
Expression in transgenic animals (mice, rabbits, and goats)	Genzyme Transgenics Corporation, One Mountain Road, Framingham, Massachusetts 01701-9322, USA. Telephone: 508-872-8400 Fax: 508-872-9080

Service	Address
Testing services for cell banks and products	Inveresk Research International Ltd., Musselburgh EH21 7UB, Scotland, UK. Telephone: 031-665-6881 Fax: 031-665-9976
	Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850, USA. Telephone: 800-553-5372 Fax: 301-738-1036
	Quality Biotech, 1667 Davis Street, Camden, New Jersey 08104, USA. Telephone: 800-622-8820 Fax: 609-342-8078
	Tektagen, 358 Technology Drive, Malvern, Pennsylvania 19355, USA. Telephone: 800-648-6682 Fax: 215-889-9028
	TSI Corporation, 5516 Nicholson Lane, Kensington, Maryland 20895, USA. Telephone: 703-642-5671 Fax: 703-642-5691

Continuous culture devices such as chemostats and turbidostats [181–184], which are excellent tools for studies of microbial and animal cell physiology [185–213], can be very useful for isolation and selection [214–222], because they allow the well-controlled application and maintenance of chemical and physical selective pressures.

Through history, and without knowing about the existence of microorganisms, people have selected microbial strains for the elaboration of many products, especially foods and beverages. With the discovery of penicillin, microbial screening became an expanded activity, and since the 1940s most of the large-scale microbial screening done has been oriented toward finding new antibiotics and other bioactive compounds [165,223,224]. The sampling of exotic ecosystems such as extreme environments and the development of culture media and physicochemical conditions to fit the requirements of the microorganism and its desired biosynthetic activity continue to lead to new and promising findings [87,134,138,139,170,174,224,225].

Once the initial selection has been made from culture collections and/or natural samples, secondary screening follows. Secondary screening is both qualitative and quantitative and has a dual purpose: to eliminate false positives and to evaluate the production potential of each microorganism [165]. Most indus-

trial screening procedures and equipment used are confidential and not described in the literature [170,226]. The success of a screening program depends on the selectivity and sensitivity of the assays developed. The convergence of several technologies and a multidisciplinary approach has revolutionized microbial screening and facilitates large-scale searches for microbial bioactive compounds and activities [8,224,226,227].

As with appropriate assays, appropriate fermentation procedures capable of providing detectable titers of active product are critical for successful screening [139,224,228]. After comparative studies are completed, one organism may finally be selected on the basis of productivity or activity. Often after the selection, development and optimization of the fermentation process will follow [229,230]. However, unless the microorganism selected obviously qualifies for a cost-effective process, it will be necessary to pursue genetic improvement by mutagenesis and/or other genetic techniques [8,231–251]. The history of penicillin production is a classic example of the impact of genetic manipulations coupled with media and fermentation development for process improvement. By a combination of mutagenesis and fermentation optimization the yields of the antibiotic have been increased three orders of magnitude from 0.06 to >26 g/L [236,252].

B. Selection of Animal Cells

Although relatively few in number, the products obtained with the use and cultivation of animal cells are of significant medical and economic importance. The main ones are virus vaccines for human and veterinary use [253–257], human interferons [258], and carcinoembrionic antigen [259]. These products are manufactured by growing cells that were screened and selected for their ability to support virus growth or to secrete the desired product, in addition of being free of adventitious agents and considered to be sufficiently stable [260–263]. Various types of cells are used for these purposes (Table 3).

III. NONTRADITIONAL PROCESSES

A. Recombinant Microorganisms

Recently, protein and pathway engineering has been employed to improve existing microbial products and processes and to develop new ones [264–266]. This has been done by increasing enzyme levels, catalytic activities, and half-lives, decreasing or eliminating the sensitivity of enzymes to feedback inhibition, and introducing new genes. By using site-directed mutagenesis, subtilisin E, a protease from *Bacillus subtilis* expressed in *Escherichia coli*, was engineered to increase its catalytic efficiency for peptide substrates by two- to sixfold [267]. A 54% yield increase in tryptophan production was reported after pathway en-

Table 3 Products from Animal Cells

Product	Cells	References
Human interferons		
Alpha leukocyte type	Periferal blood leukocytes	258
Alpha lymphoblastoid type	Namalva	258,261
Beta fibroblast type	Many cells	258
Gamma immune interferon	Leukocytes	258
	Lymphocytes	258
	Polymorphonuclear cells	258
Carcinoembrionic antigen	Several carcinoma cells	259
Hormones and enzymes	Many cells	257
Plasminogen activator	Many cells	257,260
Growth factors	Several cells	257
Viruses for human vaccines		
Live attenuated		
Measles	Chicken embryo fibroblast	255,256
	Human diploid cells	255,256
Mumps	Chicken embryo fibroblast	255,256
Rubella	Human diploid cells	255,256
Varicella	Human diploid cells	255,256
Polio	Primary monkey kidney	255,256,261
	Human diploid cells	256
	Vero	256
Killed viruses		
Polio	Primary monkey kidney	255,256,261
	Vero	255,256,261
	Human diploid cells	256
Rabies	Human diploid cells	255,256,261
	Chicken embryo fibroblast	255,256
	Fetal bovine kidney cells	256
	Primary hamster kidney	256
	Vero	256
Japanese Encephalitis	Baby hamster kidney (BHK-21)	261
Viruses for veterinary vaccines		
Over 30 vaccines	Many cells	253,254,257

gineering in *Corynebacterium glutamicum* [268]. Improvements in cephalosporin C production by *Acremonium chrysogenum* were observed after the introduction and expression of *Vitreoscilla* hemoglobin gene, and the technology is being tested in penicillin-producing strains [269]. A metabolic operon to catalyze the biosynthesis of the pigment indigo in *E. coli* has increased the possibility of a cost-effective fermentation process for this product [270].

The processes for specialty chemicals that involve the use of hazardous chemicals and generate toxic by-products are important targets for substitution with fermentation processes utilizing recombinant microorganisms yet to be developed [271]. The ability to create novel metabolites such as hybrid bioactive molecules is also showing progress [272].

B. Selection of Expression Systems for Heterologous Proteins

Advances in technology have widened the choice of expression systems available for the production of recombinant proteins. Genes of interest can be introduced into cells or whole organisms using specialized expression vectors and methods for their insertion that have been specifically developed [272–275]. As discussed recently by Hodgson [276], the potential producing organisms now span five kingdoms: Bacteria such as *Escherichia coli*, which remains by far the most frequently used microorganism [276–278], *Bacillus* [279,280], and *Streptomycetes* [281,282]; Fungi including the yeasts *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis*, *Hansenula polymorpha*, and filamentous fungi [283–293]; insect viruses and cells [294–297]; mammalian cells [298–304], among which CHO cells are most frequently used; transgenic plants [305–309]; and transgenic animals [310–315]. By making use of this availability, a wide variety of proteins are being expressed in different systems to test their activities [276].

1. Monoclonal Antibodies

For the expression and production of monoclonal antibodies (MAbs) a choice of myeloma must be made before proceeding with the fusion protocol. By far the most widely used myelomas are of murine origin and all descend from MOPC-21, a transplantable mouse plasmacytoma of BALB/c origin [316–322] (Fig. 1). Among these myelomas two variants have completely lost the ability to produce the endogenous heavy and light chains of MOPC-21. These variants are P3x63-Ag8.653 [320] and NSO/1 [321]. Sp2/O-Ag14 [322] is a nonproducer hybrid also frequently used. Other mouse, rat, and human myelomas are also used but to a lesser degree and some present difficulties [323–325]. Once the fusion of myeloma cells and splenocytes is done, hybridomas are obtained by placing the cell mixtures in selective HAT medium [323]. For screening the hybridomas an assay capable of detecting MAbs of the desired specificity that can be performed in large numbers at a reasonable cost and effort is required. The initial selection of clones should be followed by subcloning to eliminate stability problems, comparative studies of the clones to select the most productive ones, and repeated subcloning to ensure monoclonality and stability [323,324,326,327]. These strategies are similar to those employed with microorganisms in traditional processes (discussed earlier).

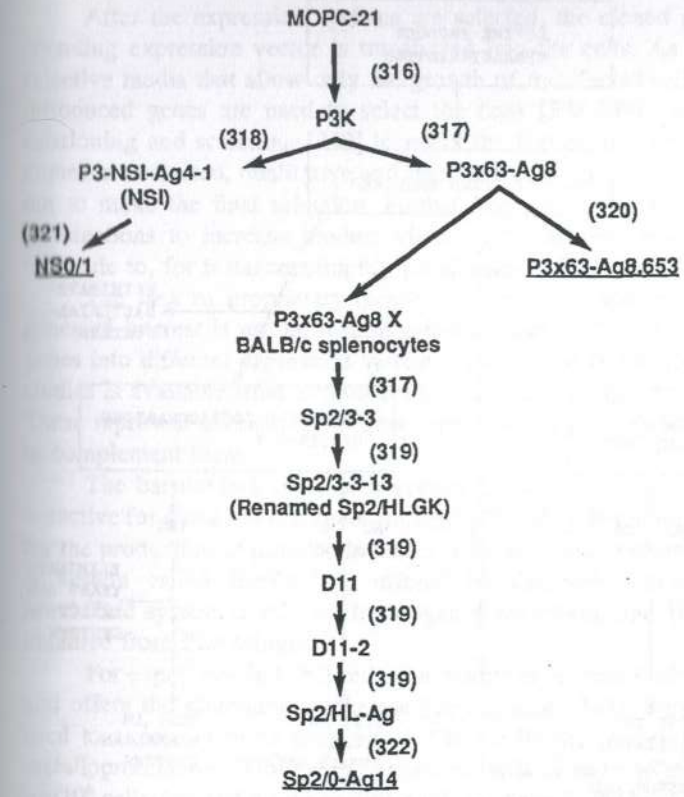


Figure 1 Derivation of mouse myelomas. References in parentheses.

2. Recombinant Proteins

The understanding, availability, and ease of use of expression systems have reached remarkable levels and continue to improve. The evolution of and advances in the field are reviewed frequently [274]. All systems have advantages and disadvantages [276,277,288,328–334] and none is optimal for the expression and production of all recombinant proteins. Marino [277] outlined a flow diagram of the steps required for determining the best expression system for a given recombinant protein (Fig. 2). If a protein is naturally glycosylated and this plays an important role in its functionality, then a bacterial system should not be used because bacteria do not glycosylate proteins. By the same token, yeasts cannot be used if complex oligosaccharides are needed, and only mammalian systems will have to be considered. If a protein is glycosylated but the

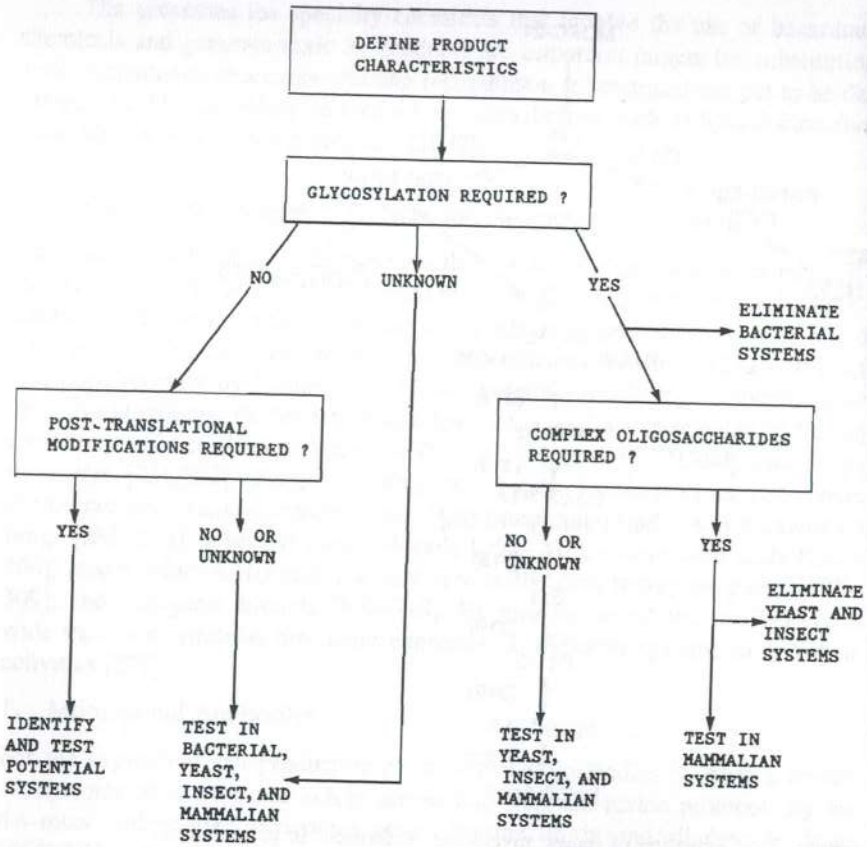


Figure 2 Flow diagram for selection of expression systems for recombinant proteins. (Adapted from Ref. 277.)

effect of glycosylation is unknown, the protein should be expressed in different systems to determine the expression levels and to make product so that the needed studies of functionality and other properties can be done [335,336]. A cost evaluation should be performed as early as possible, estimating the amounts of purified bioactive product that different alternatives may provide based on results from fermentation and cell culture runs, combined with the purification processes required in each case. Few reports of this type have been published, but a recent one on the production of tissue plasminogen activator by animal cell and bacterial fermentation represents an interesting case study [337].

After the expression systems are selected, the cloned gene in the corresponding expression vector is transfected into the cells. As with hybridomas, selective media that allow only the growth of transfected cells that express the introduced genes are used to select the cells [338,339]; this is followed by subcloning and screening [340] to select the highest producers. Once different clones are selected, qualitative and quantitative comparative studies are carried out to make the final selection. Further improvement may involve additional transfections to increase product yields or to change other properties of the molecule to, for instance, simplify purification [341].

The lack of proprietary technology for the production of proteins from genes of interest is not an insurmountable obstacle. The means of introducing genes into different expression systems to carry out feasibility and comparative studies is available from several companies either as kits or services (Table 2). These represent alternatives to developing in-house capabilities and a fast way to complement them.

The baculovirus expression system is widely used and has become very attractive for quick expression of proteins [342,343]. Baculovirus expression kits for the production of proteins in insect cells are available from three companies. A system called BacPak6 is offered by Clontech Laboratories, Inc. The MaxMBac system is sold by Invitrogen Corporation, and BaculoGold can be obtained from PharMingen.

For expression in CHO cells and murine myelomas Celltech has developed and offers the glutamine synthetase (GS) system [344]. This system has been used to express several proteins. In CHO cells the human tissue inhibitor of metalloproteinases (TIMP) was expressed with a specific productivity of 110 $\mu g/10^6$ cells/day and a titer of 180 mg/L in shake flasks [344]. A mouse-human chimeric antibody was expressed in the murine NSO myeloma at a specific productivity of 10–15 $\mu g/10^6$ cells/day and titers up to 560 mg/L in fed-batch airlift fermentations [345,346].

The yeast *Pichia pastoris* has been used to produce heterologous proteins and glycoproteins ranging from 14 to 150 kilodaltons in molecular weight [288,289] with intracellular yields of up to 12 grams for tetanus toxin fragment C [347] and up to 4 g of secreted human serum albumin per liter of culture [292]. To transform *P. pastoris*, SIBIA has developed several vectors that include a tightly regulated alcohol oxidase promoter. They have named this expression vehicle rPichia GEN. An expression system in the yeast *Hansenula polymorpha* can be licensed out from Rhein Biotech GmbH.

Three different microorganism expression systems are offered by the company Gist-brocades. The service offered is named PLUGBUG and utilizes the gram-positive bacteria *Bacillus licheniformis*, the yeast *Kluyveromyces lactis*, and the filamentous fungus *Aspergillus niger*. All three organisms enjoy the GRAS (generally recognized as safe) status for different uses in the food in-

dustry. At least one biopharmaceutical protein, human interleukin-3, produced in *B. licheniformis* as the expression system is in clinical trials [335]. According to Gist-brocades, a feasibility study contracted with them aimed at testing one gene in the three systems and selecting at least one secreting host is expected to last 4 to 6 months.

A more recent and very exciting alternative is the use of transgenic animals. Genzyme Transgenics Corporation offers development collaborations for their technology that consist of the insertion of a human gene using either genomic or complementary DNA into mammalian embryos (mice, rabbits, and goats). Upon lactation, the resulting transgenic animals secrete the human protein into their milk, from which it is purified. Nine proteins have been expressed and the levels in milk have ranged over three orders of magnitude from 30 ng/ml for protein C in mice to 7.5 mg/ml for "longer-acting" tissue plasminogen activator (tPA) in goat. The expression of at least nine other proteins, including two monoclonal antibodies, for client companies is currently ongoing. To reach the level of a manufacturing herd of goats a 2-year period is required; each animal could then be used for 5 years with one lactation per year lasting 300 days and a yield of 800 L of milk. Up to December 1993, no Investigational New Drug (IND) applications for therapeutic proteins using this technology had been filed with the Food and Drug Administration (FDA), but a "Points to Consider in the Use of Transgenic Animals for Production of Therapeutic Proteins" is being drafted at the FDA and is expected to be available for distribution in the near future.

As the potential, accessibility, and time-saving opportunities represented by these services continue to be recognized in the industry, it seems likely that the demand for them will continue to grow.

IV. MAINTENANCE OF STOCKS

A. Maintenance and Preservation of Microorganisms

During and after the selection process, maintenance and preservation of the microorganisms are important in order to ensure access to viable, homogeneous, and axenic stock cultures and to prevent genetic change that may lead to reduction or loss of the desired phenotypic properties. Techniques for short- and long-term maintenance of microorganisms that include subculturing, storage under paraffin, desiccation, lyophilization, and cryopreservation are described in detail in several publications [155,164,348–351].

B. Maintenance of Animal Cells

Animal cell stocks are maintained as frozen cell banks. The rationale for the preparation of cell banks includes the need to ensure an adequate supply of

equivalent cells for use during the entire life span of the product and to allow detailed characterization of the cell line, decreasing the likelihood of cell line cross-contamination and adventitious agent contamination. The cell bank system should consist of two tiers: a master cell bank (MCB), defined as a collection of uniform composition derived from a single tissue or cell, and manufacturer's working cell banks (MWCBs). An MCB is used to generate MWCBs. One or more vials from an MWCB is used for the production of one lot of biological product [352]. Both the MCB and the MWCB should be stored in the liquid or vapor phase of liquid nitrogen, and it is recommended that both be stored in more than one location to avoid loss of the cell line by accidents. The location, identity, and inventory of individual vials should be thoroughly documented.

Detailed protocols for freezing cells can be found in several publications [353–355]. Although controlled slow freezing (i.e., −1°C to −3°C per minute) is frequently recommended [353–357], we have found that several cell lines, including murine myelomas such as NSI, P3x63-Ag8.653, and SP2, transformants of these myelomas, murine/murine hybridomas obtained using different parental myelomas, and parental and transformed CHO-K1 and DHFR$^-$ CHO cells, all freeze and recover with high viability and stability when placed directly into the vapor phase of a liquid nitrogen freezer without following the controlled slow freezing approach. We have had consistent results when using either serum-containing or serum-free media and, for several hybridomas, even protein-free media. However, due to the heterogeneity of cells, their response to freezing, and the differences in liquid nitrogen freezers, before cell banks are prepared and to avoid losses it is advisable to determine empirically how the available equipment and freezing procedures affect each cell line of interest. In our experience key aspects resulting in successful freezing include

1. A healthy, exponentially growing culture of high viability is of importance. Do not use cultures that have grown beyond the exponential phase or that show a viability below that normally observed during healthy exponential growth.
2. After centrifugation, resuspend the cells at high concentration (i.e., 10^7 viable cells/ml) using fresh cold (2–8°C) medium to slow the cell metabolism.
3. Work fast to minimize the time during which the cells are maintained at high concentration. This will reduce nutrient depletion, the accumulation of waste products, and the decrease in pH, all of which are undesirable.
4. Mix the concentrated cell suspension in aliquots, one at the time, with fresh medium containing dimethyl sulfoxide (DMSO) at a concentration such that after mixing will be at approximately 10%. Once one aliquot is mixed, quickly dispense it into vials or ampules, seal or

close them, and transfer them into the corresponding locations in the vapor phase of the liquid nitrogen freezer. Avoid delays at this stage as in the previous ones and proceed with the next aliquot immediately until all are used.

5. Store the vials for 24 to 48 hours before testing the first and last vials for recoverability as follows: thaw vials in a 37°C water bath by holding with two fingers and agitating gently, transfer thawed cell suspension to a centrifuge tube containing fresh culture medium, centrifuge at 500–1000 × g for 5 minutes, discard supernatant containing DMSO, resuspend cells in 2 to 4 ml of fresh medium, transfer to a tissue culture flask, and place, with the caps loose, in a CO_2 incubator at 37°C. Determine the length of lag period before exponential growth resumes, follow daily growth by counting cells, and determine specific growth rate (μ) during the exponential phase by using the equation

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$$

where X_2 and X_1 represent viable cell counts at times t_2 and t_1, respectively.

Calculate doubling times (t_d) from

$$t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}$$

Determine maximum viable cell and product concentrations. Follow daily product concentrations to determine specific productivities (q_p) by using the equation

$$q_p = \frac{(p_2 - p_1)(\ln X_2 - \ln X_1)}{(t_2 - t_1)(X_2 - X_1)} \quad \text{or} \quad q_p = \frac{p_2 - p_1}{(X_2 + X_1/2)(t_2 - t_1)}$$

where p_1 and p_2 represent product concentrations at times t_1 and t_2, respectively.

The results of the tests will give an indication of the quality and homogeneity of the freezing. Extended lags and more than 20% differences in growth and product yields and kinetics between the first and last vial may imply that the cells were not in good condition when frozen or have suffered during the freeze/storage. If the quality or homogeneity of the freeze is suspect, it will be best to prepare a new cell bank before proceeding with the expensive characterization needed (see next section) and risking loss of the cell line.

C. Characterization of Animal Cell Banks

Testing of MCB and MWCB is needed to ensure identity and freedom from adventitious agents. Extensive testing of the MCB should be done once and include morphology by light and electron microscopy; species of origin by karyology or, as preferred now, DNA fingerprinting and isozyme analysis; demonstration that the cells can be used for their intended purpose, such as by showing consistency in the quantity and quality of protein they produce and the state and copy number of the expression system in repeated runs; and any other tests that may be useful for demonstrating that the MCB contains cells with the intended characteristics. The MWCB, derived from the MCB and propagated for an approved number of passages, needs to be checked for potential cell cross-contamination by identity tests. With both banks, checking for the presence of adventitious agents should include tests for bacteria and fungi [358], *Mycoplasma* (see attachment 2 in Ref. 352), and viruses by appropriate in vivo and in vitro tests. In addition, tests for endogenous retroviruses should include thin-section transmission electron microscopy, reverse transcriptase assays, and infectivity assays for ecotropic and xenotropic retroviruses. Most of these tests can be contracted out with any of several companies, and copies of the respective protocols, information on samples needed, schedules, and costs can be obtained by contacting them (Table 2).

V. CONCLUSIONS

For all microbial applications only a relatively small amount of microbes are used from the vast universe of microorganisms believed to exist [139,165,166, 170,178,359] and that, in spite of its known diversity, continues to amaze us with new findings such as *Epulopiscium fishelsoni*, a truly gigantic bacterium up to 600 μm in length and 80 μm in diameter isolated from the intestinal tract of surgeon fish species from the Red Sea and the Great Barrier Reef [360,361]. This diversity is an assurance that new and useful microbial activities await to be found. The isolation of new organisms and screening of known and new ones will remain important, expanding activities [165,170,225,359,362,363].

The continuing increase in understanding the regulatory, biochemical, and physicochemical mechanisms that affect protein translation initiation, elongation, folding, solubility, posttranslational modifications, and secretory processes [278,293,364–371] should facilitate the development of new expression systems with very specific characteristics allowing the tailoring of desired protein modifications combined with high overall process yields.

REFERENCES

1. S. C. Prescott and C. G. Dunn, *Industrial Microbiology*. McGraw-Hill, New York, 1959.

2. L. E. Casida, *Industrial Microbiology*, John Wiley & Sons, New York, 1968.
3. J. S. Harrison and J. C. J. Graham, Yeasts in distillery practice, *The Yeasts* (A. H. Rose and J. S. Harrison, eds.), Academic Press, London, 1970, pp. 283–348.
4. H. Dellweg, Biomass, Microorganisms for special applications, *Microbial Products I, Energy from Renewable Resources, Biotechnology*, Vol. 3 (H. J. Rehm and G. Reed, eds.), Verlag Chemie, Weinheim, 1983.
5. T. P. Lyons, Ethanol production in developed countries, *CRC Crit. Rev. Biotechnol. 1*: 189 (1984).
6. M. R. G. de van Broock, Industrial fermentation ethanol production in South America, *CRC Crit. Rev. Biotechnol. 1*: 209 (1984).
7. J. C. Linden, Industrially important strains and pathways. *Handbook of Anaerobic Fermentations* (L. E. Erickson and D. Y. Fung, eds.), Marcel Dekker, New York, 1988, pp. 59–80.
8. W. Crueger and A. Crueger, *Biotechnology: A Textbook of Industrial Microbiology* (T. D. Brock, ed.), Sinauer Associates, Sunderland, MD, 1990.
9. N. Nagashima, Progress in ethanol production with yeasts, *Yeasts, Biotechnology and Biocatalysis* (H. Verachtert and R. De Mot, eds.), Marcel Dekker, New York, 1990, pp. 57–84.
10. T. Benitez, E. Martinez, J. Jimenez, and A. Aguilera, Production of ethanol by yeast, *Handbook of Applied Mycology*, Vol. 4, *Fungal Biotechnology* (D. K. Arora, R. P. Elander, and K. G. Mukerji, eds.), Marcel Dekker, New York, 1992, pp. 603–680.
11. T. Lida, Fuel ethanol production by immobilized yeasts and yeast immobilization, *Industrial Application of Immobilized Biocatalysts* (A. Tanaka, T. Tosa, and T. Kobayashi, eds.), Marcel Dekker, New York, 1993, pp. 163–182.
12. J. J. H. Hastings, Acetone-butyl alcohol fermentations, *Economic Microbiology*, Vol. 2, *Primary Products of Metabolism* (A. H. Rose, ed.), Academic Press, London, 1978, pp. 31–47.
13. M. T. Walton and J. L. Martin, Production of butanol-acetone by fermentation, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 188–210.
14. D. T. Jones and D. R. Woods, *Microbiol. Rev. 50*: 484 (1986).
15. H. Bahl and G. Gottschalk, Microbial production of butanol/acetone, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 6b, *Special Microbial Processes* (H. J. Rehm, ed.), VCH, Weinheim, 1988, pp. 1–30.
16. H. J. Rehm, Microbial production of glycerol and other polyols, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 6b, *Special Microbial Processes* (H. J. Rehm, ed.), VCH, Weinheim, 1988, pp. 51–70.
17. J. E. Smith and D. R. Berry, *The Filamentous Fungi*. Vol. 1, *Industrial Mycology*, John Wiley & Sons, New York, 1975.
18. L. M. Miall, Organic acids, *Economic Microbiology*, Vol. 2, *Primary Products of Metabolism* (A. H. Rose, ed.), Academic Press, London, 1978, pp. 48–120.
19. L. B. Lockwood, Production of organic acids by fermentation, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 356–388.
20. C. P. Kubicek and M. Rohr, Citric acid fermentation, *CRC Crit. Rev. Biotechnol. 3*: 331 (1986).

21. I. Tanaka and T. Tosa, Production of L-malic acid, *Industrial Application of Immobilized Biocatalysts* (A. Tanaka, T. Tosa, and T. Kobayashi, eds.), Marcel Dekker, New York, 1993, pp. 53–65.
22. M. J. Zidwick, Organic acids, *Biotechnology of Filamentous Fungi Technology and Products* (D. B. Finkelstein and C. Ball, eds.), Butterworth-Heinemann, Boston, 1991, pp. 303–334.
23. M. Mattey, The production of organic acids, *CRC Crit. Rev. Biotechnol. 12*: 87 (1992).
24. M. Roehr, C. P. Kubicek, and J. Kominek, Industrial acids and other small molecules, *Aspergillus: Biology and Industrial Applications* (J. W. Bennett and M. A. Klich, eds.), Butterworth-Heinemann, Boston, 1992, pp. 91–132.
25. K. Yamada, S. Kinoshita, T. Tsunoda, and K. Aida, *The Microbial Production of Aminoacids*, Halsted Press, New York, 1972.
26. S. Kinoshita and K. Nakayama, Amino acids, *Economic Microbiology*, Vol. 2, *Primary Products of Metabolism* (A. H. Rose, ed.), Academic Press, London, 1978, pp. 210–262.
27. Y. Hirose and H. Okada, Microbial production of aminoacids, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 211–240.
28. E. M. Kubicek-Pranz and C. P. Kubicek, Production and biosynthesis of amino acids by fungi, *Handbook of Applied Mycology*, Vol. 4, *Fungal Biotechnology* (D. K. Arora, R. P. Elander, and K. G. Mukerji, eds.), Marcel Dekker, New York, 1992, pp. 313–356.
29. T. Sato and T. Tosa, Production of L-aspartic acid, *Industrial Application of Immobilized Biocatalysts* (A. Tanaka, T. Tosa, and T. Kobayashi, eds.), Marcel Dekker, New York, 1993, pp. 15–24.
30. S. Takamatsu and T. Tosa, Production of L-alanine and D-aspartic acid, *Industrial Application of Immobilized Biocatalysts* (A. Tanaka, T. Tosa, and T. Kobayashi, eds.), Marcel Dekker, New York, 1993, pp. 25–35.
31. A. L. Demain, Production of nucleotides by micro-organisms, *Economic Microbiology*, Vol. 2, *Primary Products of Metabolism* (A. H. Rose, ed.), Academic Press, London, 1978, pp. 187–209.
32. Y. Nakao, Microbial production of nucleosides and nucleotides, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 312–355.
33. A. Kuninaka, Nucleic acids, nucleotides, and related compounds, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 71–114.
34. N. Trivedi, Use of microorganisms in the production of unique ingredients, *Biotechnology in Food Processing* (S. K. Harlander and T. P. Labuza, eds.), Noyes Publications, Park Ridge, NJ, 1986, pp. 115–132.
35. D. H. Griffin, Nucleic acids and nucleotides, *Handbook of Applied Mycology*, Vol. 4, *Fungal Biotechnology* (D. K. Arora, R. P. Elander, and K. G. Mukerji, eds.), Marcel Dekker, New York, 1992, pp. 445–474.
36. K. Aunstrup, O. Andresen, E. A. Falch, and T. K. Nielsen, Production of microbial enzymes, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 282–211.

37. P. W. Lambert, Industrial enzyme production and recovery from filamentous fungi, *The Filamentous Fungi*, Vol. 4 (J. E. Smith, D. R. Berry, and B. Kristiansen, eds.), Edward Arnold, London, 1983, pp. 210–237.
38. W. M. Fogarty and C. T. Kelly, Developments in microbial extracellular enzymes, *Topics in Enzyme and Fermentation Biotechnology* (A. Wiseman, ed.), John Wiley & Sons, New York, 1979, pp. 45–102.
39. G. Street, Large scale industrial enzyme production, *CRC Crit. Rev. Biotechnol. 1*: 59 (1983).
40. E. Volesky, J. H. T. Luong, and A. Hutt, Microbial enzymes: production, purification, and isolation, *CRC Crit. Rev. Biotechnol. 2*: 119 (1985).
41. J. F. Kennedy, *Enzyme Technology*, Vol. 7a, *Biotechnology* (H. J. Rehm and G. Reed, eds.), VCH, Weinheim, 1987.
42. A. Paterson, Fungal enzymes, *Physiology of Industrial Fungi* (D. R. Berry, ed.), Blackwell Scientific Publications, Oxford, 1988, pp. 101–129.
43. J. K. Kristjansson, TIBTECH, *7*: 349 (1989).
44. W. M. Fogarty and C. T. Kelly, *Microbial Enzymes and Biotechnology*, Elsevier Science Publishers, New York, 1990.
45. W. Gerhartz, *Enzymes in Industry Production and Applications*, VCH, Weinheim, 1990.
46. P. Layman, *Chem. Eng. News 68*: 17 (1990).
47. M. Ward, Chymosin production in *Aspergillus*, *Molecular Industrial Mycology Systems and Applications for Filamentous Fungi* (S. A. Leong and R. M. Berka, eds.), Marcel Dekker, New York, 1991, pp. 83–106.
48. R. Bigelis, Food enzymes, *Biotechnology of Filamentous Fungi Technology and Products* (D. B. Finkelstein and C. Ball, eds.), Butterworth-Heinemann, Boston, 1991, pp. 361–416.
49. R. M. Berka, N. Dunn-Coleman, and M. Ward, Industrial enzymes from *Aspergillus* species, *Aspergillus: Biology and Industrial Applications* (J. W. Bennett and M. A. Klich, eds.), Butterworth-Heinemann, Boston, 1992, pp. 155–202.
50. R. M. Kelly and S. H. Brown, *Curr. Opin. Biotechnol. 4*: 188 (1993).
51. D. Perlman, Vitamins, *Economic Microbiology*, Vol. 2, *Primary Products of Metabolism* (A. H. Rose, ed.), Academic Press, London, 1978, pp. 303–327.
52. J. Florent and L. Ninet, Vitamin B_{12}, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 497–520.
53. D. Perlman, Microbial processes for riboflavine production, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 521–528.
54. L. Ninet and J. Rebaut, Carotenoids, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 529–544.
55. A. H. S. Onions, A. Allsopp, and H. O. W. Eggins, *Smith's Introduction to Industrial Mycology*, John Wiley & Sons, New York, 1981.
56. J. Florent, Vitamins, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 115–158.
57. A. H. Rose, Secondary products of metabolism, *Economic Microbiology*, Vol. 3, Academic Press, London, 1979.
58. D. Perlman, Microbial production of antibiotics, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 241–281.

59. G. DeTilly, D. G. Mou, and C. L. Cooney, Optimization and economics of antibiotic production, *The Filamentous Fungi*, Vol. 4 (J. E. Smith, D. R. Berry, and B. Kristiansen, eds.), Edward Arnold, London, 1983, pp. 190–209.
60. J. O'Sullivan and R. B. Sykes, β-Lactam antibiotics, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 247–282.
61. H. Kleinkauf and H. von Dohren, Peptide antibiotics, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 283–308.
62. S. Umezawa, S. Kondo, and Y. Ito, Aminoglycoside antibiotics, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 309–358.
63. S. Omura and Y. Tanake, Macrolide antibiotics, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 359–392.
64. Z. Holstalek and Z. Vanek, Tetracyclines, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 393–430.
65. G. Lancini, Ansamycins, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 431–464.
66. J. Berdy, Further antibiotics with practical application, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 465–508.
67. R. Bentley and J. W. Bennet, Biosynthesis of secondary metabolites, *Physiology of Industrial Fungi* (D. R. Berry, ed.), Blackwell Scientific Publications, Oxford, 1988, pp. 161–186.
68. J. F. Martin, T. D. Ingolia, and S. W. Queener, Molecular genetics of penicillin and cephalosporin antibiotic biosynthesis, *Molecular Industrial Mycology Systems and Applications for Filamentous Fungi* (S. A. Leong and R. M. Berka, eds.), Marcel Dekker, New York, 1991, pp. 149–196.
69. P. S. Masurekar, Therapeutic metabolites, *Biotechnology of Filamentous Fungi Technology and Products* (D. B. Finkelstein and C. Ball, eds.), Butterworth-Heinemann, Boston, 1991, pp. 241–302.
70. M. Abdel-Halim and M. El-Sayed, Production of penicillins and cephalosporins by fungi, *Handbook of Applied Mycology*, Vol. 4, *Fungal Biotechnology* (D. K. Arora, R. P. Elander, and K. G. Mukerji, eds.), Marcel Dekker, New York, 1992, pp. 517–564.
71. H. Kobel and J. J. Sanglier, Ergot alkaloids, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 4, *Microbial Products II* (H. Pape and H. J. Rehm, eds.), VCH, Weinheim, 1986, pp. 569–610.
72. H. Socic and V. Gaberc-Porekar, Biosynthesis and physiology of ergot alkaloids, *Handbook of Applied Mycology*, Vol. 4, *Fungal Biotechnology* (D. K. Arora, R. P. Elander, and K. G. Mukerji, eds.), Marcel Dekker, New York, 1992, pp. 475–516.

73. O. K. Sebek and D. Perlman, Microbial transformation of steroids and sterols, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 483–496.
74. K. Kieslich, *Biotransformations, Biotechnology*, Vol. 6a (H. J. Rehm and G. Reed, eds.), Verlag Chemie, Weinheim, 1984.
75. P. Z. Margalith, *Steroid Microbiology*, Charles C Thomas, Springfield, IL, 1986.
76. C. T. Goodhue, J. P. Rosazza, and G. P. Peruzzotti, Methods for transformation of organic compounds, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 97–121.
77. L. Sedlaczek, Biotransformations of steroids, *CRC Crit. Rev. Biotechnol. 7*: 187 (1988).
78. L. G. Copping, R. E. Martin, J. A. Pickett, C. Bucke, and A. W. Bunch, *Opportunities in Biotransformations*, Elsevier Applied Science, London, 1990.
79. H. L. Holland, Bioconversions, *Biotechnology of Filamentous Fungi* (D. B. Finkelstein and C. Ball, eds.), Butterworth-Heinemann, Boston, 1991, pp. 157–188.
80. P. S. J. Cheetham, *Tibtech 11*: 478 (1993).
81. J. H. Lichfield, Production of single-cell protein for use in food or feed, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 93–156.
82. A. H. Rose, *Economic Microbiology*, Vol. 4, *Microbial Biomass*, Academic Press, New York, 1979.
83. J. H. Litchfield, *Science 219*: 740 (1983).
84. G. L. Solomon, *CRC Crit. Rev. Biotechnol. 1*: 21 (1983).
85. C. A. Batt and A. J. Sinskey, *Food Technol. 38*(2): 108 (1984).
86. C. Rolz, Microbial biomass from renewables a second review of the alternatives, *Annual Reports on Fermentation Processes*, Vol. 7 (G. T. Tsao, M. C. Flickinger, and R. K. Finn, eds.), Academic Press, Orlando, FL, 1984, pp. 213–356.
87. I. Goldberg, *Single Cell Protein* (Biotechnology Monographs, Vol. 1), Springer Verlag, Berlin, 1985.
88. W. Harvey, *Biotechnology 6*: 488 (1988).
89. H. Boze, G. Moulin, and P. Galzy, Production of food and fodder yeasts, *CRC Crit. Rev. Biotechnol. 12*: 65 (1992).
90. C. J. Lawson and I. W. Sutherland, Polysaccharides, *Economic Microbiology*, Vol. 2, *Primary Products of Metabolism* (A. H. Rose, ed.), Academic Press, London, 1978, pp. 328–392.
91. K. S. Kang and I. W. Cottrell, Polysaccharides, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 418–482.
92. A. Vincent, Fermentation techniques in xanthan gum production, *Topics in Enzyme and Fermentation Biotechnology 10* (A. Wiseman, ed.), Ellis Horwood Limited, Chichester, 1985, pp. 109–145.
93. A. Sinskey, S. Jams, D. Easson, Jr., and C. Rha, Biopolymers and modified polysaccharides, *Biotechnology in Food Processing* (S. K. Harlander and T. P. Labuza, eds.), Noyes Publications, Park Ridge, NJ, 1986, pp. 73–114.
94. W. A. Hayes and S. H. Wright, Edible mushrooms, *Economic Microbiology*, Vol. 4, *Microbial Biomass* (A. H. Rose, ed.), Academic Press, New York, 1979, pp. 142–176.

95. P. A. Horgen and J. B. Anderson, Edible mushrooms, *Biotechnology of Filamentous Fungi Technology and Products* (D. B. Finkelstein and C. Ball, eds.), Butterworth-Heinemann, Boston, 1991, pp. 447–462.
96. K. S. Djien and C. W. Hesseltine, Tempe and related foods, *Economic Microbiology*, Vol. 4, *Microbial Biomass* (A. H. Rose, ed.), Academic Press, New York, 1979, pp. 116–141.
97. R. N. Greenshields, Acetic acid: vinegar, *Economic Microbiology*, Vol. 2, *Primary Products of Metabolism* (A. H. Rose, ed.), Academic Press, London, 1978, pp. 121–186.
98. G. Reed, Food and feed production with microorganisms, *Biotechnology*, Vol. 5 (H. J. Rehm and G. Reed, eds.), Verlag Chemie, Weinheim, 1983.
99. K. H. Steinkraus, Industrial applications of Oriental fungal fermentations. *The Filamentous Fungi*, Vol. 4 (J. E. Smith, D. R. Berry, and B. Kristiansen, eds.), Edward Arnold, London, 1983, pp. 171–189.
100. R. Buchanan, Food fermentation with molds, *Biotechnology in Food Processing* (S. K. Harlander and T. P. Labuza, eds.), Noyes Publications, Park Ridge, NJ, 1986, pp. 209–222.
101. M. Sfat, Unitization of fermented foods: an application of fermentation technology, *Biotechnology of Food Processing* (S. K. Harlander and T. P. Labuza, eds.), Noyes Publications, Park Ridge, NJ, 1986, pp. 223–236.
102. S. Hara, K. Kitamoto, and K. Gomi, New developments in fermented beverages and foods, *Aspergillus: Biology and Industrial Applications* (J. W. Bennett and M. A. Klich, eds.), Butterworth-Heinemann, Boston, 1992, pp. 133–154.
103. H. J. Peppler, Production of yeasts and yeast products, *Microbial Technology* (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 157–187.
104. S. Burrows, Baker's yeast, *Economic Microbiology*, Vol. 4, *Microbial Biomass* (A. H. Rose, ed.), Academic Press, New York, 1979, pp. 32–64.
105. N. B. Trivedi, G. K. Jacobson, and W. Tesch, Baker's Yeast, *CRC Crit. Rev. Biotechnol. 4*: 75 (1986).
106. K. Rosen, Production of baker's yeast, *Yeast Biotechnology* (D. R. Berry, I. Russell, and G. G. Stewart, eds.), Allen & Unwin, London, 1987, pp. 471–499.
107. R. F. Beudeker, H. W. van Dam, J. B. van der Plaat, and K. Vellenga, Developments in baker's yeast production, *Yeast Biotechnology and Biocatalysis* (H. Verachtert and R. De Mot, eds.), Marcel Dekker, New York, 1990, pp. 103–146.
108. A. E. Torma, Leaching of metals, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 6a, *Special Microbial Processes* (H. J. Rehm, ed.), VCH, Weinheim, 1988, pp. 367–400.
109. E. B. Lindstrom, E. Gunneriusson, and O. H. Tuovinen, Bacterial oxidation of refractory sulfide ores for gold recovery, *CRC Crit. Rev. Biotechnol. 12*: 133 (1992).
110. J. Barrett, M. N. Hughes, G. I. Karavaiko, and P. A. Spencer, *Metal Extraction by Bacterial Oxidation of Minerals*, Ellis Horwood, New York, 1993.
111. W. Burgstaller and F. Schinner, *J. Biotechnol. 27*: 91 (1993).
112. N. Kosaric, N. C. C. Gray, and W. L. Cairns, Microbial emulsifiers and deemulsifiers, *Biotechnology*, Vol. 3, *Biomass, Microorganisms for Special Applications.*

Microbial Products I, *Energy from Renewable Resources* (H. J. Rehm and G. Reed, eds.), Verlag Chemie, Weinheim, 1983, pp. 575–594.
113. J. E. Zajic and W. Seffens, Biosurfactants, *CRC Crit. Rev. Biotechnol. 1*: 87 (1984).
114. E. Rosenberg and R. Mitchell, Microbial surfactants, *CRC Crit. Rev. Biotechnol. 3*: 109 (1986).
115. N. Kosaric, W. L. Cairns, and N. C. C. Gray, *Biosurfactants and Biotechnology*, Marcel Dekker, New York, 1987.
116. A. Fiechter, *Tibtech 10*: 208 (1992).
117. N. Kosaric, *Biosurfactants: Production, Properties, Applications*, Marcel Dekker, New York, 1993.
118. T. K. Kirk, Degradation and conversion of lignocelluloses, *The Filamentous Fungi*, Vol. 4 (J. E. Smith, D. R. Berry, and B. Kristiansen, eds.), Edward Arnold, London, 1983, pp. 266–295.
119. J. M. Grainger and J. M. Lynch, *Microbiological Methods for Environmental Biotechnology*, Academic Press, London, 1984.
120. W. Schonborn, *Biotechnology*, Vol. 8, *Microbial Degradations* (H. J. Rehm and G. Reed, eds.), VCH, Weinheim, 1986.
121. G. M. Gadd, Accumulation of metals by microorganisms and algae, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 6b, *Special Microbial Processes* (H. J. Rehm, ed.), VCH, Weinheim, 1988, pp. 401–434.
122. S. Kunst and K. Mudrack, Microbial elimination of nitrogen and phosphorus, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 6b, *Special Microbial Processes* (H. J. Rehm, ed.), VCH, Weinheim, 1988, pp. 435–462.
123. U. Fischer, Sulfur in biotechnology, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 6b, *Special Microbial Processes* (H. J. Rehm, ed.), VCH, Weinheim, 1988, pp. 463–496.
124. J. Klein, M. Beyer, M. van Afferden, W. Hodeck, F. Pfeifer, H. Seewald, E. Wolff-Fisher, and H. Juntgen, Coal in biotechnology, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 6b, *Special Microbial Processes* (H. J. Rehm, ed.), VCH, Weinheim, 1988, pp. 497–568.
125. A. Mizrahi, *Biological Waste Treatment*, Alan R. Liss, New York, 1989.
126. L. E. Erickson and L. T. Fan, Anaerobic degradation of toxic and hazardous wastes, *Handbook on Anaerobic Fermentations* (L. E. Erickson and D. Y. Fung, eds.), Marcel Dekker, New York, 1988, pp. 59–80.
127. P. Morgan and R. J. Watkinson, Hydrocarbon Degradation in Soils and Methods for Soil Biotreatment, *CRC Crit. Rev. Biotechnol. 8*: 305 (1989).
128. H. Y. Neujahr, Yeasts in biodegradation and biodeterioration processes, *Yeast Biotechnology and Biocatalysis* (H. Verachtert and R. De Mot, eds.), Marcel Dekker, New York, 1990, pp. 321–348.
129. P. H. Pritchard, *Curr. Opin. Biotechnol. 3*: 232 (1992).
130. A. O. Summers, *Curr. Opin. Biotechnol. 3*: 271 (1992).
131. R. A. Herbert and R. J. Sharp, *Molecular Biology and Biotechnology of Extremophiles*, Blackie Publishing Group, London, 1992.
132. R. Nandan and S. Raisuddin, Fungal degradation of industrial wastes and wastewater, *Handbook of Applied Mycology*, Vol. 4, *Fungal Biotechnology* (A. K. Arora, R. P. Elander, and K. G. Mukerji, eds.), Marcel Dekker, New York, 1992, pp. 931–992.

133. P. Broda, *Tibtech 10*: 303 (1992).
134. R. A. Herbert, *Tibtech 10*: 395 (1992).
135. M. Kawase, Wastewater treatment by anaerobic fixed-bed reactor, *Industrial Application of Immobilized Biocatalysts* (A. Tanaka, T. Tosa, and T. Kobayashi), Marcel Dekker, New York, 1993, pp. 355–376.
136. T. Sumino, H. Nakamura, and N. Mori, Development of a high-efficiency wastewater treatment system using immobilized microorganisms, *Industrial Application of Immobilized Biocatalysts* (A. Tanaka, T. Tosa, and T. Kobayashi), Marcel Dekker, New York, 1993, pp. 377–391.
137. C. Robinson, Bioremediation, technological and commercial issues in the biological detoxification of polluted sites, *Tibtech 11*(8) (1993).
138. R. L. Monaghan and J. S. Tkacz, *Annu. Rev. Microbiol. 44*: 271 (1990).
139. Y. Tanaka and S. Omura, *Annu. Rev. Microbiol. 47*: 57 (1993).
140. C. M. Ignoffo and R. F. Anderson, Bioinsecticides, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 1–28.
141. L. A. Bulla and A. A. Yousten, Bacterial insecticides, *Economic Microbiology*, Vol. 4, *Microbial Biomass* (A. H. Rose, ed.), Academic Press, New York, 1979, pp. 91–115.
142. S. G. Lisansky and R. A. Hall, Fungal control of insects, *The Filamentous Fungi*, Vol. 4 (J. E. Smith, D. R. Berry, and B. Kristiansen, eds.), Edward Arnold, London, 1983, pp. 327–345.
143. G. E. Rowe, A. Margaritis, and H. T. Dulmage, Bioprocess developments in the production of bioinsecticides by *Bacillus thuringiensis*, *CRC Crit. Rev. Biotechnol. 6*: 87 (1987).
144. R. E. Andrews, R. M. Faust, H. Wabiko, K. C. Raymond, and L. A. Bulla, The biotechnology of *Bacillus thuringiensis*, *CRC Crit. Rev. Biotechnol. 6*: 163 (1987).
145. W. A. Gardner and C. W. McCoy, Insecticides and herbicides, *Biotechnology of Filamentous Fungi Technology and Products* (D. B. Finkelstein and C. Ball, eds.), Butterworth-Heinemann, Boston, 1991, pp. 335–360.
146. J. C. Burton, *Rhizobium* species, *Microbial Technology*, Vol. 1 (H. J. Peppler, ed.), Academic Press, New York, 1979, pp. 29–58.
147. J. C. Burton, Bacterial for azotification, *Economic Microbiology*, Vol. 4, *Microbial Biomass* (A. H. Rose, ed.), Academic Press, New York, 1979, pp. 65–115.
148. Y. Okon and Y. Hadar, Microbial inoculants as crop-yield enhancers, *CRC Crit. Rev. Biotechnol. 6*: 61 (1987).
149. A. Mizrahi, *Bacterial Vaccines*, Wiley-Liss, New York, 1990.
150. R. Keeler, *Res. Dev. Mag. 33*: 46 (1991).
151. D. J. Leak, P. J. Aikens, and M. Seyed-Mahmoudian, *Tibtech 10*: 258 (1992).
152. M. Ratafia, *Bio/Technology 5*: 692 (1987).
153. R. Spier, *Tibtech 6*: 2 (1988).
154. B. Kirsop, The current status of culture collections and their contribution to biotechnology, *CRC Current Rev. Biotechnol. 2*: 287 (1985).
155. L. T. Chang and R. P. Elander, Long-term preservation of industrially important microorganisms, *Manual of Industrial Microbiology and Biotechnology* (A. L.

Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 49–55.
156. B. Kirsop, Maintenance of yeast cultures, *Yeast Biotechnology* (D. R. Berry, I. Russell, and G. G. Stewart, eds.), Allen & Unwin, London, 1987, pp. 3–52.
157. D. Allsop and F. P. Simione, Culture collection services, *Filamentous Fungi* (D. L. Hawksworth and B. E. Kirsop, eds.), Cambridge University Press, Cambridge, 1988, pp. 162–200.
158. C. P. Kurtzman, Culture collections as sources of strains for industrial use, *Yeast Strain Selection* (C. J. Panchal, ed.), Marcel Dekker, New York, 1990, pp. 1–19.
159. I. Campbell, Principal sources of yeast cultures, *Yeast a Practical Approach* (I. Campbell and J. H. Duffus, eds.), IRL Press, Oxford, 1991, pp. 283–284.
160. D. Allsopp and F. P. Simione, Culture collection services, *Bacteria* (L. R. Hill and B. E. Kirsop, eds.), Cambridge University Press, Cambridge, 1991, pp. 134–145.
161. S. Jong and J. M. Birmingham, Culture collections: sources and management of *Aspergillus* cultures for biotechnology, *Aspergillus: Biotechnology and Industrial Applications* (J. W. Bennett and M. A. Klich, eds.), Butterworth-Heinemann, Boston, 1992, pp. 313–328.
162. S. Jong and J. M. Birmingham, Current status of fungal collections and their role in biotechnology, *Handbook of Applied Mycology*, Vol. 4, *Fungal Biotechnology* (D. K. Arora, R. P. Elander, and K. G. Mukerji, eds.), Marcel Dekker, New York, 1992, pp. 993–1024.
163. L. V. Kalakoutskii, *Tibtech 11*: 443 (1993).
164. B. E. Kirsop, Service collections: their functions, *Maintenance of Microorganisms and Cultured Cells* (B. E. Kirsop and A. Doyle, eds.), Academic Press, London, 1991, pp. 5–20.
165. D. B. Steele and M. D. Stowers, *Annu. Rev. Microbiol. 45*: 89 (1991).
166. D. M. Ward, R. Weller, and M. M. Bateson, *Nature 345*: 63 (1990).
167. J. C. Hunter-Cevera, M. E. Fonda, and A. Belt, Isolation of cultures, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 3–23.
168. J. C. Hunter, M. Fonda, L. Sotos, B. Toso, and A. Belt, *Dev. Ind. Microbiol. 25*: 247 (1984).
169. R. A. Herbert and G. A. Codd, *Microbes in Extreme Environments*, Academic Press, London, 1986.
170. P. S. J. Cheetham, *Enzyme Microb. Technol. 9*: 194 (1987).
171. N. A. Hendy, *J. Ind. Microbiol. 1*: 389 (1987).
172. F. Rodriguez-Valera, *General and Applied Aspects of Halophilic Microorganisms*, Plenum Press, New York, 1991.
173. M. A. Lachance, Yeast selection in nature, *Yeast Strain Selection* (C. J. Panchal), Marcel Dekker, New York, 1990, pp. 21–41.
174. D. A. Cowan, *Tibtech 10*: 315 (1992).
175. S. E. Lowe, M. K. Jain, and G. Zeikus, *Microbiol. Rev. 57*: 451 (1993).
176. N. R. Krieg, Enrichment and isolation, *Manual of Methods for General Bacteriology* (P. Gerhardt, ed. in chief), American Society for Microbiology, Washington, DC, 1981, pp. 112–142.

177. K. Nakayama, Sources of microorganisms for industry (general approaches), *Biotechnology*, Vol. 1, *Microbial Fundamentals* (H. J. Rehm and G. Reed, eds.), Verlag Chemie, Weinheim, 1981, pp. 357–410.
178. D. P. Labeda, *Isolation of Biotechnological Organisms from Nature*, McGraw-Hill, New York, 1990.
179. K. O'Donnell and S. W. Peterson, Isolation, preservation, and taxonomy, *Biotechnology of Filamentous Fungi Technology and Products* (D. B. Finkelstein and C. Ball), Butterworth-Heinemann, Boston, 1991, pp. 7–40.
180. M. A. Klich, L. H. Tiffany, and G. Knaphus, Ecology of the aspergilli of soils and litter, *Aspergillus: Biology and Industrial Applications* (J. W. Bennett and M. A. Klich, eds.), Butterworth-Heinemann, Boston, 1992, pp. 329–354.
181. A. Novick and L. Szilard, *Science 112*: 715 (1950).
182. J. J. Pirt, *Principles of Microbe and Cell Cultivation*, Blackwell Scientific, Oxford, 1975.
183. P. H. Calcott, *Continuous Cultures of Cells*, CRC Press, Boca Raton, FL, 1981.
184. K. Stafford, Continuous fermentation, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 137–151.
185. P. D. Cooper, J. N. Wilson, and A. M. Burt, *J. Gen. Microbiol. 21*: 702 (1959).
186. E. P. Cohen and H. Eagle, *J. Exp. Med. 113*: 467 (1961).
187. S. J. Pirt and D. S. Callow, *Exp. Cell Res. 33*: 413 (1964).
188. J. B. Griffiths and S. J. Pirt, *Proc. R. Soc. B 168*: 421 (1967).
189. C. Peraino, S. Bachhetti, and W. J. Eisler, *Science 169*: 204 (1970).
190. M. G. Tovey, G. E. Mathison, and S. J. Pirt, *J. Gen. Virol. 20*: 29 (1973).
191. M. Tovey and D. Brouty-Boye, *Exp. Cell. Res. 101*: 436 (1976).
192. M. G. Tovey and D. Brouty-Boye, *Exp. Cell. Res. 118*: 383 (1979).
193. M. G. Tovey, The cultivation of animal cells in the chemostat: applications to the study of tumor cell multiplication, *Advances in Cancer Research* (G. Klein and S. Weinhouse, eds.), Academic Press, New York, 1980, pp. 1–37.
194. A. Matin, Regulation of enzyme synthesis as studied in continuous culture, *Continuous Culture of Cells*, Vol. II (P. H. Calcott, ed.), CRC Press, Inc., Boca Raton, FL, 1981, pp. 69–97.
195. K. Toda, *J. Chem. Tech. Biotechnol. 31*: 775 (1981).
196. M. Pedrique and F. J. Castillo, *Appl. Environ. Microbiol. 43*: 303 (1982).
197. A. Gomez and F. J. Castillo, *Biotechnol. Bioeng. 25*: 1341 (1983).
198. S. Fazekas de St. Groth, *J. Immunol. Methods 57*: 121 (1983).
199. F. J. Castillo, H. Blanch, and C. R. Wilke, *Biotechnol. Lett. 6*: 593 (1984).
200. H. W. Verseveld, W. R. Chesbro, M. Braster, and A. H. Stouthamer, *Arch. Microbiol. 137*: 176 (1984).
201. E. Kromer, D. Lutz, and W. Scheirer, *Dev. Biol. Standard 55*: 129 (1984).
202. A. L. van Wezel, C. A. M. van der Velden-de Groot, H. H. de Haan, N. van den Heuvel, and R. Schasfoort, *Dev. Biol. Standard. 60*: 229 (1985).
203. J. R. Birch, P. W. Thompson, K. Lambert, and R. Boraston, The large scale cultivation of hybridoma cells producing monoclonal antibodies, American Chemical Society Meeting, Philadelphia, 1984.

204. J. R. Birch, K. Lambert, R. Boraston, P. W. Thompson, S. Garland, and A. C. Kenney, The industrial production of monoclonal antibodies in cell culture, NATO, ASI Meeting, Plenum Publishing Corporation, 1985.
205. F. J. Castillo, J. Thrift, G. Traugh, T. Bruggman, L. Mullen, and W. C. MacGregor, Growth and monoclonal antibody production by several hybridomas in continuous culture, 194th American Chemical Society Meeting, New Orleans, 1987.
206. W. M. Miller, C. R. Wilke, and H. W. Blanch, *J. Cell. Physiol. 132*: 524 (1987).
207. W. M. Miller, C. R. Wilke, and H. W. Blanch, *Bioprocess. Eng. 3*: 113 (1988).
208. G. J. MacMichael, *Hybridoma 8*: 117 (1989).
209. T. S. Shaughnessy and F. Kargi, *Enz. Microb. Technol. 12*: 676 (1990).
210. S. S. Ozturk and B. O. Palsson, *Biotechnol. Prog. 6*: 437 (1990).
211. T. I. Linardos, N. Kalogerakis, L. A. Behie, and L. R. Lamontagne, *Can. J. Chem. Eng. 69*: 429 (1991).
212. D. E. Martens, C. D. de Gooijer, C. A. M. van der Velden-de Groot, E. C. Beuvery, and J. Tramper, *Biotechnol. Bioeng. 41*: 429 (1993).
213. F. J. Castillo, L. J. Mullen, J. C. Thrift, and B. C. Grant, *Ann. N. Y. Acad. Sci. 665*: 72 (1992).
214. V. Bryson and W. Szybalski, *Science 116*: 45 (1952).
215. H. W. Jannash, *Archiv. Mikrobiol. 59*: 165 (1967).
216. W. Harder, J. G. Kuenen, and A. Matin, *J. Appl. Bacteriol. 43*: 1 (1977).
217. B. Sikyta, P. Kyslik, B. Volesky, E. Pavlasova, and E. Stejskalova, Overproduction of endoenzymes in *Escherichia coli*—selection of hyperproducing strains in a chemostat, *Overproduction of Microbial Products* (V. Krumphanzl, B. Sikyta, and Z. Yanek, eds.), Academic Press, London, 1982, pp. 593–599.
218. J. J. Kilbane, D. K. Chatterjee, J. S. Karns, S. T. Kellogg, and A. M. Chakrabarty, *Appl. Environ. Microbiol. 44*: 72 (1982).
219. D. E. Dykhuizen and D. L. Hartl, *Microbiol. Rev. 47*: 150 (1983).
220. D. Kohler-Staub and T. Leisinger, *J. Bacteriol. 162*: 676 (1985).
221. S. Keuning, D. B. Janssen, and B. Witholt, *J. Bacteriol. 163*: 635 (1985).
222. C. Wandrey, A. Aivasidis, and K. H. Bastin, *Ann. N. Y. Acad. Sci. 469*: 421 (1986).
223. R. J. White, W. M. Maiese, and M. Greenstein, Screening for new products from microorganisms, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 24–31.
224. M. J. Hall, *Biotechnology 7*: 427 (1989).
225. S. Omura, *Gene 115*: 141 (1992).
226. D. R. Kirsch, *Curr. Opin. Biotechnol. 4*: 543 (1993).
227. I. A. Field, E. de Jong, G. Feijoo-Costa, and I. A. M. de Bont, *Tibtech 11*: 44 (1993).
228. R. L. Monaghan and L. R. Koupoal, Use of Plackett & Burman technique in a discovery program for new natural products, *Novel Microbial Products for Medicine and Agriculture* (A. L. Demain, G. A. Somkuti, J. C. Hunter-Cevera, and H. W. Rossmoore, eds.), Elsevier, New York, 1989, pp. 25–32.
229. R. Greasham and E. Inamine, Nutritional improvement of process, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 41–48.

230. M. D. White, Z. P. Shalita, D. Marcus, and S. Reuveny, A systematic route for optimization of media composition and growth conditions, *Biologicals from Recombinant Microorganisms and Animal Cells* (M. D. White, S. Reuveny, and A. Shafferman, eds.), VCH, Weinheim, 1991, pp. 101–116.
231. J. R. Johnston, Strain improvement and strain stability in filamentous fungi, *The Filamentous Fungi*, Vol. 1 (J. E. Smith and D. R. Berry, eds.), John Wiley & Sons, New York, 1975, pp. 59–78.
232. R. P. Elander and A. L. Demain, Genetics of microorganisms in relation to industrial requirements, *Biotechnology*, Vol. 1 (H. J. Rehm and G. Reed, eds.), Verlag Chemie, Weinheim, 1981, pp. 235–277.
233. G. K. Jacobson, Mutations, *Biotechnology*, Vol. 1 (H. J. Rehm and G. Reed, eds.), Verlag Chemie, Weinheim, 1981, pp. 279–304.
234. R. T. Rowlands, Industrial fungal genetics and strain selection, *The Filamentous Fungi*, Vol. 4 (J. E. Smith, D. R. Berry, and B. Kristiansen, eds.), Edward Arnold, London, 1983, pp. 346–372.
235. R. H. Baltz, Strain improvement: introduction, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, p. 154.
236. S. W. Queener and D. H. Lively, Screening and selection for strain improvement, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 155–169.
237. P. Matsushima and R. H. Baltz, Protoplast fusion, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 170–183.
238. R. H. Baltz, Mutagenesis in *Streptomyces* spp., *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 184–190.
239. D. A. Hopwood, Genetic mapping by conjugation in *Streptomyces* spp., *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 191–197.
240. D. A. Hopwood, Gene cloning in *Streptomyces* spp., *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 198–203.
241. T. D. Ingolia and J. S. Wood, Genetic manipulation of *Saccharomyces cerevisiae*, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, pp. 204–213.
242. I. Russell, R. Jones, and G. Stewart, The genetic modification of brewer's yeast and other industrial yeast strains, *Biotechnology in Food Processing* (S. K. Harlander and T. P. Labuza, eds.), Noyes Publications, Park Ridge, NJ, 1986, pp. 171–196.
243. R. T. Rowlands, Strain improvement and strain stability, *Biotechnology of Filamentous Fungi* (D. B. Finkelstein and C. Ball, eds.), Butterworth-Heinemann, Boston, 1991, pp. 41–64.

244. M. F. Tuite, Strategies for the genetic manipulation of *Saccharomyces cerevisiae*, *CRC Crit. Rev. Biotechnol. 12*: 157 (1992).
245. J. F. Peberdy, Genetic manipulation, *Physiology of Industrial Fungi* (D. R. Berry, ed.), Blackwell Scientific Publications, Oxford, 1988, pp. 187–218.
246. J. P. DeWitt, J. V. Jackson, and T. J. Paulus, Actinomycetes, *Fermentation Process Development of Industrial Organisms* (J. O. Neway, ed.), Marcel Dekker, New York, 1989, pp. 1–72.
247. F. G. Priest and R. J. Sharp, Fermentation of bacilli, *Fermentation Process Development of Industrial Organisms* (J. O. Neway, ed.), Marcel Dekker, New York, 1989, pp. 73–132.
248. R. P. Elander, Bioprocess technology in industrial fungi, *Fermentation Process Development of Industrial Organisms* (J. O. Neway, ed.), Marcel Dekker, New York, 1989, pp. 169–219.
249. D. Iserentant, Methods for the genetic engineering of yeasts, *Yeasts Biotechnology and Biocatalysis* (H. Verachtert and Rene De Mot, eds.), Marcel Dekker, New York, 1990, pp. 35–55.
250. C. J. Panchal, *Yeast Strain Selection*, Marcel Dekker, New York, 1990.
251. B. C. Carlton and B. J. Brown, Gene mutation, *Manual of Methods for General Bacteriology* (P. Gerhardt, ed. in chief), American Society for Microbiology, Washington, DC, 1986, pp. 222–242.
252. C. P. van der Beek and J. A. Roels, *Antoine van Leeuwenhoek 50*: 625 (1984).
253. P. J. Radlett, T. W. F. Pay, and A. J. M. Garland, *Dev. Biol. Standard. 60*: 163 (1985).
254. P. J. Radlett, The use of BHK suspension cells for the production of foot and mouth disease vaccines, *Advances in Biochemical Engineering/Biotechnology*, Vol. 34 (A. Fiechter, ed.), Springer-Verlag, Berlin, 1987, pp. 129–146.
255. A. Mizrahi, *Viral Vaccines*, Wiley-Liss, New York, 1990.
256. A. J. Beale, The production of viruses for human vaccines from animal cells in culture, *Animal Cell Biotechnology*, Vol. 5 (R. E. Spier and J. B. Griffiths, eds.), Academic Press, London, 1992, pp. 190–200.
257. J. B. Griffiths, Cell products: an overview, *Animal Cell Biotechnology*, Vol. 2 (R. E. Spier and J. B. Griffiths), Academic Press, London, 1985, pp. 3–12.
258. A. Mizrahi, A. Lazar, and S. Reuveny, Interferons derived from human cells, *Animal Cell Biotechnology*, Vol. 4 (R. E. Spier and J. B. Griffiths, eds.), Academic Press, London, 1990, pp. 413–444.
259. N. Epstein, S. Reuveny, J. Friedman, and N. Ariel, The manufacture and use of a colon cancer antigen-carcinoembryonic antigen, *Animal Cell Biotechnology*, Vol. 4 (R. E. Spier and J. B. Griffiths, eds.), Academic Press, London, 1990, pp. 445–474.
260. J. B. Griffiths and A. Electriwala, Production of plasminogen activators from animal cells, *Advances in Biochemical Engineering*, Vol. 34 (A. Fiechter, ed.), Springer-Verlag, Berlin, 1987, pp. 147–166.
261. L. Hayflick and W. Hennessen, *Continuous Cell Lines as Substrates for Biologicals, Developments in Biological Standardization*, Vol. 70, Karger, Basel, 1989.
262. F. Horaud and F. Brown, *Virological Aspects of the Safety of Biological Products, Developments in Biological Standardization*, Vol. 75, Karger, Basel, 1991.

263. F. Brown, E. E. Esber, and M. Williams, *Continuous Cell Lines—An International Workshop on Current Issues, Developments in Biological Standardization*, Vol. 76, Karger, Basel, 1992.
264. P. L. Skatrud, *Tibtech 10*: 324 (1992).
265. G. Stephanopoulos and A. J. Sinskey, *Tibtech 11*: 392 (1993).
266. Y. Murooka and T. Imanaka, *Recombinant Microbes for Industrial and Agricultural Applications*, Marcel Dekker, New York, 1993.
267. H. Takagi, Y. Morinaga, H. Ikemura, and M. Inouye, *J. Biol. Chem. 263*: 19592 (1988).
268. R. Katsumata and M. Ikeda, *Bio/Technology 11*: 921 (1993).
269. J. A. DeModena, S. Gutierrez, J. Velasco, F. J. Fernandez, R. A. Fachini, J. L. Galazzo, D. E. Hughes, and J. F. Martin, *Bio/Technology 11*: 926 (1993).
270. D. Murdock, B. D. Ensley, C. Serdar, and M. Thalen, *Bio/Technology 11*: 381 (1993).
271. R. E. Shamel and J. J. Chow, *Bio/Technology 6*: 681 (1988).
272. D. A. Hopwood, *Curr. Opin. Biotechnol. 4*: 531 (1993).
273. R. Kaufman, Expression Systems, *Curr. Opin. Biotechnol. 3*(5) (1992).
274. A. R. Shatzman, Expression Systems, *Curr. Opin. Biotechnol. 4*(5) (1993).
275. A. Smith, *Gene Expression in Recombinant Microorganisms*, Marcel Dekker, New York, 1994.
276. J. Hodgson, *Bio/Technology 11*: 887 (1993).
277. M. H. Marino, *BioPharm 2*: 18 (1989).
278. P. O. Olins and S. C. Lee, *Curr. Opin. Biotechnol. 4*: 520 (1993).
279. G. Rapoport and A. Klier, *Curr. Opin. Biotechnol. 1*: 21 (1990).
280. C. R. Harwood, *Tibtech 10*: 247 (1992).
281. R. H. Balz, *Curr. Opin. Biotechnol. 1*: 12 (1990).
282. J. A. Fornwald, M. J. Donovan, R. Gerber, J. Keller, D. P. Taylor, E. J. Arcuri, and M. E. Brawner, *Bio/Technology 11*: 1031 (1993).
283. C. J. Panchal, Yeast plasmids and transformation, *Yeast Biotechnology* (D. R. Berry, I. Russell, and G. G. Stewart, eds.), Allen & Unwin, London, 1987, pp. 80–103.
284. M. Ratner, *Bio/Technology 7*: 1129 (1989).
285. C. L. Hershberger, S. W. Queener, and G. Hegeman, *Genetics and Molecular Biology of Industrial Microorganisms*, American Society for Microbiology, Washington, DC, 1989.
286. P. Tekamp-Olson and P. Valenzuela, *Curr. Opin. Biotechnol. 1*: 28 (1990).
287. M. J. de Wilde, Yeast as a host for the production of macromolecules of prophylactic or therapeutic interest in human health care, *Yeast Biotechnology and Biocatalysis* (H. Verachtert and R. De Mot, eds.), Marcel Dekker, New York, 1990, pp. 479–504.
288. R. G. Buckholz and M. A. G. Gleeson, *Bio/Technology 9*: 1067 (1991).
289. T. S. Vedvick, *Curr. Opin. Biotechnol. 2*: 742 (1991).
290. R. D. Brearley and D. E. Kelly, Genetic engineering techniques in yeast, *Genetically-Engineered Proteins and Enzymes from Yeasts: Production Control* (A. Wiseman, ed.), Ellis Horwood, New York, 1991, pp. 75–95.

291. J. M. Cregg, T. Vedvick, and W. C. Raschke, Recent advances in the *Pichia pastoris* expression system, 10th Intersectional Symposium of Japanese Yeast Researchers, Kyoto, Japan, 1992.
292. J. M. Cregg, T. S. Vedvick, and W. C. Raschke, *Bio/Technology 11*: 905 (1993).
293. R. G. Buckholz, *Curr. Opin. Biotechnol. 4*: 538 (1993).
294. V. A. Luckow and M. D. Summers, *Bio/Technology 6*: 47 (1988).
295. D. H. L. Bishop, *Curr. Opin. Biotechnol. 1*: 62 (1990).
296. L. A. King and R. D. Possee, *The Baculovirus Expression System—A Laboratory Guide*, Chapman & Hall, London, 1992.
297. M. F. A. Goosen, A. J. Daugulis, and P. Faulkner, *Insect Cell Culture Engineering*, Marcel Dekker, New York, 1993.
298. C. L. Propst, R. J. von Wedel, and A. S. Lubiniecki, Using mammalian cells to produce products, *Fermentation Process Development of Industrial Organisms* (J. O. Neway, ed.), Marcel Dekker, New York, 1989, pp. 221–276.
299. R. E. Spier and J. B. Griffiths, *Animal Cell Biotechnology*, Vol. 4, Academic Press, London, 1990.
300. A. S. Lubiniecki, *Large-Scale Mammalian Cell Culture Technology*, Marcel Dekker, New York, 1990.
301. C. M. Gorman, *Curr. Opin. Biotechnol. 1*: 36 (1990).
302. R. E. Kellems, *Curr. Opin. Biotechnol. 2*: 723 (1991).
303. W. G. Thilly, *Mammalian Cell Technology*, Butterworths, Boston, 1986.
304. C. P. Edwards and A. Aruffo, *Curr. Opin. Biotechnol. 4*: 558 (1993).
305. A. Hiatt, R. Cafferkey, and K. Bowdish, *Nature 342*: 76 (1989).
306. A. Caplan and M. van Montagu, *Curr. Opin. Biotechnol. 1*: 68 (1990).
307. J. Hughes and M. W. Qoronfleh, *BioPharm 4*: 18 (1991).
308. R. Fraley and J. Schell, Plant biotechnology, *Curr. Opin. Biotechnol. 4*(2) (1993).
309. E. Truve, A. Aaspollu, J. Honkanen, R. Puska, Merja Mehto, A. Hassi, T. H. Teeri, M. Kelve, P. Seppanen, and M. Saarma, *Bio/Technology 11*: 1048 (1993).
310. B. S. Minhas and S. A. Voelker, Transgenic animals, *Biotechnology* (H. J. Rehm and G. Reed, eds.), Vol. 7b, *Gene Technology* (G. K. Jakobson and S. O. Jolly, eds.), VCH, Weinheim, 1989, pp. 357–398.
311. L. Hennighausen, L. Ruiz, and R. Wall, *Curr. Opin. Biotechnol. 1*: 74 (1990).
312. A. Carver, G. Wright, D. Cottom, J. Cooper, M. Dalrymple, S. Temperley, M. Udell, D. Reeves, J. Percy, A. Scott, D. Barras, Y. Gibson, Y. Jeffrey, C. Samuel, A. Colman, and I. Garner, *Cytotechnology 9*: 77 (1992).
313. J. S. Logan, *Curr. Opin. Biotechnol. 4*: 591 (1993).
314. A. S. Carver, M. A. Dalrymple, G. Wright, D. S. Cottom, D. B. Reeves, Y. H. Gibson, J. L. Keenan, J. D. Barrass, A. R. Scott, A. Colman, and I. Garner, *Bio/Technology 11*: 1263 (1993).
315. C. A. Pinkert, *Transgenic Animal Technology*, Academic Press, New York, 1993.
316. K. Horibata and A. W. Harris, *Exp. Cell Res. 60*: 61 (1970).
317. G. Kohler and C. Milstein, *Nature 256*: 496 (1975).
318. G. Kholer, S. C. Howe, and C. Milstein, *Eur. J. Immunol. 6*: 292 (1976).
319. G. Kholer and C. Milstein, *Eur. J. Immunol. 6*: 511 (1976).
320. J. F. Kearney, A. Radbruch, B. Liesegang, and K. Rajewsky, *J. Immunol. 123*: 1548 (1979).

321. G. Galfre and C. Milstein, Preparation of monoclonal antibodies: strategies and procedures, *Methods in Enzymology*, Vol. 73 (J. J. Langone and H. van Vunakis, eds.), Academic Press, New York, 1981, pp. 3–46.
322. M. Shulman, C. D. Wilde, and G. Kohler, *Nature 276*: 269 (1978).
323. J. W. Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, London, 1983.
324. J. M. Davis, A single-step technique for selecting and cloning hybridomas for monoclonal antibody production, *Methods in Enzymology*, Vol. 121 (J. J. Langone, and H. van Vunakis, eds.), Academic Press, New York, 1986, pp. 307–322.
325. K. James, Therapeutic monoclonal antibodies—their production and applications, *Animal Cell Biotechnology*, Vol. 4 (R. E. Spier and J. B. Griffiths, eds.), Academic Press, London, 1990, pp. 206–255.
326. A. Boeye, Clonal isolation of hybridomas by manual single-cell isolation, *Methods in Enzymology*, Vol. 121 (J. J. Langone and H. van Vunakis, eds.), Academic Press, New York, 1986, pp. 332–341.
327. H. A. Coller and B. S. Coller, Poisson statistical analysis of repetitive subcloning by the limiting dilution technique as a way of assessing hybridoma monoclonality, *Methods in Enzymology*, Vol. 121 (J. J. Langone and H. van Vunakis, eds.), Academic Press, New York, 1986, pp. 412–417.
328. R. E. Spier and F. Horaud, The biotechnological future for animal cells in culture, *Animal Cell Biotechnology*, Vol. 2 (R. E. Spier and J. B. Griffiths, eds.), Academic Press, London, 1985, pp. 431–458.
329. A. Pavirani, P. Meulien, H. Harrer, F. Schamber, K. Dott, D. Villeval, Y. Cordier, M. L. Wiesel, C. Mazurier, H. van de Pol, Y. Piquet, J. P. Cazenave, and J. P. Lecocq, *Bio/Technology 5*: 389 (1987).
330. T. J. R. Harris, *Protein Production in Biotechnology*. Elsevier Science Publishers, Essex, 1990.
331. P. S. Sanders, Protein production by genetically engineered mammalian cell lines, *Animal Cell Biotechnology*, Vol. 4 (R. E. Spier and J. B. Griffiths, eds.), Academic Press, London, 1990, pp. 15–70.
332. D. Vapnek, Choosing a production system for recombinant proteins, *Biologicals from Recombinant Microorganisms and Animal Cells* (M. D. White, S. Reuveny, and A. Shafferman, eds.), CVH, Weinheim, 1991, pp. 1–14.
333. A. R. Goodney, *Tibtech 11*: 430 (1993).
334. C. E. Warren, *Curr. Opin. Biotechnol. 4*: 596 (1993).
335. R. W. van Leen, J. G. Bakhuis, R. F. W. C. van Beckhoven, H. Burger, L. C. J. Dorssers, R. W. J. Hommes, P. J. Lemson, B. Noordam, N. L. M. Persoon, and G. Wagemaker, *Bio/Technology 9*: 47 (1991).
336. M. Fountoulakis and R. Gentz, *Bio/Technology 10*: 1143 (1992).
337. R. V. Datar, T. Cartwright, and C. G. Rosen, *Bio/Technology 11*: 349 (1993).
338. L. A. Weymouth and J. Barsoum, Genetic engineering in mammalian cells, *Mammalian Cell Technology* (W. G. Thilly, ed.), Butterworths, Boston, 1986, pp. 9–62.
339. R. J. Kaufman, Use of recombinant DNA technology for engineering mammalian cells to produce proteins, *Large-Scale Mammalian Cell Culture Technology* (A. S. Lubiniecki, ed.), Marcel Dekker, New York, 1990, pp. 15–69.

340. M. Wirth, S. Y. Li, L. Schumaker, J. Lehmann, G. Zettlmeibl, and H. Hauser, Screening for and fermentation of high producer cell clones from recombinant BHK cells, *Advances in Animal Cell Biology and Technology for Bioprocesses* (R. E. Spier, J. B. Griffiths, J. Stephenne, and P. J. Crooy, eds.), Butterworth & Co. Publishers, Essex, 1989, pp. 44–51.
341. A. H. Davies, J. B. M. Jowett, and I. M. Jones, *Bio/Technology 11*: 933 (1993).
342. M. J. Browne and J. M. Balcarek, *Curr. Opin. Biotechnol. 4*: 553 (1993).
343. V. A. Luckow, *Curr. Opin. Biotechnol. 4*: 564 (1993).
344. M. I. Cockett, C. R. Bebbington and G. T. Yarranton, *Bio/Technology 8*: 662 (1990).
345. M. E. Brown, G. Renner, R. P. Field, and T. Hassell, *Cytotechnology 9*: 231 (1992).
346. C. R. Bebbington, G. Renner, S. Thomson, D. King, D. Abrams, and G. T. Yarranton, *Bio/Technology 10*: 169 (1992).
347. J. J. Clare, F. B. Rayment, S. P. Ballantine, K. Sreekrishna, and M. A. Romanos, *Biotechnology 9*: 455 (1991).
348. R. L. Gherna, Preservation, *Manual of Methods for General Bacteriology* (P. Gerhardt, ed. in chief), American Society for Microbiology, Washington, DC, 1981, pp. 208–217.
349. A. H. S. Onions, Preservation of fungi, *The Filamentous Fungi*, Vol. 4 (J. E. Smith, D. R. Berry, and B. Kristiansen, eds.), Edward Arnold, London, 1983, pp. 373–390.
350. P. H. Calcott, Cryopreservation of microorganisms, *CRC Crit. Rev. Biotechnol. 4*: 279 (1986).
351. D. Smith, Culture and preservation, *Filamentous Fungi* (D. L. Hawksworth and B. E. Kirsop, eds.), Cambridge University Press, Cambridge, 1988, pp. 75–161.
352. Center for Biologics Evaluation and Research, Food and Drug Administration, Points to Consider in the Characterization of Cell Lines used to Produce Biologicals, 1993.
353. R. J. Hay, Preservation and characterization, *Animal Cell Culture a Practical Approach* (R. I. Freshney, ed.), IRL Press, Oxford, 1989, pp. 71–112.
354. M. E. Wiebe and L. H. May, Cell banking, *Large-Scale Mammalian Cell Culture Technology* (A. S. Lubiniecki, ed.), Marcel Dekker, New York, 1990, pp. 147–160.
355. A. Doyle and C. B. Morris, Maintenance of animal cells, *Maintenance of Microorganisms and Cultured Cells* (B. E. Kirsop and A. Doyle, eds.), Academic Press, London, 1991, pp. 227–241.
356. L. L. Coriell, Preservation, storage, and shipment, *Methods in Enzymology*, Vol. 58, *Cell Culture* (W. B. Jakoby and I. H. Pastan, eds.), Academic Press, New York, 1979, pp. 29–36.
357. P. M. Daggett and F. P. Simione, *Cryopreservation Manual*, Nalge Company, Rochester, NY, 1987.
358. Food and Drug Administration, Sterility, *Code of Federal Regulations 21*, Part 610—General Biological Products Standards, Subpart B—General Provisions, 610.12, Office of the Federal Register National Archives and Records Administration, 1993.

359. D. A. Stahl, *Am. Soc. Microbiol. News 59*: 609 (1993).
360. K. D. Clements and S. Bullivant, *J. Bacteriol. 173*: 5359 (1991).
361. E. R. Angert, K. D. Clements, and N. R. Pace, *Nature 362*: 239 (1993).
362. A. L. Demain, G. A. Somkuti, J. C. Hunter-Cevera, and H. W. Rossmoore, *Novel Microbial Products for Medicine and Agriculture*, Elsevier Science Publishers, Amsterdam, 1989.
363. A. H. Goldstein, R. D. Rogers, and G. Mead, *Bio/Technology 11*: 1251 (1993).
364. J. King, *Chem. Eng. News 67*(15): 31 (1989).
365. E. U. Lee, J. Roth, and J. C. Paulson, *J. Biol. Chem. 264*: 13848 (1989).
366. C. H. Schein, *Bio/Technology 7*: 1141 (1989).
367. C. H. Schein, *Bio/Technology 8*: 309 (1990).
368. D. L. Wilkinson and R. G. Harrison, *Bio/Technology 9*: 443 (1991).
369. J. Zhu, E. Baron, and R. Greenberg, *Pharmaceutical Technol. 17*: 28 (1993).
370. R. F. Rosenberger and R. Holliday, *Tibtech 11*: 498 (1993).
371. M. A. S. Santos and M. F. Tuite, *Tibtech 11*: 500 (1993).

3

Bacterial, Yeast, and Fungal Cultures

Effect of Microorganism Type and Culture Characteristics on Bioreactor Design and Operation

Moshe D. White

Israel Institute for Biological Research
Ness-Ziona, Israel

Bernard R. Glick and Campbell W. Robinson

University of Waterloo
Waterloo, Ontario, Canada

I. INTRODUCTION

The choice of a specific microorganism for use in a fermentation process has a direct influence on the design of the bioreactor. The impact that the microorganism has on the overall process is multifaceted, in part due to its complexity as a living entity and the ensuing complexity of conditions inside the bioreactor. Consequently, no general models for fermentation scale-up exist which can readily take into account the enormous diversity of the biology of the relevant organisms. As a result, scale-up is dependent on experimental results from small and pilot plant studies.

The performance of a large number of bioreactors has been studied over the past two decades [1, pp. 626–629]. However, a substantial amount of this research has been done using model solutions and not actual fermentation broths. In most cases in which studies with microorganisms were undertaken, only one type of bioreactor was investigated.

In an effort to remedy the lack of systematic data on microbial fermentation, it was recommended that a series of studies in a few different bioreactors should be done with specific "model" organisms [2]. In fact, a few model microorganisms [3] have even been suggested based on the following criteria: (1) the medium should be well defined, with limiting components known and optimized for the test organism; (2) the organism is not pathogenic or hazardous in any way; (3) the organism must be well characterized, not too complicated to handle, genetically stable, with no separately segregating population (i.e., no plasmid stability problems), readily available from a public culture collection, and not present a biohazard; (4) analytical measurements should be automated (or simple and rapid manual methods should be available) for biomass, substrate(s), off-gas analysis, gas flow, product, and power consumption.

The recommended test organisms are listed in Table 1. However, as these authors point out, the biology of these organism is such that they can be grown

Table 1 Microorganisms that Have Been Suggested for Use as "Model" Cells for Bioreactor Research

Microorganism	Special feature of interest
Bacillus subtilis	To characterize mixing and oxygen transfer evaluated by the production of acetoin and butanediol [8]
Beauveria tenella fungi imperfecti	Useful for evaluation of wall growth
Chaetomium cellulolyticum	Fungus, used to test more highly viscous broths [4]
Candida utilus	For studying the effects of hydrostatic pressures [5]
Escherichia coli	Used as a test organism due to its role as a producer of many recombinant proteins
Methylomonas M15	Used for comparison of mass transport, growth, and product quality in CSTR and column reactors [7]
Pseudomonas fluorescens	Used to test the influence of varying hydrostatic pressure [5]
Trichosporon cutaneum	Strict aerobic yeast; model for maximal oxygen transfer
Xanthomonas campestris	Bacteria used for studying the effect of viscous media [6]

Source: Ref. 3.

in different modes. For example, the fungus *Trichoderma* can grow in a life cycle similar to a "yeast" and also in a filamentous mode of growth.

At present, the use of "standard" microorganisms for bioreactor development has not been examined to any great extent. Therefore, in this chapter results based on the use of many different organisms and bioreactors are considered.

The bioreactor that is currently favored by most workers is the stirred tank reactor (STR). It is so common that it has even been suggested [9] that it be called a fermenter while other types should be called bioreactors. Although we do not accept this idea, it emphasizes the ubiquitous nature of the STR in fermentation studies.

The main reason for the widespread use of the STR is that when most microorganisms are grown in shake flasks, they often attain only low cell densities, usually due to oxygen transfer limitations [10]. The immediate and simple scale-up of shake flask cultures is to a small mixed vessel where an impeller replaces the shaking for the purposes of mixing and bubble dispersion. This equipment is versatile and very common in laboratories. Most organisms can grow in an STR, although some equipment modifications are necessary for different types of organisms. These include aerobic and anaerobic conditions, use of light, and different agitation speeds and impeller types.

The widespread industrial use of the STR is related to several considerations, including the following [3]: (1) The classic stirred tank bioreactor is a versatile and useful design for the culture of most microorganisms, including those that develop highly viscous culture broths (e.g., filamentous microorganisms). For the cultivation of shear-sensitive cells (e.g., mammalian cells) the turbine can easily be replaced by a marine impeller. (2) Because of the high capital investment involved, there is reluctance to replace conventional equipment, with which a great deal of experience and knowledge on the pilot or large scale has been accumulated. (3) Data from fermentations carried out under comparable conditions, in differently designed bioreactors and on different scales, are lacking. This is certainly also the main reason for the "conventional wisdom" that, according to Charles [11], has led to the almost universal choice of the conventional stirred tank reactor.

This chapter discusses the effect that varying the microorganism has on the choice of bioreactor. However, bioreactor choice depends not only on the organism chosen but on many additional factors. These include parameters which affect the growth of the organism (e.g., growth rate, temperature, induction type, and duration of growth) as well as parameters concerning the product (i.e., the chemical and physical nature of the product and the kinetics of product synthesis). It should be noted that this chapter does not consider immobilized cell bioreactors (e.g., gel entrapment, surface immobilized, microcapsules), which have been described elsewhere [12–14].

II. GENERAL GROWTH CHARACTERISTICS OF MICROORGANISMS

A. Basic Description

Numerous books describe the growth of microorganisms, ranging from more basic microbiology textbooks [15–19] to detailed treatises [20–23]. Extensive lists of microorganisms and their properties can be found in the manuals of culture collections, the best known being the American Type Culture Collection (ATCC) [24], and many of them are available including special industrial collections [25,26]. In addition, numerous specific reviews on industrial microorganisms are available [27,28]. For clarity, we provide short descriptions of typical microbial growth cycles. These are only general outlines, and when working with a specific microorganism its growth cycle should be explored in detail.

There are many species of microorganisms with a large variety of characteristics. Generally, they are divided into prokaryotic and eukaryotic [19]. The former (prokaryotes) are simpler in genomic construction and number of suborganelles and include bacteria and the blue-green algae (cyanobacteria). The eukaryotic cell is typical of the mammalian kingdom and is usually larger than the prokaryotic cell. Eukaryotic microorganisms are more complex than the bacteria and include the algae and mycota (fungi and yeast). The differences in genomic structure between the prokaryotes and eukaryotes lead to different problems in their use in recombinant DNA technology (see below). From the point of view of bioreactor design, this biological distinction is not as important as the distinction between unicellular, multicellular, and budding microorganisms.

B. Unicellular Microorganisms—Most of the Bacteria

Bacteria are generally unicellular organisms with a cell size typically in the range of 0.5 to 2 μm [19]. However, among the bacteria there is a wide variety of cell morphologies; the principal ones are shown in Fig. 1. This diversity of cell structure, as seen through the light microscope, is one of the major parameters used for classification of bacteria. The other distinguishing parameters are all aspects of the organism's growth requirements including, among others, the requirement for oxygen and the sources of carbon, nitrogen, and energy (Table 2). The accepted classification of bacteria is found in *Bergey's Manual of Systematic Bacteriology* [29] and is based upon these parameters. Some of the more common bacteria used industrially are *Escherichia coli* [30,31], *Pseudomonas*, *Xanthomonas* [32], and *Bacillus* [33].

Bacteria reproduce by binary fission into two daughter cells. At the end of each division cycle there are two daughter cells, which are identical in overall composition to the former mother cell, and therefore from one generation to the next the cell mass is doubled. This is the basis for the kinetics of bacterial cell

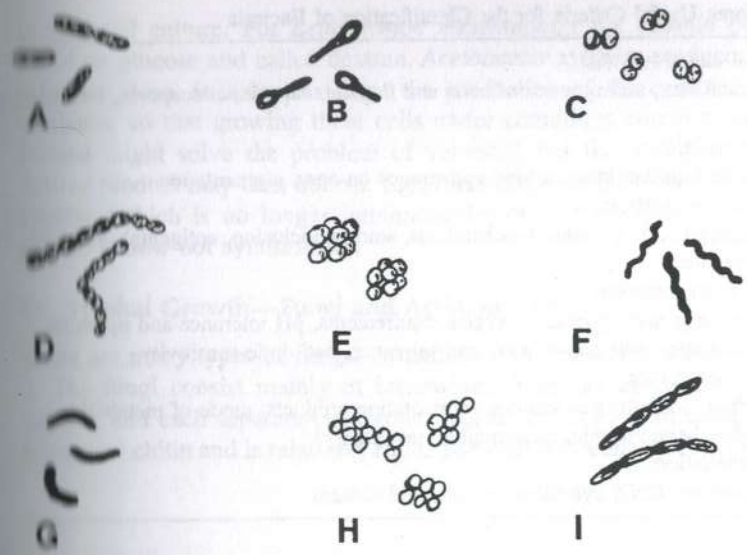


Figure 1 Principal morphological structures of bacteria. (A) Bacilli as *Bacillus cereus*; (B) *Clostridium tetani* bacilli swollen with spores; (C) *Diplococcus pneumniae*; (D) Streptobacilli; (E) *Sarcinae* (eight cocci in cubelike shape); (F) spiral shape of *Leptospira*; (G) comma shape as in *Vibrio cholera*; (H) staphylococci in various attachments; (I) *Escherichia coli* bacilli as long rods without separation when grown under limiting conditions.

growth. However, this condition holds only when the cells are grown in a constant environment, and it is then called balanced growth [34]. This situation does not always occur, which is one of the reasons for the complexity in modeling cell growth.

Some bacteria have the ability to produce spores (endospores), a dormant stage in which the bacteria are not dividing or growing. Spores are usually formed under condition of stress (such as a shortage of the carbon or the nitrogen source or extreme temperatures in the habitat). Sporulation begins by multiplication of the DNA, followed by division of the cell into two parts, a larger mother cell and a smaller forespore. The forespore is engulfed by a second membrane layer and cut off as a free protoplast in the mother cell before being covered with a cell wall (cortex) and an additional layer of peptidoglycan. In the next step a protein coat is deposited on the cortex, followed by combination of all layers to form a very rigid cell wall, followed by the dissolution of the mother cell wall and release of the mature spore. Under suitable conditions of

Table 2 Some Useful Criteria for the Classification of Bacteria

Morphological characteristics:
Cell shape and size, arrangement of cells and flagella, capsule, endospores, Gram stain
Growth characteristics:
Appearance in liquid culture, colony appearance on agar, pigmentation
Biochemical characteristics:
Cell wall composition, pigment biochemicals, storage inclusion, antigen(s) produced, RNA molecules
Physiological characteristics:
Temperature range and optimum, oxygen requirements, pH tolerance and optimum, osmotic tolerance, salt requirement and tolerance, antibiotic sensitivity
Nutritional characteristics:
Energy, carbon, and nitrogen sources, fermentation products, mode of metabolism (autotrophic, heterotrophic, fermentative, respiratory)
Genetic characteristics:
DNA composition, DNA hybridization, plasmid content

wet, rich media spores germinate and produce vegetative cells, which again proliferate by binary fission.

The spore is built with a very thick cell wall, which enables it to survive in extremely harsh environments for extended periods of time. Spores are especially noted for their stability against chemicals [35,36] and ability to withstand high temperatures. Some spore cultures are able to survive for a few hours in boiling water and require heating to 121°C for their destruction. The sterilization time required for the number of potentially viable spores to decrease by a factor of 10 (the so-called decimal reduction time) depends on the specific organism. The values for the most stable spores are in the range of a few minutes. However, microorganisms that can form more stable spores have been reported [37,38]. The existence of these spores is the reason why the absolute minimal time required for medium sterilization is 15 minutes at 121°C. However, this should be the temperature in every part in the sterilized vessel, and as most vessels have locations where heat transfer is inefficient, sterilization is usually carried out for 30 minutes. The kinetics of sterilization and possible damage to media are discussed elsewhere [10,39,40].

It has to be remembered that the specific characteristics of a bacterial strain might be quite different from those of whichever "typical" bacteria one is used to. For example, many microorganisms produce a polysaccharide slime layer called a capsule, which may be quite thick. Its existence can contribute to increasing substantially the viscosity of the culture, which usually is not viscous

in bacterial culture. For *Leuconostoc mesenteoides* the capsule polymer is a based on glucose and called dextran. *Acetobacter xylinum* produces exocellular cellulose. Also, in some instances the production of the capsule is related to virulence, so that growing these cells under conditions where a capsule is not formed might solve the problem of viscosity, but the ability to produce the desired product may then be lost, e.g., from antigenic bacteria for production of vaccine (which is no longer immunogenic) or in the synthesis of a polymer (which is now not synthesized).

C. Hyphal Growth—Fungi and Actinomycetes

There are many types of fungal structures, some of which are depicted in Fig. 2. The fungi consist mainly of intertwined filaments of attached cells called hyphae, and each separate eukaryotic hyphal cell has a wall composed of cellulose and chitin and is relatively rigid. The wall between adjacent cells (septum)

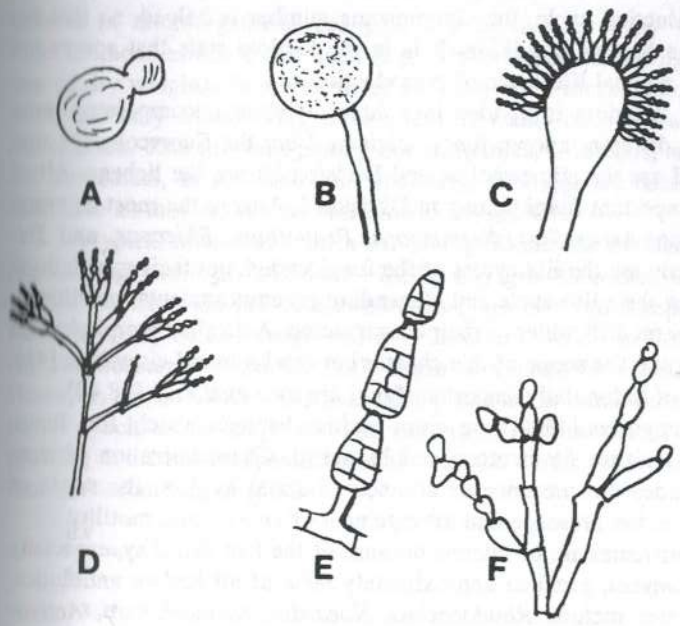


Figure 2 Shapes of fungi. (A) Budding yeast; (B) *Rhizopus*; (C) *Aspergillus*; (D) *Penicillium*; (E) *Alternaria*; (F) *Cladosporium*.

is sometimes incomplete, allowing mixing of adjacent cytoplasm. Some fungi do not have a septa at all. A large hyphal mass, which can usually be seen with a naked eye, is called a mycelium. Fungi are generally aerobic and most grow optimally at 25°C and at pH 5–6. However, their range of growth is from 4°C to 37°C and they are well known for contamination of refrigerators.

Reproduction in fungi is complex and is used as a main parameter in their classification. Reproduction can be sexual, asexual, or both. Asexual reproduction is done by producing a fruiting body, which might contain thousands of spores. Some spores develop within a sac and are called sporangiospores. Some develop on supportive structures called conidiophores. These spores are called conidia. The bread mold *Rhizopus* produces sporangiospores, and the blue-green mold *Penicillium* produces conidia. Other asexual reproduction does not involve a fruiting body. It includes spores produced by fragmentation of hyphae (arthrospores), by forming thick-walled spores along the periphery of the hypha (chlamydospores) or at its tip (oidia). Another method of reproduction is by the process of budding, as shown by yeast (see below).

The stages of sexual reproduction in fungi are accompanied by a number of diverse morphological forms. During sexual reproduction in fungi, cells of opposite mating types come together and fuse. This fusion results in a doubling of the chromosome number resulting in the formation of a diploid cell. Later in this sexual reproduction cycle, the chromosome number is halved, so that the cell returns to the haploid condition. It is in the haploid state that spores are produced for the asexual life cycle of reproduction.

The fungal kingdom is divided into three divisions encompassing more than 70,000 [41] different known fungi. Division I are the Eumycota, the true fungi; Division II are the slime molds; and Division III are the lichens. All of the industrially important fungi belong to Division I. Among the most common industrial fungi are *Aspergillus*, *Neurospora*, *Penicillium*, *Rhizopus*, and *Trichoderma*. Not only are the life cycles of the fungi varied, but their morphology can change during their life cycle and, depending on environmental conditions, often lead to inherent difficulties in their classification. A detailed morphological description is beyond the scope of this chapter but can be found elsewhere [41]. Specific reviews of industrially important fungi are also available [28,42].

The Actinomycetes [43,44] are gram-positive bacteria which, like fungi, grow as hyphae and are filamentous and branched. Characterization of their morphology includes the presence or absence of aerial hyphae, the mode of division of hyphae, the presence and arrangement of spores, and motility.

The Actinomycetes are of interest because of the fact that they, especially the genus *Streptomyces*, produce approximately 90% of all known antibiotics. Other actinomycetes include *Rhodococcus*, *Nocardia*, *Actinomedura*, *Actinoplanes*, *Micromonospora*, and *Streptoverticillium*. A concise review of the biology of industrially important actinomycetes has been published [45,46].

D. Budding Cells—Yeasts

Yeasts [22] are unicellular microorganisms that on solid media form colonies which resemble bacterial colonies. Yeast are generally larger than bacteria, with a typical diameter of 5 μm as opposed to 1 μm for bacteria. However, as eukaryotes, they belong to the fungal kingdom and have a sexual method of reproduction in which two different haploid types (designated a and α) combine to form a diploid cell in an ascus of aaαα type which later returns to the haploid form [47].

In the budding mode of reproduction, cells become swollen on one edge and produce a bud (blastospore), which later breaks off and becomes a separate cell. Yeasts, especially *Saccharomyces cerevisae* [48], are one of the most common microorganisms used in industry, notably in the production of bread, beer, or wine. This organism is also one of the most intensively studied from many biochemical and biological aspects [49] and is being used in recombinant DNA technology [50,51]. The basic knowledge that these yeasts are not at all harmful to human beings gives them an important advantage over other microorganisms for the production of recombinant pharmaceuticals.

E. Growth Kinetics

Growth of microorganisms can generally be monitored by various methods. Most methods estimate cell mass (e.g., scattering of visible light or determination of dry weight). However, these methods often measure both live and dead cells, and only enumerating the amount of viable cells gives accurate kinetic information. This involves plating out of cultures, is tedious, and the results are not immediate, so it is not done routinely, although the data can be very informative as they reflect the real kinetics of growth.

A typical microbial growth curve (Fig. 3) may be divided into six phases: (1) The *lag phase*. In this period hardly any growth occurs as the cells adapt themselves from the previous conditions (e.g., medium composition, temperature—in many cases storage was at −20°C or −70°C) to the new conditions. (2) The *acceleration phase*. Cells begin to expand in volume and to divide. However, not all of the cells reach this phase at the same time (i.e., the culture is not synchronous [52]). (3) The *exponential* (*logarithmic*) *phase*. Cells are growing at a constant specific growth rate. Cell mass (x) is increased (dx) with time (dt) according to [53,54]

$$\frac{dx}{dt} = \mu x \tag{1}$$

$$x = x_0 e^{\mu t} \tag{2}$$

where μ is the specific growth rate (h^{-1}), which is related to the doubling time (t_d) of the cells by $\mu = \ln 2/t_d$. In batch culture under conditions of no limitation

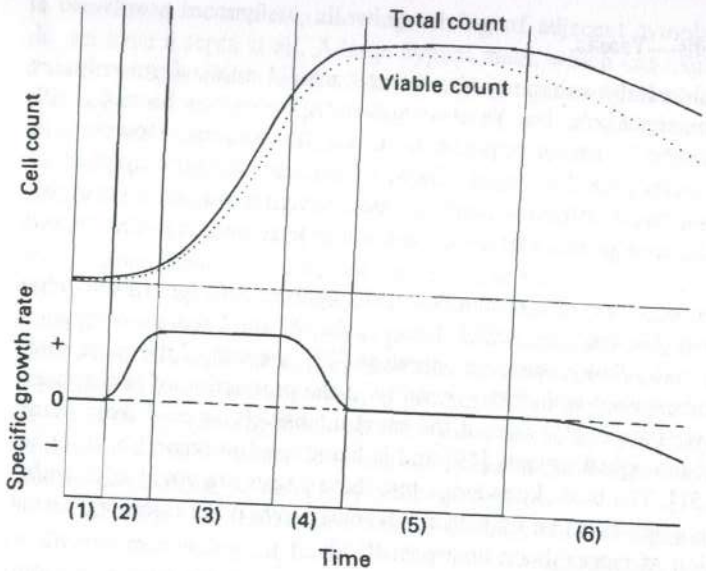


Figure 3 Kinetics of growth of microorganisms. Phases of growth: (1) lag; (2) acceleration; (3) exponential growth; (4) deceleration; (5) stationary; (6) death. See text for details. (From Ref. 15. Reproduced with permission of Blackwell Scientific Publications Company, Ltd.)

of any essential nutrient this is also the maximal specific growth rate of the culture (μ_{max}). It should be noted that this condition of constant growth rate depends on constant conditions (medium, temperature, pH, oxygen supply). Growth under these conditions is very specific for a particular organism. (4) *Deceleration phase.* Here the growth rate decreases. This change is usually gradual and is caused by either limiting levels of oxygen and/or medium components or accumulation of toxic products. With a recombinant microorganism this might also be due to the stress imposed by the high level of production of a foreign protein [55]. (5) *Stationary phase.* Cells in this phase respond with a combination of a very slow growth rate together with a slow rate of cell death. In some cases there is a slight increase in total cell mass during this phase, sometimes a slight decrease. Eventually the rate of growth decreases to zero and the rate of cell death increases. When the latter becomes predominant then the (6) *phase of decline* or the *death phase* has begun.

During the cell's life, metabolites are required for functions other than cell division. For example, cells might stay in the stationary phase for a long time, being viable but not increasing in mass. This requires maintenance energy

[33,56], which is usually small, in the range of 0.02–0.1 g of substrate consumed per gram dry cell weight per hour, depending on the specific substrate used and whether the growth is aerobic or anaerobic (where more energy is required) [57].

The specific growth rate is dependent not only on the type of substrate used but also on its concentration, in a manner analogous to the dependence of enzyme-catalyzed reactions on substrate concentration (Michaelis-Menten), and is described by the Monod equation

$$\mu = \mu_{max} \frac{S}{K_s + S} \tag{3}$$

where K_s is the saturation or Monod constant (g L^{-1}). The maximal specific growth rate, μ_{max}, is reached only in a nonlimiting culture, i.e., when $S \gg K_s$. The K_s values reported for various organisms and substrates illustrate the wide differences in required substrate concentrations [58, p. 57].

III. INFLUENCE OF MICROBIAL CHARACTERISTICS ON BIOREACTOR SELECTION

Adequate mixing of a microbial culture is essential for many aspects of a fermentation, including assuring an adequate supply of nutrients to the cells and removing any toxic materials from their vicinity. Mixing also affects the supply of oxygen by breaking large bubbles into smaller ones and dispersing them in the liquid so that their residence time in the reactor is increased, thereby bringing about increased gas holdup interfacial area for oxygen transfer. Accurate temperature control and efficient heat transfer also require good mixing for efficient heat transfer. Good mixing is essential to maintain a homogeneous culture in which the concentrations of specific metabolites can be accurately monitored. Mixing is also important to ensure the quick dispersion of any added solutions such as acid, base, or nutrients, so that there is no local high concentration buildup.

Mixing in bioreactors is very complex and has been the subject of considerable investigation and discussion [1,10,58,59]. Certainly, the greatest need for good liquid-phase mixing and gas-liquid mass transfer is the provision of a microbial culture with an adequate amount of oxygen. However, mechanical mixing causes turbulence and eddies within the fermentation medium, approximately 50–300 μm in size [60]. The transfer of solutes to the approximately 1–2-μm-long cells inside these eddies is diffusion limited, a process that is dependent on the viscosity of the medium.

As a result of these limitations, it would seem ideal to provide the culture with as much mixing as possible. However, an increase in mixing causes a concomitant increase in hydromechanical stress [61] (usually attributed to shear), which can severely impinge on the viability of the microorganisms. This phe-

nomenon has been termed turbohypobiosis [62]. The rheological properties of the medium also have an effect on the mixing, because as the viscosity increases so does the power input required for the same degree of mixing; this places very real mechanical and cost constraints on the size and structure of the bioreactor. However, for all practical purposes, these limitations do not exist for small bioreactors, so it is quite common to see small (1–2 L) bioreactors where the size of the mixer motor is up to 25% of the vessel size. This gives an efficiency of mixing which cannot be economically realized in larger bioreactors. Overall, for effective scale-up, it is essential to approximate the final fermentation as closely as possible at the research and pilot plant stage. This chapter is limited to discussing the main factors through which the microorganism affects the mixing, including (1) the production of viscous solutions, (2) the response to shear, and (3) the dissolved oxygen requirements.

A. Rheological Properties: Shear and Viscosity

Shear forces act perpendicular to the direction of fluid motion and are usually quantitated by measuring the shear rate and the shear stress, measurements that are relatively straightforward for a Newtonian suspension (viscosity independent of shear rate) but more complex for non-Newtonian solutions (effective viscosity dependent on shear rate). Detailed analyses of these phenomena, including a general analysis of the rheological properties of fermentation fluids, have been considered extensively in the literature [63–67]. In many cases, where an STR is used, the volume average of the shear rate is related to the tip speed of the impeller and is calculated as ND (m s^{-1}), where N is the angular velocity in revolutions per second and D is the impeller diameter in meters. The shear rate (γ) in bubble columns for airlift bioreactors containing non-Newtonian fermentation broths has generally been empirically correlated with the superficial gas velocity U_G (volumetric gas sparging rate per unit cross-sectional area) in the form $\gamma = \text{constant} \times U_G$. For U_G given in m s^{-1}, the value of the proportionality constant has been reported to be in the range of 1500–5000 m^{-1} [68,69].

Another source of disruptive forces is the interface of the air bubbles with the microorganism surface. This is very important for mammalian and insect cell cultures [70] and is an important determinant of hydromechanical damage in airlift bioreactors.

One of the factors that complicates the quantitative assessment of shear effects is the fact that in any bioreactor there are different shear effects in different areas, depending on the geometry of the various mechanical parts [71]. As it is impractical to measure shear at every specific point, it is difficult to model and theoretically predict the shear. This problem increases substantially with an increase in bioreactor size. However, in spite of this, and due to its importance, shear must be considered as one of the potential variables in the kinetic determinants of growth and product formation with shear-sensitive cells [65].

The main factors that influence the rheological properties of microbial fermentations [64] are the microorganism (i.e., single cells, mycelium, or pellets), any metabolites that are formed during the fermentation (e.g., biopolymers), and the substrate (e.g., starch, soyameal, or cellulose). Suspensions of single-celled microorganisms usually have low viscosity, which is similar to that of water. Even at relatively high cell concentrations (e.g., 50 g/L dry cell weight) the culture has low viscosity and exhibits Newtonian behavior [72–74]. More detailed studies show a maximal increase of viscosity of 10 centipoise (10,000 Pa · s) at high concentration of *E. coli* cells [75,76]. Of course, the essential invariance of viscosity with increasing cell density assumes that cell lysis does not occur. When cells lyse, DNA is released and the viscosity increases substantially. However, such conditions are undesirable and would in fact mean that the fermentation had gone awry.

In spite of the general nonviscous nature of bacterial cultures, some microorganisms produce very viscous broths because of polymers that are produced during the fermentation. The main polymers that have been studied in this regard are pullulan [4,77], hyaluronic acid [78,79], alginate [80], and xanthan gum [63,81], all of which generally exhibit pseudoplastic behavior—i.e., the effective viscosity decreases with increasing shear rate. An example of the changes of viscosity of the fermentation broth during the time of growth is shown in Fig. 4.

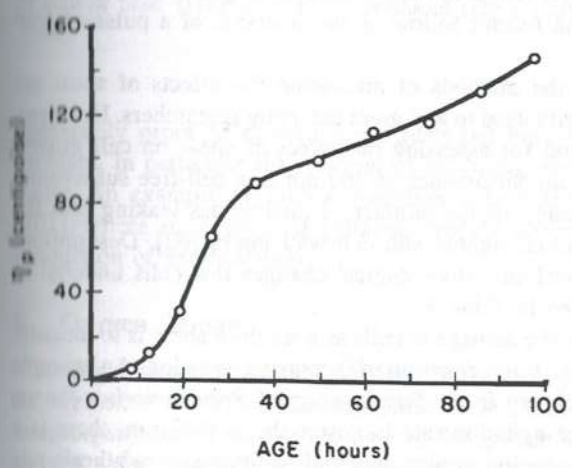


Figure 4 Plastic viscosity versus elapsed fermentation time for *Penicillium chysogenum*. (From Ref. 63. Reproduced with permission of Springer-Verlag, New York.)

Since yeast are, for the most part, single celled, they may be considered similar to bacterial cells in terms of their rheological properties [7,64,82–85]. However, for many filamentous fungi the viscosity can increase substantially during growth, and their fluid behavior is usually of a non-Newtonian character. For example, *Penicillium* has been found to have various non-Newtonian culture characteristics, e.g., pseudoplastic [6], Bingham plastic [86], and Casson plastic behavior [8,87,88]. Similarly, *Aspergillus* has been found to have a Bingham plastic [5], a pseudoplastic, or a Casson plastic [89] culture behavior. During fungal growth there is a complex dependence of viscosity on cell morphology [90]. Fungi can grow in pellets, with shoots that grow out from the pellets being cut off and forming new pellets. On the other hand, pellets might increase in size and produce a hyphal growth (mycelium). Sometimes this sort of change in cell morphology accompanies complex physiological changes within the cell and is required in order to produce a desired secondary metabolite.

Measurements of viscosity, shear rate, and shear stress are complex and interrelated. The fermentation fluid can be measured outside the fermenter by use of a viscometer [63]; however, such measurements do not take into account the air supply in the fermenter. To get around this problem, researchers have attempted to make these measurements under conditions that simulate those found in bioreactors [65]. Another means of measuring shear in fermenter-like conditions is the DASHE (Device for Assessment of Shear Effects) system [91], in which it is possible to grow cells for a long time, assuring sterility and required oxygen transfer rate.

A simple experimental approach which can give an indication of the extent of an existing rheological problem is to measure the mixing time in a bioreactor by monitoring the pH in the reactor following the injection of a pulse of acid or base (Fig. 5).

Unfortunately, all of the methods of measuring the effects of shear are rather complex, so they are not used to any great extent by researchers. However, one relatively simple method for assessing the effect of shear on cell growth and function is to measure the absorbance at 260 nm in a cell-free suspension, thereby obtaining an indication of the number of nucleotides leaking into the medium as a result of both cell rupture and cell wall injury [92]. Descriptions of some of the morphological and physiological changes that cells undergo as a result of shear are included in Table 3.

Another way to assess the damage to cells as a result of shear is to measure an indicative biological activity as a function of increasing agitation. An example of such a measurement is shown in the fermentation of *Brevibacterium flavum* to produce lysine; when the agitation rate is raised above 900 rpm, there is a sharp decrease both in the specific growth rate and in the lysine synthesis rate (Fig. 6). However, Merchuk [65, p. 82] claims that measuring deleterious changes in bacterial activity which result from an increasing agitation rate is not

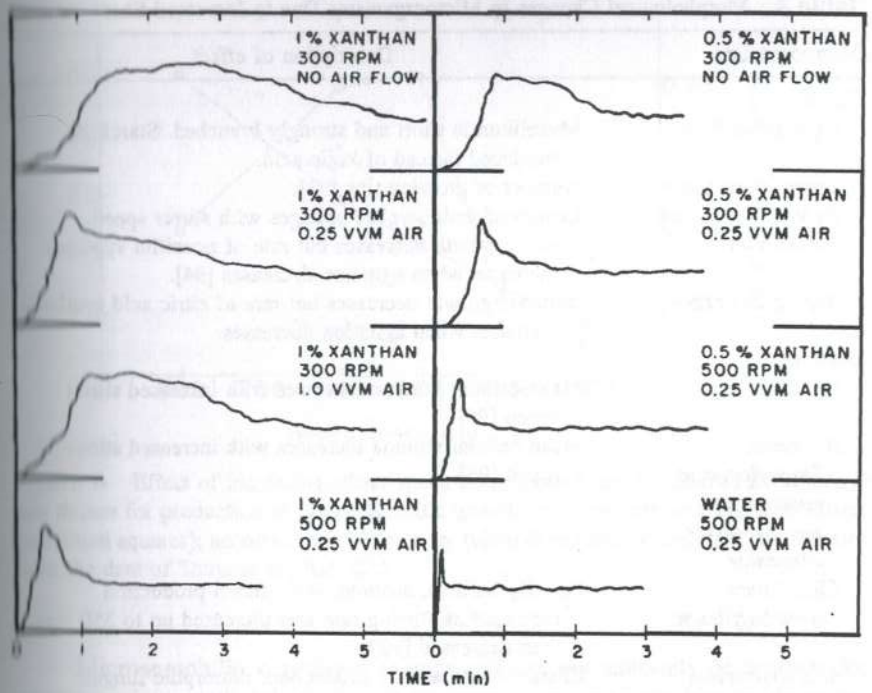


Figure 5 Mixing times for culture broths containing xanthan under various conditions of agitation and aeration. The curve represents a pH trace in response to a pulse addition of acid or base. (From Ref. 63. Reproduced with permission of Springer-Verlag, New York.)

necessarily proof of direct shear effects but may also reflect a mass transfer problem, in particular the removal of inhibiting metabolites from the cell's vicinity. An example of such a condition is in a fermentation of *Xanthomonas* [101], where an increase of agitation brought about an increase in the specific production of xanthan gum.

II. Oxygen Demand

Microorganisms may be divided into three main groups with regard to their requirement for oxygen: anaerobic, microaerophilic, and aerobic. The anaerobic microorganisms do not require oxygen for growth and in some cases are even oxygen sensitive [102]. Anaerobic culture conditions are defined as having a redox potential below 11 mV (where the indicator methylene blue changes color). Usually, after bringing the culture to this redox potential (e.g., by sparg-

Table 3 Morphological Changes in Microorganisms Due to Increased Shear

Microorganism	Description of effect
Fungi	
Aspergillus flavus	Mycellium is short and strongly branched. Starch is produced instead of kojic acid.
Filamentous fungus	Number of growing tips [93].
Penicillium chrisogen	Length of main hyphae changes with stirrer speed.
Penicillium	Biomas growth decreases but rate of pencillin synthesis increases when agitation decreases [94].
Aspergillus niger	Biomass growth decreases but rate of citric acid synthesis increases when agitation decreases.
Bacteria	
E. coli	Mean cellular volume increases with increased stirrer speed [95].
B. cereus, Staphylococcus epidermidis, Saccharomyces cerevisiae	Mean cellular volume increases with increased stirrer speed [96].
Clostridium acetobutylicum	Co_2, H_2, butanol, acetone, and ethanol production increased as stirring rate was increased up to 350 rpm, then decreased [109].
Aureobacterium pullulans	Change of metabolite production: dimorphic fungus: Above 200 rpm, culture changes from filamentous to yeast type and production of pullalam increases (it is produced only by yeast-type cells) [97].
Algae	
Spirillium platensis	Increase in agitation causes changes in structure leading to break up of cells [98,99].
Dunaliella	Increased sensitivity to bubbling rates [100].

Source: Ref. 65.

ing with nitrogen), the cells continue to reduce the redox potential by producing reducing compounds such as H_2 or H_2S. An extreme (obligate) anaerobe will grow at redox potential of −500 mV [103,104].

The main industrial uses for anaerobic organisms are wastewater treatment [105] and bioleaching [106–108]. In addition, until the end of the 1930s butanol was produced by the acetone-butanol fermentation of *Clostridium acetobutylicum*; however, this process was then replaced by chemical synthesis. Interestingly, as a consequence of recombinant DNA technology, the efficacy of the *Clostridium* fermentation process is being reexamined [109–111].

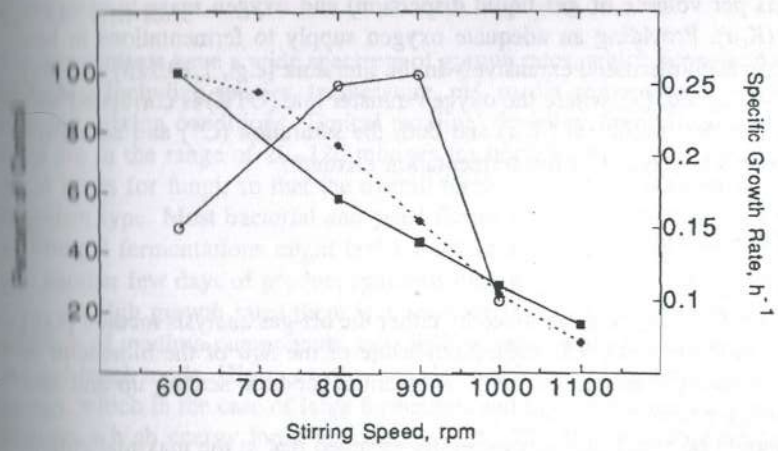


Figure 6 Effect of increasing stirrer speed on microbial fermentation of *Brevibacterium flavum* for production of lysine. Specific growth rate (open circles); lysine synthesis rate (filled squares); aconitase specific activity (filled diamonds). (Compiled and redrawn from the data of Toma et al., Ref. 62.)

Microaerophilic organisms require only minor amounts of oxygen for growth, and generally this does not impose any serious constraints on bioreactor design. This is not the case for the aerobic organisms, which often require a large amount of oxygen, depending on the substrate used and the growth rate. The maximal oxygen demand in a fermentation (Q_{max}) is dependent on cell mass (x), the maximal specific growth rate (μ_{max}), and the growth yield based on oxygen consumed (Y_{O_2}) [112,53] as described by Eq. (4):

$$Q_{max} = \frac{x\mu_{max}}{Y_{O_2}} \tag{4}$$

The maximal growth rate is dependent on all of the cultural conditions including oxygen availability (see below), while the growth yield is dependent primarily on the substrate used [54]. The higher the initial oxidation level of the substrate (e.g., glucose versus methane), the more ATP that can be produced by the cell from its oxidation. Therefore, less of the carbon source is used as an energy source and more carbon is available for biomass production.

The concentration of oxygen that can be dissolved in water is very low (i.e., 0.0084 g/L at 25°C [53]) and is even somewhat lower for fermentation media [113]. It is therefore necessary to supply oxygen continuously to a growing culture. However, as oxygen is supplied as a gas, its bubbles have to be broken up in order to provide a sufficiently large gas hold-up (volume of dis-

persed gas per volume of gas-liquid dispersion) and oxygen mass transfer coefficient (K_La). Providing an adequate oxygen supply to fermentations in bioreactors has been discussed extensively in the literature [e.g., 1,10,113] and may be described by Eq. (5), where the oxygen transfer rate (OTR) is correlated with the mass transfer coefficient (K_La) and both the saturation (C^*) and actual (C) concentrations of oxygen in the fermentation medium:

$$\text{OTR} = K_La(C^* - C) \tag{5}$$

The constant K_La may be determined by either the off-gas analysis method [114] or the dynamic method [115–118]. Knowledge of the K_La of the bioreactor in which preliminary research was done is essential for both scaling up and bioreactor design purposes.

It should be noted that it is generally assumed that at the maximal culture growth rate, the maximal OTR is equal to the maximal oxygen uptake rate (OUR), and these terms are therefore used synonymously in many cases.

It is not advisable to use all the dissolved oxygen in the medium because there is a critical oxygen concentration below which cell growth is hampered. This is usually taken to be about 30% saturation, although much lower values have been reported [119]. Some microorganisms (e.g., *S. cerevisiae* and *E. coli*) can grow in either aerobic or anaerobic conditions and are termed facultative microorganisms. It is very important that the cells are not allowed to shift from one condition to the other as the cell's metabolism can change dramatically as the oxygen concentration changes. For example, *S. cerevisiae* produces biomass under aerobic conditions and alcohol under anaerobic conditions [120]. Similarly, changing from aerobic to anaerobic conditions causes *E. coli* to produce less cell weight per gram of carbon source consumed as well as a different spectrum of gases and other metabolites.

In spite of the limitation of providing growing microbial cultures with sufficient oxygen, very high cell concentrations up to 100 g dry cell weight per liter have been achieved by a variety of researchers [73]. The means of achieving such high cell densities include sparging the culture with oxygen instead of air [72] and reducing the growth rate, either by decreasing the growth temperature [121,122] or by using a slower-growing strain, thereby reducing the oxygen uptake rate. Use of oxygen or oxygen-enriched air is generally limited to the 1000-L bioreactor scale, due to the cost of the oxygen. However, these techniques may be economical for use with larger bioreactors when growing recombinant microorganisms producing relatively expensive biochemicals [123]. A cheaper way to produce the required oxygen might be by the use of membranes that can produce selectively an enriched oxygen air supply in a continuous manner [124].

C. Growth Rate

Microorganisms have a wide spectrum of growth rates, which depend on a range of factors including species, temperature, pH, media composition, oxygen supply, and mixing conditions. Typical maximal doubling times at optimal conditions are in the range of 20–120 minutes for bacteria, 1–4 hours for yeast, and 4–24 hours for fungi, so that the overall fermentation time is dependent on the organism type. Most bacterial and yeast fermentations are short (12–24 hours), and fungal fermentations might last a week or so (i.e., 24–48 hours of growth and another few days of product synthesis during a prolonged stationary phase).

At high growth rates there is a need for sophisticated pH control and the addition of medium components, especially glucose when the fermentation is in the fed-batch mode. High growth rates also result in a high output of metabolic energy, which in the case of large fermenters and high cell density fermentations requires a high energy input for cooling [125,121]. It is therefore necessary to evaluate the maximal specific heat production (cal/min-DCW) during a fermentation process so that the cooling requirements may be precisely determined.

At low growth rates the requirements for cooling are not so severe, and in many instances it is possible that maintaining the growth temperature at 37°C might require energy input for heating. However, cultures containing cells that grow relatively slowly are much more susceptible to microbial contamination. This is for two reasons: (1) as the process is much longer, maybe a week instead of 12 hours, there are many more opportunities for contamination, and (2) as cells grow slowly, a faster-growing minor contaminant might "take over" the culture.

D. Effect of Culture pH

Most microorganisms grow in the pH 5.5–8.8 range, with optimal growth for fungi in the range pH 5–7 and yeast pH 4–5. Any growth condition outside this range will permit only a small number of microbial species to grow.

Low pH, in the range of 4–5 has been used as a selective pH in yeast fermentations for a long time. These conditions not only facilitate yeast growth but also act to prevent the proliferation of other, potentially contaminating microorganisms. The initial pH is usually 5 and is decreased during the fermentation, thereby increasing the selectivity.

Some acidophilic microorganisms grow preferentially under conditions of very low pH. For example, *Thiobacillus ferroxidans*, an organism used in bioleaching, has a pH optimum of about 1.5 and can survive at a pH of 0.7. These conditions are extremely corrosive, especially since they include a high concentration of H_2S produced as a by-product of this microorganism's metabolism [126,127]. In such cases it is impossible to build a bioreactor from steel, as even

stainless steel 316 corrodes under these conditions, and other materials (e.g., glass or plastics) should be used.

Microorganisms that proliferate under conditions of high pH are less common. *Bacillus macerans*, an organism that produces cyclodextrins, is generally grown above pH 9.0 [128]. In fact, most organisms reduce the pH during growth. However, if the growth rate of an organism that grows at high pH is relatively high and the decrease of pH is toward the end of the growth, the number of contaminating microorganisms can be kept to a minimum.

E. Growth Temperature

Microorganisms are often defined according to their growth temperature, as either thermophiles (growth at 40 to 80°C, optimum 55 to 75°C), mesophiles (growth at 10 to 47°C, optimum 30 to 45°C), or psychrophiles (growth at −5 to 35°C, optimum 15 to 30°C).

To date, the growth of psychrophilic organisms (below 15°C) [129] has not been used extensively in the fermentation industry. This is due to the usual requirement for faster-growing organisms. However, psychrophilic microorganisms may be useful in open, large area, and large volume fermentations in colder regions. They may also be used for the production of molecules that are especially susceptible to proteolytic hydrolysis or "cold"-specific proteins, e.g., ice nucleation or antifreeze proteins.

The psychrophiles have begun to be used in recombinant DNA technology. For example, a toluene-degrading plasmid from the bacterium *Pseudomonas putida* was transferred and expressed at 0°C in a psychrophilic strain of the same species [130]. It has been suggested [131] that psychrophilic microorganisms will be used to an increasing extent in the future because (1) their temperature adaptability could be used to increase survival of the recombinant strains in the environment; (2) use of psychrophilic bacteria in the milk industry will enable enzymatic conversions to be done at lower temperatures, thereby reducing contamination by mesophyllic bacteria; and (3) at lower temperatures proteases are much less active so that recombinant psychrophiles are good candidates for the production of protease-sensitive proteins.

Thermophilic organisms [132] were originally known primarily as causative agents of contamination in the fermentation industry. However, most recently they have been used for the production of a variety of products. They have several advantages: (1) Cooling is easy following fermentation because of the high temperature differential between the culture and the supply water. (2) Due to the high fermentation temperature it is relatively easy to collect volatile products (e.g., ethanol [133]) and thereby avoid the decreased yield often associated with product inhibition. (3) Among the thermophiles there are no known pathogenics, probably reflecting the fact that these organisms do not

grow at body temperature. This is an important point to consider when developing organisms for use as hosts for foreign genes. Also, growing cells at these high temperatures is a very selective condition and can be done without excessive concern for sterile conditions. (4) Growth at high temperatures is generally fast and results in a large turnover for the fermenter.

One of the main advantages of thermophilic bacteria is that they often produce thermostable proteins, such as proteases, which are used in household detergents or amylases used in the industrial production of alcohol from starch [134]. There is currently an effort to transfer some genes for thermostable proteins from thermophilic bacteria into some more commonly used bacterial hosts for large-scale production of these proteins [135].

F. Medium Composition and Preparation

Medium composition has a large effect on growth rate [54]. For example, the growth rate is likely to be high when cells are grown on a rich (complex) medium, which contains an abundance of nutrients, and relatively slow in a minimal medium, which contains only salts, buffer, and a carbon and nitrogen source [136]. The main factors in the medium that affect growth rate are the sources of carbon and nitrogen, as well as vitamins, and any limiting elements such as potassium or phosphorus, and minor minerals.

The most commonly used carbon sources for cell growth are simple sugars such as glucose or sucrose. In addition, some more unusual carbon sources should be mentioned. These include hydrocarbons and carbon dioxide. With hydrocarbons the fermentation kinetics are biphasic due to the limited solubility of hydrocarbons in water. These fermentations are not especially common, but they have been used for the production of biosurfactants [101,137–139] and might be used in the future for some biotransformations. Autotrophic microorganisms [140] which can produce all of their carbon needs from CO_2 are not commonly used in the fermentation industry. However, they are attractive for the bioleaching industry and for the production of single-cell protein. Using these microorganisms requires knowledge and control of the minimal and maximal allowable concentrations of dissolved CO_2 in the fermentation medium.

Although usually neglected in laboratory-scale work, a number of minerals (e.g., Cu, Zn, Fe, Co, Mn, Mo) are necessary (usually in trace amounts) for cell growth. In addition, some of these minerals are inhibitory when they are present in high concentrations in the growth medium. Consequently, numerous microorganisms usually display a growth optimum that is dependent on mineral concentration. Suggested optimum concentrations of the various minerals have been published for yeast [141] and bacteria [53] and are of common use in the development of an optimal medium composition.

Some microorganisms are able to grow in the presence of very high concentrations of specific minerals, including Cd, As, Fe, and S, and may be used

in bioleaching processes [108]. Due to their highly selective growth conditions, which prevent the proliferation of other microorganisms (i.e., high mineral concentration and, usually, very low pH), high-volume systems are made possible and the bioleaching processes are usually carried out in large open pools.

In an industrial setting, e.g., in the large-scale production of a relatively low-value product, the cost of the medium can reach up to 70% of the cost of the fermentation process. Therefore, it is necessary to optimize the medium composition carefully. This may be done by the use of mass balance calculations [142] together with statistical experimental design [143]. To this end, a detailed analysis of industrial media has been published [144].

Although laboratory media tend to be quite consistent from one batch to the next, media used for industrial-scale fermentations often vary considerably in their elemental composition and pH. In addition, the medium might be viscous or contain particulate matter, affecting the methods of medium transportation, filtration, and sterilization. This impact includes equipment design, flow rates (i.e., residence time), and sterilization temperature [40]. Obviously, such industrial-scale media are not suitable for use in membrane-type bioreactors or in fermentations for the production of recombinant proteins. The latter limitation is due mainly to the requirement that the fermentation media shall not contribute to the complexity of the downstream processing.

G. Requirement for Light

Several special groups of bacteria—the cyanobacteria and the anoxygenic phototrophic bacteria (including the purple bacteria and the green bacteria)—are chemolithotrophic and can assimilate CO_2 by photosynthesis [145–147]. Thus, the growth of these organisms requires both the addition of CO_2 and the presence of light. The main problem in introducing light into bioreactors is that the light intensity dissipates within a few centimeters.

Therefore, the growth of these organisms has a profound impact on the reactor design. In order to grow them, quite a few novel reactor designs have been implemented, including continuous culture through glass tubes which are proximal to fluorescent lamps or a reactor in which fluorescent lamps are installed inside the reactor and other designs [147–152].

H. Foam Production

Production of foam is a very common phenomenon in microbial fermentations. It arises from the flow of air through the liquid fermentation medium and the subsequent formation of small bubbles as a consequence of mixing. If the bubble film is not strong enough, the bubble is easily destroyed and no foam is formed. This strength is dependent mainly on the surface tension of the liquid. The surface tension (σ) of pure water is 0.072 N m^{-1}; this is always higher than the

surface tension of a fermentation medium, which is usually in the range 0.060–0.065 N m^{-1} [153]. Compounds that contribute to lowering the surface tension include proteins and protein hydrolysates, as well as oils and fats.

In large-scale industrial fermentations, e.g., of yeast, which use large concentrations of corn steep liquor and molasses, the production of foam is potentially quite problematic, so most research dealing with foaming problems has been done under these conditions [153]. However, even with the less complex media commonly used with recombinant bacteria, foam can form, usually in the last stages of the fermentation involving high airflow, high-speed mixing, and, occasionally, some cell lysis. This cell lysis, even to a small extent, brings about an increase in the protein concentration in the medium which enables foam production.

Except for beer production, foam is usually considered to be a negative phenomenon. It can fill the head space of the bioreactor and clog filters, thereby causing a significant and possibly dangerous increase in pressure. Wetting of filters also stops them from functioning properly and as a consequence makes the bioreactor more susceptible to contamination. The stable foam can also cause entrapment of oxygen so that the dissolved oxygen level increases substantially. However, the increased dissolved oxygen level is immediately decreased when the foam is broken. Such large and sudden changes in measured dissolved oxygen can play havoc if the fermentation is automatically controlled depending on the dissolved oxygen concentration.

Viesturs et al. [153] emphasized that the presence of microorganisms in the liquid fermentation medium and their dispersity can affect the formation and character of the foam. This makes it difficult to study the phenomenon of foaming in bioreactors, because different organisms are likely to affect the foaming differently. Some options for this problem are discussed in Ref. 153 and are summarized below.

One of the traits that could be characterized is foam stability. The breakdown of the foam is caused by liquid escape from foams, a multistep process called syneresis. Syneresis consists of (1) liquid running over the films surrounding the bubbles, into the capillary interbubble channels (Gibbs plateau channels); (2) diffusion of gas from smaller bubbles into larger ones; (3) redistribution of the liquid along the height of the foam column; (4) escape of liquid from the foam; and (5) destruction of some interbubble films.

It has been found that a critical height for a foam column exists, above which the syneresis increases dramatically. The more stable the foam, the higher is the foam column. Therefore, it is possible to measure the maximal height of foam produced under defined conditions. One recommended simple way to measure foam characteristics is to use a foam generator, which consists of a cylinder connected to a flask containing medium into which air is sparged in a

controlled manner. The foam that is formed rises into the cylinder and its height can be measured.

Another method is to measure the average lifetime of an air bubble in the foam. Interestingly, it was observed that an increase in the velocity of the air bubbled through a liquid medium had no effect on the average lifetime of a bubble in the foam (foam stability). However, the airflow increase caused a decrease in the syneresis time, i.e., a decrease in the lifetime of the column of foam. These seemingly contradictory results indicate the complexity of foam measurements. In another experiment the addition of corn steep extract and salts decreased the hazard of foam; in fact, the effect increased with increasing molasses concentration.

In large bioreactors it may be advantageous to install mechanical foam breakers. Ideally, these breakers [153] should be compact and placed either inside or outside the apparatus. The foam is first suppressed in cyclones and then in centrifugal fields. If necessary, the final destruction takes place using injections of a chemical antifoam solution which is sprayed into the foam flow or introduced onto the rotor of the mechanical foam breaker. The liquid phase is returned to the bioreactor. It is necessary for the homogeneity of the solution in the bioreactor that the velocity of the liquid circulation be 10 or more times its actual volume per hour.

In smaller bioreactors, breaking the foam is generally done with chemical antifoam agents. The antifoaming agent is added (usually in pulses) when the foam level goes above a sensor level and stops only when the level of the foam drops below the sensor level. This approach often leads to excessive use of antifoam, a condition which can decrease the dissolved oxygen level in the culture medium. Also, many chemical antifoaming agents can decrease the performance of microfiltration or ultrafiltration membranes that are used in subsequent downscale processing steps [154]. On the positive side, it has been found that in some cases, silicone-based antifoaming agents are less harmful to ultrafiltration membranes than polyethylene glycol–based antifoams.

An interesting finding has been reported [153] that 3.5% of the yeast strains used in industry do not lead to the formation of foam. Thus, where possible, the use of mutant strains that do not result in foaming might provide an alternative to the use of chemical antifoaming agents.

It should be noted that certain current Good Manufacturing Practices require that none of the antifoam will be detected in the final product. This requirement is increasing the use of mechanical foam breakers instead of the use of chemical antifoam agents in pharmaceutical fermentations.

I. Sterility

Although most fermentation research is done under sterile conditions, this is not necessarily the case for larger-scale studies. This is especially true for very large

volumes of culture, where selective conditions make it worthwhile risking a rare contamination problem while at the same time saving the enormous investment required to provide completely sterile conditions. Selective conditions are those which enable the growth of only a limited number of microbial species, including pH, temperature, and medium composition.

In terms of sterility, there are three main ways in which a fermentation run may be approached: (1) there are no selective conditions, so the fermentation has to be performed under strict aseptic conditions. In addition, according to existing regulations [155], fermentation of recombinant microorganisms requires strict aseptic conditions; (2) partly selective conditions exist, so it is unlikely that other organisms will proliferate (e.g., yeast growth at pH 3.5); and (3) very selective conditions exist, so there is hardly any chance of contaminating organisms (e.g., bioleaching at a combination of high copper content and very low pH). Cell growth in a selective condition might require steam cleaning of the reactor at 90–100°C (without steam pressure). As this does not require the vessel to withstand high pressure, the cost of this type of vessel is much lower than the cost of a vessel used in strictly aseptic conditions.

Generally, the longer the duration of the fermentation, the more stringent the demand on the bioreactor design for aseptic conditions. The main design considerations are the agitator seal, valves, O-rings, inoculation, sampling and addition ports, antifoam breakage connections, and the construction materials.

J. Safety and Regulation

Growth of microorganisms is subject to rules and regulations [156]. Complying with some of these regulations is often a significant cost factor. In each case one or more of the following might be applicable.

1. First, the microorganism must be defined according to its pathogenicity. The lower level are the GRAS (generally regarded as safe) microorganisms. This is a U.S. Food and Drug Administration (FDA) classification; equivalent classifications exist in most industrial countries. These organisms are the ones most commonly used in industry. However, all microorganisms are further classified into four levels, where P1 includes nonpathogenic microorganisms and P4 extremely pathogenic ones. P2 and P3 conditions are often required for the growth of microorganisms for the production of vaccines and might also be used for recombinant microorganisms which are nonvirulent but produce toxic proteins (e.g., tetanus toxin [157]). The regulations are strict, and as the level changes from P1 to P4, the level of containment of the bioreactor increases as do other safety measures (e.g., clothing, building).

2. Recombinant DNA microorganisms were originally thought to be intrinsically dangerous, in part because of the fear that the recombinant organism might produce hitherto unknown toxins that might rapidly destroy a plant or animal species. However, since the mid-1970s, researchers have gained considerable experience with recombinant DNA technology. Today this technology is safely practiced by tens of thousands of laboratories worldwide, most operating at the P1 (or lowest) level of containment. Most of the large-scale growth of recombinant microorganisms is at the LS P1 (large scale P1) level, where the main physical containment is the bioreactor itself. Guidelines for conducting recombinant DNA research have been published in the United States [155] and other countries.
3. In the production of pharmaceuticals and cosmetics there are complex guidelines for the operation called current Good Manufacturing Procedures (cGMPs). These regulations, published by the FDA in the United States and by equivalent authorities in other countries, are very strict concerning procedures and recordkeeping and usually require "top of the line" bioreactors. The main implications for bioreactor design concern the design of seals, entry and sampling ports, and air sterilization.
4. Large amounts of waste microorganisms, especially those suspended in culture medium, will increase substantially the biological oxygen demand (BOD) in municipal wastewater systems. Therefore, all microorganisms, especially recombinant ones, should be sterilized by a verified procedure before they are discharged from the facility. Solutions with extreme BOD and pH should also be treated before being discharged to city sewage. This should be taken into consideration especially when using very large bioreactors, as the bioreactor itself might be used for treatment of the discharged liquid (usually after centrifugation).

IV. RECOMBINANT ORGANISMS

The advent of genetic engineering techniques has led to the development of a large number of recombinant microorganisms which produce a variety of biochemicals [158,159]. In contrast to the classic genetic approach to improving the performance of a specific microorganism, in which a wild-type strain is modified by nonspecific chemical or physical mutation and then selected for the desired phenotype, recombinant DNA techniques are highly specific. The gene(s) of interest is introduced into the host organism so that a specific protein or metabolite may be overproduced. To date, most genetic manipulation has involved the use of *E. coli* [160]. However, during the last few years many other

microorganisms have been used as hosts for foreign DNA. The bacteria used include *Bacillus subtilis*, various *Pseudomonas* species, *Corynebacterium*, and many others. In addition, foreign DNA has been expressed in various yeasts [50,51,161] and fungi [162,163]. These latter organisms can both glycosylate and secrete mature proteins, functions that are often lacking in bacterial hosts.

The initial products of recombinant DNA technology were mainly pharmaceuticals, but many other products, such as antibiotics, biopolymers, and vaccines, are likely to be produced using this approach.

In response to the need to increase the level of foreign gene expression, many specialized expression vectors have been created. The molecular biological features that may be manipulated to modulate gene expression experimentally in prokaryotes include: (1) the nature of the transcriptional promoter and terminator; (2) the ribosome binding site; (3) the number of copies of the cloned gene and whether it is plasmid-borne or integrated into the genome of the host cell; (4) the final cellular location of the synthesized foreign protein; (5) whether manipulation of the codons that are used by the cloned gene is required to guarantee efficient translation in the host organism; and (6) the intrinsic stability of the cloned gene protein within the host cell. Unfortunately, there is no simple strategy for achieving maximal expression of every cloned gene. Different genes have distinctive molecular properties that require specific conditions to achieve high levels of expression. In addition, each host organism has specific features that must be considered when developing an expression strategy. For example, prokaryotes, but not eukaryotes, are subject to translational control of gene expression, and gram-positive but not gram-negative bacteria can secrete proteins directly into the growth medium.

A priori it might seem that a good way to optimize the expression of a cloned gene would be to insert it into a plasmid under the control of a continuously transcribed strong promoter. However, a high level of ongoing expression of a cloned gene is usually detrimental to the host cell because it creates an energy drain that can impair many host cell functions. Often, with this type of metabolic stress all or a portion of the plasmid carrying the gene of interest is lost during the long-term growth of the transformed organism. Such plasmid instability may prevent the efficient production of a plasmid-borne gene product on a large scale or during a continuous fermentation. To overcome this drawback, regulatable strong promoters are preferred so that the cloned gene is expressed only at a specific stage in the host cell growth cycle and only for a specified duration. The most widely used strong and regulatable prokaryotic promoters are from the *E. coli lac* and *trp* operons, the *tac* promoter, an in vitro construct that includes elements from both the *lac* and *trp* promoters, and the leftward, or *p*L, promoter from bacteriophage lambda. However, the strategy of using strong and regulatable promoters is not limited to prokaryotes. The expression of foreign proteins in eukaryotic hosts is often controlled in this manner.

Plasmid-containing cells generally grow slower than the same cells without plasmids. As a result, the longer the fermentation, the greater the proportion of non-plasmid-containing cells. This segregational plasmid instability is dependent on growth rate, medium composition, the metabolic state of the cells, and the plasmid itself. For example, plasmids that impose a greater metabolic load on the host cells are more likely to be unstable. Such a metabolic load will be especially deleterious to the functioning of the host if the target gene is expressed constitutively. Detailed mathematical models of plasmid loss in growing cultures have been developed [164], although it is probably better to avoid this phenomenon altogether than to be able to describe it in precise terms. Generally, the level of gene expression is proportional to the number of copies of the transcribed gene in the host cell. An obvious inference is that by increasing the plasmid copy number there will be a concomitant increase in the amount of the cloned gene protein product that is synthesized. However, in addition to the cloned gene, plasmids contain other transcribed genes such as antibiotic resistance genes which act as selective markers, so that as the plasmid copy number is increased, the energy resources of the cell may become limiting. Thus, increasing plasmid copy number is not necessarily effective in maximizing the yield of the cloned gene product. Perhaps the simplest means of assuring the highest level of gene expression while at the same time avoiding entirely the problem of plasmid instability is to integrate the target gene directly into the chromosomal DNA of the host cell. It is our experience that a single copy of the gene for T4 DNA ligase, under the transcriptional control of the *pL* promoter, integrated into the host *E. coli* chromosomal DNA can result in the synthesis of approximately 5% of the protein of the cell as T4 DNA ligase [165]. In addition, when an α-amylase gene from *Bacillus amyloliquefaciens* was inserted into the *B. subtilis* chromosomal DNA, transformants that contained multiple copies of the α-amylase gene were identified and the level of enzyme activity expressed from transformants with nine chromosomally integrated α-amylase gene copies exceeded by approximately sixfold the activity levels that occurred when the α-amylase gene was present on a multicopy (about 20–40 copies per cell) *B. subtilis* plasmid [166].

The use of a strong and regulatable promoter requires that the fermentation be divided into two separate stages; first the cells are grown under optimum conditions to a relatively high density and then the activity of the strong promoter is induced, usually either by shifting the temperature or by adding a metabolite to the medium. While this sort of two-stage system may be performed in a single STR, it may be more conveniently and efficiently undertaken in a two-stage fermenter system, several of which have been described [167].

In prokaryotic cells, different mRNAs are not translated with the same efficiency. In fact, they may be expressed at very different levels from one another (up to several hundredfold) as a consequence of differences in their translation initiation signals. Differences in translational efficiency, along with transcriptional regulation, enable the cell to produce hundreds or even thousands of copies of some proteins but only a few of others. In part, the molecular basis for this differential translation is the presence of a translation initiation signal, ribosome binding site, in the transcribed mRNA. Generally, the stronger the binding of the mRNA to the ribosome, the greater the efficiency of translation initiation. Consequently, many expression vectors have been created that ensure that the mRNA of a cloned gene contains a strong host-specific ribosome binding site, thereby ensuring that heterologous genes can be translated readily.

V. EFFECT OF PRODUCT CHARACTERISTICS

A. Product Inhibition

The cessation of the logarithmic phase of growth, when not due to a lack of dissolved oxygen, is usually attributable to either a shortage of a substrate or the accumulation of a toxic product. In many cases, the precise reason for the cessation of cell growth is not studied. Supplementing the culture with all required nutrients sometimes does not result in an increase in cell density, implying that the problem is the accumulation of a growth-inhibitory or toxic product. The effects of product inhibition have been demonstrated directly through the use of dialysis bioreactors. In this system, the toxic medium components, which otherwise would accumulate in the broth, are continuously removed during the fermentation so that it is possible to reach very high cell densities [167–169]. In cases in which product inhibition is clearly shown to reduce the fermentation yield, modern perfusion systems should be considered. However, these reactor systems are for the most part still in the experimental stage and are not yet suitable for large-scale fermentations. Another way to reduce product inhibition is to optimize the medium composition in order to reduce the use of potentially problematic medium components.

An example of product inhibition is the production of alcohol by yeasts that have varying degrees of sensitivity to alcohol [170,171]. This situation led to the design of systems in which the volatile ethanol is continuously removed under vacuum, thereby lowering the amount of ethanol present in the medium and, as a result, achieving a higher conversion rate of the substrate to alcohol [172].

B. Rate and Phase of Product Synthesis

Fermentation products can be divided into growth-associated products and non-growth-associated products. The growth-associated products are generally called primary metabolites and include organic acids, nucleotides, amino acids, lipids, vitamins, and polysaccharides [173]. As these products are produced in parallel

to growth, they can, in principle, be produced in continuous culture [53]. Usually, for low-priced products, this will be economical only if the growth and production rates are relatively large. Commercial continuous culture is practiced only with cultures having a doubling time (i.e., maximal dilution time) of 2 hours or less.

Non–growth-associated products, also termed secondary metabolites, include antibiotics, alkaloids, microbial toxins, and mycotoxins [174]. These products are usually produced in the late stationary phase of growth. This situation causes a complication for continuous culture, as a two-stage continuous culture is much more complicated for control purposes than a one-stage system.

Products from recombinant microorganisms are often grown in a two-stage process. The first stage includes the growth of the microorganism, usually to the end of exponential phase, and the second stage includes the induction of the expression of the product. In this situation the time differences are usually much smaller than in the fermentation of secondary metabolites, where stationary phase is typically a few days long. Although not common practice yet, this difference has enabled the production of recombinant products by two-stage continuous culture [167].

VI. SUMMARY—DATA REQUIRED FOR BIOREACTOR SELECTION AND DESIGN

Many of the factors that have been described in this chapter and influence the choice of bioreactor design are interrelated. We have separated these factors into a checklist of questions that may be useful to consider before undertaking the development of a process based on a particular fermentation. Acquiring the information to answer these questions should give the bioreactor designer the data necessary for selecting and designing the most appropriate type of bioreactor.

Microorganism Species

1. What special properties are involved?
2. Are hyphae produced? Are pellets formed?
3. Does cell lysis occur under certain conditions?
4. Are exopolysaccharides produced?
5. Are the substrates soluble?
6. Is the fermentation performed in liquid, semisolid, or slurry medium?

Growth and Oxygen Requirements

1. Oxygen requirements: aerobic/anaerobic/microaerophilic?
2. Minimum level of oxygen required (in mg/L or mmol/L; state temperature)?

3. Maximum cell density desired (g DCW/L)?
4. Maximum specific growth rate (μ_{max})?
5. Maximum specific oxygen requirement (Q_{max})?
6. Cell sensitivity to CO_2 concentration?
7. What is the optimal pH for growth? For product formation? What is the sensitivity of growth/product formation kinetics to variation in pH?

Shear and Rheology Effects

1. Are cells shear sensitive?
2. At what agitation rate (tip speed) is damage evident?
3. How does growth medium viscosity affect production?
4. Is the medium nonviscous with Newtonian viscosity or is it viscous with a non-Newtonian viscosity? Consider the effects on heat and mass transfer and on mixing.

Sterility

1. Are there any selective conditions (extremes of pH, temperature, or heavy metal concentration)?
2. Are there any pharmaceutical/current Good Manufacturing Practice (cGMP) requirements?
3. Is the final product required to be sterile?

Volumetric Output

1. What is the final plant daily/yearly output?

Light

1. Is there a requirement for light?

Foam

1. Is foaming deleterious to the microbe or to the final product?
2. Will the use of antifoam agents affect the gas-liquid oxygen and mass transfer rate?
3. Will the use of antifoam agents affect the growth of the organism?
4. Will the use of antifoams affect the recovery of the final product?
5. Are some antifoam agents preferred over others?
6. Can the foam be broken mechanically?

Final Use

1. What is the use of the final product?

Cleaning and Sterilizing

1. Is there wall growth in the bioreactor?
2. Are spores formed?
3. What special cleaning problems exist?

Heating and Cooling

1. What is the optimum growth temperature?
2. What is the optimum induction temperature?
3. Is there a requirement for close temperature control? Can sufficient heat transfer capability be provided on scale-up?
4. What is the maximum allowable time period needed to reach the induction temperature, in temperature-induced recombinant organism batch cultures?
5. In temperature shift–induced recombinant organism batch cultures, can rapid heating/cooling be achieved on scaling up?
6. Is the bioreactor to be cooled following the fermentation? How quickly must this occur?

Materials of Construction

1. What are the extremes of temperature in the bioreactor? Is the raw fermentation medium to be heat sterilized in situ?
2. What are the pH extremes in the bioreactor (including the pH of growth, medium preparation, and final treatment)?
3. Are corrosive chemicals (e.g., sulfides) produced or used in the fermentation?
4. Are organic solvents produced?

Measurement Systems

1. What measurements are essential to make (e.g., pH, temperature, dissolved oxygen, foam level, or liquid level)?
2. What measurements might be desirable to make (e.g., exit gas analysis or specific metabolite concentration)?

PROBLEMS

1. Describe and discuss the main factors which affect the growth of microorganisms.
2. How is the growth of fungi different from the growth of bacteria?
3. *Bacillus thuringiensis* produces an insecticidal toxin during sporulation. Would it be an advantage or a disadvantage from the point of view of ease and

cost of fermentation if this organism could be manipulated to produce the insecticidal toxin during vegetative growth, but not during sporulation? Why?
4. After heat sterilizing your bioreactor, you realize that a number of heat-stable bacterial spores may not have been killed by this treatment. What sorts of fermentation conditions would put your mind at rest and convince you that these spores will not begin to grow and contaminate your culture? Give some specific examples.
5. If we inoculate a 10,000 L bioreactor with a 1% (v/v) inoculum of cells with an optical density at 600 nm of 1.2, what amount of time is required for fermentation if the final dry cell mass required is 125 kg? Assume that (a) the cells go through a 2-hour lag phase, (b) the specific growth rate (μ) is 0.52 h^{-1}, and (c) a culture of OD 3.2 at 600 nm has a cell density of 1 g of dry cell weight per liter. How does this change when $\mu = 0.26\ h^{-1}$?
6. *Xanthomonas campestris* is a gram-negative bacterium that secretes copious amounts of an extracellular polysaccharide (xanthan gum). How might the production of this polymer affect the growth of this bacterium?
7. Why is it important to know about the shear sensitivity of cells, and why is it difficult to measure?
8. Recombinant microorganisms are often grown in two stages, a growth stage and an induction stage. Why? In addition, once recombinant cells are induced they often change their growth rate and physiological properties. Why? What parameters can an experimenter control in order to obtain an optimal yield of product from a fermentation of such recombinant cells?
9. Describe and discuss the role that oxygen plays in the fermentation of microbial cells. Give several examples.
10. Describe a bioreactor that you could use to grow a cyanobacterium which is able to fix CO_2 but not nitrogen, on a scale of 10 L and on a scale of 1000 L.
11. Compare the relative advantages and disadvantages of using STR or airlift bioreactors for large-scale growth of various types of microorganisms.

NOMENCLATURE

C	Concentration of oxygen in liquid (mol O_2 L^{-1})
C^*	Saturation concentration of oxygen in liquid (mol O_2 L^{-1})
D	Impeller diameter (m)
DCW	Dry cell weight (g)
DO	Dissolved oxygen (percent of saturation)
t_d	Doubling time (h)
K_La	Oxygen mass transfer coefficient (h^{-1})

K_s	Saturation constant (g L^{-1})
N	Angular velocity of agitation (rpm, min^{-1})
OTR	Oxygen transfer rate (mol O_2 L^{-1} h^{-1})
OUR	Oxygen uptake rate (mol O_2 L^{-1} h^{-1})
Q_{max}	Maximal oxygen required by cells (g oxygen L^{-1} h^{-1})
STR	Stirred tank reactor
U_G	Superficial gas velocity (m s^{-1})
x	Cell concentration (g DCW L^{-1})
Y_{O_2}	Growth yield on oxygen (g g^{-1}, DCW/oxygen)
Y_p	Specific product yield (g g^{-1}, product/DCW)
μ	Specific growth rate (h^{-1})
μ_{max}	Maximal specific growth rate (h^{-1})
σ	Surface tension (N m^{-1})
γ	Shear rate (s^{-1})

REFERENCES

1. J. E. Baily and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2nd ed., McGraw-Hill, New York, 1986.
2. W. Crueger (ed.), *Physical Aspects of Bioreactor Performance*, Working Party on Bioreactor Performance of the European Federation of Biotechnology, DECHEMA, Frankfurt am Main, 1987.
3. H. P. Meyer, *Physical Aspects of Bioreactor Performance* (W. Crueger, ed.), DECHEMA, Frankfurt am Main, 1987, p. 144.
4. B. Wagner, I. Adler, and A. Fiechter, Annual meeting of the DECHEMA, Frankfurt, June 9, 1986.
5. U. Onken, T. Jostmann, and P. Weiland, Proc. 3rd European Congress on Biotechnology, 1984, vol. 2, p. 481.
6. H.-P. Meyer and M. Charles, *Biotechnol. Bioeng. 24*: 190 (1982).
7. J. K. Lehmann, A. Reng, A. Strijewski, and F. Wagner, DECHEMA Monographien, *Biotechnologie* (H.-J. Rehm, ed.), Vol. 81, Verlag Chemie, Weinheim, 1977, p. 137.
8. J. Moes, M. Griot, E. Heinzle, I. J. Dunn, and J. R. Bourne, Proceedings, 3rd European Congress on Biotechnology, 1985, Vol. 2, p. 285.
9. B. J. Spalding, *Bio/Technology 9*: 338 (1991).
10. D. I. C. Wang, C. L. Cooney, A. L. Demain, P. Dunnill, A. E. Humphrey, and M. D. Lilly, *Fermentation and Enzyme Technology*, John Wiley & Sons, New York, 1979.
11. M. Charles, *Trends Biotechnol. 3*: 134 (1985).
12. I. Chibata, T. Tosa, and T. Sato, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986.
13. R. E. Midgett, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986.

14. M. Moo-Young, *Bioreactor Immobilized Enzymes and Cells: Fundamentals and Applications*, Elsevier Science Publishing, New York, 1988.
15. T. F. Wilkinson, I. R. Booth, G. W. Gooday, N. A. R. Gow, W. A. Hamilton, and J. I. Prosser, *Introduction to Microbiology*, 3rd ed., Blackwell Scientific Publications, Oxford, 1989.
16. R. F. Boyd, *General Microbiology*, 2nd ed., Times/Mirror/Mosby College Publishing, St. Louis, 1988.
17. I. E. Alcamo, *Fundamentals of Microbiology*, 3rd ed., Benjamin/Cummings Publishing Company, California, 1991.
18. R. M. Atlas, *Microbiology—Fundamentals and Applications*, 2nd ed., Macmillan Publishing Company, New York, 1988.
19. T. D. Brock and M. T. Madigan, *Biology of Microorganisms*, 6th ed., Prentice-Hall, Englewood Cliffs, NJ, 1991.
20. J. N. Abelson and M. I. Simon (eds.) (S. P. Colowick and N. O. Kaplan, founding eds.), *Methods in Enzymology*, Academic Press, New York, 1992, 185 vols.
21. S. L. Neidleman and A. I. Laskin (eds.), *Advances in Applied Microbiology*, Academic Press, New York, 1992, 35 vols.
22. A. H. Rose and J. S. Harrison, *The Yeasts*, 2nd ed., Academic Press, London, 1987.
23. J. Grinsted and P. M. Bennett (eds.) (J. Norris, prev. ed.), *Methods in Microbiology*, Academic Press, London, 1992, 21 vols.
24. R. Gherna, P. Pienta, and R. Cote (eds.), American Type Culture Collection, *Catalogue of Bacteria and Phages*, 17th ed., ATCC, Rockville, MD, 1989.
25. T. R. Dando (ed.), *Catalogue of Strains*, The National Collection of Industrial and Marine Bacteria (NCIMB), 4th ed. The National Collections of Industrial and Marine Bacteria, Aberdeen, UK, 1986.
26. M. J. Edwards (ed.), *ATCC Microbes and Cells at Work—An Index to ATCC Strains with Special Application*, 1st ed., American Type Culture Collection, Rockville, MD, 1988.
27. A. Kockova-Kratochvilova, *Biotechnology—A Comprehensive Treatise* (H.-J. Rehm and G. Reed, eds.), Vol. 1, Verlag Chemie, Weinheim, 1982, p. 7.
28. A. L. Demain and N. A. Solomon (eds.), *Biology of Industrial Microorganisms*, Benjamin/Cummings Publishing Company, Menlo Park, CA, 1985.
29. J. G. Holt and N. R. Krieg (eds.), *Bergey's Manual of Systematic Bacteriology*, Williams & Wilkins, Baltimore, MD, 1984–1986.
30. A. L. Koch and M. Schaechter, *Biology of Industrial Microorganisms* (A. L. Demain and N. A. Solomon, eds.), Benjamin/Cummings Publishing Company, Menlo Park, CA, 1985.
31. Neidhardt, F. C. (ed.), *Escherichia Coli and Salmonella Typhimurium—Cellular and Molecular Biology*, Vols. 1 and 2, American Society for Microbiology, Washington, DC, 1987.
32. N. J. Palleroni, *Biology of Industrial Microorganisms* (A. L. Demain and N. A. Solomon, eds.), Benjamin/Cummings Publishing Company, Menlo Park, CA, 1985, p. 27.
33. L. A. Bulla and J. A. Hoch, *Biology of Industrial Microorganisms* (A. L. Demain and N. A. Solomon, eds.), Benjamin/Cummings Publishing Company, Menlo Park, CA, 1985, p. 57.

34. J. L. Ingraham, O. Maaloe, and F. C. Neidhardt, *Growth of the Bacterial Cell*, Sinauer Associates, Sunderland, MA, 1983.
35. S. S. Block, *Disinfection, Sterilization and Preservation*, 2nd ed., Lea & Febiger, Philadelphia, 1977.
36. A. D. Russel, *The Destruction of Bacterial Spores*, Academic Press, London, 1982.
37. L. S. Donnelly and F. F. Busta, *Appl. Environ. Microbiol. 40*: 721 (1980).
38. H. H. Hyun, J. G. Zeikus, R. Longin, J. Millet, and A. Ryter, *J. Bacteriol. 156*: 1332 (1983).
39. A. L. Demain and N. A. Solomon (eds.), *Manual of Industrial Microbiology and Biotechnology*, American Society for Microbiology, Washington, DC, 1986.
40. P. F. Stanbury and A. Whitaker, *Principles of Fermentation Technology*, Pergamon Press, Oxford, 1984.
41. B. Kendrick, *The Fifth Kingdom*, 2nd ed., Focus Texts, Focus Information Group, MA, 1992.
42. J. W. Bennett, *Biology of Industrial Microorganisms* (A. L. Demain and N. A. Solomon, eds.), Benjamin/Cummings Publishing Company, Menlo Park, CA, 1985, p. 359.
43. J. P. DeWitt, J. V. Jackson, and T. J. Paulus, *Fermentation Process Development of Industrial Microorganisms* (J. O. Neway, ed.), Marcel Dekker, New York, 1989, p. 1.
44. M. Goodfellow, M. Mordarski, and S. T. Williams (eds.), *The Biology of Actinomycetes*. Academic Press, London, 1984.
45. C. F. Hirsch and P. A. McCann-McCormick, *Biology of Industrial Microorganisms* (A. L. Demain and N. A. Solomon, eds.), Benjamin/Cummings Publishing Company, Menlo Park, CA, 1985, p. 291.
46. M. P. Lechevalier and H. Lechevalier, *Biology of Industrial Microorganisms* (A. L. Demain and N. A. Solomon, eds.), Benjamin/Cummings Publishing Company, Menlo Park, CA, 1985, p. 315.
47. D. R. Berry, *Fermentation Process Development of Industrial Microorganisms* (J. O. Neway, ed.), Marcel Dekker, New York, 1989, p. 22.
48. G. G. Stewart and I. Russel, *Biology of Industrial Microorganisms* (A. L. Demain and N. A. Solomon, eds.), Benjamin/Cummings Publishing Company, Menlo Park, CA, 1985, p. 511.
49. G. G. Stewart, I. Russell, R. D. Klein, and R. R. Heibsch, *Biological Research on Industrial Yeasts*, Vol. 3, CRC Press, Boca Raton, FL, 1985.
50. R. G. Buckholz and M. A. G. Gleeson, *Bio/Technology 9*: 1067 (1991).
51. A. De Baetselier, A. Vasavada, P. Dohet, V. Ha-thi, M. De Beukelaer, T. Erpicum, L. De Clerck, J. Hanotier, and S. Rosenberg, *Bio/Technology 9*: 559 (1991).
52. J. Mandelstam, K. McQuillen, and I. Dawes, *Biochemistry of the Bacterial Cell*, 3rd ed., Blackwell Scientific Publications, Oxford, 1982.
53. S. J. Pirt, *Principles of Microbe and Cell Cultivation*, Blackwell Scientific Publications, Oxford, 1975.
54. U. Wanner and T. Egli, *FEMS Microbiol. Rev. 75*: 19 (1990).
55. W. E. Bently, N. Mirjalili, D. C. Andersen, R. H. Davis, and D. S. Kompala, *Biotechnol. Bioeng. 35*: 668 (1990).

56. J. Fieschko and H. A. Humphrey, *Biotechnol. Bioeng. 26*: 394 (1984).
57. P. N. Levett, *Anaerobic Bacteria—A Functional Biology*, Open University Press, Milton Keynes, PA, 1990.
58. A. H. Scragg, *Bioreactors in Biotechnology—A Practical Approach*, Ellis Horwood, New York, 1991.
59. S. Aiba, A. E. Humphery, and N. F. Millis, *Biochemical Engineering*, 2nd ed., Academic Press, New York, 1973.
60. E. H. Dunlop and S. J. Ye, *Biotechnol. Bioeng. 36*: 854 (1990).
61. R. I. Fox, *Physical Aspects of Bioreactor Performance* (W. Crueger, ed.) DECHEMA, Frankfurt am Main, 1987, p. 134.
62. M. K. Toma, M. P. Ruklisha, J. J. Vanags, M. O. Zeltina, M. P. Leite, N. I. Galinina, U. E. Viesturs, and R. P. Tengerdy, *Biotechnol. Bioeng. 38*: 552 (1991).
63. M. Charles, *Adv. Biochem. Eng. 8*: 1 (1978).
64. W. Crueger, *Physical Aspects of Bioreactor Performance* (W. Crueger, ed.), DECHEMA, Frankfurt am Main, 1987, p. 121.
65. J. C. Merchuk, *Adv. Biochem. Eng. 44*: 64 (1991).
66. M. Moo-Young and H. W. Blanch, *Adv. Biochem. Eng. 19*: 1 (1981).
67. A. D. Grant and C. W. Robinson, *Chem. Eng. Sci. 45*: 37 (1990).
68. M. Nishikawa, H. Kato, and K. Hashimoto, *Ind. Eng. Chem. Process Res. Dev. 16*: 133 (1977).
69. A. Schumpe and W.-D. Deckwer, *Bioprocess Eng. 2*: 79 (1987).
70. B. Metz, *Biotechnol. Bioeng. 19*: 781 (1977).
71. A. Fiechter, *Adv. Biochem. Eng. Biotechnol. 30*: 8 (1984).
72. S. Bauer and J. Shiloach, *Biotechnol. Bioeng. 16*: 933 (1974).
73. J. Shiloach, M. Van De Walle, J. B. Kaufman, and R. Fass, *Biologicals from Recombinant Microorganisms and Animal Cells: Production and Recovery* (M. D. White, S. Reuveny, and A. Shafferman, eds.), Balaban Publishers, Rehovot, Israel, and VCH Publishers, Weinheim, Proceedings of the 34th Oholo Conference, Eilat, Israel, 1990, pp. 33–45.
74. J. M. Cutayer and D. Poillon, *Biotechnol. Lett. 11*: 155 (1989).
75. D. N. Wood, Harvesting of *E. coli* using cross-flow membrane filtration, M.Sc. thesis, University of Waterloo, Ontario, Canada, 1988.
76. K. H. Kroner, Zum Eissats der Querstronfiltration bei der Aufarbeitung von Enzmen, *BTF Biotech. Forum 3*: 20 (1986).
77. Y. Miura, K. Fukushima, K. Sambuichi, and S. Ueda, *J. Ferment. Tech. 54*: 166 (1976).
78. J. Van-Brunt, *Bio/Technology 4*: 780 (1986).
79. I. W. Sutherland, *Biotechnology of Microbial Polysaccharides*, Cambridge University Press, Cambridge, 1990.
80. G. W. Pace and R. C. Righelato, *Adv. Biochem. Eng. 15*: 41 (1980).
81. G. W. Pace, *Chem. Eng.* (1978), p. 833.
82. S. Aiba, S. Kitai, and N. Ishida, *J. Gen. Appl. Microbiol. 8*: 103 (1962).
83. B. W. Shimmons, W. Y. Sverck, and J. E. Zajik, *Biotech. Bioeng. 18*: 1793 (1976).
84. C. R. Pearly, J. R. Swartz, and C. L. Cooney, *Biotech. Bioeng. 21*: 519 (1979).
85. J. K. Lehmann, *Biotechnology—A Comprehensive Treatise* (H.-J. Rehm and G. Reed, eds.), vol. 2, Verlag Chemie, Weinheim, 1984, p. 607.

86. F. H. Deindorfer and E. L. Gaden, *Appl. Microbiol. 3*: 253 (1955).
87. H.-P. Meyer, O. Kappeli, and A. Fiechter, *Annu. Rev. Microbiol. 39*: 299 (1985).
88. B. Metz, N. W. F. Kossen, and J. C. van Suiijdam, *Adv. Biochem. Eng. 11*: 103 (1979).
89. N. Blakebrough, W. J. McNamaney, and R. T. Tart, *J. Appl. Chem. Biotechnol. 28*: 453 (1978).
90. J. C. Van Suijdam and P. J. B. Dusseljee, *Physical Aspects of Bioreactor Performance* (W. Crueger, ed.), DECHEMA, Frankfurt am Main, 1987, p. 107.
91. N. Edwards, S. Beeton, A. T. Bull, and J. C. Merchuk, *Appl. Microbiol. Biotechnol. 30*: 190 (1989).
92. H. Tanaka, J. Takahashi, and J. Ueda, *J. Ferment. Technol. 53*: 18 (1975).
93. D. E. Pitt and A. T. Bull, *Trans. Br. Micol. Soc. 78*: 97 (1982).
94. B. Konig, C. H. Seewald, and K. Schugerl, *Eur. J. Appl. Microb. Biotechnol. 12*: 205 (1981).
95. J. D. Wase and A. M. Ratwate, *Appl. Microbiol. Biotechnol. 22*: 325 (1985).
96. J. D. Wase and Y. R. Pate, *J. Gen. Microbiol. 131*: 725 (1985).
97. B. McNeil and B. Kristiansen, *Biotechnol. Lett. 9*: 41 (1987).
98. R. Bronnenmeier and M. Markl, *Biotechnol. Bioeng. 24*: 553 (1982).
99. H. Markl and R. Bronnenmeier, *Biotechnology—A Comprehensive Treatise* (H.-J. Rehm and G. Reed, eds.), vol. 2, VCH, Weinheim, 1985, p. 369.
100. H. J. Silva, T. Cortinas, and J. R. Ertola, *J. Chem. Tech. Biotechnol. 40*: 41 (1987).
101. Z. Duvnjak and N. Kosaric, *Biotechnol. Lett. 9*: 39 (1987).
102. P. N. Levett, *Anaerobic Microbiology: A Practical Approach*, Practical Approach Series (D. Rickwood and B. D. Hames, ser. eds.), IRL Press at Oxford University Press, Oxford, 1991.
103. L. E. Erickson and D. Y.-C. Fung (eds.), *Handbook of Anaerobic Fermentations*, Marcel Dekker, New York, 1988.
104. S. P. Srinivas, G. Rao, and R. Mutharasan, *Handbook of Anaerobic Fermentations* (L. E. Erickson and D. Y.-C. Fung, eds.), Marcel Dekker, New York, 1988.
105. A. Wheatley (ed.), *Anaerobic Digestion: A Waste Treatment Technology*, Critical Reports on Applied Chemistry, vol. 31, Elsevier Applied Science, London, 1990.
106. R. A. Guedes de Carvahlho, M. Celeste Cruz, M. Ceu Goncalves, M. Moura, and O. Neves, *Hydrometallurgy 24*: 263 (1990).
107. S. N. Grodev and V. I. Grodeva, *Biotechnol. Bioeng. Symp. 16*: 91 (1986).
108. B. H. Bellivear, M. E. Starodvb, C. Cotter, and J. T. Trevors, *Biotech. Adv. 5*: 101 (1987).
109. L. Yerushalmi and B. Volesky, *Biotechnol. Bioeng. 27*: 1297 (1985).
110. T. T. Jones, M. T. N. Chauveau, J. S. Youngleson, and D. R. Woods, *Fermentation Technologies, Industrial Applications* (Yu, Pak-lam, ed.), Elsevier Applied Science, London, 1991, p. 22.
111. L. D. Mermelstein, N. E. Welker, G. N. Bennett, and E. T. Papoutsakis, *Bio/Technology 10*: 190 (1992).
112. S. Bauer and E. Ziv, *Biotechnol. Bioeng. 18*: 81 (1976).
113. U. Onken and E. Liefke, *Adv. Biochem. Eng. Biotechnol. 40*: 137 (1989).
114. H. Y. Wang, C. L. Cooney, and D. I. C. Wang, *Biotechnol. Bioeng. Symp. 9*: 13 (1978).

115. P. D. G. Wilson, *Biotechnol. Techniques 1*: 151 (1987).
116. X. M. Yang, Z. X. Mao, S. Z. Yang, and M. Y. Mao, *Biotechnol. Bioeng. 31*: 1006 (1988).
117. V. Linek, J. Sinkula, and P. Benes, *Biotechnol. Bioeng. 38*: 323 (1991).
118. H. Taguchi and A. E. Humphrey, *J. Ferment. Tech. (Jpn.) 44*: 881 (1966).
119. J. M. Cutayar and D. Poillon, *Biotechnol. Lett. 11*: 155 (1989).
120. H. Y. Wang, C. L. Cooney, and D. I. C. Wang, *Biotechnol. Bioeng. 19*: 69 (1977).
121. S. Bauer and M. D. White, *Biotechnol. Bioeng. 18*: 839 (1976).
122. J. Shiloach and S. Bauer, *Biotechnol. Bioeng. 17*: 227 (1975).
123. J. Shiloach, M. van de Walle, J. B. Kaufman, T. R. Clem, and R. Fass, *Fermentation Technologies, Industrial Applications* (Yu, Pak-lam, ed.), Elsevier Applied Science, London, 1991, p. 79.
124. R. Fass, T. R. Clem, and J. Shiloach, *Appl. Environ. Microbiol. 55*: 1305 (1988).
125. A. Trilli, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, p. 277.
126. R. Fass, Y. Geva, J. Fleming, J. Propes, M. D. White, S. Reuveny, A. Mizrahi, and Z. P. Shalita, Bioleaching of metals and toxic elements from coal fly ash by *Thiobacillus thiooxidans*, Poster presentation, 48th SIM Annual Meeting, Philadelphia, 1991.
127. R. Fass, Y. Geva, M. D. White, Z. P. Shalita, and J. Y. Fleming, A bioleaching method to extract metals from coal fly ash using *Thiobacillus thiooxidans*, Israel Patent, Registration No. 100950, Feb. 2, 1992, Pending.
128. K. Horikoshi, Enzymology and molecular genetics of CD-forming enzymes, *Proceedings of the 4th International Symposium on Cyclodextrins* (O. Huber and J. Szejtli, eds.), Kluwer Academic Publishers, Boston, 1988, p. 7.
129. A.-M. Gounot, *J. Appl. Bactriol. 71*: 386 (1991).
130. J. C. Merchuk, *Trends Biotechnol. 8*: 66 (1990).
131. Commentary, *Bio/Technology 10*: 7 (1992).
132. B. Sonnleitner, *Adv. Biochem. Eng. 28*: 69 (1983).
133. G. E. Slapack, I. Russell, and G. G. Stewart, *Thermophilic Microbes in Ethanol Production*, CRC Press, Boca Raton, FL, 1987.
134. T. Godfrey and J. Reichelt, *Industrial Enzymology—The Application of Enzymes in Industry*, Nature Press, 1983.
135. K. Peek, P. H. Janssen, H. W. Morgan, and R. M. Daniel, *Fermentation Technologies, Industrial Applications* (Pak-lam, Yu, ed.), Elsevier Applied Science, London, 1991, p. 97.
136. A. G. Marr, *Microbiol. Rev. 55*: 316 (1991).
137. N. Kosaric, W. L. Cairns, and N. C. C. Gray (eds.), *Surfactants and Biotechnology*, Marcel Dekker, New York, 1987.
138. S. Lang and F. Wagner, *Surfactants and Biotechnology* (N. Kosaric, W. L. Cairns, and N. C. C. Gray, eds.), Marcel Dekker, New York, 1987, p. 21.
139. C. Syldatk, *Surfactants and Biotechnology* (N. Kosaric, W. L. Cairns, and N. C. C. Gray, eds.), Marcel Dekker, New York, 1987, p. 89.
140. H. G. Schleged and B. Bowien (eds.), *Autotrophic Bacteria*, Springer-Verlag, Berlin, and Science Tech Publishers, Madison, WI, 1989.

141. T. Egli and A. Fiechter, *J. Gen. Microbiol. 123*: 365 (1981).
142. M. D. White, Z. P. Shalita, D. Marcus, and S. Reuveny, *Biologicals from Recombinant Microorganisms and Animal Cells: Production and Recovery* (M. D. White, S. Reuveny and A. Shafferman, eds.), Balaban Publishers, Rehovot, Israel and VCH Publishers, Weinheim, Proceedings of the 34th Oholo Conference, Eilat, Israel, 1990, pp. 101–116.
143. R. Greasham and E. Inamine, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, p. 41.
144. D. W. Zabriskie, W. B. Armiger, D. H. Phillips, and P. A. Albano, *Traders Guide to Fermentation Media Formulation*, Traders Protein, Memphis, TN, 1980.
145. P. Fay and C. Van Baalen (eds.), *The Cyanobacteria*, Elsevier, Amsterdam, 1987.
146. L. Packer and A. N. Glaze (eds.), Cyanobacteria, *Methods Enzymol. 167*: (1988).
147. N. W. Kerby and W. D. P. Stewart, The biotechnology of microalgae, *Biochemistry of the Algae and Cyanobacteria*, Proceedings, Phytochem. Soc. Eur., 1989, Vol. 28, pp. 319–334.
148. D. J. Douglas, A modified fermentor system for process-controlled culture of photosynthetic marine microorganisms, Second International Marine Biotechnology Conference, Program and Abstracts, 1991, p. 74.
149. T. Matsunaga, Production of useful chemicals from CO_2 by marine microalgae using a biosolar reactor employing light diffusing optical fibers, Second International Marine Biotechnology Conference, Program and Abstracts, 1991, p. 61.
150. E. T.-Y. Lee and M. J. Bazin, *New Phytol. 116*: 331 (1990).
151. F. Bocci, G. Torzillo, M. Vincenzini, and R. Materassi, *Algal Biotechnology* (T. Stadle, Y. Karamanos, J. Mollion, H. Morvan, M.-C. Verdus, and D. Christiaen, eds.), 1988, p. 219.
152. P. Pohl, M. Kohlhase, and M. Martin, *Algal Biotechnology* (T. Stadle, Y. Karamanos, J. Mollion, H. Morvan, M.-C. Verdus, and D. Christiaen, eds.), 1988, p. 209.
153. U. E. Viesturs, M. Z. Kristapsons, and E. S. Levitans, *Adv. Biochem. Eng. 21*: 169 (1980).
154. W. C. McGregor, J. F. Weaver, and S. P. Tansey, *Biotechnol. Bioeng. 31*: 385 (1988).
155. *Guidelines for Research Involving Recombinant DNA Molecules*. Department of Health and Human Services, NIH, Federal Register, Part III, Vol. 51, No. 88, p. 16958 (1986).
156. D. F. Liberman and R. Fink, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, p. 402.
157. J. J. Clare, F. B. Rayment, S. P. Ballantine, K. Sreekrishna, and M. A. Romanos, *Bio/Technology 9*: 455 (1991).
158. Products in the US Pipeline, Pharmaceutical Manufacturers Association, *Bio/Technology 9*: 947 (1991).
159. B. R. Glick and J. J. Pasternak, *Molecular Biotechnology*, American Society for Microbiology, Washington, DC, 1994.

160. B. R. Glick and G. K. Whitney, *J. Ind. Microbiol. 1*: 277 (1987).
161. V. Kumar, S. Ramakrishnan, T. T. Teeri, J. K. C. Knowles, and B. S. Hartley, *Bio/Technology 10*: 82 (1992).
162. N. S. Dunn-Coleman, P. Bloebaum, R. M. Berka, E. Bodie, N. Robinson, G. Armstrong, M. Ward, M. Przetak, G. L. Carter, R. LaCost, L. J. Wilson, K. H. Kodama, E. F. Baliu, B. Bower, M. Lamsa, and H. Heinsohn, *Bio/Technology 9*: 976 (1991).
163. J. T. Fayerman, *Bio/Technology 4*: 786 (1986).
164. D. K. Summers, *Trends Biotechnol. 9*: 273 (1991).
165. G. K. Whitney, B. R. Glick, and C. W. Robinson, *Biotechnol. Bioeng. 33*: 991 (1989).
166. P. Kallio, A. Palva, and I. Palva, *Appl. Microbiol. Biotechnol. 27*: 64 (1987).
167. T. H. Park, J.-H. Seo, and H. C. Lim, *Biotechnol. Bioeng. 37*: 297 (1991).
168. P. Landwall and T. Holme, *J. Microbiol. 103*: 345 (1977).
169. P. Landwall and T. Holme, *J. Microbiol. 103*: 353 (1977).
170. N. van Uden (ed.), *Alcohol Toxicity in Yeasts and Bacteria*, CRC Press, Boca Raton, FL, 1989.
171. C. J. Panchal (ed.), *Yeast Strain Selection*, Marcel Dekker, New York, 1990.
172. G. R. Cysewski and C. R. Wilke, *Biotechnol. Bioeng. 19*: 1125 (1977).
173. A. H. Rose, *Primary Products of Metabolism*, Economic Microbiology, vol. 2, Academic Press, London, 1979.
174. A. H. Rose, *Secondary Products of Metabolism*, Economic Microbiology, vol. 3; Academic Press, London, 1979.

4

Design, Formulation, and Optimization of Media

Rodolfo J. Ertola

University of La Plata
La Plata, Argentina

Ana Maria Giulietti

University of Buenos Aires
Buenos Aires, Argentina

Francisco Javier Castillo

NOMA Corporation
Berkeley, California

I. INTRODUCTION

Growth and production of metabolites by an organism in a bioprocess are the result of the interactions between intra- and extracellular effectors [1]. The intracellular effectors are specific to the organism considered and include the genome and the control mechanisms of replication, transcription, and translation of the genetic information. The extracellular effectors are of physical and chemical nature. The physical parameters are related to the culture systems and the reactor employed and are mainly represented by the temperature, viscosity,

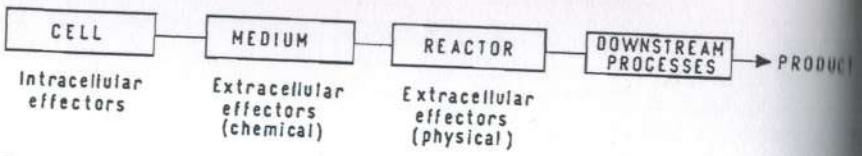


Figure 1 Fundamental steps of a typical bioprocess.

aeration/agitation conditions, etc., and the chemical effectors include all the nutrients used. Figure 1 shows the fundamental steps of a typical bioprocess.

Bioprocesses are characterized by complex relationships among the different steps. The consideration of the interactions between the physical, chemical, and biochemical aspects and their integration and the selection of the best course of action among the different alternatives for optimization are complicated by the difficulties of analyzing comparatively the input and output of the different stages [2].

Advances reported in the past years concerning the role of some medium ingredients have provided evidence that nutrient deficiencies rather than metabolic or other constraints can be the major limiting steps in many bioprocesses. According to Marr [3], experimental evidence supports the conjecture that the rate of growth of *Escherichia coli* is met by the flux of precursors, metabolites, and the monomers derived from them, rather than the flux of ATP or the rate of protein synthesis. In that case *E. coli* is considered a system in which the capacity to use the precursors exceeds the capacity of intermediary metabolism and the medium to provide these compounds. For this reason, the growth rate of many bacteria generally increases when the medium is supplemented with amino acids, nucleosides, or vitamins.

Reports concerning the stimulatory effects produced by using high concentrations of some organic nitrogen sources in microbial processes, such as yeast extract, are providing new concepts about the metabolic roles of such sources.

The use of elicitors to enhance metabolite production in many plant cell bioprocesses has introduced in this field new ideas for the design of media. Finally, the tremendous advances achieved in the clarification of roles of medium components used for mammalian cell cultures and the introduction of serum-free media demonstrate the paramount importance of adequate strategies for the design, formulation, and optimization of media in bioprocesses.

II. MICROBIAL PROCESSES

In a nutritionally and physically appropriate medium a microbial culture will grow at a defined and characteristic rate. The growth is unrestricted as long as

the concentration of nutrients does not become limiting and that of toxic compounds does not reach an effective level, conditions which normally occur in the early steps of a batch culture. Growth becomes restricted by changes in the environment imposed either by the growth of the microorganisms themselves or by external alterations in the chemical or physical conditions.

Media used in microbial processes can be grouped into two general types: defined or synthetic and undefined or complex. Defined media are those whose compounds are chemically definable. These media, which allow the limiting factor to be clearly defined, are very useful for physiological studies. A medium composed of glucose, an ammonium compound, mineral salts, and pure vitamins is an example of a defined medium. The addition of a peptone or another undefined source to this medium makes it a complex one. Undefined or complex media are generally used for production purposes. If elucidation of biosynthetic pathways or regulatory mechanisms is required in a commercial process, then defined media should be used. An interesting example of a defined-medium strategy for studying the biosynthesis of cephalosporin C has been reported by Huber and Tietz [4].

Medium design is a complex subject which was generally neglected in the past. What was commonly done in many cases was merely a selection of compounds based on a combination of experimentation and logic, taking into account previously successful media.

The word *design* will be used here to define the first step in medium development, which ends with the election of the ingredients. The design should consider the functions of the ingredients for promoting growth and product formation and also take into account all the information available about the microorganism, substrates, and process as well as the influence they exert on the different steps. The aim of any medium design for production purposes is generally focused on product formation with the highest yield and productivity and at the minimum cost. In some cases, the purity of the final product is also very important. In our opinion the term *formulation* refers to quantitative aspects and is related to the calculation of the component's concentration. Finally, *optimization* has the role of checking and modifying (if necessary) the nature and concentration of the ingredients. This sequence of steps can lead to a redesign or to a reformulation of the medium.

The first and obvious question to be answered is, what is the function of the medium to be designed? Different media are required in many operations, both in a laboratory (microbiological or physiological studies, productivity testing, etc.) and in an industrial plant. As the aim of each step is different, the design has to be considered accordingly. Some steps require rapid growth to give a high concentration of cells in the optimum physiological state, whereas others, like the production step, generally require the maximum productivity at the lowest possible cost. The second question concerns the approach to follow

for the medium design. One way is to select a medium from the literature and then optimize it according to the purpose of the process. Another approach is to base the design on the information available about the microorganism, substrates, and process. Table 1 lists the essential topics which will be considered in connection with medium development.

A. Design and Formulation

1. Nutritional Requirements for Growth and Product Formation

The nutrients required for growth are those which provide the macroelements, generally needed in concentrations greater than 10^{-4} mol L^{-1}, and those which provide the micro- or trace elements and growth factors, usually used in concentrations lower than 10^{-4} mol L^{-1}. The main functions related to macroelements and some microelements are given in Table 2.

Macroelements. The macroelements, which constitute 90–95% of the dry weight of microbial biomass, are carbon, oxygen, hydrogen, sulfur, phosphorus, potassium, and magnesium. In this section some consideration will be given to carbon and nitrogen sources.

According to carbon nutrition, microorganisms are classified as heterotrophs (which obtain the cell carbon from organic compounds) and autotrophs (which obtain the cell carbon from CO_2). In the majority of industrial processes the microorganisms used belong to the former group.

When a carbon substrate acts as the carbon and energy source, as normally happens, part of it is assimilated to provide cell carbon and the rest provides

Table 1 Steps and Essential Topics Related to Medium Development

Step	Topics
Design	Nutritional requirements for growth and product formation
	Other additives
	Biochemical mechanisms
	Upstream and downstream processes
	Bioavailability of ions
	Cost, availability, and suitability of substrates
Formulation	Mass composition of cells, stoichiometry of growth and product formation
	Yield coefficients and other fermentation parameters
Optimization	Optimization methodologies

Table 2 Functions, Elemental Composition of Microorganisms, and Growth Yields Related to Macro- and Microelements

Element	Function or constituent of	Elemental composition of microorganisms	Growth yield (g dry biomass/g element)
C	C, energy	44–53[a,b,c]	1.1[d]
O	Proteins, nucleic acids, cell wall polymers	10–14[a] 7–10[b,c]	8.75[d] 9.09[e]
H	Nucleic acids, phospholipids, cell wall polymers	2.0–3.0[a] 0.8–2.6[b] 0.4–4.5[c]	39.1[d] 27.7[e]
S	Sulfur amino acids, biotin, coenzyme A	0.2–1.0[a] 0.01–0.24[b] 0.1–0.5[c]	333[d]
P	RNA, enzyme cofactor, principal cation	1.–4.5[a,b] 0.2–2.5[c]	59.5[d] 161.3[e]
Mg	Ribosomes, enzyme cofactor	0.1–1.2[a,b,c]	430[d] 128[e]
Ca	Bacterial spores, enzyme cofactor	0.01–1.1[a,b] 0.1–1.4[c]	3.3×10^{3}[d] 8.3×10^{2}[e]
Fe	Cytochromes, enzyme cofactor	7×10^{-3}–0.9[a,b,c]	6.7×10^{3}[d] 1.7×10^{3}[e]
Zn	Enzyme cofactor	8×10^{-3}–2.4×10^{-2}[a,b]	2×10^{4}[d] 2.7×10^{5}[e]
Mn	Bacillus spores, enzyme cofactor	7×10^{-4}–4.8×10^{-2}[a,b]	2×10^{4}[d] 7.7×10^{5}[e]

Sources: [a] Bacteria, Refs. 5 and 6; [b] Yeasts, Refs. 5 and 7; [c] Fungi, Ref. 8; [d] Assumed values for *Klebsiella aerogenes*, Ref. 9; [e] calculated values for a *Pseudomonas* sp. as determined by continuous culture [10].

energy, which is used for synthesizing cell material and for maintenance functions such as turnover of cell materials, osmotic work to maintain concentration gradients, and cell motility.

As the maintenance energy consumed can be a significant part of the total carbon and energy source employed, it is essential to consider its influence in the formulation of media. In the case of penicillin production, for example, of the total amount of glucose consumed, 10% represents the amount present as carbon in the biomass, 10% is consumed as energy for biomass synthesis, 10% for penicillin carbon and energy, and the rest (70%) for maintenance energy [11]. The parameter related to the maintenance energy is the maintenance coefficient (m), generally expressed as grams of energy source per gram of dry biomass per hour (g g^{-1} h^{-1}). In the case of recombinant strains, the burden

placed on the microorganism by the existence of a plasmid in the host is reflected as an increase in its maintenance requirements.

The range of carbon compounds that can be utilized is large: carbohydrates, alcohols, organic acids, hydrocarbons, and also organic nitrogen compounds.

The nitrogen requirements of microorganisms can be met by inorganic or organic sources. The majority of microorganisms utilize ammonia as a nitrogen source, generally supplied as $(NH_4)_2SO_4$, which is the preferred source used both in the laboratory and in industry. Many complex organic sources, such as proteins and peptides, can be utilized if the microorganism is able to hydrolyze these compounds into smaller units.

In connection with some organic nitrogen sources, like yeast extract and peptones, it is interesting to mention that they seem to serve other functions apart from being employed for their organic nitrogen or vitamin content. For example, yeast extract appears to fulfill complex functions such as protection from proteolitic degradation of a human insulin growth factor in a recombinant *E. coli* strain [12], stabilization of plasmids [13], maintenance of alcohol production rate [14], and enhancement of hepatitis B virus preantigen formation [15].

L-Amino acids, which can be also employed as nitrogen sources, may have functions of growth factors as well. Some bacteria grow faster when are provided with one or more of the amino acids in peptide form, which is ascribed to the antagonistic effect of some amino acids on the uptake of others. In the case of *Lactobacillus delbrueckii*, for example, the uptake of serine is antagonized by D- and L-alanine [16]. In connection with the use of amino acids as ingredients of media, it is interesting to mention the kinetic model proposed by Shu and Shuler [17] for predicting growth response to a variety of amino acid supplements.

Microelements. Some microelements are essential for growth, such as Ca, Mn, Fe, Cu, Co, and Zn, whereas B, Cr, Mo, and others are rarely required.

Microelements are commonly added as mineral salts. In the case of complex media it is also possible that some raw materials may contribute with several trace elements. The composition of yeast extract, for example, reveals that the content of some elements may be sufficient to support growth if a high enough concentration of yeast extract is used. Some microelements may be also incorporated by solubilization from the vessel. In this respect Hutner [18] has pointed out that inorganic nutrition can present problems when the process is scaled up owing to the decrease of the vessel surface/vessel volume ratio, which reduces the availability of some metals.

There are many publications concerning the specific role of a number of ions. Jones and Greenfield [19] have published a complete list of cations and their role in yeast growth. In the case of secondary metabolite production, Weinberg [20] reported the key metal functions of Fe^{2+}, Mn^{2+}, and Zn^{2+}.

Trace element deficiencies in batch cultures are reflected by limitation of the growth rate and in continuous cultures by the decrease observed in the steady-state biomass concentration [9].

Although a suitable list of trace elements required can be selected for a particular process, it is important to consider the interactions because the optimum concentration of any ion is dependent on the concentrations of the others. The interactions reported by Jones and Greenfield [19] in the case of yeast growth include, among others, (1) antagonism of Zn^{2+} to Cu^{2+} or the additive effect of Zn^{2+} plus Mn^{2+}, which antagonize the effect of Cu^{2+} and Cd^{2+}; (2) synergism, such as the toxic effect of Cu^{2+} and Cd^{2+}; and (3) stimulation, such as the Ca^{2+} and Mn^{2+} growth-enhancing effects, which depend on the presence of Zn^{2+}.

The best way to detect deficiencies or toxic effects of trace elements in a medium is by pulse addition of the ionic species using a defined medium in steady-state continuous culture. An example of this approach is reported by Antier et al. [21], who clearly showed the detrimental effects of some trace elements.

Growth Factors. Some microorganisms require specific nutritional factors which they can not synthesize, called growth factors. Certain protists and eukaryotes like yeasts have an absolute necessity for some of these factors. There are three groups of growth factors: vitamins, amino acids (or some peptides instead), and miscellaneous compounds.

The vitamins important in microbial nutrition are the water solubles with the exception of ascorbic acid. Most of them are components of coenzymes. Vitamins can be used as pure ingredients in defined media or can be incorporated with some component like yeast extract. Cane molasses and beet molasses also contain several vitamins, with the biotin content higher in cane molasses and pantothenic acid content higher in beet molasses. Antagonistic effects have also been described in the case of vitamins, like that observed between thiamine and pyridoxal in a yeast strain [22].

The use of amino acids has already been mentioned (Section A.1). Concerning other compounds, fatty acids like oleic acid for certain bacteria and ergosterol for yeasts [23] can be cited.

Differences in Nutrient Requirements for Growth and Product Formation. The nutrients required to obtain the maximal yield or productivity of a metabolite can be different from those required to achieve optimum growth, particularly in the case of non-growth-associated processes. There are many examples of this in the literature. Secondary metabolite production is a typical process affected by the presence of phosphates or ammonium salts in spite of the good growth attained when these compounds are employed without control. According to Silva et al. [24], the use of cheese whey produced optimum growth of a

Clostridium perfringens strain but little deltatoxin compared with the use of common dextrin medium. Silva et al. [25] also reported that the nutrients used for attaining the highest content of c-phycocyanin of a *Nostoc* species did not correspond to those required for obtaining the maximal cell concentration.

Influence of Physical Factors. The nutritional requirements are affected by the physical conditions of the environment. Temperature and water activity are considered here.

If a higher temperature than usual is used for growing *Saccharomyces cerevisiae*, for example, 37°C instead of 30°C, the pantothenic acid content of the medium has to be increased [26]. Also, *Pasteurella pestis* requires more amino acids and vitamins for growth at 37°C than for growth at 28°C [27].

The water activity or the tonicity (osmolarity) is the parameter related to the osmotic pressure of the medium. Molds in general can tolerate lower water activity than bacteria. Among the microbes most tolerant of lower water activity are the osmophilic yeasts, which can grow at 0.6 osmols. The osmotic pressure can have a dramatic influence on growth and product formation. An interesting example is related to industrial L-amino acid fermentation carried out with a high concentration of glucose, in which the osmotic pressure was the limiting factor affecting productivity [28]. The modification in the medium design includes the addition of glycine betaine (*N,N,N*-trimethyl glycine), which acts as an osmotic protectant. The osmotic pressure can also affect growth and product formation in different ways. Arcas et al. [29] observed that the toxin production of a *Bacillus thuringiensis* strain was more affected than growth when the osmolarity of the medium was increased from 0.808 to 1.068 osmols.

2. Other Additives

In many cases it is necessary to supply compounds other than those required for growth and product formation. These compounds can serve different functions, such as increasing yields or avoiding precipitations, foam formation, or inhibitory effects of other components present in the medium or produced by the cells. These additives can be classified according to their functions as precursors, stimulants, protectants, antifoams, chelators, stabilizers, and neutralizing agents.

Phenylacetic acid, a precursor in penicillin biosynthesis, is a well-known example of a precursor. A whole range of side chain precursors have been shown to be incorporated with directed synthesis of the final product, although not all of them have proved to be as useful as phenylacetic acid. Another example is the addition of chloride in chlortetracycline production.

Examples of stimulants to enhance the production of metabolites are methanol in citric acid production and surfactants in enzyme production [30]. An interesting case is the use of methionine to enhance antibiotic titers with *Cephalosporium acremonium*, an effect which was ascribed to the influence of the amino acid on *Cephalosporium* differentiation [4].

In some cases it is convenient to add specific compounds to avoid toxic, denaturation, or osmotic effects. Imaisumi et al. [31] proposed the addition of methylcyclodextrin for sequestering compounds that are inhibitors in *Bordetella pertussis* cultures. Another example is the addition of osmoprotectants (see Section A.1).

To control foam formation in bioprocesses it is a common practice to use a chemical agent to fulfill the function of an antifoam. Currently, the compounds employed most are fatty acids, polyglycols, higher alcohols, and silicones. In order to select the most appropriate antifoam it is important to consider not only its efficiency but also its possible inhibitory effects and its cost and availability.

Problems caused by precipitation of certain elements can be overcome by adding a chelating agent (particularly in the case of defined media) which acts as metal buffer. Pirt [9], for example, recommended the addition of EDTA in a defined medium for culturing a *Klebsiella aerogenes* strain. EDTA has the advantage that it is not metabolized, whereas other chelators like citrate are assimilated by many bacteria and fungi. The addition of chelating agents has to be done carefully because they can be inhibitory for certain microorganisms, as EDTA is for several yeasts [23], or they can lower the yields; for instance, in a process of proteinase production by a *Thermus* species [32] inorganic phosphate was found to reduce the concentration of Ca^{2+} available to the culture. The bioavailability of ionic species in connection with the presence of chelators is discussed in Section A.5.

Stabilizers are commonly required with recombinant microorganisms to avoid loss of plasmids. The use of some antibiotics or uracil is recommended in these cases.

When changes in pH during a process (due to NH_4^+, NO_3^-, or amino acid consumption or production of acids) affect growth or product formation, a neutralizing agent has to be added to the medium. Apart from the use of mixtures of phosphates as buffers, when it is possible or sufficient, urea, or calcium carbonate, it is common to control the pH by the addition of ammonium or hydroxides, in which case their calculated consumption rates can be useful parameters for monitoring the process.

3. Biochemical Mechanisms

Knowledge of several fundamental aspects of microbial metabolism and its regulation is essential for medium design. The effects of carbon catabolite repression and phosphate and nitrogen metabolite regulation can be considered in terms of nutritional repression. The Crabtree effect and feedback regulation are other important mechanisms.

Carbon catabolite repression is significant in commercial practice because many metabolites, like enzymes and antibiotics, are subject to this type of regulation. Glucose or any other rapidly assimilated carbon substrate such as glycerol or citrate produces carbon catabolite repression.

The effect of ammonia, a rapidly assimilable nitrogen source, in the biosynthesis of antibiotics is another example of nutritional repression [33].

Phosphate concentration is a crucial factor in many antibiotic fermentations, as in the case of candicidin and tetracycline production. If an excess of phosphate is present (≥10 mM) the extracellular phosphate is not depleted and no antibiotic is synthesized. For this reason the industrial production of many antibiotics is carried out at growth-limiting concentrations of inorganic phosphate [33].

The Crabtree effect is an important regulatory mechanism related to glucose metabolism in some eukaryotic microorganisms. In the case of *S. cerevisiae*, when the glucose concentration is above 0.15 g L^{-1}, alcohol formation occurs even under aerobic conditions. To prevent alcohol formation the growth rate and glucose concentration must be below 0.22 h^{-1} and 0.15 g L^{-1} respectively [34]. Ethanol excretion, now considered the result of an overflow reaction at the point of pyruvate, is due to the carbon flux exceeding the capacity of oxidative breakdown [35].

The alteration of cell permeability by biotin limitation in coryneform bacteria with glutamate overproduction is a strategy of medium design based on avoiding feedback regulation by altering the permeability barrier to facilitate the exit of the amino acid [9].

Knowledge of the regulatory effects facilitates selection of the most appropriate strategy for achieving restricted growth in connection with the substrate responsible for the undesirable effect. If the process is conducted in batch mode, the mechanism dictates the nature of the substrate (and the medium design), as in the case of penicillin production, which requires a slower assimilable carbon source like lactose. If glucose is used its concentration has to be restricted, for example, by a fed-batch operation.

4. Upstream and Downstream Processes

For adequate medium design it is necessary to examine and integrate the knowledge of all the steps which are carried out before the medium is ready to use (upstream processes) and also the separation and purification stages (downstream processes) which follow the fermentation step. In some cases it is also important to consider the pollution problems associated with the effluents produced.

The upstream processes are related to the pretreatment of raw materials (if necessary), such as the conversion steps of starch and cellulosic material (liquefaction and saccharification), and also the effects of sterilization on medium composition.

Sterilization, normally carried out by heating with pressurized steam, often produces undesirable chemical changes in the medium. Interactions of ingredients may occur as the result of obligate components or impurities present in them or as result of compounds produced during the sterilization. The most common cause of precipitation is the reaction between di- and trivalent metals and phosphates, which can be prevented by the addition of chelating agents, and the Maillard reaction, which can be prevented by a high-temperature, short-time sterilization process or by sterilizing carbohydrates and nitrogenous compounds separately. Many other alterations of components should be predicted for medium design. The amino acids tryptophan, glutamine, and asparagine, for example, are labile and should be sterilized by filtration. Among the water-soluble vitamins, thiamine, riboflavin, and pyridoxine are the most labile [9]. It is also known that, depending on the sterilization conditions, the presence of trace elements in the medium may influence the solubilities of the nitrogen sources, the degree of which may have an effect on the productivity of the process [36]. It is interesting to mention a kinetic model proposed by Boeck et al. [37] based on a general-purpose Arrhenius "pseudo" rate equation designed to evaluate the influence of temperature and heating time on chemical reactions taking place in the medium.

In connection with downstream processes it is important to consider the influence of medium components (or compounds produced from them) on the separation and purification steps. A medium ingredient like whey can result in high yields in the fermentation stage but prove to be inadequate for the downstream processes, owing to the higher cost of purification. For this reason defined media are sometimes preferred (mainly for high-value products) in spite of the higher cost of the ingredients because of the separation and purification facilities.

The purity of the final product is of paramount importance in the case of proteins which are given parenterally or compounds used as food additives. Immunological complications [38] can result from administration of compounds contaminated with a medium component or with products derived from it. Nutritional intoxication can also occur, as in a case reported in Japan due to the administration of impure L-tryptophan [39] which produced a disease affecting blood cells. These effects demonstrate the importance of considering the interrelations between medium design and downstream processes.

5. Bioavailability of Ions

The metallic ions have to be available to the cells in order to facilitate growth and metabolic activities. In complex media an ion concentration can be reduced by several compounds, such as humic acids, amino acids, proteins, and other insoluble or colloidal materials which regulate the concentration of the ionic species by chelating and sequestering the various ions with different affinities and rates of association [19].

The dynamic of complex formation is determined by the equilibrium constant formation of the metal ligand (ML) and also by the rate at which the equilibrium is reached. The equilibrium constant for the metal chelate formation can be expressed as follows:

$$K = \frac{[ML]}{[M]\,[L]} \tag{1}$$

where K is the equilibrium constant of formation of (ML). The square brackets denote concentration. The value of K depends mainly on the metal ion. The greater the value of K, the greater is the affinity of the ligand for the metal ion. A list, in terms of decreasing values of K reported by Jones and Greenfield [19], can be written as follows: $Fe^{3+} > Cu^{2+} > Co^{3+} > Zn^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+} > Na^{2+} > K^{+}$. This means that Cu^{2+}, for example, will be mostly complexed, whereas Ca^{2+} and K^{+} will be mostly free ions. On the other hand, the rate at which the equilibrium is reached also has different values according to the ion. A list in decreasing order of reaction shows that $Ca^{2+} > Zn^{2+} > Mn^{2+} > Fe^{2+} > Co^{2+} > Mg^{2+}$. According to the dynamics of complex formation it follows that when the equilibrium is reached the ion Ca^{2+} will be almost free and if it is complexed it will be quickly available, whereas Mg^{2+} will also be generally free but if it is complexed it will be available very slowly. By the same reasoning Co^{2+} will generally be complexed and also available at a very low rate. For this reason this element is always potentially limiting to complex media.

The potential limiting cations can be detected in continuous culture by pulse additions of solutions containing them. Application of this criterion allowed an increase of the dilution rate from 0.25 to 0.35 h^{-1} in a continuous culture process for producing *Candida utilis* from stillage by feeding a solution containing Fe^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+} [40].

6. Cost, Availability, and Suitability of Medium Ingredients

Cost and availability of a regular supply are essential aspects affecting the suitability of any raw material used in medium preparation. It is also important to take into account the stability of the material as well the ease of handling and in some cases the approval of the regulatory authorities [36]. Many raw materials of animal or vegetable origin are subject to price fluctuations depending on many factors which are difficult to control. As these fluctuations usually have a dramatic effect in the economy of the process, it is important to test other raw materials.

It is also essential to consider variability in the composition of raw materials which may occur seasonally or because of differences found among var-

ious suppliers, as is common with cane molasses. The presence of herbicides or pesticides employed in the cane plantation as well as other impurities may produce alterations in the composition of molasses affecting its quality and suitability for microbial processes [41]. As regular analysis of the raw materials may fail to detect the level of impurities which affect yield or productivity, they should be tested in small-scale cultures before the lot is accepted for use on the large scale, even in the case of mineral salts. In this respect it can be mentioned that a higher than usual level of arsenic in a noncontrolled lot of ammonium sulfate caused serious problems in a penicillin production plant some years ago [42].

Table 3 shows a list of carbon and nitrogen sources used in typical industrial processes. Nitrogen and carbon sources must be stable and uniform from lot to lot because lack of uniform composition may affect dramatically the yields or productivity. Complex substrates like many peptones and corn steep liquor are not stable enough for storage for extended periods, whereas others, such as dried yeast are generally uniform from batch to batch.

7. Mass Composition of Cells, Yield Coefficients, and Stoichiometry of Growth and Product Formation

A rational approach for medium formulation is mainly based on mass composition of cells, yield coefficients, and the stoichiometry of growth and product formation. Although the elemental composition of cells can vary according to the conditions of growth, the data given in Table 1 can be used as a first attempt for formulation of a minimal growth medium. Suppose, for example, it is desired to produce 20 g L^{-1} (dry weight) of a bacteria which can be grown in a defined and simple medium. Considering the nitrogen content of 11–12% of many bacteria, it can be estimated that the medium will require 2.4 g L^{-1} of assimilable nitrogen, which means about 12 g L^{-1} of $(NH_4)_2SO_4$. Similar estimations can be done for other elements. However, in some cases the requirement for one element can be met in excess owing to the use of a mineral salt containing more than one element, as in the case of $(NH_4)_2SO_4$ or K_2HPO_4. The trace element concentrations can be estimated approximately in the range of 10^{-4} to 10^{-5} M. Concerning the carbon source it is necessary to consider the amount needed for energy and maintenance, apart from the employed in biomass synthesis.

The stoichiometry of biomass and product formation can also be a useful guide for the formulation of minimal media for growth and product formation. A general equation for the stoichiometry of growth and product formation can be written as follows:

$$CH_aO_b + A(O_2) + B(NH_3) \rightarrow y_{x/s}CH_\alpha O_\beta N_\gamma + y_{p/s}CH_{\alpha'}O_{\beta'}N_{\gamma'} + y_{CO_2/s}CO_2 + DH_2O \tag{2}$$

Table 3 Carbon and Nitrogen Sources Used in Typical Microbial Processes

Process	Carbon sources	Nitrogen sources	References
Single-cell protein	Methanol Ethanol Molasses[a] Whey[b] *n*-Paraffins	Ammonia Urea	43
Citric acid	Molasses[a] Sucrose Glucose *n*-Alkanes	Ammonium nitrate	44
Penicillin	Glucose Lactose Molasses[a] Vegetable oils	Ammonium sulfate Corn-steep liquor[c] Cottonseed meal[d] Soybean meal[e]	45

[a]Cane molasses (sucrose, 30–40%; reducing sugars, 15–20%); beet molasses (sucrose, 48–55%).
[b]Sweet whey, dried (protein, 13%; lactose, 75%); acid whey, dried (protein, 12.5%; lactose, 67%).
[c]Total solids, 50%; proteins, 24%.
[d]Proteins, 32%.
[e]Proteins, 42%.

where $y_{x/s}$, $y_{p/s}$, and $y_{CO_2/s}$ are the coefficients related to biomass, product, and CO_2 formation, respectively, expressed as C mole of biomass, product, and CO_2 per mole of carbon and energy source. Considering that the yields are referred to one C mole of the carbon and energy source it is clear that

$$y_{x/s} + y_{p/s} + y_{CO_2/s} = 1 \tag{3}$$

The values of these coefficients are related to the actual coefficients measured in practice ($Y_{x/s}$ and $Y_{p/s}$) by the following relationships:

$$y_{x/s} = Y_{x/s}\frac{\sigma_x}{\sigma_s} \tag{4}$$

$$y_{p/s} = Y_{p/s}\frac{\sigma_p}{\sigma_s} \tag{5}$$

where σ_x, σ_s, and σ_p are the carbon fractions of the biomass, substrate, and product, respectively. The $Y_{x/s}$ and $Y_{p/s}$ are expressed as g dry weight of biomass or product per g of substrate consumed. The value of σ_x in the case of the elemental biomass formula $CH_{1.64}O_{0.52}N_{0.16}$ is 0.459 considering an ash content of 8%.

Although more elaborate equations including other elements can be written, it is not necessary to do so owing to the obligate incorporation in excess of some elements, as was mentioned.

Table 4 gives some values of growth yield coefficients. The values of the recombinant strain indicate a decreased yield as the result of carrying a plasmid burden. A yield coefficient is not constant for a given microorganism and medium owing to changes due to variation in the growth rate and also because the carbon source is derived for different functions.

In practice, the growth yield coefficients are calculated by measuring the mass of cells or product and substrate consumed over some period of time. It is also possible to calculate theoretical yields which provide useful guide for designing experiments and evaluating results. The theoretical yields on carbon and energy source can be calculated from the available electrons or from the ATP yields in the case of anaerobic cultures. The mean yield of cell mass per available electron is 3.14 g cells per mole av e^- [5] using ammonia as nitrogen source. The ATP yield means that the amount of energy required to synthesize a given amount of biomass should be directly proportional to the number of moles of ATP produced per mole of the energy source. The values can vary from 5 to 32 g dry biomass per mole ATP depending on the nature of the carbon source [51].

Stoichiometric considerations for product formation by direct catabolism of some compounds give theoretical yields which are approximately attained in practice. For example, in the case of ethanol from glucose the stoichiometry

Table 4 Growth Yield Coefficients of Different Microorganisms on Various Sources (g dry weight g^{-1})

Microorganism	Substrate	$Y_{x/s}$	References
Aerobacter aerogenes	Glucose	0.40	46
	Glycerol	0.45	46
Pseudomonas fluorescens	Glucose	0.38	46
	Ethanol	0.49	46
Candida utilis	Glucose	0.51	47
	Ethanol	0.68	47
Penicillium chrysogenum	Glucose	0.43	48
Candida intermedia	*n*-alkanes	0.81	48
Methylococus sp.	Methane	1.01	49
Escherichia coli	Glucose	0.35[a]	50
(recombinant strain)		0.25[b]	

[a]Host strain.
[b]Host strain carrying a plasmid.

predicts a yield of 0.51 g per g of glucose. However, this value cannot be achieved because part of the glucose is consumed for biomass formation, and yields of 90–95% are frequently obtained. For products derived from anabolic metabolism the stoichiometry is much more complex. In some cases theoretical yields have been calculated, as in the case of penicillin, which, according to Cooney and Acevedo [52], gives a yield of 1.1 g of penicillin per g of glucose, which represents the maximal yield assuming that no cells or other products accumulate. In practice, the actual yields are much lower (0.1 g g^{-1}), which indicates that a significant part of the substrate supports growth, maintenance, or formation of other products.

Apart from the yield coefficients other parameters such as the saturation constant (K_s), the specific growth rate (μ), and the rates of O_2 uptake and CO_2 production as well as the respiratory quotient are also very useful in evaluating the performance of media to support full growth. In addition, the evaluation of the maintenance coefficient (m) is of particular importance for formulation of media in processes in which the maintenance energy is high (see Section A.1).

The specific growth rate depends on the nature and concentration of the substrate and also on the limiting substrate selected. In complex media, after a step of rapid growth a limitation in one or more substrates (not generally known) occurs and consequently the growth rate decreases, which favors the biosynthesis of the secondary compounds.

When a high concentration of biomass is desired, as in the case of high-density cultures, the formulation of media has to be considered accordingly. The simplest way to formulate a medium for high-density culture is by increasing the concentration of all the components in the same proportion. Using this procedure, Arcas et al. [29] succeeded in increasing the biomass up to seven times in a batch culture of *B. thuringiensis*. This procedure, of course, does not allow evaluation of those components which do not have to increase in the same proportion. A better approach to determining specifically the ingredients that should be increased is by pulse addition of the different components in continuous culture. A more rigorous solution was proposed by Suzuki et al. [7] for producing 150 g L^{-1} of a *Candida* strain in a fed-batch operation. The authors developed an automatic continuous feeding schedule of minerals based on equations derived from the mass balance principle to calculate the required concentration of each mineral ion in the mineral solution fed.

In spite of the basic information available about many microorganisms and processes, complete rational design and formulation of media is still a problem due to (1) interaction effects among medium ingredients and also among components and the physical effectors which are difficult to predict; (2) bioavailability of some nutrients (particularly ions) which is not always assured even when the requirements are theoretical satisfied; (3) release of products which may be inhibitory to cell growth; (4) difficulties in the evaluation of minimal

and maximal concentration of some ingredients responsible of particular biological actions. Some of these difficulties can be overcome by applying adequate optimization techniques.

B. Medium Preparation

Although medium preparation seems to be a simple step, it is essential to consider that careless preparation of media may cause complete failure of the process productivity. Corbett [36] has emphasized the main aspects that have to be taken into account for successful commercial operation. The crucial point is to be sure about the reproducibility of the medium composition of each batch. In order to achieve this objective it is important (1) to ensure the reproducibility of the ingredients' quality considering the influence of storage and handling of the raw materials used; (2) to avoid adding components in the wrong order; and (3) if the medium contains solids in suspension and if it is prepared in a separate tank, to ensure that the medium is adequately agitated when it is transferred to the fermentor.

C. Optimization

Several methodologies can be applied for the optimization of media. The classical approach involves changing one component while fixing the others at a certain level. This procedure is simple but time consuming, particularly for a large number of components, and it does not generally guarantee that the optimum found is the true optimum. Also, it is not possible with this technique to detect interactions which normally occur among media ingredients. More adequate and rigorous methodologies are based on the use of continuous cultures and on statistical techniques.

Addition of ingredients to a steady-state continuous culture, which can be done either to the growth vessel or to the incoming medium, has become a well-known approach for the optimization of growth media. It was proposed by Mateles and Batat [10] in 1974 and is now considered a very useful, simple, and practical methodology. The response obtained is informative because absence of reaction to the addition of a particular nutrient means that the nutrient is not limiting whereas an increase of biomass after the addition shows that the nutrient considered was growth limiting. Figure 2 shows results obtained in a chemostat [10]. As can be seen, stimulatory or inhibitory effects are easily detected when different ingredients are added to the growth vessel or to the incoming medium. Another advantage of the technique is related to the calculation of the stoichiometric requirements for the nutrients considering the cell concentration and the concentration of the limiting nutrients (see Table 1). The authors also mentioned that the optimized medium supports a high concentration in a batch culture as well. Experimental evidence, however, has shown that this is not always the case.

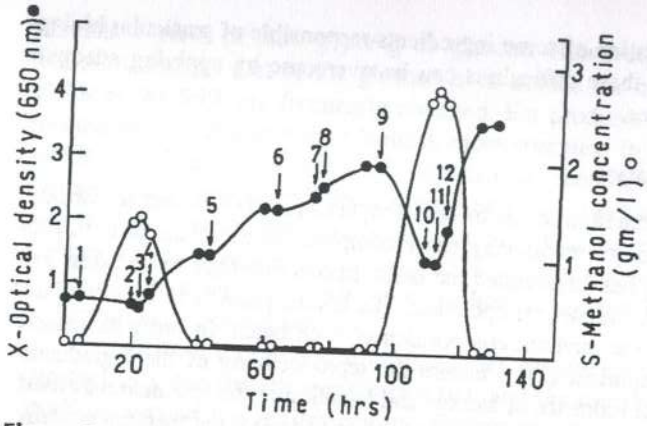


Figure 2 Changes in cell and methanol concentrations during growth of *Pseudomonas* C cells in a chemostat. *Pseudomonas* C cells were grown for 20 h in a chemostat with MSM medium containing methanol (1 g/L), $(NH_4)_2SO_4$ (1 g/L) as nitrogen source, and $CuSO_4 \cdot 5H_2O$ (10 μg/L) at 35°C. Dilution rate was 0.35/h, with stirring of 600 rpm and aeration of 0.5 v/v min. Optical density of cell suspension was measured at 650 nm, and the methanol concentration was that in the growth vessel (S). Numbers indicate additions as follows: 1, Methanol (1 g/L) added to the medium in the reservoir (S_o methanol = 2 g/L); 2, $MnSO_4 \cdot 5H_2O$ (10 μg/L) added to the growth vessel; 3, $CuSO_4 \cdot 5H_2O$ (10 μg/L) added to the growth vessel; 4, $CuSO_4 \cdot 5H_2O$ (70 μg/L) added to the medium in the reservoir; 5, methanol (1 g/L) added to the medium in the reservoir (S_o methanol = 3 g/L); 6, methanol (1 g/L) added to the medium in the reservoir (S_o methanol = 4 g/L); 7, $(NH_4)_2SO_4$ (0.5 g/L) added to the growth vessel; 8, $(NH_4)_2SO_4$ (1.5 g/L) added to the medium in the reservoir; 9, methanol (1 g/L) added to the medium in the reservoir (S_o methanol = 5 g/L); 10, $FeSO_4 \cdot 7H_2O$ (1 mg/L) added to the growth vessel; 11, $MgSO_4 \cdot 7H_2O$ (0.1 g/L) added to the growth vessel; 12, $MgSO_4$ $7H_2O$ (0.1 g/liter) added to the medium in the reservoir. The same procedure was continued until S_o was 10 g/L. (From Ref. 10.)

The statistical experimental designs consider all the possible combinations of factors to be studied at levels decided by the worker. For n variables n main effects can be inferred and also interactions of order less or equal to $(n - 1)$ can be estimated. The full factorial design is useful only for a small number of components (n) and levels (k) because the number of the experiments evolved is k^n. In order to decrease the number of experiments without affecting too much the accuracy and precision of the results, two different methodologies can be used, namely fractional (or incomplete) factorial design and the so-called heuristic methods.

Fractional design allows reduction of the number of experiments because the experimental error is estimated from the interactions of higher orders considering that it is not generally necessary to attribute a biological meaning to interactions higher than those of third order [53]. Estimation of the first-order interactions requires that the number of variables to be considered be no less than six. Available tables for fractional factorial designs are those of Plackett and Burman [54] for two levels and Box and Wilson [55] and others for three levels.

Concerning heuristic methods the simplex technique [56], which is most commonly used, consists of a sequential program of experimentation. The optimum response is achieved by moving the simplex (an analogue algorithm of a geometric figure defined by the experimental points) in the direction of the improved response. This technique is very useful on a large scale when the number of experiments has to be low.

When the key components of the medium have been identified a model of the system is formulated by means of a polynomial equation:

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_i \beta_{ii} x_i^2 + \sum_i \sum_j \beta_{ij} x_i x_j + \epsilon_{ij} \quad (6)$$

where Y is the global response; β_0 an independent term; β_i the regression coefficient of first order; β_{ii} the regression coefficient of second order; β_{ij} the multiple regression coefficient, and ϵ_{ij} the experimental error. The values of the coefficients can be calculated by no linear regression techniques [57]. Higher terms than those of second order are not generally considered.

The analytical solution of Eq. (6) by calculation of the derivative or its graphical representation by surface response [58] techniques allows to locate the zone of the presumptive optimum, which should be confirmed by experimentation for its confirmation or to study the model sensitivity. A logical sequence could be to reserve the fractional factorial for the first step and then to use the simplex technique for the second, although the reverse approach can also be used.

Table 5 gives several examples of optimization techniques for growth and product formation which were employed by different workers. In some cases, considerable reductions in the concentrations of some components in the optimized media are achieved (Mg in the case of *E. coli*), whereas in others increase of a microelement is essential, such as Cu for the *Pseudomonas* strain.

In connection with the most appropriate methodology to be used for the optimization of media, it can be concluded that the continuous culture technique appears to be a simple and practical method which can be recommended for the optimization of growth media, particularly for detecting limiting nutrients and inhibitory effects of some compounds. In addition, this technique is useful for calculating the nutrient requirements. Interactions among medium components, however, are not easy to detect. On the other hand, statistical methods are the most complete and rigorous techniques for the optimization of media for growth

Table 5 Examples of Optimization of Media

Optimization objective [Ref.]	Technique	Elements of Medium	Composition (L⁻¹) Initial	Optimum
Growth and aroma production by *C. fimbriata* [59]	Fractional factorial design	Glucose (g)	20	50
		Urea (g)	0.75	1.5
		$(NH_4)_2SO_4$ (g)	2.25	4.5
		KH_2PO_4 (g)	1	1
		$(CaNO_3)_2 \cdot 4H_2O$	0.5	1
		$MgSO_4 \cdot 7H_2O$	0.5	0.5
		Trace element solution (ml)	2	2
		Thiamine (μg)	—	150
		Biotin (μg)	—	10
Pseudomonas cultivation to give higher concentration than 2–3 g L^{-1} [10]	Continuous culture	$(NH_4)_2SO_4$ (g)	1.0	2.5
		$MgSO_4 \cdot 7H_2O$ (g)	0.1	0.3
		Na_2HPO_4 (g)	2.1	0.3
		$NaH_2PO_4H_2O$ (g)	0.9	0.15
		KCl (mg)	40	60
		$CaCl_2$ (mg)	15	20
		$FeSO_4 \cdot 7H_2O$ (mg)	1	2
		$CuSO_4 \cdot 5H_2O$ (μg)	5	80
		$ZnSO_4 \cdot 7H_2O$ (μg)	70	80
		H_3BO_3 (μg)	10	20
		$MnSO_4 \cdot 5H_2O$ (μg)	10	20
		Na_2MO_4 (μg)	—	20
		MoO_2 (μg)	10	—
E. coli B/r cultivation to give 1 g L^{-1} [60]	Continuous culture	Glucose (g)	2.5	2.5
		NH_4Cl (g)	0.8	0.77
		KH_2PO_4 (g)	0.2	0.12
		$MgSO_4 \cdot 7H_2O$ (g)	410	17
		$CaCl_2$ (mg)	10	4
		$FeSO_4 \cdot 7H_2O$ (mg)	18	0.64
		Trace element solution (ml)	2.5	2.5
Optimization of two components in candicidin production [61]	Surface response	Soybean (g)	15.9–44.1	29–32
		Cerelose (g)	31.7–88.6	62–71

and product formation including the detection of interactions and also consideration of other variables of the process.

III. PLANT CELL PROCESSES

Higher plants are potential sources for natural products such as flavors, fragrances, and pharmaceuticals. Since it is possible to cultivate plant cells in vitro in large amounts, plant cell bioprocesses have received considerable attention.

Plant cells may be cultured in several ways and with different objectives: (1) tissue culture consisting of the cultivation of seedlings, embryos, and organs; (2) cell suspension cultures, which generally represent submerged cultivation of individual cells and aggregates (or clumps) in liquid media; and (3) immobilized plant cell cultures consisting of physical restriction of cells on a fixed support. Table 6 shows some characteristics of plant cells compared with microbial cells. As can be seen, plant cells are characterized mainly by larger size, a longer doubling time, and a lower shear stress tolerance.

The classical approach to initiating a cell culture is to place explants of tissue or seeds on a solidified nutrient medium. After a certain period of time, a cell mass or callus is formed on the surface of the medium. This callus material may be cultured on a solid medium which, adequately designed, can regenerate the whole plant (micropropagation), or it can be subcultured on a solid medium again to give a new mass of cells, or it can be placed in a liquid medium to obtain a suspension. This culture can be used for larger-scale suspension culture or can be immobilized in an appropriate matrix.

Plant cell bioprocesses are based on cell suspension cultures, immobilized cell cultures, and, in some special cases, organ cultures such as root (particularly

Table 6 Some Characteristics of Plant Cells Compared with Microbial Cells

Characteristic	Microbial cells	Plant cells
Size	1–2 × 2–7 μm (Bacterial) 3.5 μm radius (yeast)	20–40 × 20–200 μm
Aggregation	Often single cells	Normally in clumps
Doubling time	<1h	>24h
Water content	75–80%	90–95%
Shear stress	Insensitive	Often sensitive
Stability	Normally stable	Unstable
Oxygen consumption	5–90 mmol L^{-1} h^{-1}	1–4 mmol L^{-1} h^{-1}
Product	Normally extracellular	Generally intracellular

Source: Taken, in part, from Refs. 62 and 63.

hairy root) cultures. Both cell suspension cultures and immobilized cell cultures are conducted similarly to their microbial counterparts, that is, submerged and immobilized microbial processes. In this respect the experience gained in microbial processes has been very useful. The bioreactors used are also the well-known stirred tank, airlift, and bubble column reactors as well as the immobilized column reactors of the microbial processes.

Several constraints have been mentioned with the process design of cell suspension cultures [64]: (1) low reactor rated and productivity, (2) instability of cell lines presents problems not easy to overcome, (3) danger of microbial contamination, (4) the secondary metabolite is often retained in the biomass, (5) plant cells are often shear sensitive, and (6) in some cases secondary compounds should be removed rapidly. Some of these difficulties can be overcome by changing to immobilized plant cell bioprocesses because then (1) the reactor can be operated at a high flow rate, (2) the problems with cell line instability can be minimized, (3) the cells are more protected from shear forces, and (4) it is easy to change the medium for induced growth or product formation.

Table 7 shows some parameters of growth of several species of plant cells in culture. The fast growing cells have doubling time values between 24 to 60 hours, whereas for the slow-growing cells doubling times of 100 to 150 hours are normally observed. A number of workers have reported yields of biomass concentration between 5 and 20 g L^{-1} dry weight in the case of *Catharanthus roseus* [71] or *Nicotiana tabacum* [72]. The productivity values reported were

Table 7 Specific Growth Rates, Growth Yield Coefficients, and Maintenance Coefficients of Various Species of Plant Cells

Species	Specific growth rate μ (d^{-1})	Growth yield coefficient (g g^{-1})	Maintenance coefficient m (h^{-1})	References
Catharantus roseus	0.26 0.45	0.58[a]	0.0084[b]	63,65,66
Nicotiana tabacum	0.68	0.43[a]	0.0076[b]	63,66
Digitalis lanata	0.15–0.20	0.29–0.40[b]	—	67
Dioscorea deltoidea	0.11–0.32	0.41[a]	—	68
Strawberry (*Fragaria ananassa* co-Brighton)	0.12–0.14	0.39–0.44[a]	—	69
Armoracia rusticana (hairy roots)	0.31	0.25[a] 0.36[a]	—	70

[a]Sucrose.
[b]Glucose.

in the range of 3 to 13 g L^{-1} d^{-1} [73], although Golstein et al. [74] suggested that higher productivities (about 30 g L^{-1} d^{-1}) may be possible with enhanced technologies. According to the $Y_{x/s}$ values reported, a high carbon conversion efficiency can be expected.

In spite of the research efforts on the production of secondary compounds, very few products are obtained commercially, among which shikonin is the most cited. The main reasons for the paucity of products are the low yields attained, cell line instability, and the necessity (for economic reasons) of applying these processes to high-value products.

One promising strategy for improving yields or productivity is by appropriate medium design. Media used in plant cell cultures can be of two types, growth media for producing plant cell biomass and production media for enhancing secondary metabolite accumulation. This generally implies a modification of the growth medium (when the step of biomass production is finished) by adding specific compounds, or by replacing the growth medium by the production one. All media used in plant cell cultures are synthetic or defined and coconut milk, yeast, and malt extract as undefined nitrogen sources are not normally employed.

The growth medium has been considered by Barz et al. [75] as part of the extracellular compartment and metabolically tied to the cell wall. In this respect it is evident that changes in the chemical composition of the medium will affect cellular metabolism. Wink [76] has also proposed a functional and metabolic relation between vacuoles (where the secondary products are generally stored) and the growth medium.

As the scientific knowledge associated with plant cell bioprocesses is still limited, all the studies related to medium development have been done on empirical bases. Generally, one starts with a classical medium such as that proposed by Murashige and Skoog [77] or by Gamborg et al. [78] and then introduce modifications in its composition.

We will focus the subject of medium design on secondary metabolite production in cell suspension cultures, considering the knowledge available. In this context the requirements for growth and product formation will be considered first and then the addition of specific compounds such as precursors and elicitors, which have proved to be a useful strategy for improving yields, will be discussed. We will consider next the needs for basic data and also for the fundamental understanding of biosynthesis and degradation of secondary compounds. Finally, an attempt will be made to give some guidelines for medium design.

A. Medium Requirements for Growth and Product Formation

Plant cells require for growth several sources which have to provide macroelements, microelements, vitamins, and phytohormones. Table 8 gives a list of the constituents of the commonly used media.

Table 8 Elements and Compound Composition of Comon Plant Cell Culture Media

Element or compound	Main Sources	Element or compound concentrations ($mg\ L^{-1}$) in media[a]		
		MS	B5	W
C	Sucrose, glucose	12.600	8.400	8.400
N	KNO_3,$(NH_4)_2SO_4$,NH_4Cl	834.4	374.6	47
S	$(NH_4)_2SO_4$,Na_2SO_4,$MgSO_4 \cdot 7H_2O$	48.1	64.9	93.6
P	KH_2PO_4,$NaH_2PO_4 \cdot H_2O$	38.76	33.60	4.7
K	KH_2PO_4,KNO_3,K_2SO_4	782	965	64.8
Mg	$MgSO_4 \cdot 7H_2O$	36.26	24.1	70.5
Ca	$CaCl_2 \cdot 2H_2O$,$Ca(NO_3)_2 \cdot 4H_2O$	119.8	41.1	50.8
I	IK	0.63	0.57	0.57
B	BO_3H	1.05	0.51	0.25
Mn	$MnSO_4 \cdot H_2O$	5.35	3.20	1.72
Zn	$ZnSO_4 \cdot 7H_2O$	1.97	0.45	0.68
Mo	$Na_2MoO_4 \cdot 2H_2O$	0.10	0.10	—
Cu	$CuSO_4 \cdot 5H_2O$	0.006	0.006	0.006
Co	$CoCl_2 \cdot 6H_2O$	0.006	0.006	—
Fe	$Fe(SO_4) \cdot 7H_2O$,$Fe_2(SO_4)_3$	5.56	5.56	0.34
EDTA·$2H_2O$	—	37.2	37.2	—
Inositol	—	100	100	—
Nicotinic acid	—	0.5	1.0	—
Pyridoxine	—	0.5	1.0	—
Thiamine HCl	—	0.1	10.	—
Glycine	—	2	—	—
Indolylacetic acid	—	1–30	—	—
Kinetin	—	0.04–10	0.1	—
2,4-D	—	—	0.1–1.0	—

[a]MS, Murashige and Skoog [77]; B5, Gamborg et al. [78]; W, White [79].

1. Macroelements

Carbon and Nitrogen Sources. Plant cell cultures are generally heterotrophic in connection with the utilization of the carbon source. This provides carbon for the cell components and also for energy for growth and maintenance.

Several workers have investigated the influence of carbon sources on growth and product formation. Sucrose is, largely, the source preferred for both growth and secondary product formation. The level of sugar supply can also influence secondary metabolism but not always in a predictable manner. High sucrose concentration (10% w/v), for example, stimulates anthraquinone synthesis in many species of *Galium*, but inhibits its production in others [80].

Sucrose (180 mM) produced the highest biomass and also the maximum productivity when several carbohydrates were tested for growth and solasodine production in batch suspension cultures of *Solanum eleagnifolium* Cav. [81] Mizukami et al. [82] have reported that the formation of shikonin derivatives in cultures of *Lithospermum* increases with increasing sucrose concentration in the range 1 to 5%. Zenk et al. [83] showed that increasing the sucrose level up to 7% increased both growth and rosmarinic acid formation (a case of a growth-associated process), but a further increase in the sucrose concentration was deleterious for both growth and product formation.

As with microorganisms, carbon catabolite repression effects have been reported by Rhodes et al. [62] when high sucrose levels (above 10%) are used. Concerning the maintenance requirements, the data of Ten Hoopen et al. [66] are among the few reported so far (see Table 7).

The nitrogen requirements are generally provided by a mixture of NO_3^- and NH_4^+. When these sources are supplied separately, both growth and product formation are generally affected in a negative way. The use of NO_3^- imposes a high energy demand on the assimilation of nitrogen. The use of NH_4^+ is energetically more efficient. Removal of NH_4^+ from the medium led to a rapid pH drop owing to the weak buffering usually present. In this connection it has been mentioned that ammonia can be used as the sole nitrogen source provided the medium is buffered with an organic acid such as succinate [84]. Utilization of NO_3^- is induced by a low level of NH_4^+ as shown in soybean cultures, where nitrate reductase developed only in the presence of NH_4^+ [85].

There are many examples in the literature of the influence of the nitrogen sources (mainly provide as NO_3^- and NH_4 mixtures) on growth and secondary product formation. In the case of solasodine production in suspension cultures of *S. eleagnifolium* Cav. [81] the productivity was influenced by the NO_3^-/NO_4^+ ratio, 2:1 being the optimum value found. Another example is concerned with berberine production by *Thalictrum* cells reported by Nakagawa et al. [86]. They showed that NH_4Cl used as the sole nitrogen source suppressed growth, NO_3^- in increasing concentrations improved growth, but NO_3^- as the only nitrogen source decreased berberine production. Finally, a mixture of both sources in the ratio 1:2 (KNO_3/NH_4Cl) was the best combination.

Concerning the levels of nitrogen sources employed, Zenk et al. [87] reported the case of anthraquinone formation in *Morinda citrifolia*, which is sensitive to the concentration of NO_3^- in the medium, the optimum being 25–40 mM. In the case of shikonin production, when the NH_4^+ ion concentration was above 3% of the total nitrogen content shikonin synthesis stopped. The optimal NO_3^- concentration for shikonin production was found to be 6.7 mM [88].

The ratio of carbon to nitrogen sources is also important because it represents an environmental control mechanism for product formation. Yamakawa

et al. [89] showed that anthocyanin production in suspension cultures of *Vitis* is influenced by the nitrogen/sucrose ratio of the culture medium.

Phosphorus, Calcium, Sulfur, Magnesium, and Potassium Sources. The influence of inorganic phosphate (the source employed to supply phosphorus) on secondary metabolite production by plant cells shows a behavior similar to that mentioned for microorganisms. Phosphate limitation has been used extensively to increase the accumulation of secondary products [90]. This has been referred to as an inverse relationship between growth rate and product formation. The accumulation of cinnamol putresine, for example, in *N. tabacum*, is increased three- to fourfold when the phosphate level is maintained below the optimum for growth [91]. Low phosphate concentration also stimulates the alkaloid production in *C. roseus* [92]. Generally the phosphate is stored in the vacuole from which it is taken during the growth phase. Secondary metabolite biosynthesis starts when the phosphate is depleted in the vacuoles, which suggests a regulatory role of phosphate.

Calcium is generally used in higher concentrations than in microbial media (for this reason it is included among the macroelements). It is interesting to mention the effect of the Ca^{2+} on cell adhesion (Fig. 3). Decreasing the Ca^{2+} level in the medium (1/30 of the level ordinarily used) diminished not only the adhesion of the cells to the wall of the bioreactors but also the foam formation [93], aspects which have technological importance.

In connection with the rest of the macroelements very little is known about their particular effects, apart from being essential components of the growth media.

2. Microelements

The microelements required for growth are listed in Table 8. Few studies have been carried out concerning the influence of microelements on growth and product formation. The nature and concentrations of these elements were recommended more than 30 years ago and are still maintained without modification. Some references related to specific effects of trace elements are concerned with copper and boron. Increasing the Cu^{2+} concentration 30-fold, increased shikonin production without affecting cell growth [94], which can be attributed to an abiotic stress condition which induced secondary product formation. A similar effect of increased Cu^{2+} concentration has been found in *Thalictrum rugosum* cultures for berberine production [95], a level of 200–500 μM proving to be the optimal to improve yields. Boron deficiency has been reported to increase the production of certain phenolic compounds [96].

As with microorganisms, it is apparent that trace element nutrition can affect growth of plant cells and also production of secondary metabolites. It is likely that significant effects can be expected in the future in this respect. Another aspect is related to interactions which may exist among ions and their effects on plant cells.

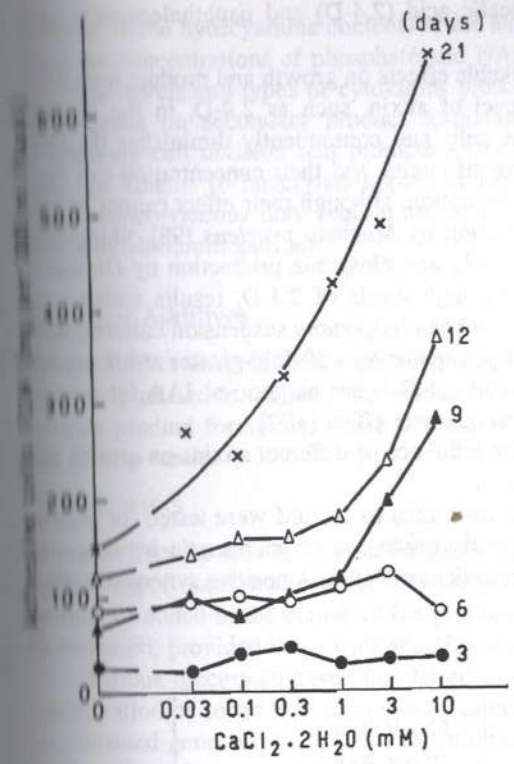


Figure 3 Effect of $CaCl_2 \cdot 2H_2O$ on cell adhesion of *Agrostemma githago* cell suspension culture. Adhesion of cells on the surface of a 300-ml Erlenmeyer flask. (From Ref. 126.)

3. Vitamins

Although the majority of cell cultures can synthesize vitamins, the rate of growth can be very slow if these compounds are not present in the medium. For this reason several vitamins like those included in Table 8 are used. Several reports related to vitamin requirements demonstrated that thiamine seems to be particularly important [97].

4. Phytohormones

Phytohormones or plant growth regulators such as auxins, cytokinins, and other compounds like gibberellic acid and abscisic acid are essential components of plant cell culture media. Auxins can be natural, like indoleacetic acid (IAA), or

synthetic, like dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA).

Phytohormones have remarkable effects on growth and product formation. In general, an increase in the level of auxin, such as 2,4-D, in the medium stimulates dedifferentiation of the cells and consequently diminishes the level of secondary metabolites. The type of auxins and their concentration can exert a particular influence on product formation, although their effect cannot be predicted. For example L-dopa production by *Mucuma pruriens* [98], ubiquinone production by *Nicotiana tabacum* [99], and diosgenin production by *Dioscorea deltoidea* [100] were stimulated by high levels of 2,4-D, results which were totally unexpected. In the case of *Cinchona ledgeriana* suspension cultures, Robins et al. [101] found that alkaloid production was 50-fold greater at 0.1 mg L^{-1} than at 2.0 mg L^{-1} 2,4-D. In general 2,4-D is not as good as IAA for product synthesis, with NAA having an intermediate effect [102].

An interesting example of the influence of different auxins on growth and product formation is shown in Fig. 4.

NAA concentrations varying from zero to 10 mM were tested for tropane alkaloid formation. A decrease in cell growth and an increase in hyoscyamine content were found at lower concentrations of NAA. A positive synergistic effect

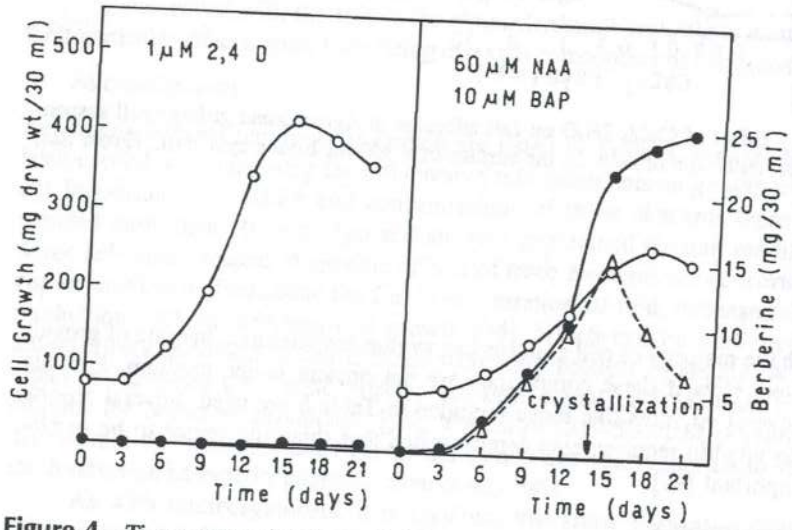


Figure 4 Time course of cell growth (○) and berberine production (●): total amount of berberine; (Δ): the amount of berberine that dissolved in the medium in *Thalictrum minus* cell suspension cultures grown in LS medium containing 1 $\mu M_{2,4}$-D (left) and in LS medium containing 60 μM NAA and 10 μM BAP (right). (From Ref. 227).

increase in the hyoscyamine content) of the auxin plus phosphate was observed when the concentrations of phosphate and NAA were lowered together [103].

The levels and types of cytokinins present in the medium may also have an influence on secondary product formation [104]. Cytokinins are known to stimulate cell division and promote shoot formation. The most commonly used are kinetin (6-furfurylaminopurine) and 6-benzyladenine. Cell cultures from monocotyledons may require an exogenous supply of zeatin (methyl-*R*-oxymethylallylaminopurine).

B. Other Additives

Other additives different from those required for growth and product formation are mainly related to the supplementation of precursors and elicitors which can enhance product formation. Some comments about the addition of antibiotics will be also made.

1. Precursors

In some cases the flux through a biosynthetic pathway may be limited by the availability of some intermediates. If these compounds are supplied with the medium or added in the course of the process, the rate of product synthesis can be increased, provided they reach the site of synthesis.

Various precursors have been tested in an attempt to further increase the concentration of secondary compounds. Lindsey and Yeoman [105], for example, obtained good results in the stimulation of capsicin synthesis in some lines of *Capsicum frutescens* by supplying phenylalanine and isocapric acid. *N*-Methylputrescine, phenylalanine, and tropic acid effectively increase hyoscyamine and scopolamine production when they are added from the beginning of incubation, as has been reported by Hashimoto and Yamada [103].

In connection with precursor additions, several considerations should be pointed out. A precursor should be easily available and not expensive to use. In addition, its concentration, time of incorporation in the medium, and biosynthetic relation to the product should also be considered [106]. Finally, it is important to mention that the feeding of precursor distant from the product may have little or no effect on the final yield.

2. Elicitors

Microbe-derived molecules which stimulate secondary metabolism have been called elicitors. The term has been extended to others compounds which fulfill the same function of producing some sort of stress on the cell which in turn enhances secondary metabolite formation.

Elicitors can be grouped into two classes, biotics and abiotics. The biotic elicitors include whole fungal mycelium, fungal cell wall material, glucan poly-

mers, glycoproteins, and low-molecular-weight organic acids. Abiotic elicitors include ultraviolet irradiation, salts of heavy metals, and chemicals, some of which have affinity for double-stranded DNA, while others disturb cell membrane integrity.

A number of plant products, phytoalexins, and related compounds can be induced in their synthesis by the addition of elicitors. The mode of action of elicitors is still not known, although there is evidence that they induce the synthesis of specific mRNAs.

According to Di Cosmo and Towers [107], there is a possible correlation between the stress to which the cell plant is subjected (when an elicitor is added) and the secondary metabolite formation.

In addition to phytoalexins, the concentration of other compounds may also increase after elicitor treatment. For example, anthraquinone concentration in *C. ledgeriana* suspension cultures can be doubled by treating them with autoclaved mycelia of *Phytophthora cinnamomi* [108] and diosgenin can also be increased after treatment with autoclaved mycelia of *Rhizopus arrhizus* [109]. Among the abiotic elicitors tested it is important to mention that about 10 metal ions were found to be effective in cell suspension cultures. The active ions were (in order of increasing effectiveness) $Al^{3+} \simeq Co^{2+} \simeq Zn^{2+} < Cd^{2+} < Ni^{2+} < Cu^{2+} \simeq Cr^{3+} < Pb^{2+} < Ag^{+} \ll Au^{3+}$ [110]. Gold in particular (0.1 mM) was found to be a very effective elicitor.

In some cases the use of elicitors has an additional advantage, which is related to the excretion of the product to the medium. For example, Brodelius et al. [111] have shown that in cultures of *Eschscholtzia californica* a number of different alkaloids are produced by elicited cells and that 70–80% of the alkaloids can be isolated from the medium.

It is important to point out that not all compounds are induced in their biosynthesis by elicitors and also that the production of elicited compounds is transient and some of these compounds may disappear rapidly by the action of extracellular peroxidases [112].

3. Antibiotics

In some cases antibiotics are used as additives to plant cell culture media because they have shown beneficial effects for controlling microbial contamination. However, it has to be pointed out that the use of antibiotics is not intended to be a substitute for adequate aseptic techniques. In addition, many antibiotics have proved to be cytotoxic to certain plant cell species. For these reasons, antibiotics (if strictly required) should be used carefully by adequate selection of compounds and working concentrations. The antibiotics most normally cited are gentamicin, kanamycin, and ampicillin.

E. Medium Preparation

Several authors have given recommendations for preparation of media, particularly for the laboratory scale. Gamborg [113] proposed the preparation of stock solutions for micronutrients, vitamins, $CaCl_2$, IK, and phytohormones. The rest of the components are added and dissolved in distilled (or demineralized) high-purity water, in a specific order. The heat-stable growth hormones such as kinetin and 2,4-D are added at the end, the pH is adjusted, and the medium is autoclaved. Heat-labile compounds like indoleacetic acid, indolebutyric acid, or gibberellic acid are added to the medium after autoclaving.

D. Possibilities for Improving Medium Design

1. Need for Basic Data and Understanding of Secondary Compound Biosynthesis and Degradation

Although there have been many studies of growth and production kinetics in plant cell cultures, most of the data reported are not suitable for the design of large-scale bioprocesses because the majority of studies were carried out using shake flasks. As pointed out by Ten Hoopen et al. [66], growth rate and product formation may change completely in either a positive or a negative direction when one wants to translate the results of shake flasks to a larger scale. A convenient way to obtain useful data is by means of continuous culture studies in a bioreactor of the type to be used for the larger scale. In this respect very few studies have been performed. Ten Hoopen et al. [66] reported values of specific growth rate, growth yields, and maintenance coefficients of *C. roseus* and *N. tabacum* suspension cultures in glucose-limited continuous processes (see Table 7). It is evident that many more studies on different cell plant species in bioreactors are needed to determine growth and production process parameters.

The accumulation of secondary compounds occurs through a complex series of steps involving biosynthesis, transport, storage, and degradation. The major routes of biosynthesis for many secondary compounds are now understood. What is now required is the complete elucidation of pathways by determining each intermediate and the characterization of the enzymes involved in its formation. As pointed out by Wink [114], future work in the field of accumulation processes will have to focus on their identification and also on their dependence on the physiological state of the cells. We can conclude that we need a better understanding of the molecular, biochemical, and regulatory basis of secondary compound formation. It is likely that advances in these aspects will contribute to the transition from empirical to rational medium and process design in plant cell cultures.

E. Some Guides for the Design and Formulation of Media for Plant Cell Suspension Culture Bioprocesses

Although it is generally accepted that several components of media for plant cell cultures behave in a nonpredictable manner, the majority of research studies have shown that

1. The use of two-step processes, the first using a growth medium and the second a production medium, seems to be the best approach to a plant cell bioprocess.
2. Concerning carbon sources, sucrose is preferentially used. Increasing sucrose levels up to a certain value favors growth and product formation, but further increase (above 10%) may produce carbon catabolite repression.
3. NO_3^- or NH_4^+ as the sole nitrogen sources may produce inhibition of growth and also in some cases inhibition of product formation. For production purposes the nitrogen compounds of media are generally reduced or even suppressed.
4. PO_4^{3-} acts as a regulatory compound of secondary metabolite formation and consequently its concentration has to be reduced in the production step.
5. Selection of the most efficient phytohormones and their relationship (not yet possible to predict) is essential for improving yields or productivity.
6. Addition of precursors or elicitors is a proven strategy for enhancing secondary metabolite formation.

Taking these considerations into account, the following guides can be given for the design, formulation, and optimization of growth and production media for plant cell bioprocesses.

Growth medium

1. Select a basal medium such as MS, B5, or other.
2. Test the medium in shake flasks and in a bioreactor and optimize it for maximal biomass production in continuous culture, testing different levels of main components.

Production medium

Maintaining the same carbohydrate concentration of the growth media (generally 20–30 g L^{-1}), test the following modifications:

1. Reduce the phosphate and nitrogen content. In some cases the nitrogen content can be reduced to nil.
2. Modify the vitamins and the iron (chelated) contents.
3. Find adequate phytohormones levels and their ratios.

4. Add a precursor (if it is suitable according to the knowledge of the biosynthetic pathways) and find the correct time for its addition.
5. Test a biotic or an abiotic elicitor.
6. Optimize the medium by a fractional factorial statistical methodology followed by a simplex technique.

IV. MAMMALIAN CELL PROCESSES

During the past decade the use of animal cells as part of manufacturing processes for the production of biopharmaceuticals has become well established [115–119]. However, a process that uses animal cells as the expression system is frequently more expensive than one using lower eukaryotes, such as yeasts, or prokaryotes, mainly because of higher medium costs, longer runs, and lower yields. This would suggest that animal cells should be used only when there is no other alternative. In most cases the alternatives are narrowed down to mammalian cells because of very specific posttranslational modifications of the proteins of interest that only certain mammalian cells can provide [120–123] and that are required for their desired functionality, including stability and pharmacokinetics [124–132].

To minimize production costs when using mammalian cells, it becomes important to maximize yields and simplify the culture medium. The yield of a mammalian cell process is a function of the interactions of factors from three main components: the cells, the bioreactor, and the medium. The bioreactor factors include design, mode of operation, and the physicochemical environment it provides to the cells. The medium factors include composition and the way in which the different components are fed or are available to the cells.

As with any other process using living cells, a mammalian cell process may be worked on to increase product yields [133]. Process development may continue indefinitely and involve cell and strain selection, bioreactor operation, and medium modifications, all potentially translating into productivity increases. However, in an industrial setting it is more the rule than the exception that limited time and resources are available for efforts to optimize any fermentation including those using mammalian cells. In general, once the cell line and its appropriate subclone have been selected based on a target unoptimized yield, scaling up may or may not start with comparative runs with different bioreactors, i.e., stir tank or airlift vessels for batch/fed-batch operations versus perfusion systems for extended cultures. The thoroughness of these studies will depend on the investigator's and/or institution's bias, experience, time, and resources including available bioreactor capacity. Regardless of the bioreactor chosen, in all cases one or more media have to be selected and lead to practical and cost-effective choices. The issue of medium cost is multifaceted and includes the prices paid up front for the medium components, availability in required quan-

tities to proper specifications at all times and from several suppliers, simplicity, and ease of preparation, handling, storage, testing, and delivery to points of use. Additional factors related to media that contribute to the overall manufacturing cost and that should also be evaluated are the effects of medium components on downstream operations based on their intrinsic potential to contaminate the product at all stages of the purification process [134,135], the apparent effects of media on some cells such as loss of specific productivity in time [136], the release of unwanted cellular components that will need to be removed, and the effects of medium components on product homogeneity [121,137,138]. Thus the selection, development, and optimization of media, although targeted at maximizing productivity in the bioreactor, should also address the homogeneity of the product, the quantity and type of contaminants, and their impact on the purification process, because purification may represent as much as 80% of the manufacturing cost [139].

Media for the isolation, maintenance, and growth of practically any type of animal tissue and cells are either commercially available or can be formulated from easily obtainable components. The development and availability of all these formulations originated in the elegant work of pioneers in the field and many investigators who improved the definition of media, identified nutritional requirements of many cells, and determined factor requirements. Many excellent reviews have described the evolution of this field and serve as ideal sources of valuable information [140–144]. In addition, the increased demand in terms of variety, quality, and quantity of media for research and commercial uses has resulted in the expansion of services of established medium suppliers and proliferation of new suppliers [141,145].

A. Initial Medium Selection

The selection of a basal medium for a recombinant cell line may start with a basal medium that supports the growth of the parental cell line. Widely used basal formulations for most, if not all, of the commonly used cell lines are listed and described in the catalogs of medium suppliers (see also Ref. 140).

Often the initial medium contains fetal bovine serum for practical reasons, and this supplementation is an easy, convenient one to implement for initial production of research quantities of the desired product. However, there are multiple advantages—logistic, economic, and regulatory—to the elimination of serum in any large-scale process. Thus, it should be one of the first priorities in medium development to eliminate serum use. All mammalian cells used for manufacturing purposes can be adapted to serum-free media [145–151] and most even to protein-free media [152–158]. There are multiple commercially available serum-free media (Table 9) [116,147,158–160] which represent convenient alternatives for fast and easy adaptations to serum-free media and for

Table 9 Serum-Free and Protein-Free Media for Mammalian Cells Available from Commercial Suppliers

	Media	Protein (mg/L)		Suppliers[c]
[illegible]	Ultra-CHO	<300	(L,P)[a]	Bio-Whittaker
	CHO-S-SFM	<400	(L)	
	CHO-S-SFM II	<100	(L)	Gibco
	CCM-5	<400	(L)	HyClone
	HB-CHO	320	(P)	Irvine Scientific
	Ex-Cell 301	100	(L,P)	
	DMRL-25[b]	0	(L,P)	JRH Biosciences
Hybridomas and Cybridomas	Ultradoma	30	(L)	
	Ultradoma-P.F.[b]	0	(L,P)	Bio-Whittaker
	Hybridoma-SFM	20	(L)	
	PFHM-II[b]	0	(L,P)	Gibco
	CCM1	2100	(L,P)	HyClone
	HB 101	780	(L,P)	
	HB 104	780	(L,P)	
	HB PRO	1	(L,P)	
	HB GRO	50	(L,P)	Irvine Scientific
	Ex-Cell 300	7–10	(P)	
	Ex-Cell 309	10	(P)	
	KC2000	250	(P)	
	APROtain-1[b]	0	(L,P)	JRH Biosciences
	QBSF-52	45	(L,P)	
	QBSF-55	65	(L,P)	
	Hybri-Max[b]	0	(L,P)	Sigma
Various cell lines	Ultraculture	3000	(L)	
	AIM V	>2500	(L)	Gibco
	Ex-Cell 320	250	(P)	JRH Biosciences
	QBSF-51	45	(L,P)	
	QBSF-53	450	(L,P)	
	QBSF-56	430	(L,P)	Sigma

[a] Available in liquid form; P, available in powdered form.
[b] Protein-free formulations.
[c] To order or obtain technical service from listed suppliers contact at:
Bio-Whittaker, Inc., 8830 Biggs Ford Rd. Walkersville, MD 21793, USA. Tel. 800-638-8147/301-[illegible]; Fax. 301-845-8338; International Fax. 301-845-8291.
Gibco Life Technologies Inc., P.O. Box 9418, Gaithersburg, MD 20898, USA. Tel. 800-828-6686; Fax. [illegible]-2286; International Fax. 301-258-8238.
HyClone Laboratories, Inc., 1725 South HyClone Rd., Logan, UT 84321-6212, USA. Tel. 800-492-[illegible]; International Tel. 801-753-4584; Fax. 800-533-9450; International Fax. 801-753-4589.
Irvine Scientific, 2511 Daimler Street, Santa Ana, CA 92705-5588, USA. Tel. 800-437-5706; Fax. [illegible]-361-6522.
JRH Biosciences, P.O. Box 14848 Lenexa, KS 66215-0848, USA. Tel. 800-255-6032, International Tel. 913-469-5580; Fax. 800-441-1561.
Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178-9916, USA. Tel. 800-325-3010/[illegible]-7791; International Tel. 314-771-5750; Fax. 800-325-5052, International Fax. 314-771-5757.

Table 10 Composition of Two Protein-Free Media for Hybridomas

Component	Hybri-Max[a] (mg/L)	ABC[b] (mg/L)	Component	Hybri-Max[a] (mg/L)	ABC[b] (mg/L)
Amino acids			Inorganic salts		
L-Alanine	9.0	8.9	AgCl	4.4×10^{-6}	$4.4 \times$ [illegible]
L-Arginine	211.0	211.0	$AlCl_3 \cdot 6H_2O$	0.001	[illegible]
L-Asparagine·$2H_2O$	15.01	34.0	$BaCl_2$	0.002	[illegible]
L-Aspartate	13.3	13.3	$CaCl_2 \cdot 2H_2O$	44.1	44.0
L-Citrulline	5.0	5.0	$CoCl_2 \cdot 6H_2O$	0.002	[illegible]
L-Cysteine·HCl·H_2O	35.0	35.1	$CrK(SO_4)_2$	0.001	[illegible]
L-Glutamic acid	14.7	14.7	$CuSO_4 \cdot 5H_2O$	0.0025	[illegible]
L-Glutamine	396.0	396.0	$FeSO_4 \cdot 7H_2O$	0.834	[illegible]
Glycine	7.51	7.5	GeO_2	0.0005	[illegible]
L-Histidine·HCl·H_2O	71.0	71.0	KBr	0.0001	[illegible]
L-Isoleucine	164.0	164.0	KCl	224.0	223.6
L-Leucine	133.0	133.0	KI	0.0001	[illegible]
L-Lysine·HCl	109.0	109.0	LiCl	10.0	10.0
L-Methionine	15.0	15.0	$MgCl_2 \cdot 6H_2O$	123.0	122.0
L-Ornithine	8.0	8.0	$MnCl_2$	0.0001	[illegible]
L-Phenylalanine	55.0	55.0	$Na_2MoO_4 \cdot 2H_2O$	0.0001	[illegible]
L-Proline	34.5	34.5	NaCl	7599.0	7599.0
L-Serine	51.0	51.0	NaF	0.004	[illegible]
L-Threonine	11.9	11.9	$NaHCO_3$	2250.0	2250.0
L-Tryptophan	5.0	5.0	Na_2HPO_4	397.39	—
L-Tyrosine 2Na·$2H_2O$	31.2	—	$Na_2HPO_4 \cdot 7H_2O$	—	750.0
L-Tyrosine	—	26.0	Na_2SeO_3	0.03	0.03
L-Valine	91.0	91.0	NH_4VO_3	0.0006	[illegible]
			$Ni(NO_3)_2 \cdot 6H_2O$	0.0002	[illegible]
Other components			RbCl	10^{-5}	10^{-5}
Adenine	0.4	0.4	$SnCl_2$	0.0001	[illegible]
Citric acid·H_2O	25.0	25.0	$TiCl_2$	0.001	[illegible]
Dilinoleoyl phosphatidylcholine	0.5	0.5	$ZnSO_4 \cdot 7H_2O$	0.863	0.863
Distearoyl phosphatidylcholine	0.5	0.5	Vitamins		
Ethanolamine·3HCl	3.0	3.0	Biotin	0.11	0.11
EDTA	5.5	5.5	$Ca_{1/2}$ pantothenate	3.5	3.5
Glucose	5202.0	5202.0	Choline chloride	13.96	14.0
Hypoxanthine	4.08	4.1	FAD	0.02	0.02
Linoleic acid	0.084	0.084	Folic acid	1.32	1.3
Lipoic acid	0.21	1.21	*myo*-Inositol	18.0	18.0
MOPS	3135.0	3135.0	Niacinamide	6.5	6.5
Na nitroprusside	5.7	5.7	Pyridoxine·HCl	0.2	0.2
Na pyruvate	110.0	110.0	Riboflavin	0.038	0.038
			Thiamine·HCl	0.34	0.34

Component	Hybri-Max[a] (mg/L)	ABC[b] (mg/L)	Component	Hybri-Max[a] (mg/L)	ABC[b] (mg/L)
[illegible] components]			[Vitamins]		
[illegible]acetate	5.0	5.0	Alpha-tocopherol acetate	0.15	0.15
[illegible] red	4.2	4.2	Vitamin B_{12}	1.36	1.36
[illegible]gesterone	0.006	0.006			
[illegible]$2H_2O$	0.161	0.161			
[illegible]dine·3HCl	0.5	0.5			
[illegible]	30.0	30.0			
[illegible]dine	0.8	0.8			
[illegible] 80	0.2	0.2			

[a] Copied from the Sigma Cell Culture Catalog, pp. 243–244, 1993.
[b] Copied from Ref. 155.

the preparation of intermediate, master, and manufacturer's working cell banks. However, these media are in most cases intended for research or bench work and are high in cost, ranging from tens to hundreds of U.S. dollars per liter [159–160]. For scaling up to manufacturing applications cheaper media (i.e., < U.S.$5.00/L) are desirable and can and need to be developed. The formulations of two very similar and effective protein-free media for hybridomas have been published and are presented in Table 10.

B. Medium Development

During the scaling-up of a process utilizing mammalian cell culture there are four specific areas that, when changed, have the potential to introduce changes in the product which will require revalidation of purification procedures and, if the product is a biopharmaceutical, costly repeating of safety or efficacy trials. These four areas are fermenter size, fermentation time, purification procedures, and medium composition [161]. Obviously, these areas need to be defined as early as possible and any changes in them after the initial phases of clinical investigation (Phases I and II) have to be considered and evaluated very carefully before implementation.

Optimization of growth conditions does not necessarily always translate into optimization of product expression [162,163]. Medium development may be an intensive, extended exercise. In many instances medium vendors are willing to work with customers in developing custom formulations. These types of collaborations may represent an alternative or complement to in-house efforts.

One of the early, easy decisions in the development work may consist of eliminating phenol red from medium formulations. This compound is not needed when electrodes are used to monitor and control pH, it does not contribute to

growth or protein production, and it fouls up the columns used downstream in product purification.

After selection of a preliminary serum-free formulation, the next tasks may involve iterative quantitative analyses of spent medium to balance the formulation for components such as amino acids, carbohydrates, and lipids [164] and systematic study of the effects of the different components, individually and in combination [165–170], on growth rates, cell yields, culture longevity, specific productivity, product yields, and product consistency. The studies may also include nonnutritional supplements [171–172]. The effort of medium optimization will also depend on the type of bioreactor selected and its mode of operation, either batch/fed-batch or continuous. For batch/fed-batch operations selective feeding of key nutrients has been most successful as a way to extend the longevity of hybridoma cultures after exponential growth which has resulted in higher product yields [168,170,173,174]. For continuous perfusion operations the chemostat is a simple and valuable tool for studying cell performance and for media optimization [175–197]. However, the cells and media formulations must be tested under high-density perfusion conditions because unpredictable effects may arise that affect cell stability and/or productivity [198–203].

The work on medium development should also include the study of effects of metabolic by-products and physicochemical parameters such as temperature, pH, and agitation, because these may significantly affect the cells' ability to take up nutrients and their overall performance [204–214].

C. Medium Preparation and Use

Once a medium formulation has been developed, most medium suppliers will be willing to prepare batches of different sizes in liquid, powdered, or concentrated form. It is advisable to negotiate this manufacture with several vendors to ensure more than one source and low competitive prices and to qualify the vendor in relation to quality, consistency, and responsiveness. Use of a powdered medium is common practice in the industry [215,216]. Most of the medium suppliers have built and validated new facilities with the capacity to produce powdered media in batches of up to hundreds of thousands of liters [160]. If powdered medium is used for a manufacturing process, the medium preparation capacity of the mammalian culture facility will define its production capacity. The preparation involves reconstitution of the medium using high-quality water (water for injection [217] or the equivalent [218–220]), low in endotoxin (<0.25 USP endotoxin units/ml), sterilization by microfiltration, testing, storage, and delivery to points of use. All of this requires comprehensive quality control of components and the prepared media [221,222], including determining the stability of components such as glutamine [223]. A medium preparation system that obviates the need for mixing tanks has been introduced into the market

[224]. It consists of a cylindrical, dual-chambered, molded-plastic device with an inlet in the bottom and an outlet at the top. Powdered medium is contained in the lower chamber and sodium bicarbonate in the upper one. A water source is connected to the inlet port and the outlet port is connected to a sterile filter and the final sterile container. As water enters the lower chamber it mixes and dissolves the powdered medium and then the sodium bicarbonate. As the medium dissolves it flows out of the upper chamber and is sterilized by the in-line filter into the final container. This device simplifies the process of medium preparation but at this time is available only at relatively small lots of medium (up to 200 L). A prototype capable of handling between 500 and 1700 L, depending on the complexity of the medium formulation, is on test and expected to reach commercialization in 1994.

Concentrated medium formulations are available and their use is increasing [225]. Concentrated medium can be reconstituted to single strength either in batches or in a continuous mode using a steady supply of sterile water and eliminating the need for large mixing and storage tanks.

An increasingly viable alternative to the preparation of medium beginning with powdered or concentrated formulations is to purchase standard or custom formulations in liquid form delivered in disposable containers of up to 1200 L, as being offered by HyClone, Gibco BRL (Life Technologies, Inc.), JRH Biosciences, and other suppliers. This has the advantage of eliminating the testing of raw materials, all the medium preparation steps, and the infrastructure required for both. This translates into a simpler facility, easier to install and expand as manufacturing needs increase. With this alternative cold storage (2 to 8°C) for liquid media will have to be available at sufficient capacity to accommodate the demand of sustained manufacturing schedules.

V. SUMMARY AND CONCLUSION

The fundamental knowledge available about the microorganism, substrates, and process represent the basis on which a rational approach for the design and formulation of media for microbial processes can be attempted. In this respect, it is necessary to analyze critically the main objective to be optimized (yield, productivity, quality of final product, etc.). The first step of medium development is concerned with the decision to be taken about the adequate components to be used, followed by the calculation of their concentrations. After medium preparation and experimentation, the application of the most appropriate statistical optimization methodology will make it possible to attain the optimal medium.

Medium design and formulation for plant cell processes are still carried out on an empirical basis owing to the lack of fundamental knowledge. However, some general guidelines can be given for growth and production media considering the experimental evidence available about the functions and influence of

the medium components for promoting growth and product formation. Adequate manipulation of the carbon, nitrogen, and phosphorus sources and phytohormones and the inclusion of precursors and elicitors in the medium represent the best strategy for improving medium development for plant cell processes.

The development of cost-effective medium supplies for mammalian cell culture production of proteins is a key element for a commercially successful process. Medium development is a task that requires experience, time, and resources in its solutions, which may be several. An optimal formulation is elusive because there will always be room for improvement, but through systematic, comprehensive work, practical combinations of nutrients, factors, and feeding schedules can be developed in a reasonable time. A properly developed formulation can bring the burden of the medium below 10% of the total cost of a mammalian cell process.

PROBLEMS

It is desired to design and formulate a medium for producing 862 g of press baker's yeast (29% dry matter) with 53% protein and 2.5% P_2O_5 (dry basis) with good storage stability by conducting a process in a fermentor without O_2 limitation.

1. Select adequate sources for carbon, nitrogen, phosphorus, and the rest of the yeast requirements and give the reason for the election. Take into consideration that according to the sources chosen a significant decrease in the pH may occur.
2. Is it necessary to restrict the growth rate and the carbon source? If so, give the reasons and recommend the best strategy for conducting the process.
3. Calculate $Y_{x/s}$ from the stoichiometric equation of biomass formation. No products are formed. For one carbon mole of biomass produced the $y_{x/s}$ value is 0.543 and for the yeast formula $CH_{1.64}O_{0.52}N_{0.16}$ considering an ash content of 8%.
4. Calculate the amounts of carbon, nitrogen, and phosphorus sources.
5. After experimenting with the medium a low value (<0.40) of $Y_{x/s}$ is obtained. Give the possible reasons for the low yield attained and suggested how the medium can be improved.
6. The storage stability of the yeast produced is poor. What modifications in the medium design can be recommended? It is known that storage stability is improved by increasing the trehalose content of the yeast, which can be enhanced by increasing the temperature of growth.

Solutions

1. Molasses, NH_4OH, and H_3PO_4. 2. Yes, fed-batch process. 3. $Y_{x/s}$ = 0.50. 4. Molasses (50% total sugars), 1000 g; NH_4OH solution (10% nitrogen), 212 ml; and H_3PO_4 (density, 1.64; 72.4% P_2O_5 w/w), 5.24 ml. 5. An element (Mg, Zn) or a vitamin (biotin) deficiency. 6. The modifications include the increase of pantothenic acid content.

ACKNOWLEDGMENTS

R. E. and A. M. G. wish to thank Dr. C. Mignone for his comments and for the stimulating discussions during the preparation of this chapter.

REFERENCES

1. A. Fiechter, *Adv. Biochem. Eng. 30*: 8 (1984).
2. M. Reuss, *Plenary Lecture*, VIIth, International Biotechnology Symposium, New Delhi, India, February 1984.
3. A. G. Marr, *Microbiol Rev. 55*: 316 (1991).
4. F. Huber and A. Tietz, *Biotech. Lett. 5*: 385 (1983).
5. D. Herbet, *Continuous Culture, 6: Applications and New Fields* (A. C. Dean, D. C. Ellwood, C. G. Evans, and J. Mellin, eds.) Ellis Horwood, Chichester, 1976, p. 1.
6. M. A. Rouf, *J. Bacteriol. 85*: 1545 (1964).
7. T. Suzuki, H. Mori, T. Yamame, and S. Shimizu, *Biotech. Bioeng. 27*: 192 (1985).
8. S. Aiba, A. E. Humphrey, and N. F. Millis, *Biochemical Engineering*, 2nd ed. Academic Press, New York, 1973, p. 29.
9. S. J. Pirt, *Principles of Microbe and Cell Cultivation*, Blackwell Scientific, London, 1975, pp. 117–136.
10. R. I. Mateles and E. Batat, *Appl. Microbiol. 28*: 901 (1974).
11. J. J. Heijnen, J. A. Roels, and A. H. Stouthamer, *Biotech. Bioeng. 21*: 2175 (1979).
12. L. B. Tsai, M. Mann, F. Morris, C. Rotgers, and D. Fenton, *J. Ind. Microbiol. 2*: 181 (1987).
13. B. C. Nudel, C. Corradini, E. Fraile, and A. M. Giulietti, *Biotech. Lett. 9*: 77 (1987).
14. K. H. Dombek and L. O. Ingram, *J. Ind. Microbiol. 1*: 219 (1986).
15. M. R. de Roubin, L. Bastein, S. H. Shen, and D. Groleau, *J. Ind. Microbiol. 8*: 147 (1991).
16. J. M. Prescott, V. J. Peters, and E. Snell, *J. Biol. Chem. 202*: 533 (1953).
17. J. Shu and M. L. Shuler, *Biotech. Bioeng. 37*: 708 (1991).
18. S. Hutner, *Annu. Rev. Microbiol. 26*: 313 (1972).
19. R. P. Jones and P. F. Greenfield, *Process Biochem. 19*: 48 (1984).
20. E. O. Weinberg, *Adv. Microb. Physiol. 4*: 1 (1970).
21. P. Antier, G. Moulin, and P. Galzy, *Process Biochem. 25*: 9 (1990).
22. E. E. Snell, *Annu. Rev. Microbiol. 3*: 97 (1949).

23. G. M. Dunn, *Comprehensive Biotechnology* (M. Moo Young, ed.), pergamon Press, Oxford, 1985, 1, pp. 113 and 126.
24. H. Silva, A. M. Giulietti, R. Segovia, and R. Ertola, *Rev. Argentina Microbiol 14*: 85 (1982).
25. H. Silva, T. Cortinas, and R. Ertola, *Appl. Microbiol. Biotech. 31*: 293 (1989).
26. W. J. Beque and H. C. Lichstein, *Proc. Soc. Exp. Biol. Med. 114*: 625 (1963).
27. G. M. Hills and E. D. Spurr, *J. Gen. Microbiol. 6*: 64 (1952).
28. Y. Kawahara, Y. Yoshihara, S. Ikeda, H. Yoshi, and Y. Hirose, *Appl. Microbiol Biotech. 34*: 87 (1990).
29. J. A. Arcas, O. Yantorno, and R. Ertola, *Biotech. Lett. 9*: 105 (1987).
30. E. T. Reese and A. Maquire, *Appl. Microbiol. 17*: 242 (1969).
31. A. Imaisumi, Y. Suzuki, Y. Ginnaga, S. Sakuma, and Y. Sato, *J. Microbiol. Methods 2*: 339 (1984).
32. P. Janssen, H. W. Morgan, and R. Daniel, *Appl. Microbiol. Biotech. 34*: 789 (1991).
33. J. F. Martin, *Physiological and Genetic Modulation of Product Formation* (L. Alberghina, L. Frontali, and G. Hamer, eds.), *Dechema Monographs 105*: 55 (1987).
34. S. L. Chem and M. Chiger, *Comprehensive Biotechnology* (M. Moo-Young, ed.), Pergamon Press, Oxford, 1985, 3, p. 430.
35. A. Fiechter and F. Gmunder, *Adv. Biochem. Eng. 34*: 1 (1988).
36. K. Corbett, *Comprehensive Biotechnology* (M. Moo-Young, ed.), Pergamon Press, Oxford, 1985, 1, p. 127.
37. L. D. Boeck, J. S. Alford, R. L. Pieper, and F. M. Huber, *J. Ind. Microbiol. 4*: 247 (1989).
38. S. B. Primrose, *J. Appl. Bacteriol. 61*: 99 (1986).
39. Anonymous, *Biotech Forum Europe 7*: 262 (1990).
40. R. Ertola, Unpublished.
41. E. Zauner, W. K. Bronn, H. Dellweg, and R. Tressl, *Branntweinwirtschaft 119*: 154 (1979).
42. W. Preschell, Personal communication.
43. D. Tuse, *CRC Crit. Rev. Food Sci. Nutr. 19*: 273 (1984).
44. P. E. Milson and J. L. Meers, *Comprehensive Biotechnology* (M. Moo-Young, ed.), Pergamon Press, Oxford, 1985, 3, p. 365.
45. R. W. Swartz, *Comprehensive Biotechnology* (M. Moo-Young, ed.), Pergamon Press, Oxford, 1985, 3 p. 7.
46. S. Nagai, *Adv. Biochem Eng. 11*: 53 (1979).
47. M. J. Johnson, *Science 155*: 1515 (1967).
48. S. J. Pirt and D. S. Callow, *J. Appl. Bacteriol 23*: 87 (1960).
49. J. H. Hardwood and S. J. Pirt, *J. Appl. Bacteriol. 35*: 597 (1972).
50. J. Seo and J. E. Bailey, *Biotech. Bioeng. 27*: 1668 (1985).
51. S. J. Pirt, *Principles of Microbe and Cell Cultivation*, Blackwell Scientific, London, 1975, p. 74.
52. C. L. Cooney and F. A. Acevedo, *Biotech. Bioeng. 19*: 1449 (1977).
53. L. Lison, *Estadistica Aplicada a la Biología Experimental* (EUDEBA, ed.), Bs. As., Argentina, 1976, p. 163.

54. R. L. Plackett and J. P. Burman, *Biomatrika 33*: 305 (1946).
55. G. E. Box and R. B. Wilson, *J. R. Stat. Soc. (B) 13*: 1 (1951).
56. S. L. Morgan and S. N. Deming, *Anal. Chem. 46*: 1170 (1974).
57. J. A. Nelder and R. Mead, *Comput. J. 7*: 308 (1965).
58. N. J. Hill and W. G. Hunter, *Technometrics 8*: 571 (1966).
59. P. Christen and M. Raimbault, *Biotech. Lett. 13*: 521 (1991).
60. H. E. Reiling, H. Laurita, and A. Fiechter, *J. Biotech. 2*: 191 (1985).
61. L. E. McDaniel, E. G. Bailey, S. Ethiraj, and H. P. Andrews, *Dev. Ind. Microbiol. 17*: 91 (1976).
62. M. J. Rhodes, R. J. Robins, A. J. Parr, and J. Hamill, *J. Appl. Bact. Symp. Suppl. 16*: 105 S (1987).
63. R. A. Taticek, M. Moo-young, and R. L. Legge, *Plant Cell, Tissue and Organ Culture 24*: 139 (1991).
64. J. M. Engasser, *Chem. Eng. Sci. 43*: 1739 (1988).
65. A. Scragg, S. Ashton, A. York, P. Bond, G. Stephan-Sarkission, and D. Grey, *Enzyme Microbiol. Tech. 12*: 292 (1990).
66. H. J. Hoopen, W. M. van Gulik, and J. J. Meijer, *Progress in Plant Cellular and Mollecular Biology* (H. J. Nijkamp, L. H. vander Plas, J. van Aartrijk, eds.), Kluwer Academic Publishers, Boston, 1990, p. 673.
67. H. Spieler, A. W. Alfermann, and E. Reinhard, *Appl. Microbiol. Biotech. 23*: 1 (1985).
68. D. Drapeau, A. W. Blanch, and C. Wilke, *Biotech Bioeng. 28*: 1555 (1986).
69. Y. C. Hong, T. P. Labuza, and S. K. Harlander, *Biotech. Prog. 5*: 137 (1989).
70. M. Taya, A. Yoyama, O. Kondo, and T. Kobayashi, *J. Chem. Eng. (Jpn.) 22*: 84 (1989).
71. M. W. Fowler, *Plant Biotechnology* (S. H. Mantell and H. Smith, eds.), Cambridge University Press, 1983, p. 4.
72. A. Kato, A. Asakura, K. Tsuji, F. Ikeda, and M. Injima, *J. Ferment. Technol. 58*: 373 (1980).
73. A. Kato, S. Kawozoe, M. Injima, and Y. Shimiza, *J. Ferment. Technol. 54*: 52 (1976).
74. W. E. Goldstein, M. B. Ingle, and L. Lasure, *Plant Tissue Cultures as a Source of Biochemicals* (E. J. Staba, ed.), CRC Press, Florida, 1980, p. 191.
75. W. Barz, A. Beimen, B. Dräger, U. Jaques, Ch. Otto, E. Süper and B. Upmeier, *Secondary Products from Plant Tissue Cultures* (B. Charlwood and M. J. Rhodes, eds.) Oxford Science Publ., New York, 1990, p. 79.
76. M. Wink, *Naturwissenschaften 71*: 635 (1984).
77. T. Murashige and F. Skoog, *Physiol. Plant. 15*: 473 (1962).
78. O. L. Gamborg, R. A. Miller, and K. Ojima, *Exp. Cell. Res. 50*: 151 (1986).
79. P. R. White, *The Cultivation of Animal and Plant Cell* (P. R. White, ed.) Ronald Press, New York, 1963, p. 228.
80. V. Schulte, H. El-Shagi, and M. H. Zenk, *Plant Cell Rep. 3*: 51 (1984).
81. H. M. Nigra, M. A. Alvarez, and A. M. Giulietti, *Plant Cell Tissue Organ Culture 21*: 55 (1990).
82. H. Mizukami, M. Konoshima, and M. Tabata, *Phytochemistry 16*: 1183 (1977).

83. M. H. Zenk, H. El-Shagi, and B. Ulbrich, *Naturwissenschaften 64*: 585 (1977).
84. M. J. Rhodes and R. J. Robins, *The Biochemistry of Plants* (D. D. Davies, ed.), Academic Press, New York, 1987, 13, p. 65.
85. J. M. Bayley, J. King, and O. L. Gamborg, *Planta 105*: 15 (1972).
86. K. Nakagawa, A. Konagai, H. Fukui, and M. Tabata, *Plant Cell Rep. 3*: 254 (1984).
87. M. H. Zenk, H. El-Shagi, and V. Schutte, *Planta Med (Suppl.) 79* (1975).
88. Y. Fujita, Y. Hara, T. Ogino, and C. Suga, *Plant Cell Report 1*: 59 (1981).
89. T. Yamakawa, S. Kato, K. Ishida, T. Kodama, and Y. Minoda, *Agric. Biol. Chem. 47*: 2185 (1983).
90. K. Knobloch and J. Berlin, *Plant Cell Tissue Organ Culture 2*: 333 (1983).
91. O. Schiel, K. Jarcow-Redeck, G. W. Piehl, J. Lehmann, and J. Berlin, *Plant Cell Rep. 3*: 18 (1984).
92. K. H. Knoblock and J. Berlin, *G. Don. Z. Naturforsch. 35C*: 551 (1980).
93. S. Takayama, M. Misawa, K. Ko, and M. Misato, *Physiol. Plant. 41*: 313 (1977).
94. Y. Fujita, Y. Hara, C. Suga, and T. Morimoto, *Plant Cell Report 1*: 61 (1981).
95. D. Kim, H. Pedersen, and C. Chin., *Biotech. Lett. 13*: 213 (1991).
96. J. Dear and S. Aronoff, *Plant Physiol. 40*: 458 (1965).
97. S. Takayama, *Physiol. Plant. 41*: 313 (1977).
98. K. R. Brain, *Plant Sci. Lett. 7*: 157 (1976).
99. T. Ikeda, T. Matsumoto, and M. Noguchi, *Phytochemistry 15*: 586 (1976).
100. E. J. Staba and B. Kaul, *U.S. Patent 3,628287* (1971).
101. R. J. Robins, J. Payne, and M. J. Rhodes, *Planta Med. 52*: 220 (1986).
102. M. H. Zenk, H. El-Shagi, H. Arens, J. Stockigt, E. W. Weiler, and B. Deus, *Plant Tissue Culture and Its Biotechnological Application* (W. Barz, E. Reinhard, and M. H. Zenk, eds.), Springer-Verlag, New York, 1977, p. 27.
103. T. Hashimoto and Y. Yamada, *Agric. Biol. Chem. 51*: 2769 (1987).
104. M. J. Rhodes, J. Payne, and R. J. Robins, *Planta Med.* 226 (1986).
105. K. Lindsey and M. M. Yeoman, *Planta 162*: 495 (1984).
106. J. S. Rokens and I. Goldberg, *Adv. Biotechnol. Proc. 4*: 241 (1985).
107. F. Di Cosmo and G. H. Towers, *Phytochemical Adaptations to Stress* (B. Timmermann, C. Steelink, and F. A. Loewus, eds.), Plenum Publishing, New York, 1984, p. 91.
108. R. Wijnsma, J. T. Go, I. N. van Weerden, P. A. Harkes, R. Verpoorte, and A. B. Svendsen, *Plant Cell Rep. 4*: 241 (1985).
109. J. S. Rokem, J. Schwarzberg, and I. Goldberg, *Plant Cell Rep. 3*: 159 (1984).
110. D. R. Threlfall and I. M. Whitehead, *Manipulating Secondary Metabolism in Culture* (R. J. Robins and M. J. C. Rhodes, eds.), Cambridge University Press, Cambridge, 1988, p. 51.
111. P. Brodelius, M. A. Collinge, C. Funk, K. Gugler, and J. Marques, *Primary and Secondary Metabolism of Plant Cell Cultures* (W. G. W. Kurz, ed.), Springer-Verlag, Berlin, 1989, p. 191.
112. W. Barz, A. Beimen, B. Drager, U. Jaques, C. H. Otto, E. Supper, and B. Upmeier, *Secondary Products from Plant Tissue culture* (B. V. Charlwood and M. J. C. Rhodes, eds.), Oxford Science Publications, Oxford, 1990, p. 79.

113. O. L. Gamborg, *Manual of Industrial Microbiology and Biotechnology* (A. L. Demain and N. A. Solomon, eds.), American Society for Microbiology, Washington, DC, 1986, p. 263.
114. M. Wink, *Secondary products from plant tissue culture* (B. V. Charlwood and M. J. C. Rhodes, eds.), Oxford Science Publications, Oxford, 1990, p. 23.
115. R. E. Spier and J. B. Griffiths (eds.), *Animal Cell Biotechnology*, Academic Press, London, 1985.
116. R. I. Freshney (ed.), *Animal Cell Culture, a Practical Approach*, IRL Press, Oxford, 1986.
117. G. W. Thilly (ed.), *Mammalian Cell Technology*, Butterwoths, Boston, 1986.
118. S. S. Seaver (ed.), *Commercial Production of Monoclonal Antibodies*, Marcel Dekker, New York, 1987.
119. A. S. Lubiniecki (ed.), *Large-Scale Mammalian Cell Culture Technology*, Marcel Dekker, New York, 1990.
120. R. E. Spier and F. Horaud, The biotechnological future for animal cells in culture, *Animal Cell Biotechnology*, Vol. 2, Academic Press, London, 1985, p. 431.
121. C. F. Goochee, M. J. Gramer, D. C. Andersen, J. B. Bahr, and J. R. Rasmussen, *Bio/Technology 9*: 1347 (1991).
122. C. F. Gooche, M. J. Gramer, D. C. Andersen, and J. B. Bahr, The oligosaccharides of glycoproteins: factors affecting their synthesis and their influence on glycoprotein properties, *Frontiers in Bioprocessing II* (P. Todd, M. Bier, and S. K. Sikdar, eds.), American Chemical Society, Washington, DC, 1991, p. 199.
123. M. H. Marino, *Biopharm 2*: 18 (1988).
124. E. Goldwasser, K. Charles, H. Kung, and J. Eliason, *J. Biol. Chem. 249*: 4202. (1974).
125. M. S. Dordal, F. F. Wang, and E. Goldwasser, *Endocrinology 116*: 2293 (1985).
126. S. Dube, J. W. Fisher, and J. S. Powell, *J. Biol. Chem 263*: 17516 (1988).
127. M. Takeuchi, S. Takasaki, M. Shimanda, and A. Kobata, *J. Biol. Chem. 265*: 12127 (1990).
128. M. Takeuchi, N. Inoue, T. W. Strickland, M. Kubota, M. Wada, R. Shimizu, S. Hoshi, H. Kozutsumi, S. Takasaki, and A. Kotaba, *Proc. Natl. Acad. Sci. USA 86*: 7819 (1989).
129. M. Goto, K. Akai, A. Murakami, C. Hashimoto, E. Tsuda, M. Ueda, G. Kawanishi, N. Takahishi, A. Ishimoto, H. Chiba, and R. Sasaki, *Bio/Technology 6*: 67 (1988).
130. J. C. Paulson, *TIBS 14*: 272 (1989)
131. A. Kobata, T. Mizuochi, T. Endo, and K. Furukawa, Function and pathology of the sugar chains of human immunoclobulin G, *Carbohydrate Recognition in Cellular Function* (G. Bock and S. Harnett, eds.), Wiley, Chichester, UK, 1989, p. 224.
132. M. Fountoulakis and R. Gentz, *Bio/Technology 10*: 1143 (1992).
133. R. E. Spier, *Enzyme Microb. Technol. 14*: 591 (1992).
134. L. S. Hana, P. Pine, G. Reuzinsky, S. Nigam, and D. R. Omstead, *Biopharm 4*: 33 (1991).
135. H. Graf, J. N. Rabaud, and J. M. Egly, *J. Immunol. Methods 139*: 135 (1991).
136. S. S. Ozturk and B. O. Palsson, *Hybridoma 9*: 167 (1990).

137. J. M. Megaw and L. D. Johnson, *Proc. Soc. Exp. Biol. Med. 161*: 60 (1979).
138. B. J. Boellering, J. L. Tedesco, R. R. Townsend, M. R. Hardy, R. W. Scott, and C. P. Prior, *Biopharm 3*: 30 (1990).
139. P. Knight, *Bio/Technology 7*: 243 (1989).
140. R. G. Ham and W. L. McKeehan, Media and growth requirements, *Methods Enzymol. 58*: 44 (1979).
141. D. W. Jayme and K. E. Blackman, Culture media for propagation of mammalian cells, viruses, and other biologicals, *Advances in Biotechnological Processes*, Vol. 5 (A. Mizrahi and A. L. van Wezel, eds.), Alan R. Liss, New York, 1985, p. 1.
142. J. Litwin, *Dev. Biol. Standard 42*: 37 (1979).
143. C. Waymouth, R. G. Ham, and P. J. Chapple (eds.), *The Growth Requirements of Vertebrate Cells in Vitro*, Cambridge University Press, London, 1981.
144. K. J. Lambert and J. R. Birch, Cell growth media, *Animal Cell Biotechnology*, Vol. 1 (R. E. Spier and J. E. Griffiths, eds.), Academic Press, New York, 1985, p. 85
145. M. Ratafia, *Bio/Technology 7*: 574 (1989).
146. G. H. Sato, A. B. Pardee, and D. A. Sirbasku (eds.), *Growth of Cells in Hormonally Defined Media*. Books A and B, Cold Spring Harbor Conferences in Cell Proliferation, Vol. 9, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
147. H. Murakami, Serum-free media used for cultivation of hybridomas, *Monoclonal Antibodies: Production and Application*; Advances in Biotechnological Processes, Vol. II, Alan R. Liss, New York, 1989, p. 107.
148. E. Shacter, *TIBTECH 7*: 248 (1989).
149. H. R. Maurer (ed.), Special issue: Serum-free media and cell cultures, *Cytotechnology 5*: 1 (1991).
150. M. V. Caple, T. R. Fletcher, W. J. Owens, S. Harada, T. J. Murphy, and B. J. Walthall, *Biopharm 5*: 52 (1992).
151. U. Bjare, *Pharmacol. Ther. 53*: 355 (1992).
152. W. G. Hamilton and R. G. Ham, *In Vitro 13*: 537 (1977).
153. W. L. Cleveland, I. Wood, and B. F. Erlanger, *J. Immunol. Methods 56*: 221 (1983).
154. J. Kovar and F. Franek, *Biotechnol. Lett. 9*: 259 (1987).
155. F. J. Darfler, *In Vitro Cell. Dev. Biol. 26*: 769 (1990).
156. F. J. Darfler, *In Vitro Cell. Dev. Biol 26*: 779 (1990).
157. R. L. Tarleton and A. M. Beyer, *Biotechniques 11*: 590 (1991).
158. R. M. Fike, J. L. Pfohl, D. A. Epstein, D. W. Jayme, and S. Weiss, *Biopharm 4*: 26 (1991).
159. K. J. Morrow, *Genet. Eng. News 11*: 8 (1991).
160. K. J. Morrow, *Genet. Eng. News 12*: 6 (1992).
161. C. N. Durfor and C. L. Scribner, An FDA perspective of manufacturing changes for products in human use, *Biochemical Engineering VII* (H. Pedersen, R. Mutharasan, and D. DiBiasio, eds.), *Ann. N. Y. Acad. Sci. 661*: 356 (1992).
162. D. F. Fink, L. M. Curran, and B. R. Allen (eds.), *Research Needs in Nonconventional Bioprocesses*, Battell Press, Columbus, OH, 1983.
163. S. S. Seaver, *Can J. Chem. Eng. 69*: 403 (1991).

164. D. W. Jayme, *Cytotechnology 5*: 15 (1991).
165. E. C. Jo, H. J. Park, J. M. Park, and K. H. Kim, *Biotechnol Bioeng 36*: 717 (1990).
166. G. Schmid, H. Zilg, U. Eberhard, and R. Johannsen, *J. Biotechnol. 17*: 155 (1991).
167. R. H. Goodwin, *Nature 347*: 209 (1990).
168. Y. T. Luan, R. Mutharassan, and W. E. Magee, *Biotechnol. Lett. 9*: 691 (1987).
169. B. Schummp and E. J. Schlaeger, *J. Cell Sci. 97*: 639 (1990).
170. D. Duval, C. Demangel, K. Munier-Jolain, S. Miossee, and I. Geabel, *Biotechnol. Bioeng. 38*: 561 (1991).
171. M. Dalili and D. F. Ollis, *Biotechnol. Lett. 10*: 781 (1988).
172. D. P. Palermo, M. E. Degraaf, K. R. Marotti, E. Rehberg, and L. E. Post, *J. Biotechnol. 19*: 35 (1991).
173. F. J. Castillo, J. Thrift, L. Mullen, G. Bostic, and W. C. McGregor, Growth and antibody production by murine hybridomas in serum-free medium, 196th American Society Meeting, Los Angeles, CA, 1988.
174. S. Reuveny, D. Velez, F. Riske, J. D. Macmillan, and L. Miller, *Dev. Biol. Standard. 60*: 185 (1985).
175. P. D. Cooper, J. N. Wilson, and A. M. Burt, *J. Gen. Microbiol 21*: 701 (1959).
176. E. P. Cohen and H. Eagle, *J. Exp. Med. 113*: 467 (1961).
177. S. J. Pirt and D. S. Callow, *Exp. Cell Res. 33*: 413 (1964).
178. J. B. Griffiths and S. J. Pirt, *Proc. R. Soc. B. 168*: 421 (1967).
179. C. Peraino, S. Bacchetti, and W. J. Eisler, *Science 169*: 204 (1970).
180. M. G. Tovey, G. E. Mathison, and S. J. Pirt, *J. Gen. Virol. 20*: 29 (1973).
181. M. G. Tovey and D. Brouty-Boye, *Exp. Cell Res 101*: 346 (1976).
182. M. G. Tovey and D. Brouty-Boye, *Exp. Cell Res. 118*: 383 (1979).
183. M. G. Tovey, The cultivation of animal cells in the chemostat: application to the study of tumor cell multiplication, *Advances in Cancer Research* (G. Klein and S. Weinhouse, eds.), Academic Press, New York, 1980, Vol. 33, p. 1.
184. S. Fazekas de St. Groth, *J. Immunol. Methods 57*: 121 (1983).
185. E. Kromer, D. Lutz, and W. Scheirer, *Dev. Biol. Standard 55*: 129 (1984).
186. A. L. van Wezel. C. A. M. van der Velden-de Groot, H. H. de Haan, N. van den Heuvel, and R. Schasfoort, *Dev. Biol. Standard. 60*: 229 (1985).
187. J. R. Birch, P. W. Thompson, K. Lambert, and R. Boraston, The large scale cultivation of hybridoma cells producing monoclonal antibodies, American Chemical Society Meeting, Philadelphia, 1984.
188. J. R. Birch, K. Lambert, R. Boraston, P. W. Thompson, S. Garland, and A. C. Kenney, *The Industrial Production of Monoclonal Antibodies in Cell Culture*, NATO, ASI Meeting, Plenum, New York, 1985.
189. F. J. Castillo, J. Thrift, G. Traugh, T. Bruggman, L. Mullen, and W. C. McGregor, Growth and monoclonal antibody production by several hybridomas in continuous culture, 194th American Chemical Society Meeting, New Orleans, LA, 1987.
190. F. J. Castillo, Continuous culture applications in biotechnology, Fermentation in the 1990s Symposium, La Jolla, CA, 1989.
191. W. M. Miller, C. R. Wilke, and H. W. Blanch, *J. Cell Physiol. 132*: 524 (1987).
192. W. M. Miller, C. R. Wilke, and H. W. Blanch, *Bioprocess Eng. 3*: 113 (1988).
193. G. J. MacMichael, *Hybridoma 8*: 117 (1989).

194. T. S. Shaughnessy and F. Kargi, *Enz. Microb. Technol. 12*: 676 (1990).
195. S. S. Ozturk and B. O. Palsson, *Biotechnol. Prog. 6*: 437 (1990).
196. T. I. Linardos, N. Kalogerakis, L. O. Behie, and L. R. Lamontagne, *Can J. Chem. Eng. 69*: 429 (1991).
197. D. E. Martens, C. D. de Gooijer, C. A. M. van der Velden-de Groot, E. C. Beuvery, and J. Tramper, *Biotechnol. Bieng. 41*: 429 (1993).
198. S. Seaver, Culture method affects antibody secretion of hybridoma cells, *Commercial Production of Monoclonal Antibodies; A Guide for Scale-Up* (S. Seaver, ed.). Marcel Dekker, New York, 1987, p. 49.
199. W. R. Kidwell, *Bio/Technology 7*: 462 (1989).
200. G. M. Lee and B. O. Palsson, *Biotechnol. Bioeng. 36*: 1049 (1990).
201. F. J. Castillo, L. J. Mullen, J. C. Thrift, and B. C. Grant, Perfusion cultures of hybridoma cells for monoclonal antibody production, *Biochemical Engineering VII* (H. Pedersen, R. Mutharasan, and D. DiBiasio, eds.), *Ann. N. Y. Acad. Sci. 661*: 72 (1992).
202. F. J. Castillo, L. J. Mullen, J. C. Thrift, B. C. Grant, J. DeLeon, and D. Burke, Extended perfusion cultures for MAb production, 203rd American Chemical Society Meeting, San Francisco, CA, 1992.
203. F. J. Castillo, L. J. Mullen, J. C. Thrift, B. C. Grant, and D. Burke, Extended perfusion culture of hybridoma in hollow fiber bioreactor, 9th International Biotechnology Symposium, Crystal City, VA, 1992.
204. J. R. Birch and D. J. Edwards, *Dev. Biol. Standard. 46*: 59 (1980).
205. O. W. Merten, S. Reiter, and H. Katinger, *Dev. Biol. Standard. 60*: 509 (1985).
206. S. Reuveny, D. Velez, J. D. Macmillan, and L. Miller, *J. Immunol. Methods 86*: 53 (1986).
207. S. Reuveny, D. Velez, L. Miller, and J. D. Macmillan, *J. Immunol. Methods 86*: 61 (1986).
208. A. McQueen, E. Meilhoe, and J. E. Bailey, *Biotechnol. Lett. 9*: 831 (1987).
209. G. M. Lee, T. K. Huard, M. S. Kaminski, and B. O. Palsson, *Biotechnol. Lett. 10*: 625 (1988).
210. S. K. W. Oh, A. W. Nienow, M. Al-Rubeai, and N. A. Emery, *J. Biotechnol. 12*: 45 (1989).
211. A. R. Gardner, J. L. Gainer, and D. J. Kirwan, *Biotechnol. Bioeng. 35*: 940 (1990).
212. J. F. Petersen, L. V. McIntire, and E. T. Papoutsakis, *Biotechnol. Prog. 6*: 114 (1990).
213. G. M. Lee and B. O. Palsson, *Biotechnol. Bioeng. 36*: 1049 (1990).
214. G. K. Sureshkumar and R. Mutharasan, *Biotechnol. Bioeng. 37*: 292 (1991).
215. J. Baker, *Am. Biotechnol. Lab. 9*: 24 (1991).
216. D. Kern and B. Fujimoto, *Am. Biotechnol. Lab. 10*: 48 (1992).
217. United States Pharmacopoeal Convention, USP XXII, NF XVIL USP, Rockville, MD, 1989, p. 1456.
218. J. Mather, F. Kaczarowski, R. Gabler, and F. Wilkins, *Bio Techniques 4*: 56 (1986).
219. Danforth, Della Piana, and Smith, *Am. Biotechnol. Lab. 5*: 58 (1987).
220. Hyclone Labs., *Art to Science 11*: 1 (1992).
221. R. W. Johnson, *Biopharm 3*: 40 (1990).

222. L. Brim, K. Kiefer, G. Hanson, and B. Fujimoto, *Am. Biotechnol. Lab. 9*: 33 (1991).
223. G. L. Tritsch and G. E. Moore, *Exp. Cell Res. 28*: 360 (1962).
224. T. J. Murphy and M. V. Caple, *Am. Biotechnol. Lab 10* (1992).
225. D. W. Jayme, Improved bioreactor productivity through implementation of liquid medium concentrates, Bio East '93, Washington, DC, 1993.
226. M. Misawa, Production of useful plant metabolites, *Adv. Biochem. Eng. 31*: 59 (1985).
227. M. Tabata, Secretion of secondary products by plant cell cultures, *Proceedings, 8th International Biotechnology Symposium* (G. Durand, L. Bobichon, and J. Florent, eds.), Vol. I, Société Française de Microbiologie, Paris, 1988, p. 167.

5

Fundamentals of Bioreactor Design

José C. Merchuk*

Ben Gurion University of the Negev
Beersheva, Israel

Juan A. Asenjo

University of Chile
Santiago, Chile
and University of Reading
Reading, England

I. INTRODUCTION

The range of possible yield of a reactor in which a process is due to take place is limited by basic principles derived from the conservation of mass (stoichiometry) and energy (thermodynamics). Those limits remain valid as well in the case of the biological and biochemical processes, which are the subject of this book. The design of the reactor for a given output within the range mentioned must be based on knowledge of the rate at which the process takes place (kinetics).

Although the design of the reactor for any process, be it chemical, biochemical, or biological, must be based on those three elements (stoichiometry,

*Current affiliation: Visiting professor, University of Reading, Reading, England.

thermodynamics, and kinetics), the complexity of the relationships may change by orders of magnitude when one deals with living cells. The biochemical engineer must keep in mind, when using simple relationships to calculate and design, that the reality of the process he is dealing with is immensely more complex and sophisticated than the simplified models he will be using.

The kinetic understanding of the process will allow the design of the bioreactor, and it is an absolutely essential element for the design of the peripheral services as well as control and heat exchange (Chapters 15 and 16). The stoichiometry and the thermodynamics constitute the link between the bioreactor, the upstream stages of the bioprocess (Chapters 3–5), and the downstream stages of product separation and purification.

II. STOICHIOMETRY

Stoichiometry is one of the pillars of reactor design. It gives the basic elements for the rational design of the medium to be used in a process. Coupled with mass balance, it gives means of checking the satisfactory function of a process and may be used to measure indirectly a variable otherwise elusive or difficult to measure (gases absorbed or desorbed, for example). Coupled with thermodynamic considerations, it renders the range of possible yields for a given system.

All of this holds in principle for bioreactor design as well. But whereas in chemical reactions stoichiometry is usually relatively easy to determine and can be known accurately, biological reactions are full of uncertainties in this area. It is almost impossible to follow all the components of a complex medium, and only part of the products generated are recognized. In addition, the activity of the culture changes during the different phases of growth.

A. The Overall Equation for the Biochemical Reaction

The first authors to propose a sound approach to the stoichiometry of biological reactors were Minkevich and Eroshin [1]. Their model establishes a general relationship between a single carbon- and energy-yielding substrate, biomass and product formation (including water and CO_2), and nitrogen and oxygen requirements. Cells, substrate, and products are defined using a single-carbon chemical formula, or single-carbon formula (SCF). A single product is considered, and the stoichiometric balance is

$$CH_mO_l + aNH_3 + bO_2 \rightarrow Y_b^c CH_pO_nN_q(\text{biomass}) + Y_p^c CH_rO_sN_t(\text{product}) + cH_2O + dCO_2 \quad (1)$$

Here Y_b^c and Y_p^c are the yields of biomass and product, respectively, per single-carbon formula weight of the substrate. The requirements for oxygen and nitrogen are expressed by a and b respectively. All these variables are thus interrelated in an organic way by elemental conservation relationships. Since only one carbon enters the biochemical reaction, the carbon balance is

$$1 = Y_b^c + Y_p^c + d \quad (2)$$

The balances on nitrogen, oxygen, and hydrogen are

$$a = qY_b^c + tY_p^c \quad (3)$$

$$l + 2b = nY_b^c + sY_p^c + c + 2d \quad (4)$$

$$m + 3a = gY_b^c + rY_p^c + 2c \quad (5)$$

This implies that if the yields are known, the amounts of ammonia and oxygen used and carbon dioxide and water produced are univocally given by these equations. In the same way, carefully done measurements of the in- and off-gases and of nitrogen consumed can help in identifying the yields.

The degree of reductance of substrate and products can be used to formulate available electron balances, which establish additional relationships. The concept of available electrons was first related to yields on substrates by Payne [2] and can be defined as the amount of electrons transferred to molecular oxygen during complete combustion of the organic substrate. There are 4 per atom of C, 1 per atom of hydrogen, −3 per atom of nitrogen, and −2 per atom of oxygen. Therefore, there are none in NH_3, CO_2, or H_2O. The reductance degree, ν, is defined as the amount of available electrons in a SCF of an organic compound.

The available electrons are conserved during a biochemical reaction, and a balance on reaction (1) can be written [3] as

$$\nu_s + (-4)b = Y_b^c\nu_b + Y_b^c\nu_p \quad (6)$$

Equation (6) can be rewritten as

$$\eta_h + \eta_b + \eta_p = 1 \quad (7)$$

where

$$\eta_h = \frac{4b}{\nu_s}, \quad \eta_b = \frac{Y_b^c\nu_b}{\nu_s}, \quad \eta_p = \frac{Y_p^c\nu_p}{\nu_s} \quad (8)$$

The first term in Eq. (7), η_h, is the fraction of the available electrons of the substrate which are transferred to oxygen; but since the amount of heat evolved per available electron, Q_o, is approximately constant [1], it follows that η_h can also be considered the fraction of the energy originally present in the substrate which is transformed into heat. The second term, η_b, is the energetic yield coefficient for biomass production. It is the ratio of the energy content of the produced biomass to the energy content of the substrate consumed. Similarly,

η_p, the energetic yield for product formation, is the fraction of the chemical energy originally in the organic substrate which is retained by the product. Thus, Eq. (7) can be useful in combining mass and energy balances. Erickson et al. [3,4] show how the stoichiometric ratios, such as the ammonia-to-oxygen ratio, the ammonia-to-carbon ratio, and the respiratory quotient, can be used to improve the quality of measurements during culture growth and product formation, checking the consistency of data.

All of this is valid for the case of NH_3 being the source of nitrogen. A generalized degree of reduction was later defined by Roels [5], to take into account a source of nitrogen with valence other than 3 and also other elements such as phosphorus and sulfur.

A factor which may be of help in the design of a bioreactor system is the recognition that some parameters remain almost constant for a wide range of biochemical reactions. As mentioned above, Minkevich and Eroshin showed that the heat effect per available electron can be taken as constant (26.95 kcal/eq available electron transferred to oxygen). Also, the biomass produced per available electron is fairly constant (3.14 g dry wt./eq. electron), as long as the biological process is energy controlled.

This last limitation stems from the fact that a single substrate is used, which is the sole source of both energy and carbon. Under these conditions, a critical degree of reduction of substrate can be defined, above which carbon supply becomes limiting and the growth yield remains constant. Below this limit, the growth yield increases as the degree of reduction of the substrate does. Since the substrate is poor in energy (low reductance degree), an increase in ν_s will increase the amount of the substrate carbon which can be assimilated, and the biomass yield will increase. Growth rate is limited by energy supply [6]. But after a certain substrate-reductance degree (which is, significantly, very close to the typical reductance degree of biomass, approximately 4.8), more energy than necessary for fixing one atom of carbon will be available in each SCF of substrate, and therefore an increase in ν_s will not have any effect on the yield (and the energetic yield, for biomass production, η_b, will consequently decrease). This limitation, with the additional limitation of the second law of thermodynamics, can be seen in Fig. 1 [6]. The data shown correspond to a series of substrates ranging from oxalic acid ($\nu_s = 1$) to methane ($\nu_s = 8$).

Another regularity is that ATP production is closely related to yields. Bauchop and Elsden [7] first concluded that the amount of biomass produced was directly proportional to the ATP that could be produced by degradation of the energy-yielding substrate. Practical exploitation of this conclusion, however, requires an exact knowledge of the catabolic pathways. But even in the case of anaerobic processes, where the pathways are better understood, production of energy in addition to ATP has ben proposed. Stouthamer and van Verseveld [8] have produced a very useful review of this subject. Figure 2 shows a general

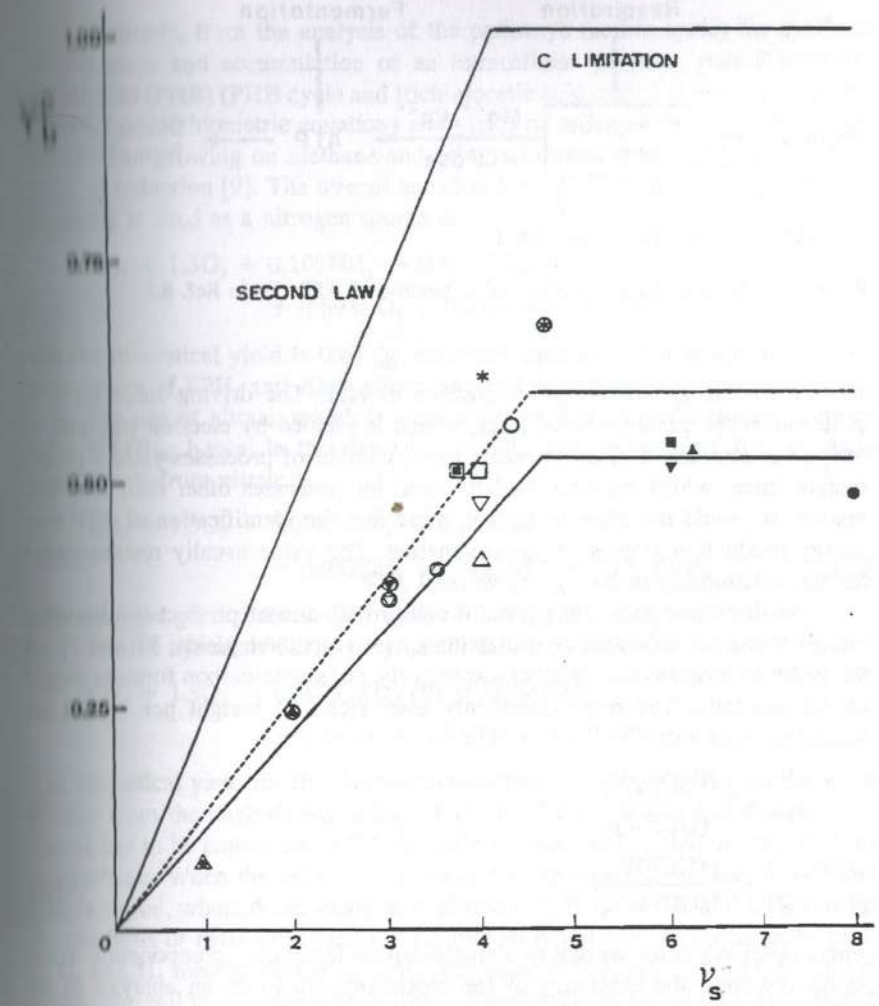


Figure 1 Influence of the degree of reductance of the substrate on yield coefficient, including the limits established by carbon limitation and the second law of thermodynamics. (From Ref. 5.)

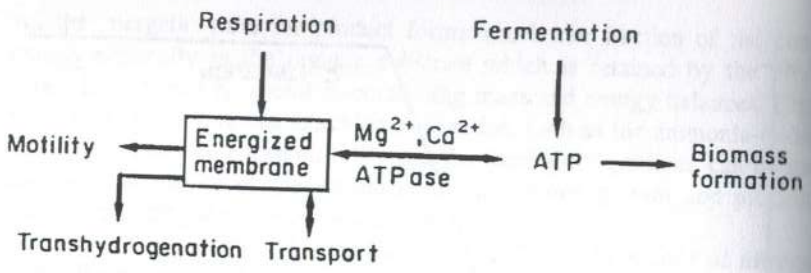


Figure 2 Scheme for generation and utilization of ATP. (From Ref. 8.)

scheme for the generation and utilization of ATP. The driving force for ATP synthesis is the proton motive force, which is yielded by electron transport to oxygen or alternative electron acceptors. A number of processes yield a proton motive force, which may be partially used for processes other than ATP formation. It should therefore be kept in mind that the identification of ATP with energy production is only an approximation. The value usually recommended for this relationship is 10.5 g dry wt./mol ATP.

As discussed above, the rates of cell growth and of product synthesis are related to the rate of substrate utilization by the respective yields; Y_b^c and Y_p^c are the yields in biomass and product respectively, per single-carbon formula weight of the substrate. The more commonly used yields in weight per weight are related to these by

$$Y_{x/s} = Y_b^c \frac{(\mathrm{UCFW})_b}{(\mathrm{UCFW})_x} \tag{9}$$

$$Y_{p/s} = Y_p^c \frac{(\mathrm{UCFW})_p}{(\mathrm{UCFW})_x} \tag{10}$$

where $(\mathrm{UCFW})_i$ is the weight of a single-carbon formula of component i. These yields represent the efficiency of the bioconversion. From an analysis of the metabolic pathways it is possible to calculate the maximum theoretical yield when synthesizing a product. This can be a very useful guide in the evaluation of results. For instance, from the analysis of the pathways in the anaerobic fermentation of glucose to ethanol by yeast the following stoichiometric equation is found:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \tag{11}$$

and thus the maximum theoretical yield is 2 mol ethanol per mol of glucose or 0.51 g/g. In practice this maximum is not possible due to some formation of cellular mass, but it is possible to reach 90–95% of this value.

Similarly, from the analysis of the pathways (serine cycle) for synthesis of cell mass and accumulation of an intracellular polymer, poly-β-hydroxybutyric acid (PHB) (PHB cycle and trichloracetic acid cycle), it has been possible to find the stoichiometric equations and yields of cell growth and polymer synthesis when growing on methane and using nitrogen sources with different degrees of reduction [9]. The overall equation for cell mass growth on methane if ammonia is used as a nitrogen source is

$$CH_4 + 1.5O_2 + 0.102NH_3 \rightarrow 0.408CH_2O_{0.5}N_{0.25} + 0.593CO_2 + 0.61ATP + 0.136FPH_2 \tag{12}$$

and the theoretical yield is 0.65 (g biomass/g methane). For simplicity the oxidized form of FPH_2 and ADP, phosphate, and H_2O have been omitted.

The use of nitrate, which is a more oxidized source of nitrogen, requires more $NADH_2$; hence, in this case the overall equation obtained for cell mass biosynthesis from nitrate is

$$CH_4 + 1.5O_2 + 0.072HNO_3 \rightarrow 0.289CH_2O_{0.5}N_{0.25} + 0.952CO_2 + 1.012ATP + 0.096FPH_2 \tag{13}$$

This equation gives a theoretical yield of 0.46 (g biomass/g methane).

For the synthesis of the polymer PHB the overall equation was

$$CH_4 + 1.5O_2 \rightarrow 0.5CH_{1.5}O_{0.5}\ (\text{PBH monomer}) + 0.5CO_2 + 1.5ATP + 0.125FPH_2 \tag{14}$$

The theoretical yield for this bioconversion then is 0.67 (g PHB/g methane). It is clear from the analysis that a large fraction of the methane and oxygen consumed has to be converted to CO_2 in order to generate the $NADH_2$ required for biosynthesis. When the cells are synthesized using ammonia 0.61 mol ATP/mol CH_4 is stored, whereas the value with nitrate is 1.01 mol ATP/mol CH_4 and for the synthesis of PHB from methane 1.5 mol ATP/mol CH_4. In all cases the ratio of O_2 to CH_4 metabolized is constant and equal to 1.5, whereas CO_2 evolution varies. For cell mass biosynthesis from ammonia the moles of CO_2 synthesized per mole of CH_4 and O_2 used is 0.237. From nitrate it is 0.323 and for PHB biosynthesis it is 0.222 [9].

Yield coefficients based on other substrates such as oxygen may be defined. For organisms growing aerobically on glucose, $Y_{X/S}$ is typically 0.4 to 0.6 g/g for most yeast and bacteria, while $Y_{X\,O_2}$ is 0.9 to 1.4. Anaerobic growth is less efficient and the yield coefficient is reduced substantially (see Fig. 3). With substrates that are more or less reduced than glucose, the value of the apparent yield coefficient will change. For methane, $Y_{X/S}$ would assume values of 0.6 to 1.0 g/g, with the corresponding $Y_{X\,O_2}$ decreasing to about 0.2 g/g. In most cases the yield of biomass on a carbon-energy source is 1.0 ± 0.4 g biomass per g

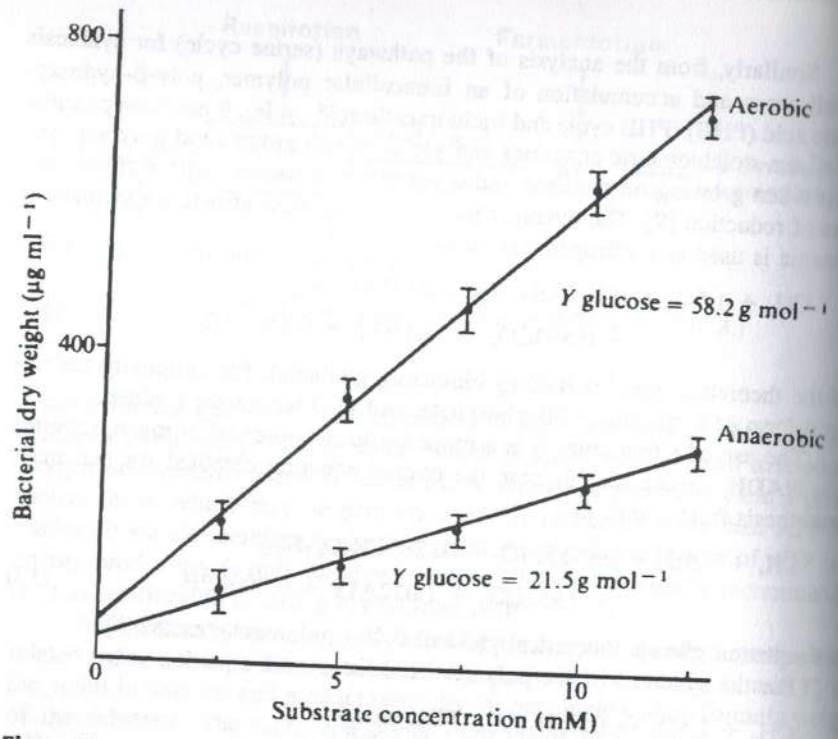


Figure 3 Aerobic and anaerobic growth yields of *Streptococcus faecalis* with glucose as substrate. (From Ref. 36.)

of carbon consumed. Table 1 lists some examples of $Y_{X/S}$ and $Y_{X\,O_2}$ for a variety of substrates and organisms.

B. Maintenance

Ample evidence shows that yields on substrate are dependent on the specific growth rate μ. This phenomenon has been explained as due to maintenance. This is the energy required for the replacement of denatured proteins, preservation of the optimal intracellular pH, active transport which counterbalances the leaks through the cytoplasmic membrane, ATP consumption in futile cycles, motility, etc. From the thermodynamic point of view, the concept of maintenance is very relevant. It is energy invested in keeping the cell in an ordered state, compensating for the creation of entropy in the system, and avoiding the equilibrium state, which means cell death. Herbert [10], and later Pirt [11] assumed that the consumption of the energy-yielding substrate was partially growth dependent (substrate utilized for biomass generation) and partially growth independent, but dependent on the

Table 1 Summary of Yield Factors for Aerobic Growth of Different Microorganisms on Various Carbon Sources

Organism	Substrate	$Y_{X/S}$ g/g	$Y_{X/S}$ g/mol	$Y_{X/S}$ g/(g−C)	$Y_{X\,O_2}$[a] g/g
Aerobacter aerogenes	Maltose	0.46	149.2	1.03	1.50
	Mannitol	0.52	95.2	1.32	1.18
	Fructose	0.42	76.1	1.05	1.46
	Glucose	0.40	72.7	1.01	1.11
Candida utilis	Glucose	0.51	91.8	1.28	1.32
Penicillium chrysogenum	Glucose	0.43	77.4	1.08	1.35
Pseudomonas fluorescens	Glucose	0.38	68.4	0.95	0.85
Rhodopseudomonas spheroides	Glucose	0.45	81.0	1.12	1.46
Saccharomyces cerevisiae	Glucose	0.50	90.0	1.25	0.97
Enterobacter aerogenes	Ribose	0.35	53.2	0.88	0.98
	Succinate	0.25	29.7	0.62	0.62
	Glycerol	0.45	41.8	1.16	0.97
	Lactate	0.18	16.6	0.46	0.37
	Pyruvate	0.20	17.9	0.49	0.48
	Acetate	0.18	10.5	0.43	0.31
Candida utilis	Acetate	0.36	21.0	0.90	0.70
Pseudomonas fluorescens	Acetate	0.28	16.8	0.70	0.46
Candida utilis	Ethanol	0.68	31.2	1.30	0.61
Pseudomonas fluorescens	Ethanol	0.49	22.5	0.93	0.42
Kitbsiella sp.	Methanol	0.38	12.2	1.01	0.56
Methylomonas sp.	Methanol	0.48	15.4	1.28	0.53
Pseudomonas sp.	Methanol	0.41	13.1	1.09	0.44
Methylococcus sp.	Methane	1.01	16.2	1.34	0.29
Pseudomonas sp.	Methane	0.80	12.8	1.06	0.20
Pseudomonas sp.	Methane	0.60	9.6	0.80	0.19
Pseudomonas methanica	Methane	0.56	9.0	0.75	0.17

[a] $Y_{X\,O_2}$ is the yield factor relating grams of cells formed per gram of O_2 consumed.
Source: Ref. 20.

amount of biomass present in the system (substrate providing the energy for maintenance). Following different approaches, both arrived at the following equation, which can be considered the definition of maintenance:

$$\frac{1}{Y_{xs}} = \frac{1}{Y_{xs}^{max}} + \frac{m_s}{\mu} \tag{15}$$

Equation (15) indicates that the yield of biomass on substrate will increase as the reaction rate increases. If the second term on the right-hand side becomes

negligible, the yield reaches its maximal value, Y_{xs}^{max}. It should be kept in mind that while Y_{xs} is the ratio of experimentally observed changes in the concentrations of cell mass and substrate, Y_{xs}^{max} is only the parameter of a model.

If both terms in Eq. (15) are multiplied by μ, the following expression, sometimes called the linear equation for substrate consumption, can be obtained:

$$\sigma = \frac{\mu}{Y_{xs}^{max}} + m_s \tag{16}$$

where σ is the rate of substrate consumption per unit biomass synthesized. Roels and Kossen [12] proposed a generalization of the linear equation of biomass growth, including the case of product formation:

$$\sigma = \frac{\mu}{Y_{xs}^{max}} + \frac{\pi}{Y_{ps}^{max}} + m_s \tag{17}$$

where π is the rate of product formation per unit biomass. A similar expression was formulated for oxygen consumption.

The linear equations for substrate and oxygen consumption are important elements for the design of bioreactors. Rates can be predicted, and the change of the yield coefficient during the history of the culture can be modeled as a function of the biomass specific growth rate.

The form of the linear Eq. (16) described above implies that the substrate is consumed by two independent reactions and therefore cannot be reconciled with the simple scheme presented in Eq. (1). On the other hand, this is to be expected. If the definition of different phases of growth means that the anabolic/catabolic pathways change during the history of a culture, it seems reasonable to consider that stoichiometry may change as well, new products may appear, etc. It cannot be expected that the same stoichiometric relationship will be valid for the complete growth and production phases. The analysis by Erickson et al. [3,4] is valid only as long as the stoichiometric coefficients do not change. In spite of this apparent lack of generality, it represents with fair accuracy the operation range of industrial interest and is therefore very useful from the engineering practical point of view of bioreactor design.

Andrews [13] recently proposed a more sophisticated model including four biochemical pathways, each with a defined reaction rate: anabolism, catabolism, respiration, and product formation. In this scheme, the number of independent rate equations is constrained by the balances of elements, available electrons, and energy in the form of ATP. The author proposes the use of this approach in order to select the minimum number of rate equations needed in order to describe accurately the bioprocess in the formulation of a control strategy.

III. KINETICS OF CELL POPULATION GROWTH, PRODUCT FORMATION, AND SUBSTRATE UTILIZATION

The rate of the changes in the concentrations of biomass, substrates, and products are all required in order to design and optimize the operation of a bioreactor. Data on the kinetics of microbial growth, cell population distribution, product synthesis, and substrate utilization are essential for the prediction of the performance and control, as well as system optimization. It is important to know how the environmental parameters (e.g., pH, temperature, chemical composition) affect the system kinetics. In some cases the use of simplified models can be sufficient for system design. However, in others there may be advantages in the use of structured and segregated models. This is the case in the description of enzyme synthesis by induction and repression; the detailed modeling of a metabolic pathway can be used to overcome bottlenecks of metabolism, the modeling of recombinant cells to account for plasmid stability, the modeling of mammalian cell cultures to account for active versus total cells, or even the distribution of cells to account for product distribution within a culture [14], and the modeling of a plant cell culture to account for cell viability and its effect on secondary metabolite production [15].

Since the process is driven by the cells, it is only natural that the classification of the systems has been centered on the question of the relationship of all other rates to the rate of growth of a cell population. The main approaches that have been used for modeling the kinetics of growth of a cell population are discussed next.

A. Cell Population Kinetics

When cells grow in a batch culture, as they do generally in nature and in most industrial processes, a series of phases can usually be identified: a lag phase after inoculation, an exponential growth phase in which cell numbers and usually biomass are at their maximum specific growth rate on a particular substrate, a deceleration phase, a stationary phase, and a decline phase. Figure 4 [16] shows a typical growth curve, as observed by measurements of optical density, particle counter, and plate count. The figure reveals that there are some differences depending on the method used to follow the increase in biomass. The curves shown describe the history of a bacterial culture in which neither product inhibition nor transport limitations occur. The nature of the first phenomenon is relatively well known and understood: A certain concentration of the product formed inhibits the growth rate. The second one, the limitation by transport phenomena, is less well known and is sometimes inaccurately understood. Microbiologists are familiar with limitations in growth rate caused by depletion of a necessary substrate. Such limitation, being concentration dependent, is, in a batch culture, also time dependent. The growth rate will decrease as the limiting substrate

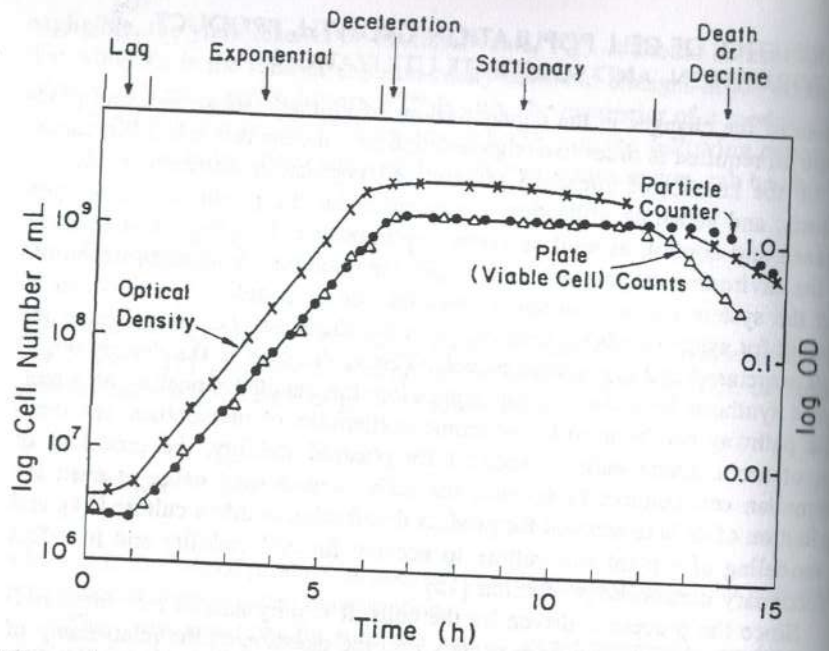


Figure 4 Typical growth curve for a bacterial population, showing the influence of the variable measured. (From Ref. 16.)

becomes scarcer. When this nutrient is depleted the cells will turn to another possible nutrient (e.g., carbon source) until all the nutrients available are depleted and growth stops altogether. On the other hand, transport limitation is usually related to external phenomena which determine the maximal rate of the process. The observable result is stabilization of the growth at a rate which remains constant over quite a wide range of biomass and substrate concentration and is lower than the maximum potential growth rate of the cells on a particular substrate. This rate will remain constant as long as the conditions do not change. Eventually one of the substates will be depleted to such a level that the possible growth rate is in the same range as the rate of the limiting transport process. The growth rate will begin to decrease as substrate limitation is reached, until it finally stops. A typical example is light limitation during algal growth (Chapter 12). The biomass will increase at a constant rate which depends on the rate of photon uptake, until the source of nitrogen becomes limiting. A similar situation has been reported in the case of oxygen transport limitation [17]. By increasing the aeration rate, a linear growth phase previously found was eliminated and a conventional exponential phase, with a higher growth rate, was shown. These

examples correspond in fact to fed-batch operation, when the rate of the process is controlled by the feed rate of one substrate (see Chapter 13).

Sometimes transport limitation may also occur in closed batch systems, if the rate of transport from the bulk of the medium to the microorganism is controlled by diffusion. In this case the period of constant growth rate may be shortened because the mass transfer is a function of the concentration of the key component transferred. Mass transfer limitation in immobilized systems (Chapters 9 and 10) will produce similar effects. Rotem et al. [18] showed the influence of the mass transfer of nutrients on the growth of *Porphyridium* sp. by increasing the viscosity of the broth. Lower growth rates were obtained as the initial viscosity of the broth increased.

During the exponential growth phase (Fig. 4) the cell population grows at the maximum specific growth rate on that particular substrate. This increase in biomass is characterized by the time required to double the cell mass or the number of cells, also called doubling time, t_d, which is constant. During exponential growth, Eq. (18) is valid, where μ is the specific growth rate and X is the biomass concentration in g/L.

$$\frac{dX}{dt} = \mu X \tag{18}$$

This equation can also be written in terms of cell number (N):

$$\frac{dN}{dt} = \mu N \tag{19}$$

where it has been assumed that N is directly proportional to X.

Integrating Eq. (18) and calling X_0 the biomass concentration at time zero:

$$\ln \frac{X}{X_0} = \mu t \tag{20}$$

and hence the doubling time, t_d, is

$$t_d = \frac{\ln 2}{\mu} \tag{21}$$

This simplified model is largely true for bacteria and yeast. Deviations observed when using fungi (linear instead of exponential growth) or mammalian cells (rise of cell numbers and not biomass in g/L) will be analyzed later in this chapter. The deceleration growth phase which follows the exponential phase (Fig. 4) is usually due to either depletion of one or more essential nutrients or accumulation of inhibitory products or by-products of growth.

The effect of both substrate depletion and product inhibition will be analyzed in the next sections. However, if the decelerating growth phase is due to

substrate depletion an equation that has been used successfully in a number of cases is the so-called Monod equation:

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{22}$$

where μ_{max} is the maximum specific growth on a particular substrate when $S >> K_s$, and K_s is a saturation constant and is equal to the value of S at which $\mu = \mu_{max}/2$. This equation is semiempirical, as discussed in the next sections, and in practice K_s is very small (see Table 2).

When toxic metabolites accumulate a number of product inhibition models can be used as will be discussed in Section III.D. However, a simple equation that has been used successfully in many cases is the semiempirical logistic

Table 2 Some Values for K_s in the Monod Model for Growth on Different Substrates

Microorganism	Substrate	K_s (mg L^{-1})
Aspergillus	Arginine	0.5
	Glucose	5.0
Candida	Glycerol	4.5
	Oxygen	0.45
Cryptococcus	Thiamine	1.4×10^{-7}
Enterobacter (Aerobacter) aerogenes	Ammonia	0.1
	Glucose	1.0
	Magnesium	0.6
Escherichia coli	Glucose	2.0–4.0
	Lactose	20.0
	Mannitol	2.0
	Phosphate	1.6
	Tryptophan	0.001
Hansenula polymorpha	Methanol	120.0
	Ribose	3.0
Klebsiella sp.	Carbon dioxide	0.4
	Magnesium	0.56
	Potassium	0.39
	Sulfate	2.7
Pseudomonas sp.	Methane	0.7
	Methanol	0.4
Saccharomyces cerevisiae	Glucose	25.0
Tetrahymena sp.	Bacteria	12.0

Source: Ref. 36.

equation:

$$r_X = kX\left(1 - \frac{X}{X_{max}}\right) \tag{23}$$

Has such application was in penicillin fermentation [19]. It should be noted that the only variable in this equation (in addition to time) is X, the biomass. In its integrated form, taking $X(0) = X_0$, the logistic curve is obtained:

$$X = \frac{X_0 e^{(kt)}}{1 - (X_0/X_{max})(1 - e^{(kt)})} \tag{24}$$

Filamentous organisms such as molds often form microbial pellets in suspension culture. Cells growing inside pellets may be subject to diffusion limitations. Hence growth models of mold usually include the simultaneous diffusion and consumption of nutrients within the pellet at large pellet sizes (similar to entrapped or gel-immobilized cells). Filamentous cells can also grow on the surface of a moist solid. Such growth is usually a complicated process, involving growth kinetics and diffusion of nutrients and toxic metabolic by-products. On the other hand, for an isolated colony growing in liquid medium some of these complications can be ignored. The equations for the growth of mold, particularly spherical pellets in submerged fermentation, have been analyzed in some detail by Bailey and Ollis [20] and by Shuler and Kargi [16].

B. Kinetic Approaches

Figure 5 shows the different approaches to the modeling of cell growth kinetics, as presented by Bailey and Ollis [20]. It shows how it is possible to move (via a bizarre series of assumptions) from the actual case of a growing culture, which implies a multicomponent description of the biochemical processes taking place inside each cell, and from the heterogeneity of the population in a batch culture, toward the simple expressions which are usually adopted for the design of a bioreactor. Assuming that all the population can be represented by an "average" cell, the treatment of the segregated population is avoided, and the potential that population analysis offers for the understanding of the biological process [21] is lost. Assuming that the internal composition of the cells is constant, all the concentrations of components inside the cells are eliminated as variables and the system can be represented by models that take into account only abiotic variables, which are much easier to measure and monitor. These simplified models are called nonstructured models because they ignore the internal structure of the biomass. Although this approach is easy to dismiss on absolute scientific grounds, proving that both the "average cell" and "balanced growth" assumptions are not true, the role of the biochemical engineer is usually to simplify reality and use models that have the necessary level of structure according to

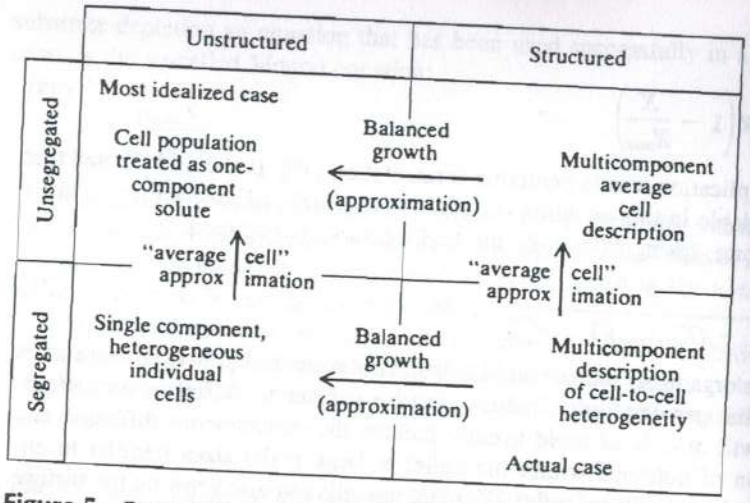


Figure 5 Perspectives for the modeling of cell population growth. (From Ref. 20, with permission of McGraw-Hill.)

the actual use or application. Simpler, unstructured models will usually be restricted, because the constants appearing in the kinetic equation seldom have a real physical meaning. On the other hand, development of completely structured models, taking into account the changes in cell composition, will give a clear biochemical meaning to all constants, which will be independent of the specific process [22]. However, the use of fully rigorous models cannot usually be justified, particularly when the final aim is a bioreactor system design. Kinetic models for this use will thus be based on reasonable simplifications, and the constants have an "approximate" meaning. Thus most kinetic models considered in this chapter will be unstructured and unsegregated, but recent developments in more rigorous modeling will be discussed and their application in a bioreactor system design will be dealt with in Sections III.G and III.H.

C. Single Substrate Limitation

1. The Monod Equation and Mass Transfer

Monod [24] considered that "it is both convenient and logical to adopt a hyperbolic equation similar to an adsorption isotherm or to the Michaelis equation." That is, he was unconvinced by the attempts to use the concept of a master reaction to give a physical meaning to the constants in the equation for growth rate which bears his name. Indeed, Michaelis-Menten kinetics applied to enzymatic reactions give pseudomechanistic meaning to the constants. The constant v_{max} is the rate of an elementary reaction of product formation by dis-

sociation of the enzyme-substrate complex, and K_m is either the equilibrium constant for the enzyme-substrate system (rapid equilibrium assumption) or a combination of the constants corresponding to the elementary reactions of reversible formation of the complex and its dissociation (quasi–steady-state assumption). None of those meanings can be given to a substrate-cell system.

On the other hand, the substrate limitation phenomenon can be seen as a pure mass transfer effect. If k_L is the mass transfer coefficient valid for the substrate being consumed by the cell, the flux of the limiting substrate from the bulk of the liquid into the cell can be given as

$$N_s = k_L(S - S_c) \tag{25}$$

where S and S_c are the substrate concentration in the bulk and on the surface of the cell, respectively.

Assuming that the cells can be taken as spherical, so that the area-to-volume ratio of the cell is $(6/d_c)$, where d_c is the characteristic cell diameter, the area of cells per unit reaction volume (A_c/V) is expressed as

$$\frac{A_c}{V} = \frac{6X}{\rho_c d_c} \tag{26}$$

where X is the biomass concentration, V is the volume of the culture, and ρ_c the density of the cell. For cell forms different from a sphere, the area-to-volume ratio will change accordingly. Equation (25) can be now converted into the limiting rate of S uptake, which depends on S:

$$(-r_S)_{lim} = N_S\frac{A_c}{V} = \frac{6k_L}{\rho_c d_c}X(S - S_c) \tag{27}$$

Using the definition of biomass yield on substrate, the rate at which the process will take place when completely controlled by this flux will be

$$\mu_{lim} = \frac{6Y_{xs}k_L}{\rho_c d_c}(S - S_c) \tag{28}$$

where μ_{lim} indicates the specific growth rate under substrate limitation control and Y_{xs} the yield of biomass in substrate S. When the substrate concentration is high, this rate will be much higher than the maximum potential growth rate of the cell at the given conditions (temperature, pH, nature of the substrate, etc.), μ_{max}. Being the mass transfer and the bioreaction in the cell in series, the influence of μ_{lim} on the overall rate is negligible in this phase, and $\mu = \mu_{max}$. As the substrate concentration diminishes, so does μ_{lim}, until it becomes rate controlling.

In the general case, the inverse of the overall rate can be obtained summing the resistances of the two consecutive steps:

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{1}{\mu_{lim}} \tag{29}$$

and replacing Eq. (22) in Eq. (23),

$$\mu = \frac{\mu_{max}(6y_{xs}k_L/\rho_c d_c)(S - S_c)}{\mu_{max} + (6y_{xs}k_L/\rho_c d_c)(S - S_c)} \quad (30)$$

The concentration of substrate at the cell wall, S_c, is unknown. If we assume that it is much smaller than at the bulk, $S >> S_c$, then Eq. (30) becomes equivalent to the Monod equation (22), with

$$K_S = \frac{\mu_{max}}{6y_{xs}k_L/\rho_c d_c} \quad (31)$$

In the range of substrate limitation μ_{lim} becomes much smaller than μ_{max}. The substrate concentration which leads to this situation is

$$(S - S_c) << K_S = \frac{\mu_{max}}{6y_{xs}k_L/\rho_c d_c} \quad (32)$$

Introducing typical values in Eq. (31), $\mu_{max} = 1\ h^{-1}$, $d_c = 2 \times 10^{-6}$ m, $\rho_c = 10^4$ kg/m^3, $Y_{xS} = 0.5$, $k_L = 1$ m/h, it is found that $K_s = 0.66 \times 10^3$ kg/m^3, which is within the range of values reported for this parameter.

Equations (30) and (31) are equivalent to Monod's equation but have the advantage that whereas K_s in Monod's equation is a completely empirical constant, k_L has a clear meaning. Approximate values of K_s and the influence of changes in physical characteristics and operational variables can be predicted using Eq. (31). This simplifies considerably the task of obtaining an approximate kinetic expression, since only a value of μ_{max} must be experimentally obtained.

2. Other Kinetic Formulations

The Monod equation form describes substrate-limited growth only when growth is slow and population density is low. Under these circumstances growth rate can be related simply to S. At high population levels the accumulation of toxic metabolic products becomes more important. In addition to the Monod equation, several others have been proposed to describe substrate limited growth, such as the Blackman equation, which simply doubles the value of K_s and eliminates the smooth transition between exponential growth and deceleration given by Monod's equation:

$$\mu = \mu_{max} \quad \text{if } S > 2K_S$$
$$= \frac{\mu_{max}}{2K_S} \quad \text{if } S < 2K_S \quad (33)$$

the Tessier equation, which uses an exponential rather than hyperbolic form:

$$\mu = \mu_{max}(1 - e^{-K_S S}) \quad (34)$$

and the Moser equation, which adds a third kinetic parameter, n:

$$\mu = \frac{\mu_{max}S^n}{K_S + S^n} \quad (35)$$

On the other hand, the Contois equation:

$$\mu = \frac{\mu_{max}S}{K_S X + S} \quad (36)$$

appears more appropriate for high-density cultures as the specific growth rate decreases with increasing cell mass.

3. Multiple Substrates

Cultures can usually grow on different substrates, but even if several of those are available only one of them at the time is used as the main energy and/or carbon source. Only when this one is consumed will the enzymatic system required for the consumption of another substrate be developed, at the cost of a new lag time (diauxic growth). If in each of these instances the kinetic behavior can be described by one of the kinetic forms mentioned before for a single substrate, the simplest approach would be a global expression, $F(S_i)$, which reduces to the particular one for the substrate S_i when it becomes the limiting one.

Although several possible ways of combining the rate expressions have been described [16], it appears that when one is dealing with more than one carbon source, which in batch culture are used sequentially and in continuous culture simultaneously, the solution found by Imanaka et al. [25,26] follows this experimental evidence extremely well. They modeled batch cell growth on glucose and galactose by Eq. (37).

$$\frac{dX}{dt} = (\mu_1 + \mu_2)X \quad (37)$$

where

$$\mu_1 = \frac{\mu_{max1}S_1}{K_1 + S_1} \quad (38)$$

$$\mu_2 = \frac{\mu_{max2}S_2}{K_2[1 + (S_1/K_i)] + S_2} \quad (39)$$

in which the subscript 1 stands for glucose and 2 for galactose. In Eq. (39), when glucose is present in batch culture S_1 inhibits the growth on galactose and K_i is very small, hence $\mu_2 \rightarrow 0$. This results in the following overall expression

for μ:

$$\mu = \mu_1 + \mu_2 = \frac{\mu_{max1} S_1}{K_1 + S_1} + \frac{\mu_{max2} S_2}{K_2[1 + (S_1/K_i)] + S_2} \tag{40}$$

This corresponds to what has been classified as an "additive" expression for the specific growth rate.

On the other hand, when there have not been any changes of nutrients, and the problem is that a different one of the originally present substrates becomes limiting (e.g., C, N, O_2), the value of μ_{max} does not change and the behavior has been described well [27] by Eq. (41), which corresponds to the product of several Monod-type expressions:

$$\mu = \mu_{max}\left(\frac{G}{K_G + G}\right)\left(\frac{N}{K_N + N}\right)\left(\frac{O}{K_O + O}\right)\cdots \tag{41}$$

or

$$\mu = \mu_{max} \prod_{i=1}^{n} \left(\frac{S_i}{K_i + S_i}\right) \tag{42}$$

where i denotes the different nutrients which may limit growth. This corresponds to an "interactive" expression for the specific growth rate [16].

4. Product Formation

Metabolic products and proteins are released into the growth medium or accumulated intracellularly. Product formation can be classified into several different groups:

1. The main product is a result of energy metabolism, for example, ethanol synthesis during anaerobic growth of yeast (type 1 in Gaden's classification).
2. The main product is an indirect result of energy metabolism, as in the synthesis of citric acid during aerobic growth of mold or intracellular accumulation of PHB in bacteria (type 2 in Gaden's classification).
3. The product is a secondary metabolite, as in the production of penicillin in aerobic fermentation of mold (type 3 in Gaden's classification).
4. The product is an extracellular or intracellular protein. This belongs to the field of protein synthesis and can be subjected to regulation by induction and catabolite repression, as in the synthesis of enzymes.

These four types of kinetics for the synthesis of products by the cell can be simply classified in two groups. In one group product synthesis occurs during growth, so-called growth associated as shown in Fig. 6 and given by Eq. (43)

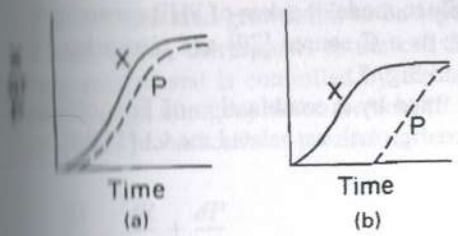


Figure 6 Kinetic patterns of growth and product formation in batch fermentations: (a) growth-associated product synthesis and (b) non-growth-associated product synthesis (From Ref. 16.)

or (44):

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} \tag{43}$$

$$\pi = \alpha\mu \tag{44}$$

where π is the specific rate of product formation. This corresponds mainly to groups 1 (Gaden's type 1) and 4. The synthesis of enzymes (i.e., regulated by induction and repression) and recombinant proteins will be discussed in more detail in Sections III.G and III.H of this chapter.

The other group, in which product synthesis usually occurs after cell growth has finished (or is related to a low growth rate), is called non-growth-associated and is shown in Fig. 6 and Eqs. (45) or (46).

$$\frac{dP}{dt} = \beta X \tag{45}$$

$$\pi = \beta \tag{46}$$

However, in practice these equations do not follow adequately the fact that product synthesis usually does not occur during growth but after it, as happens for the synthesis of citric acid [28] and PHB [29], group 2 above (type 2 in Gaden's classification), or in many cases in the synthesis of penicillin in group 3 above (type 3 in Gaden's classification).

In Fig. 6 it can be seen that if Eq. (45) is valid some product will be synthesized in the period between $t = 0$ and $t = t_{end\ of\ growth}$, which will be proportional to the concentration of cells present. In order to overcome this limitation of the model, a term for inhibition of product synthesis by the substrate controlling growth (usually N) can be added, resulting in Eq. (47) [29].

$$\frac{dP}{dt} = \beta X\left(\frac{K_N}{K_N + N}\right) \tag{47}$$

this equation has been used satisfactorily to model the data of PHB accumulation in *Metholycistis parvus* using methane as a C source [29] and also using CO_2 with *Alcaligenes eutrophus* as shown in Fig. 7.

In some cases the data are best fitted by a combination of Eqs. (43) and (45), in which case one refers to a mixed-growth-associated model [16,30], and this is given by Eqs. (48) or (49):

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \tag{48}$$

$$\pi = \alpha\mu + \beta \tag{49}$$

In the case of synthesis of an intracellular polymer such as PHB the biomass includes the nonproduct biomass plus the product, which can be substantial as

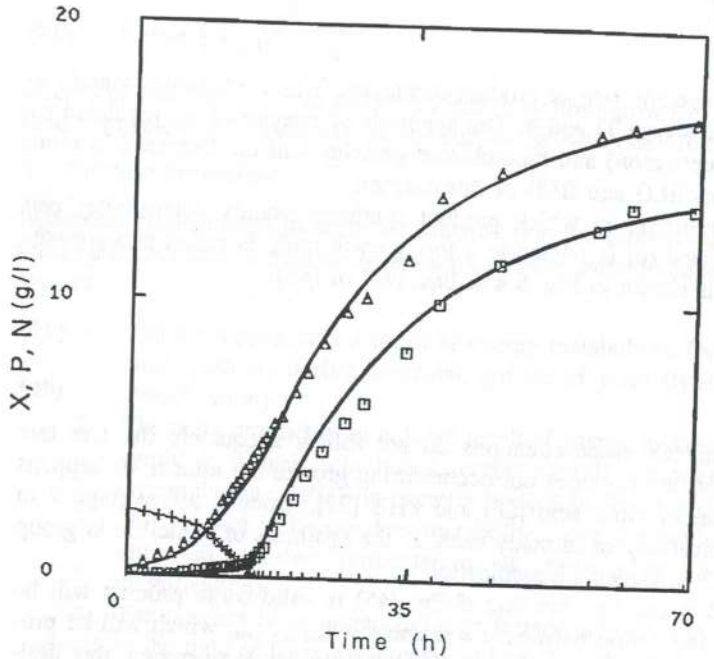


Figure 7 Experimental data and computer simulation for cell growth of *Alcaligenes eutrophus* including intracellular product (PHB) accumulation. Non-growth-associated product formation with term for nitrogen inhibition of product synthesis [Eq. (47)]. (+) Nitrogen in medium; (□) product accumulation; (△) total cell mass, which includes residual (R) and product (P): $X = R + P$ [Eq. (50)].

in Fig. 7, hence total growth has to be separated into two terms [Eq. (50)]. The first term, dR/dt, corresponds to the cell fraction which is proportional to the protein content and is controlled by the level of a nutrient that limits protein synthesis (e.g., nitrogen) in the medium (R stands for residual). The second term corresponds to intracellular product accumulation, dP/dt [29]:

$$\frac{dX}{dt} = \frac{dR}{dt} + \frac{dP}{dt} \tag{50}$$

$$\frac{dR}{dt} = \mu_{max}R\left(\frac{N}{K_S + N}\right) \tag{51}$$

and dP/dt is given by Eq. (46).

ii. Growth and Product Synthesis Inhibitors

Very high substrate concentrations can be inhibitory to growth and to product synthesis. For instance, in order to obtain very high biomass or product concentration it is necessary to add substrate (e.g., carbon source) during the fermentation (fed-batch) as it is not possible to begin the fermentation with all the substrate necessary for cell growth and/or product synthesis. If glucose is used as a carbon source a fermentation is usually not started at concentrations above 150 g/L (above 350 g/L most microorganisms will not grow because of an osmotic effect that results in cell dehydration). This phenomenon is known as substrate inhibition. A number of equations have been proposed to describe this phenomenon and have been reviewed [31] Two of the most important ones seem to be those for noncompetitive inhibition:

$$\mu = \frac{\mu_{max}S}{K_S + S + S^2/K_I} \tag{52}$$

and the one proposed by Aiba and Shoda [32]:

$$\mu = \frac{\mu_{max}S}{K_S + S}e^{(-S/K_I)} \tag{53}$$

The equation for competitive inhibition is similar to that derived for enzyme kinetics [16].

It is extremely common that metabolic end products are inhibitory at high concentrations. These inhibitions affect both the growth rate and the specific rate of product synthesis. The three most common equations for inhibition of

growth are [32–34]

$$\mu = \frac{\mu_{max} S}{K_S + S} e^{-Kp} \tag{54}$$

$$\mu = \frac{\mu_{max} S}{K_S + S}\left(\frac{K_I}{K_I + P}\right) \tag{55}$$

$$\mu = \mu_{max}\left(1 - \frac{P}{P_{max}}\right) \tag{56}$$

If product synthesis, on the other hand, is expressed by using a mixed-growth associated model with dependence on substrate concentration,

$$\frac{dP}{dt} = \left(\alpha\frac{dX}{dt} + \beta X\right)\left(\frac{S}{K_S + S}\right) \tag{57}$$

product inhibition terms can be included in several ways in Eq. (57) as shown in Eqs. (58)–(60) [27].

$$\frac{dP}{dt} = \left(\alpha\frac{dX}{dt} + \beta X\right)\left(\frac{S}{K_S + S}\right)e^{-kP} \tag{58}$$

$$\frac{dP}{dt} = \left(\alpha\frac{dX}{dt} + \beta X\right)\left(\frac{S}{K_S + S + (K_S/K_P)P}\right) \tag{59}$$

$$\frac{dP}{dt} = \left(\alpha\frac{dX}{dt} + \beta X\right)\left(\frac{S}{K_S + S}\right)\left[1 - \left(\frac{P}{P_{max}}\right)^{n_1}\right]^{n_2} \tag{60}$$

Equation (60) shows a more general inhibition term where the value of n_1 (usually >1) or n_2 (usually <1 but >0) will depend on the type of inhibition effect.

When product inhibition is observed, product concentration is usually so high that synthesis is virtually turned off:

$$\frac{dP}{dt} = 0 \quad \text{at} \quad P > P_{max} \tag{61}$$

Equations (60) and (61) have been used successfully to model the synthesis of ethanol and citric acid by yeast [27].

E. Effect of Temperature and pH

The kinetics of microbial growth and product formation are influenced by environmental conditions such as temperature and pH. Temperature is a critical factor that affects the performance of cells. Although most microorganisms used in bioreactors are mesophiles (20°C < T_{opt} < 50°C), organisms can also be psychrophiles (T_{opt} < 20°C) or thermophiles (T_{opt} > 50°C).

Figure 8 shows a typical curve of growth rate as a function of the reciprocal of the absolute temperature. As the temperature is increased toward the optimum, the growth rate approximately doubles every 10°C. Above the optimum the growth rate decreases and subsequently thermal death will occur. The equation that has been proposed for the net growth rate is

$$\frac{dX}{dt} = (\mu - k_d')X \tag{62}$$

where both μ and k_d' can be expressed as functions of temperature following the Arrhenius equation.

$$\frac{dX}{dt} = (Ae^{(-E_a/RT)} - A'e^{(-E_d/RT)})X \tag{63}$$

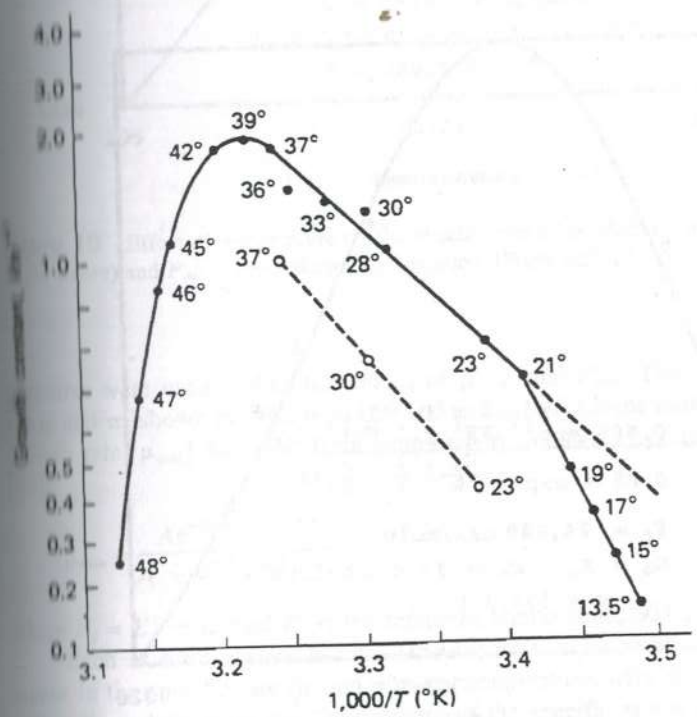


Figure 8 Arrhenius plot of growth rate of *E. coli* B/r. Individual data points are marked with corresponding degrees Celsius. *E. coli* B/r was grown in a rich complex medium (●) and a glucose–mineral salts medium (○). (From Ref. 16.)

The first term represents the increase in specific growth rate with temperature. E_a/RT represents the slope of the right-hand side of the curve in Fig. 8. The value of E_a is typically 10 to 20 kcal/mol. The second term in Eq. (63) represents thermal death, which becomes important at temperatures above the optimum, and E_d has values much higher than E_a, typically 60 to 80 kcal/mol. Therefore thermal death is usually much more sensitive to temperature changes than cell population growth. E_d represents the slope of the left-hand side of the curve shown in Fig. 8.

The effect of temperature on the rates of microbial growth and product formation has been reported for a number of different fermentations. The effect on the specific growth rate, μ, the specific rate of product formation, π, and the maximum product accumulation, P_{max}, in an ethanol fermentation using yeast, where production is coupled to growth, is shown in Figs. 9 and 10. The experimental data on the kinetics of growth and product formation at different temperatures were used to find the values of μ, π, and P_{max}. The equations used for μ and π [shown in Figs. 9 and 10 and as Eq. (64) for the maximum specific growth rate, μ_{max}] have the form proposed by Johnson, Eyring, and Polissar [35]:

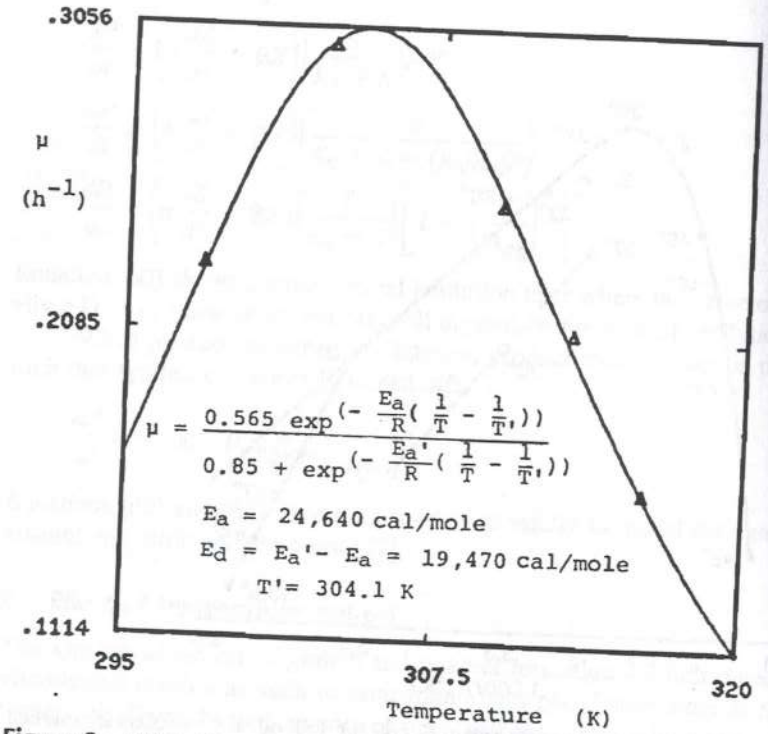


Figure 9 Effect of temperature on specific growth rate μ in an ethanol fermentation. (From Ref. 27.)

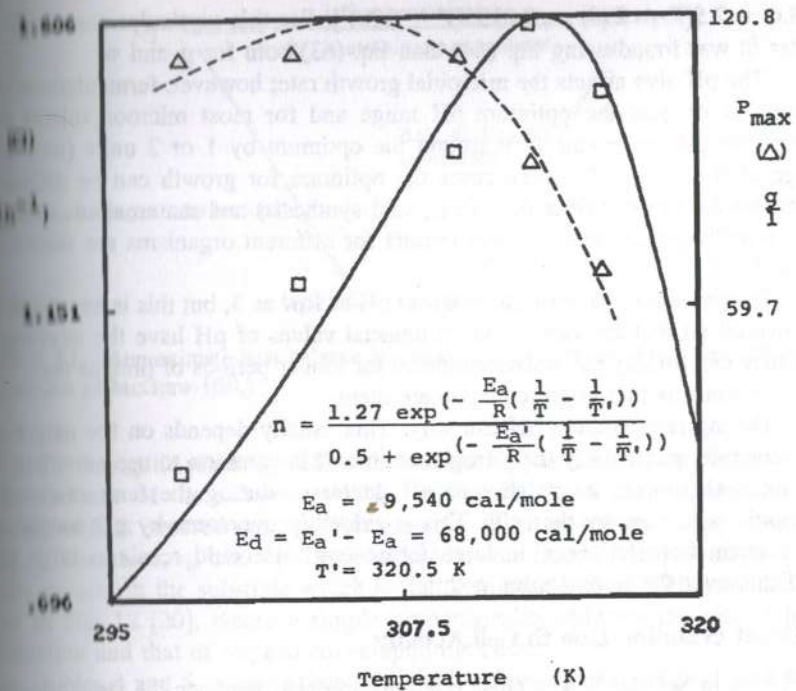


Figure 10 Effect of temperature on the specific rate of product formation π ($\pi = n$ in the figure) and P_{max} for an ethanol fermentation. (From Ref. 27.)

$$\mu_{max} = \frac{Ae^{-E_a/RT(1/T - 1/T')}}{B + e^{-E_d/RT(1/T - 1/T')}} \tag{64}$$

where $E_d = E_a' - E_a$ and T' is the reference temperature, 304.1 K.

Each of them results in two Arhenius-type expressions, one for the increase in the specific rate (μ and π) with temperature, with an energy of activation E_a, and the other for the decrease of the specific rate with temperature, at high temperature, with an energy of activation $E_d = (E_a' - E_a)$. For μ the maximum was found at 303–304 K. For π the maximum was at 313 K with P_{max} having a maximum below 308 K. For P_{max} a polynomial was used [P_{max} =

$108.6 + 2.5(T - 298) - 0.0155(T - 298)^3$]. For this particular case a much better fit was found using Eq. (64) than Eq. (63) both for μ and π.

The pH also affects the microbial growth rate; however, fermentations are usually at or near the optimum pH range and for most microorganisms the acceptable pH range can vary around the optimum by 1 or 2 units (total pH range of 3–4 units). In some cases the optimum for growth can be different from that for product formation (e.g., acid synthesis) and mammalian cells are very sensitive to pH changes. pH optima for different organisms are shown in Table 3.

In some cases bacteria can grow at pH as low as 3, but this is exceptional. Fermentations that are carried out at unusual values of pH have the advantage that they can usually run uncontaminated for longer periods of time as there are fewer organisms that might contaminate them.

During fermentation pH can vary. This usually depends on the nature of the substrate, particularly the nitrogen source. It is common to use ammonia as the nitrogen source, in which case pH decreases during the fermentation as ammonia is utilized by the cells. This is evidently overcome by pH control of the fermentation; however, in large fermenters this could result in large pH gradients over the fermentation culture.

F. Heat Evolution Due to Cell Activity

As shown in Section II.A, a close relationship exists between the stoichiometry of the bioreaction and the evolution of heat. A gross balance of the energy content in the substrate, as shown in Fig. 11, shows the energetic equivalence of an aerobic fermentation process and simple substrate combustion. Oxidation of the substrate is the source of the energy required for growth as well as for maintenance. From the pathways shown, a fair approximation to the heat generated per gram of substrate consumed can be obtained. Since the maintenance is frequently negligible during stages of rapid cell growth, it follows that the rate of heat release is proportional to the rate of growth.

Table 3 pH Optima for Different Cells

Cells	pH range
Bacteria	4–8
Yeast	3–6
Molds	3–7
Plant	5–6
Animal	6.5–7.5

Source: Based on Ref. 16.

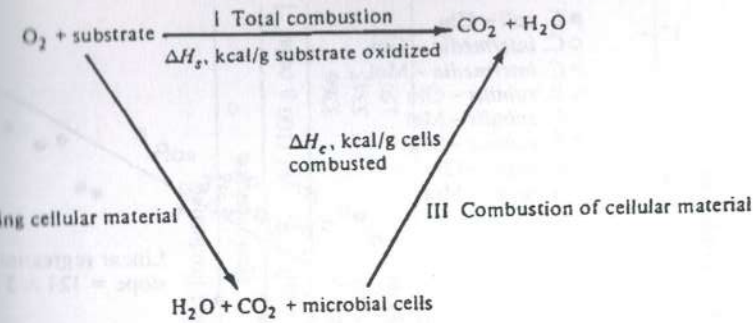


Figure 11 Approximate heat balance in substrate consumption. (From Ref. 20, with permission of McGraw-Hill.)

The energetic yield η_h, defined in Section II.A as the fraction of the available electrons of the substrate which are transferred to oxygen, is also the amount of heat evolved per available electron, Q_0. It is approximately constant [1,3,4]. Therefore, η_h can also be considered as the fraction of the energy originally present in the substrate which is transformed into heat. This can also be seen in Fig. 12 [20], where a simple proportionality between the rate of heat production and that of oxygen consumption is clear.

Tables 4 and 5, adapted from [36], give relevant values of heat evolution for different microorganisms and substrates. If no data are available for the specific system considered, a fair approximation can be obtained from the stoichiometric data, using the regularity found by Minkevich and Eroshin [1] (26.95 kcal/eq. available electron transferred to oxygen).

G. Structured and Segregated Kinetic Models

As discussed in Section III.B, Fig. 5 classifies the approaches to modeling cell population systems according to the number of components used in the cellular representation and whether or not the cells are represented as a heterogeneous collection of discrete units or instead as an average cell which is conceptually similar to a component in solution [20]. Multicomponent cellular representations are called structured and single-component representations of the cell (such as the Monod and similar models already discussed) are called unstructured. On the other hand, in a culture there is evidently cell heterogeneity. If this culture distribution of cells does not substantially affect the kinetic processes of interest, it is possible to introduce the concept of the average cell approximation. Hence in many situations models that ignore the distribution of components and of kinetic parameters in the population (such as the models already described) are used to describe the kinetics of a fermentation culture.

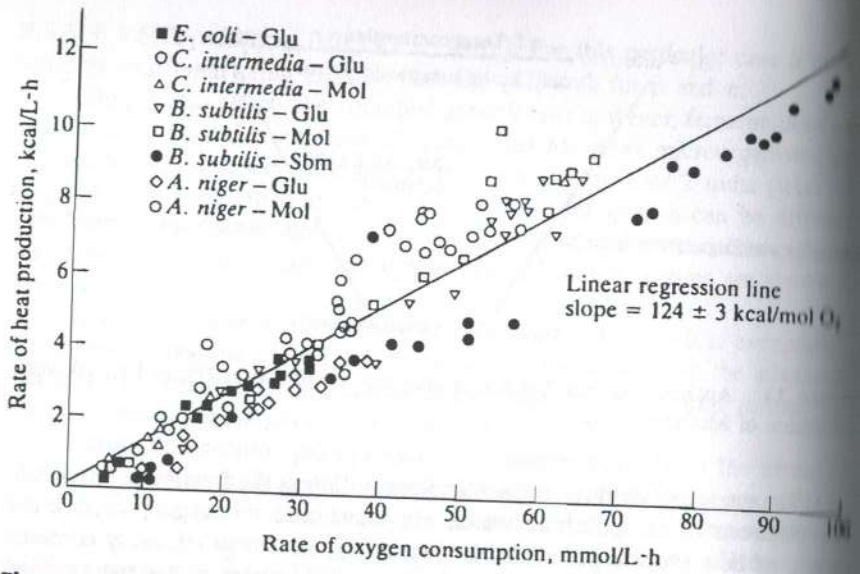


Figure 12 Correlation between heat production and oxygen consumption for several microorganisms grown in different media (Glu, glucose medium; Mol, molasses medium; Sbm, soy bean medium). (From Ref. 20, with permission of McGraw-Hill.)

In the simplest structured models, the biomass is compartmentalized into a small number of components (these are also called compartmental models) [20]. An interesting structured model is that of enzyme regulation by induction and catabolite repression [25,26,37–40]. The model proposed by Imanaka et al. was used to simulate the experimental data of the synthesis of α-galactosidase in *Monascus* sp. in batch, continuous, and transient cultures extremely well. This work has been reviewed in detail by Bailey and Ollis [20] and it includes, in addition to cell mass and two substrates, mass transfer of galactose into the cell, concentration of repressor, concentration of the galactose-repressor complex, mRNA for galactose, and enzyme synthesis.

More sophisticated models with 20 to 40 components are being used by many groups today. One of the best-known ones is that developed by Shuler and co-workers shown in Fig. 13. This model, which was initially developed as the model of a single bacterial cell [41] and later generalized for a cell population, has been described extensively in the literature [22]. A very important point regarding structured models is that all reactions should be expressed in terms of intrinsic concentrations. An intrinsic concentration is the amount of a compound per unit cell mass or cell volume. An extrinsic concentration is the amount of a compound per unit reactor volume and cannot be used in the kinetic

Table 4 Influence of Substrate and Cell Yield on Oxygen Requirement and Heat Production

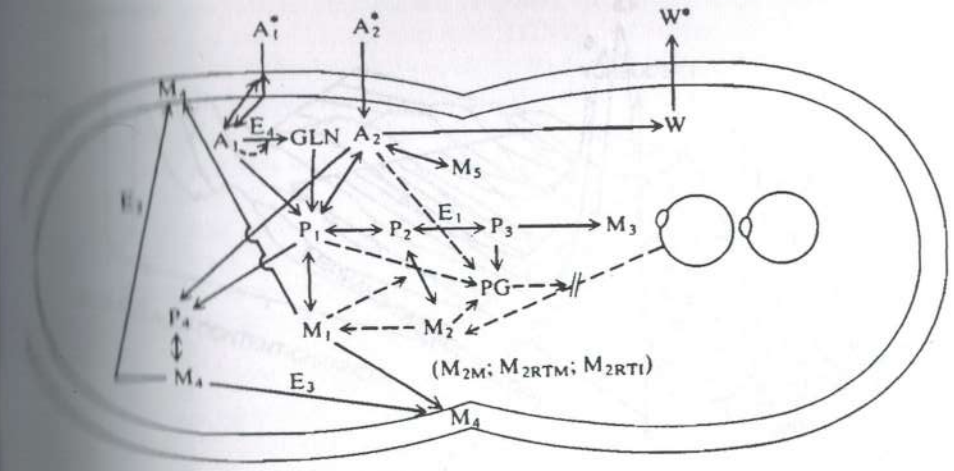
Microorganism	Substrate	Cell yield ($g\ L^{-1}$)	Oxygen required [$g\ (100\ g\ cells)^{-1}$]	Heat released [$kcal\ (100\ g\ cells)^{-1}$]	Heat released [$kJ\ (100\ g\ cells)^{-1}$]
Bacteria	*n*-Alkanes	1.0	172	780	3266
Yeasts	*n*-Alkanes	1.0	197	799	3345
	Carbohydrates	0.5	67	380	1591

Source: Ref. 36.

Table 5 Heat Released During the Continuous Culture of Microorganisms Using Different Substrates[a]

Substrate	Cell yield (% of substrate used)	Heat released [kcal (100 g cell)$^{-1}$]	Heat released [kJ (100 g cell)$^{-1}$]	Rate of heat released (kcal h^{-1})	Rate of heat released (kJ h^{-1})
n-Alkanes (C_{12}–C_{18})	100	780	3270	12	50
Methane	60	1800	7550	30	125
Sucrose	50	380	1590	6	25

[a]Dilution rate, 0.10 h^{-1}; cell density, 15 gL^{-1}.
Source: Ref. 36.



A_1 = ammonium ion
A_2 = glucose (and associated compounds in the cell)
W = waste products (CO_2, H_2O, and acetate) formed from energy metabolism during aerobic growth
P_1 = amino acids
P_2 = ribonucleotides
P_3 = deoxyribonucleotides
P_4 = cell envelope precursors
M_1 = protein (both cytoplasmic and envelope)
M_{2M} = immature "stable" RNA
M_{2RTM} = mature "stable" RNA (rRNA and tRNA—assume 85% rRNA throughout)
M_{2RTI} = messenger RNA
M_3 = DNA
M_4 = nonprotein part of cell envelope (assume 16.7% peptidoglycan, 47.6% lipid, and 35.7% polysaccharide)
M_5 = glycogen
PG = ppGpp
E_1 = enzymes in the conversion of P_2 to P_3
E_3, E_4 = molecules involved in directing cross-wall formation and cell envelope synthesis
GLN = glutamine
E_4 = glutamine synthetase
[ ] = the material is present in the external environment

Figure 13 Schematic diagram of a highly structured single-cell model for growth of *E. coli* B/r A on glucose–ammonium salts medium. (From Ref. 22.)

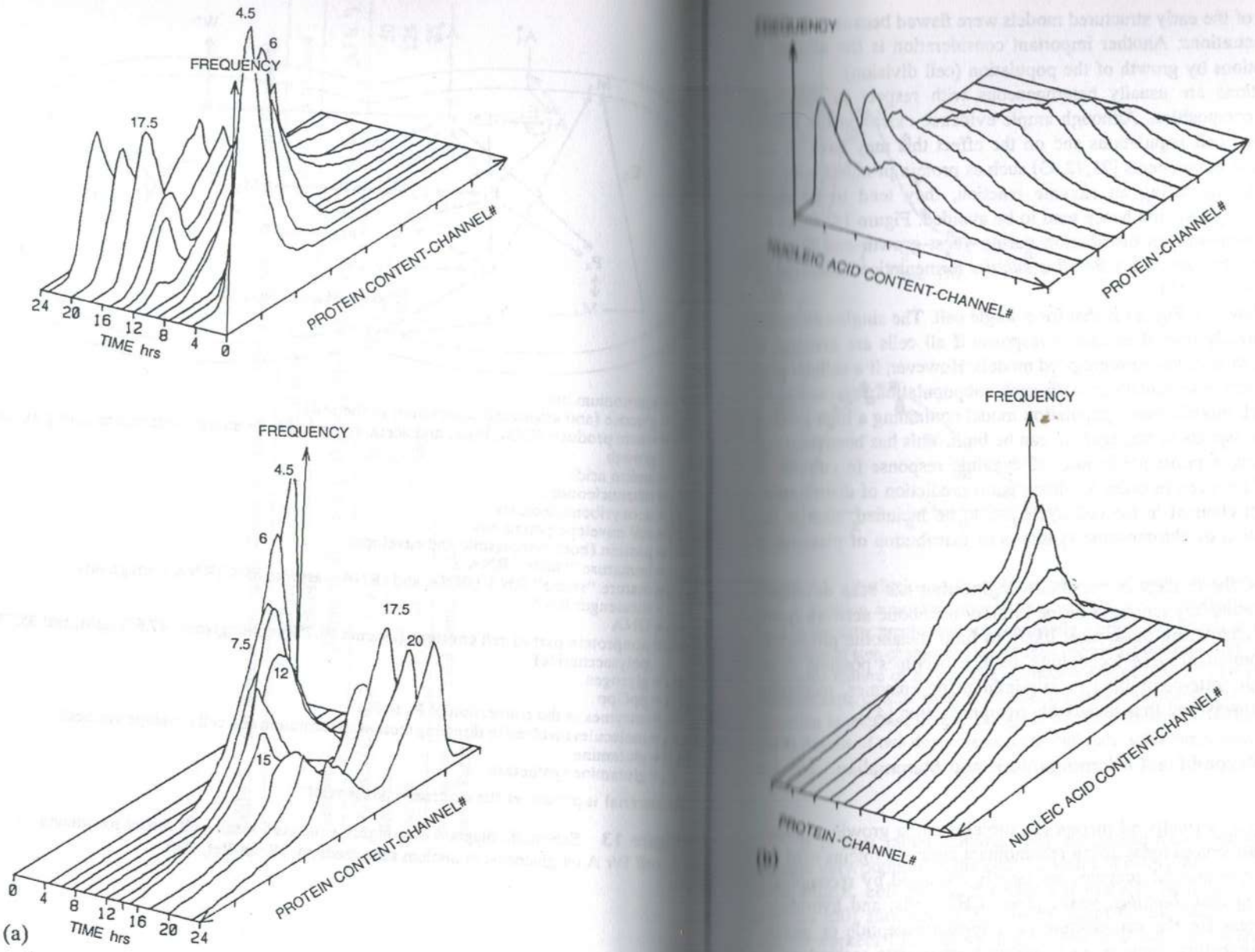


Figure 14 (a) Change in yeast population protein frequency function with time during diauxic batch growth. (b) Two different views of the joint protein–nucleic acid frequency surface for a *B. subtilis* population in exponential growth. From Ref. 42.

expressions. Most of the early structured models were flawed because of the use of extrinsic concentrations. Another important consideration is the dilution of intrinsic concentrations by growth of the population (cell division).

Cell populations are usually heterogeneous with respect to size, age, growth rates, and composition. Although ample evidence exists on the heterogeneity of individual cell populations and on the effect this may have on distribution of particular components [21,42,43] such as protein products both during a fermentation and during an enzyme reaction, they tend to be mathematically complex to solve and hence tend to be avoided. Figure 14 shows the change in protein composition distribution during yeast growth and the joint protein–nucleic acid frequency for *Bacillus subtilis* fermentation measured by flow microfluorometry (FMF).

The model shown in Fig. 13 is that for a single cell. The single-cell model response can be directly related to culture response if all cells are assumed to behave identically (structured, nonsegregated model). However, if a cellular population is divided into subpopulations with each subpopulation represented by a separate single-cell model, then a population model containing a high level of structure, as well as aspects of segregation, can be built. This has been used and is capable of making a priori predictions of dynamic response in cultures as shown in Fig. 15. However, in order to obtain good prediction of distributions, at least one random element in the cell cycle has to be included, such as the timing of the initiation of chromosome synthesis or distribution of plasmids at division [46].

More recently, the concept of metabolic engineering has been developed with the aim of locating key junctions in the primary metabolic network (pathway of a cell) and identifying those most likely to limit metabolite production [44]. By using recombinant DNA technology, then, it becomes possible to increase the titer of the rate-controlling or rate-influencing enzymes, increasing flux through the pathway and thus metabolite overproduction [45].

H. Modeling of Recombinant Microorganisms and Mammalian Cells

As stated in Chapter 1, virtually all therapeutic proteins and a growing number of vaccines are manufactured today using recombinant strains. Proteins used for diagnostics and for commercial enzymes are largely produced by recombinant cells like *Escherichia coli, Bacillus,* yeast, mold, CHO cells, and hybridoma cells. If a new process for the manufacture of a specialty peptide or protein (therapeutic, for diagnostics, enzyme, vaccine) with up-to-date technology is being considered, it would be a virtual sine qua non to choose a recombinant strain in order to be competitive. Moreover, if such a recombinant strain was not readily available, it would become a top priority to develop it quickly.

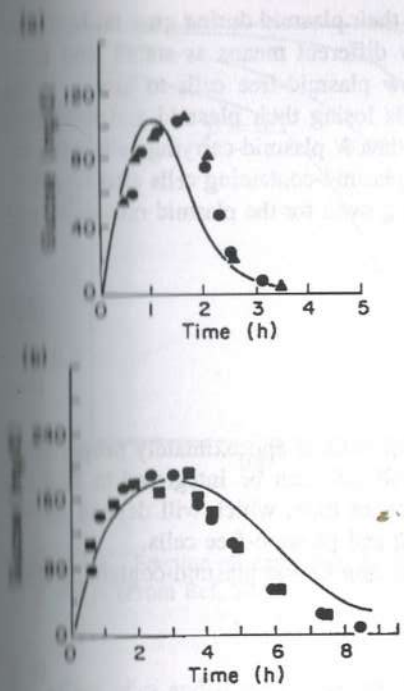


Figure 15 Prediction of transient response in a continuous-flow system to perturbations in limiting substrate concentration or flow rate. Note that the predictions are not fitted to the data; predictions are completely a priori. (a) Transient glucose concentration in response to step change in glucose feed concentration from 1.0 to 1.88 g/L in anaerobic continuous culture of *E. coli* B/r at dilution rate of 0.38 h^{-1}. (● and ▲) Results of two duplicate experimental runs; solid line, computer prediction. (b) Transient glucose concentration in response to step change in dilution rate from $D = 0.16$ to $D = 0.55$ h^{-1}. (● and ■) Results of two duplicate experimental runs; solid line, computer prediction. (From Ref. 46.)

It is thus rather obvious why the modeling of recombinant strains has become very popular in the past 12 years [47–49]. Most of the early work was done with *E. coli* but more recently it has also included other strains such as *Bacillus* [50] and yeast [51]. An excellent review has been published by Ryu and co-workers [52]; hence the subject will not be covered in detail in this chapter. Concise summaries of the subject have been covered by Bailey and Ollis [20], Shuler and Kargi [16], Lee [53], and Patnaik [54], among others.

A basic concept in the modeling of recombinant strains is the presence of both plasmid-containing cells (X^+) and plasmid-free cells (X^-) and the fact that

some of the plasmid-containing cells lose their plasmid during growth; however, this can be controlled and minimized by different means as stated later (e.g., presence of antibiotics that will not allow plasmid-free cells to grow). If the probability of the plasmid-containing cells losing their plasmid and producing plasmid-free cells is p after one division, then N plasmid-carrying cells will produce Np plasmid-free cells and $N(1 - p)$ plasmid-containing cells after one division. Hence the expression for exponential growth for the plasmid-containing and plasmid-free cells is

$$\frac{dX^+}{dt} = (1 - p)\mu^+X^+ \tag{65}$$

$$\frac{dX^-}{dt} = p\mu^+X^+ + \mu^-X^- \tag{66}$$

These expressions assume that the mass of cells is approximately proportional to the number of cells. Equations (65) and (66) can be integrated to find the concentrations of X^+ and X^- as a function of time, which will depend on the initial concentration of plasmid-containing and plasmid-free cells.

An important parameter is f, the fraction of the plasmid-containing cells in the total population, defined as

$$f = \frac{X^+}{X^+ + X^-} \tag{67}$$

which evidently decreases as a function of the number of generations. During exponential growth, the number of generations, n, of the plasmid-carrying cells can be calculated by

$$n = \frac{\mu^+ t}{\ln 2} \tag{68}$$

The equation found for f [53] can be used together with Eq. (68) to find the equation for f for the nth generation

$$f_n = \frac{1 - \alpha - p}{1 - \alpha - p(2^{n(\alpha+p-1)})} \tag{69}$$

where α is the ratio of the specific growth rates:

$$\alpha = \frac{\mu^-}{\mu^+} \tag{70}$$

Equation (69) can be used to predict the change in f with respect to the number of generations for a series of batch fermentations. Assuming it takes about 25 generations to scale up from a slant culture to a 33,000-L production-scale fermenter [53], f_{25} would be an important value to follow. Figure 16 shows

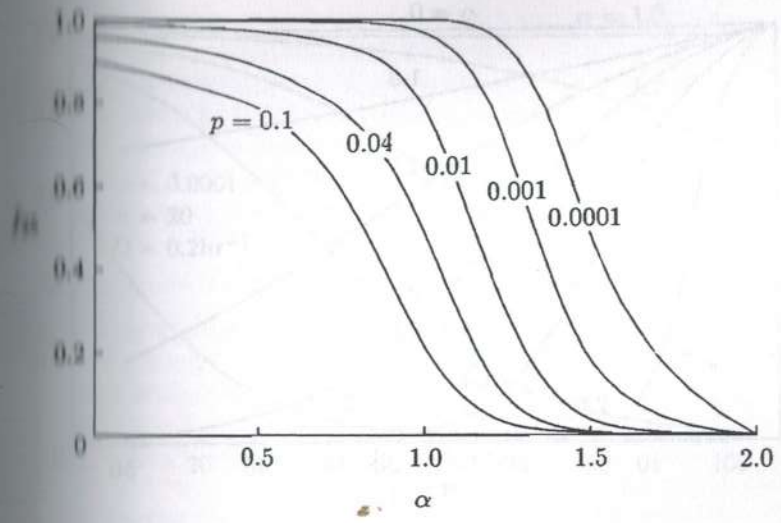


Figure 16 Fraction of plasmid-containing cells after 25 generations (f_{25}) as a function of α and p. (From Ref. 53.)

the effect of p and α on f_{25}, which decreases as α and p increase. Figure 17 shows the change in f as a function of n and α at a constant p ($p = 0.01$). When α is 1.4 all the plasmid-containing cells will lose their plasmids in just over 30 generations.

As recombinant cells lose their plasmid as a function of time, a considerable effort has been devoted to studying continuous culture fermentations. It is interesting to observe how the fraction of plasmid-containing cells changes with time (df/dt). Values of f at different dilution rates as a function of time are shown in Fig. 18. These simulations were done for a value α of 1.2. However, if α increases to 1.4 a continuous fermenter will lose almost all plasmid-containing cells in 100 hours, as can be seen in Fig. 19. This evidently results in the fact that virtually all recombinant fermentations are operated in a batch or more commonly fed-batch mode for production purposes. In parallel, though, several methods for stabilizing recombinant cultures have been proposed. Among the more important ones are the use of selective pressure against plasmid-free cells, such as the use of antibiotic-resistant plasmids or *auxotrophic mutants*. These are mutants which require a specific growth substance beyond the minimum required for normal metabolism and reproduction. In general, medium that favors the growth of plasmid-containing cells over plasmid-free cells is sought. Other stabilization systems for recombinant cultures include the use of temperature-dependent mutant plasmids, temperature-dependent control of

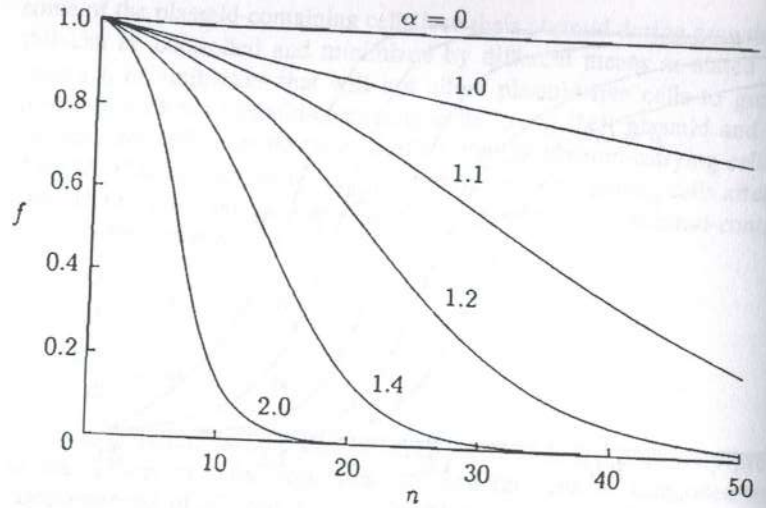


Figure 17 Fraction of plasmid-containing cells (f) versus the number of generations n ($p = 0.01$). (From Ref. 53.)

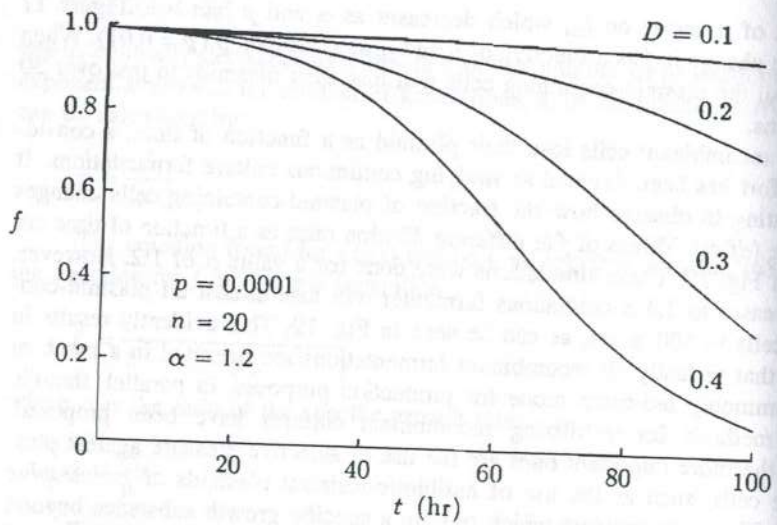


Figure 18 Effect of dilution rate (D) on the fraction of plasmid-containing cells during steady-state operation of a continuous fermentation. The initial value of f was calculated by assuming that the number of generations required for the step inoculation and initial batch and unsteady-state continuous fermentation was 20. (From Ref. 53.)

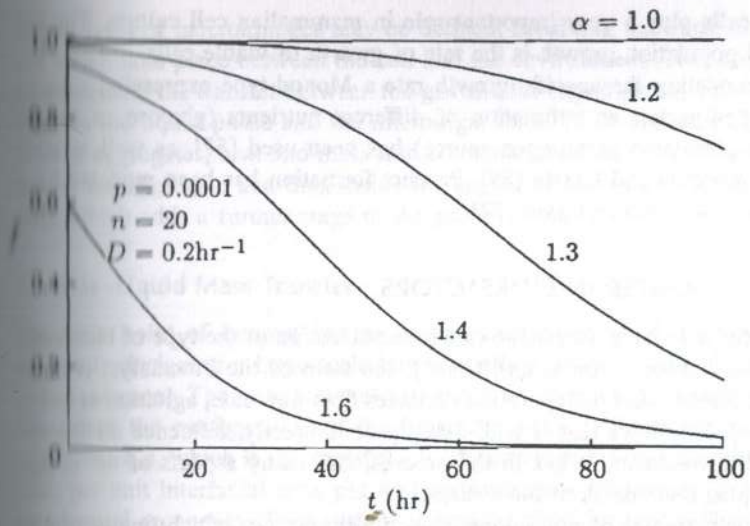


Figure 19 Effect of α on the fraction of plasmid-containing cells during steady-state operation of a continuous fermentation. The initial value of f was calculated by assuming that the number of generations required for the step inoculation and initial batch and unsteady-state continuous fermentation was 20. (From Ref. 53.)

gene expression, plasmids with no transposable element, or recombination deficient strains [53].

More structured models of recombinant strains will predict the distribution of plasmids within a population as well as describe individual regulatory and genetic functions (e.g., *tac* promoter induction, runaway plasmid replication) and population dynamics of the system [55]. Interestingly, it has been found that it is reasonable to assume that sufficient antibiotic can be added to a continuous culture to prevent plasmid-free cells from dominating. The use of a two-stage culture where the first stage is used for growth and the second for protein induction and synthesis has been investigated in some detail [55,56].

The development of models and quantitative aspects of animal cell growth is far less advanced than that of recombinant microorganisms. Animal cells grow slowly with maximum specific growth rates of the order of days^{-1} and they are extremely sensitive to pH. It is important to differentiate between submerged and attached cell lines and also between viable and nonviable cells. Continuous culture studies have been carried out using carbon (e.g., glucose or glutamine) or oxygen as the limiting substrate [57–60]. Several Monod-type models can be found in the literature; the main difference from microbial cultures is that

nonviable cells play a very important role in mammalian cell culture. The net rate of cell population growth is the rate of growth of viable cells.

For modeling the specific growth rate a Monod-type expression such as that described earlier as a function of different nutrients (glucose as carbon source and glutamine as nitrogen source) has been used [57], as well as inhibition by ammonia and lactate [59]. Product formation has been modeled by a non-growth-associated equation [57].

IV. MASS TRANSFER IN BIOREACTORS

Mass transfer may have a decisive role in the selection of the type of bioreactor (stirred tank, bubble column, airlift, etc.), the state of the biocatalyst (free or immobilized cells), and the operation variables (gas flow rate, agitation velocity, temperature). It follows that it will, directly or indirectly, influence all the upstream and downstream stages in the process, and many aspects of the design of the peripheral elements of the system.

All the activities of microorganisms in a bioreactor, which finally lead to an increase of biomass or to the formation of a desired product, are linked to the environment via transfer of mass to or/and from the microorganism and dissipation of heat from it. Substrata must reach the bulk of the broth, metabolites are disposed, and all this occurs at rates which must satisfy stoichiometry and mass balances over the biomass and the bioreactor as a whole. In gas-liquid reactors in general, the transfer of low-solubility gases is one of the most obvious problems because of the need for continuous provision of a substrate that would otherwise be almost instantaneously depleted in the liquid and become the rate-limiting reactant. In the case of aerobic bioprocesses, the provision of oxygen has been recognized as a key problem, and the rate of provision of oxygen is often considered the main issue in bioreactor selection and design.

The rates of transfer of materials to and from the point in space where the actual chemical reactions take place may thus influence and sometimes mask the real rate of the chemical transformations. In these cases we actually measure the overall rate, obtaining what was called by van Kervelen [61] "macrokinetics" of the process. In the specific case of biological processes, where thousands of chemical reactions are involved, the actual microkinetics of a single reaction is not only difficult to observe and follow but also of almost no use. Even the most sophisticated structural models are only crude approximations to the complexity of the living cell. In most of the practical applications, we are actually interested in the rates at the level of the whole cell, under given environmental conditions. Those rates, which represent a macrokinetics for the hypothetical observer able to distinguish the phenomena at the molecular level, become the basic or microkinetic information for the observer at the cellular level. In this chapter the cell as a whole is considered the catalytic active point, and again a

second level of macrokinetics may be defined, involving the transport phenomena which take place between the cell and the environment. Two stages can be distinguished: the transfer between the gas and the liquid phase, and the transfer between the liquid phase and the microorganisms. When the cells occur in the form of aggregates, heat and mass transfer must take place to or from the liquid toward the aggregate and then inside the aggregate to or from the immobilized cells, which adds a further stage to the process [62].

A. Gas–Liquid Mass Transfer

The main point of interest for the biochemical engineer is the possibility of accurately designing a large-scale bioreactor from data obtained in laboratory-scale equipment. The most relevant parameter which has to be chosen and which determines the configuration of the bioreactor is the volumetric mass transfer coefficient $k_L a$, which is the specific rate of mass transfer (gas absorbed per unit time, per unit interfacial area, per unit concentration difference). It depends on the physical properties of the system and on the fluid dynamics. The volumetric mass transfer coefficient is the product of two terms: (1) the mass transfer coefficient k_L, which depends on the physical properties of the liquid and fluid dynamics near the liquid surface, and (2) the area of bubbles per unit volume of the aerated reactor. It is well understood today that the dependence of k_L on the power input is rather weak, while the interfacial area is a strong function of physical properties, the geometric design, and hydrodynamics. The interfacial area is a lumped parameter and cannot be defined at a point. On the other hand, the mass transfer coefficient is in fact a proportionality factor between the mass flux of the substrate (or other chemical being transferred), N_S, and the gradient that drives the phenomenon, which is the difference of concentrations:

$$N = k_L(S_1 - S_2) \tag{71}$$

where 1 and 2 indicate the two points between which mass is being transferred. In actual reactors a wide range of values of the gradient may coexist simultaneously, and therefore a value representing the whole reactor must be chosen.

It follows that the value of the mass transfer coefficient depends on the concentrations adopted for its definition in Eq. (71). This means deciding which is the fluid dynamic model representing the reactor. Lack of awareness of this fact is the reason for erroneous data which can be sometimes found in the literature, where mass transfer coefficients have been calculated using a wrong driving force. The most common error is assuming perfect mixing (usually because of the simplicity of the calculations) without assuring the validity of the assumption.

It is obvious that the larger the demand for oxygen by the biological system, the more relevant becomes the rate of oxygen absorption into the liquid.

But even in systems of relatively small oxygen demand, such as animal cell culture, the problem of oxygen transfer is of relevance. An example of this can be seen in Shi et al. [63], where it has been shown that enhancement of the oxygen transfer rate leads to an increase in the final concentration of murine hybridoma cell line 62′D3 and monoclonal antibody product.

Data on k_La can be obtained from published correlations, keeping in mind that those are generalizations based on a limited number of experimental data. The closer the designed equipment is to the geometric configuration as well as to the physical properties of the original experimental system, the safer the design. Extrapolations will always be subject to uncertainty.

B. Mechanically Stirred Bioreactors

The specific rate of mass transfer depends in general on the energy input to the system. This energy is invested in shear for the creation of a large interfacial area, by generation of small bubbles or breakage of larger ones, and in the circulation and mixing of the liquid.

Since energy may enter the system both as the shaft work in the impeller and the expansion work of the gas used for aeration, the general form of the equations proposed is

$$k_La = a_1\left(\frac{P_i}{V_L}\right)^{\alpha}(J_G)^{\beta} \tag{72}$$

where the constants α and β depend on the geometry of the system and the rheology of the liquid.

Robinson and Wilke [64] proposed a modification taking into account the effect of the liquid properties:

$$k_La = a_1\left(\frac{P_i}{V_L}\right)^{\alpha}(J_G)^{\beta}\xi \tag{73}$$

where

$$\xi = (\rho_L)^{0.533}(D_L)^{2/3}\sigma^{-0.6}(\mu_L)^{-.33} \tag{74}$$

The authors gave different sets of values of a_1, α, and β, for water and for a salt solution, because effects of ionic strength are not included in ξ. In addition, this correction holds for low viscosities. Van't Riet [65,66] reviewed mass transfer rates in agitated vessels in the same range of viscosities and recommended a modification of Eq. (72) taking into account the static pressure at the height of the impeller:

$$k_La = a\left(\frac{P_i}{V_L}\right)^{\alpha}\left(\frac{J_G p_a}{p_s}\right)^{\beta} \tag{75}$$

where p_a and p_s indicate the static pressure in the atmosphere and at the height of the stirrer. They gave different values of the constants for coalescing and noncoalescing liquids. This way of presenting results, i.e., considering separately coalescent and noncoalescent liquids, is rather common in the literature [67,68]. The reason for this seems to be our lack of knowledge about the variables controlling this complex phenomenon.

The influence of viscosity on mass transfer rates may be important in stirred tanks, especially at high viscosities. Figure 20, taken from [66], shows that up to viscosities of the order of 5×10^{-2} Pa s the mass transfer coefficient is almost independent of the liquid viscosity, but after that the dependence is quite strong. Cooke and Middleton [69] studied mixing and gas–liquid mass transfer rates in laboratory and pilot scale fermenters, using a paper fiber suspension rheologically matched to a filamentous fermentation culture. They found that the best fit to their data was obtained by adding to Eq. (72) a term including the viscosity of the suspension with an exponent (−1).

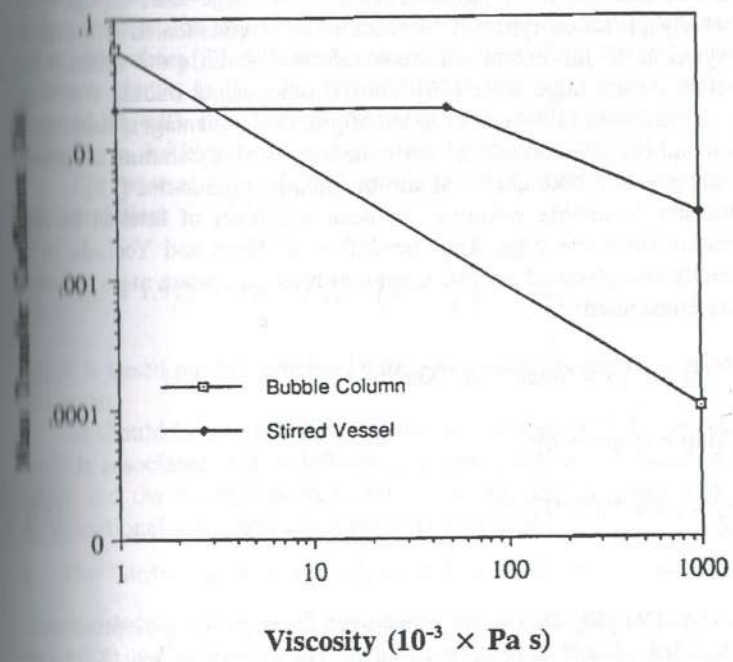


Figure 20 Dependence of the mass transfer coefficient on liquid viscosity in stirred vessels and in bubble columns. (Adapted from Ref. 66.)

Highly viscous liquids are very difficult to aerate adequately, and in those cases multiple impeller stirrers and special designs of the impellers may be required [70–72]. Chapter 10 in this book gives a deeper analysis of the problem of designing mechanically stirred bioreactors.

C. Pneumatically Stirred Bioreactors

1. Bubble Columns

Bubble columns are the simplest reactors from the point of view of their construction and operation and belong to the general category of pneumatically agitated reactors. They are simply vessels in which gas is sparged into the liquid. They have no moving parts, because all of the energy needed for the agitation of the contents of the vessel and the oxygen required for the culture are provided by the gas, usually air, sparged into it.

Bubbling systems have been considered harmful for animal cell and other sensitive cultures because of shear-related damage [73–76]. Due to this, systems which avoid direct contact between gas and the cell-containing medium have been suggested [77]. Although this holds true for laboratory-scale processes, bubble reactors remain the most viable alternative for large-scale equipment. Most of the newly proposed types of bioreactors are sophisticated and rather complicated systems, to the extent that some of them seem quite difficult to build and operate on the large scale [78]. On the other hand, bubble columns show an impressive record in the achievement of practical advantages, simplicity of construction and operation at a large scale. Indeed, bubble columns are gaining an important place in both chemical and biochemical industries [79].

Mass transfer in bubble columns has been a subject of interest in the technical literature for some time. The correlation of Akita and Yoshida [67] has been generally accepted and became a point of reference when mass transfer coefficients are considered:

$$\frac{k_L a D_c^2}{D_L} = (\mathrm{Sh})(aD_c) = 0.6\mathrm{Sc}^{0.5}\mathrm{Bo}^{0.62}\mathrm{Ga}^{0.31}\epsilon^{1.1} \tag{76}$$

where the holdup ϵ is given by

$$\frac{\epsilon}{(1-\epsilon)^4} = c_1\mathrm{Bo}^{1/8}\mathrm{Ga}^{1/12}\mathrm{Fr}^{1.0} \tag{77}$$

2. Airlift Reactors

Airlift reactors (ALRs) [80] offer some advantages for large-scale bioprocesses, in particular for animal and plant cell cultures. The reason seems to be the difference in fluid dynamics between ALRs and more conventional bioreactors. In conventional stirred tanks or bubble columns, the energy needed for the movement of the liquids enters focally, at one point of the reactor, via the stirrer or sparger, respectively. In ALRs there are no such points of high energy dissipation rate, and as a consequence the field of shear is much more homogeneous. A cell or a cell aggregate moving in the bioreactor will not have to endure sharp changes in the dynamics of the environment, and this may be important for shear-sensitive cultures.

Since the fluid flow has a predominant role in the performance of ALRs, the geometric design of the equipment is especially important. In particular, the bottom clearance (which is an indication of the resistance to flow at the bottom of the reactor) and the design of the gas separator seem to have a strong influence on the mass transfer rate. A correlation taking into account these factors for the case of internal loop ALRs is recommended here [81]:

$$\mathrm{Sh} = 6.82 \times 10^4 \mathrm{Fr}^{0.9} M^{-3.4} \mathrm{Ga}^{0.13} X_{dr}^{-0.07} Y^{-0.18} (1 + A_d/A_r)^{-1} \tag{78}$$

In addition to the well-known dimensionless groups Sh, Fr, and Ga, three geometric ratios are introduced: M reflects the effect of the design of the gas separation zone, X_{dr} accounts for the design of the bottom, and Y accounts for the top clearance. The effect of variations in cross-sectional areas of riser and downcomer which appears in Eq. (78) is based on the dependence found by other researchers [82–84]. A comparison between Eq. (78) and the experimental results is shown in Fig. 21. In addition to the experimental data obtained on both ALRs with the three liquids used, the results predicted by correlations available in the literature are shown for comparison [85,86].

For external loop ALRs, Popovic and Robinson [84] proposed the following correlation:

$$k_L a = 1.911 \times 10^{-4} (J_G)_r^{0.525} \left(1 + \frac{A_d}{A_r}\right)^{-0.853} \mu_{app}^{-0.89} \tag{79}$$

which is based on data obtained with carboxymethyl cellulose (CMC) solutions and water.

It should be stressed that, contrary to correlation (78), Popovic and Robinson's is associated with a defined geometry, because the shape of the gas separator and the clearances were not taken into account, and only the ratio of cross-sectional areas was considered as a variable.

3. The Global Approach for Bubble Columns

Non-Newtonian behavior of liquids is a phenomenon often encountered in fermentation broths. Several authors have studied the issue. Nishikawa et al. [87] analyzed a problem of heat transfer in a bubble column with non-Newtonian liquids. They proposed a simple proportionality between shear rate γ and su-

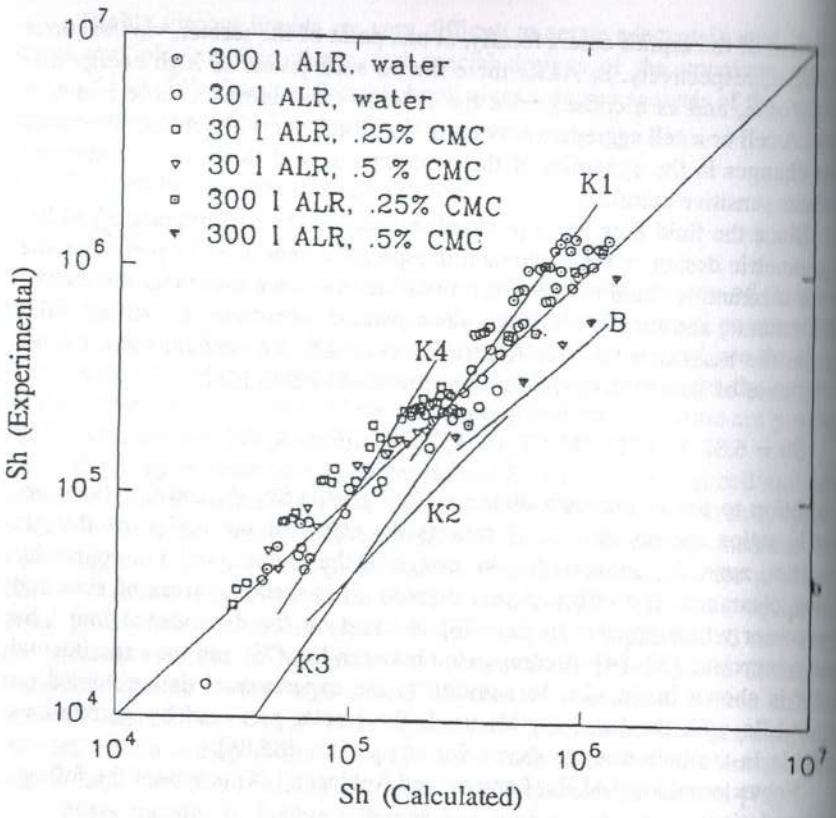


Figure 21. Experimentally obtained values of the mass transfer coefficient in ALRs presented as Sherwood number, Sh, versus those calculated from Eq. (78) [81]. The lines represent the predictions made using other correlations, as follows: B, Bello et al. [82]; K1 to K4, Koide et al. [86].

perficial gas velocity J_G, which for J_G larger than 0.04 m/s was

$$\gamma = 5000 J_G \tag{80}$$

If the rheological behavior of the liquid is known, this global shear rate can be used in order to calculate a global viscosity. In shear-sensitive cultures, the definition of a global shear rate is of great importance by itself, since it may be related to cell damage.

Henzler [88], Kawase and Moo-Young [89], Schumpe and Deckwer [90], and Zaidi et al. [91] followed Nishikawa's approach but suggested different proportionality constants relating the global shear rate to superficial gas velocity.

This approach is questionable from a rheological point of view because it will predict the same shear rate for a certain superficial gas velocity, no matter which liquid is used. El-Tamtamy et al. [92] introduced an improvement by calculating the shear rate from the bubble velocity divided by bubble diameter. An accurate evaluation is, however, difficult for both parameters.

The preceding relations predict different shear rates which vary up to three orders of magnitude. This rather disturbing fact was one of the motivations for the proposal of a new approach [93], called the global approach. This approach considers the general equation describing the rheology of Newtonian and non-Newtonian liquids:

$$\tau = \mu\gamma \tag{81}$$

where τ is the shear force applied per unit area and γ is the shear rate. The viscosity μ is constant in Newtonian liquids. In non-Newtonian liquids the viscosity is shear rate dependent and decreases with increasing shear rate for the case of pseudoplastic liquids. In order to describe the viscosity behavior, a constitutive equation should be chosen.

The shear stress in a bubble column was defined as equal to the acting force, which can be calculated from the power input divided by the sum of the areas of all bubbles:

$$\tau = P_i / H\pi a_b \tag{82}$$

The power input can be calculated assuming ideal gas isothermal expansion, as follows:

$$P_i = nRT \ln\left(\frac{p_1}{p_2}\right) \tag{83}$$

where the molar flow rate n is taken at the inlet point.

The specific interfacial area was adopted from Schumpe and Deckwer's [94] correlation for non-Newtonian liquids which can be written as follows:

$$a = 4.65 \times 10^{-2}[(J_G)^{n/\kappa}]^{0.51} \tag{84}$$

Equation (84) takes into account the gas superficial velocity and power law liquid rheology and is probably the most reliable correlation available for non-Newtonian liquids.

Equation (84) allows the calculation of a global shear force acting on the liquid. Using a constitutive equation, a global shear rate can be calculated. Using the power law model:

$$\gamma = \left(\frac{p_1 J_G \ln(p_1/p_2)}{aH^2\kappa}\right)^{1/n} \tag{85}$$

Equation (85) gives a global shear rate which is a function of both the fluid dynamics and rheology.

The mass transfer coefficient k_La can be seen as the product of two terms: the mass transfer coefficient k_L and the specific interfacial area. They both depend on a series of variables which can be grouped into three categories: (1) static properties of the liquid such as density, diffusivity and surface tension; (2) dynamic properties of the liquid (related to liquid flow), such as the rheological parameters; and (3) liquid dynamics. In general the variables in group 1 do not change very drastically. The groups of variables 2 and 3 vary over a wide range. The shear rate acting on the system is also an expression of the effect of these variables. Based on this observation, the global approach predicts that k_La will show a simple dependence on γ independent of the liquid tested.

Volumetric mass transfer coefficient versus global shear rate for both Newtonian and non-Newtonian liquids showed a single line, which can be represented by the following surprisingly simple equation [93]:

$$k_La = 1.4 \times 10^{-6}\gamma^{1.7} \tag{86}$$

Comparison with data published by other authors can be seen in Fig. 22. There is good agreement with the data of Deckwer et al. [95], with some of the data

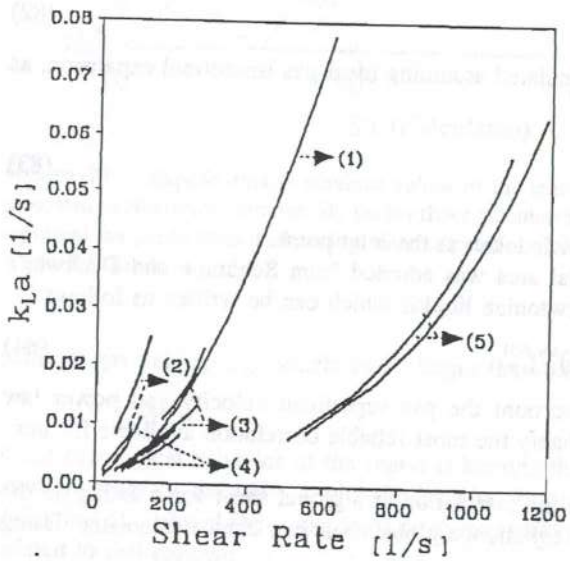


Figure 22 Volumetric mass transfer coefficient versus shear rate. 1, Merchuk and Ben-Zvi [93]; 2, Godbole et al. [96]; 3, Schumpe and Deckwer [94]; 4, Deckwer et al. [95]; 5, Nakanoh and Yoshida [97]. (From Ref. 93.)

of Godbole et al. [96], and with Schumpe and Deckwer [90]. The data of Nakanoh and Yoshida [97] show the same trend but are shifted toward higher shear rates. Since the plot of mass transfer coefficients for Newtonian and non-Newtonian liquids against the global shear rate showed that all of them behave in a similar way, this approach offers a possibility of developing general correlations for mass transfer rate, applicable to any liquid of known rheological behavior.

(ii) Fundamental Approach

Although most of the literature on the subject reports empirical correlations, which are usually valid for only a certain range of the variables, attempts have been made to formulate design methods based on more fundamental principles. Such an approach tends toward generalization and broadening of the range of applicability of the findings [98–101].

The first step in this direction is the separation of k_La into the two elements which constitute it: (1) the mass transfer coefficient k_L, which depends on the physical properties of the liquid and the fluid dynamics near the liquid surface and describes the rate of mass transfer at a point of this surface, and (2) the interfacial area per unit volume a, which is a property of the bioreactor as a whole or of a region of it. Since a is a lumped variable, it cannot be referred to a small domain (for instance, a region smaller than a bubble in a gas sparged reactor).

One of the latest models for the a priori prediction of the mass transfer coefficient k_L has been presented by Kawase et al. [101]. It is applicable to Newtonian and power law fluids. It uses Higbie's model as the functional basis for the dependence of k_L on diffusivity and exposure time of a liquid element in the gas. The latter is obtained by applying the transitional sublayer model [102]. They assumed that the turbulent flow near a free interface is governed by the same mechanisms as near a solid interface but with fourfold higher velocity fluctuations. They finally propose the following expression:

$$k_L = 2^{2/n}\pi^{-0.5}T^{-1}D^{0.5}\left(\frac{\kappa\epsilon'}{\rho}\right)^{-0.5(1+n)} \tag{87}$$

where the dimensionless bursting period T is given by [102]

$$t_e = T^2\left[\frac{(\kappa/\rho)^{1/n}}{\nu_0^{2/n}}\right] \tag{88}$$

and t_e is the exposure time of the liquid element.

The expression shown above was compared very satisfactorily with results obtained on both bubble columns and stirred reactors [101]. In Fig. 23 the original figure presented by Kawase et al. has been redrawn schematically add-

ing other data from the literature [98,104–108]. This seems to be a very encouraging step in the direction of the prediction of mass transfer rates based on fundamental principles. It should be remembered, however, that it is still far from enabling the design of large-scale equipment by itself. It is well understood today that the dependence of k_L on the power input is rather weak [109], while the interfacial area is a strong function of the physical properties, the geometric design, and the hydrodynamics. In any case, Yoshida's rule of thumb [110] which advocates a constant value of 1 m/h for k_L seems to give an excellent approximation within the range of interest. This is also shown in Fig. 23.

It should be kept in mind that k_L may be studied by a fundamental approach, which would be applicable to any free gas-liquid surface irrespective of type of bioreactor considered, because it is related only to the hydrodynamics in this point. This is not the case for a, the specific interfacial area, which is a global parameter of the system and will necessarily depend on the type and geometry of the system. Furthermore, the dependence of a on the power input is much stronger than that of k_L. The design of bioreactors, therefore, must

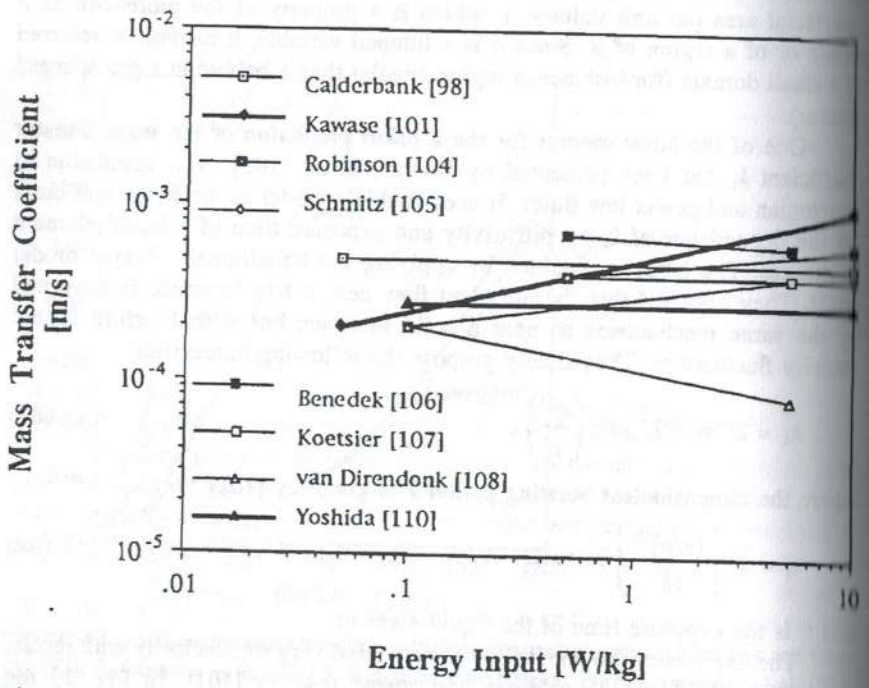


Figure 23 Mass transfer coefficient in free surfaces.

generally rely on empirical knowledge of the interfacial area or the volumetric mass transfer coefficient.

E. Liquid–Microorganism Mass Transfer

The substrates required by the cells must diffuse trough the boundary layer around it and then penetrate the cell in order to react. One of the most important questions that must be answered at this stage is whether the controlling step is the transport inside the cell or around it. This knowledge will enable us to predict the possible influence of the physical properties of the liquid on the rate of the process.

Rotem et al. [111] studied the influence of viscosity on the rate of growth of the unicellular red alga *Porphyridium* sp. The alga was cultured in a medium containing increasing concentrations of the soluble fraction of its own cell wall polysaccharide. As the polysaccharide concentration in the culture medium increased, algal growth rate and maximum cell number progressively decreased. The increasing polysaccharide concentration also inhibited the rate of carbon uptake by the cells and as a result also inhibited photosynthesis. In vitro experiments showed that the mass transfer coefficient for nitrate, bicarbonate, phosphate, and potassium decreased as the polysaccharide concentration increased. The conclusion was that the reduction of the growth rate was due to the hindering of nutrient transfer by the highly viscous polysaccharide.

In case the microorganisms appear as aggregates or are immobilized on a solid support, use of the following expression by Sanger and Deckwer [112] is recommended:

$$\frac{k_L d_p}{D_L} = 2.0 + 0.545\left(\frac{\mu_L}{\rho_L D_L}\right)^{1/3}\left(\frac{\epsilon' d_p^4}{\nu_L^3}\right)^{0.264} \tag{89}$$

F. Enhancement of the Absorption Rate Due to Microbial Activity

When gas is absorbed into a liquid and reacts there, the concentration profiles of the absorbed gas change due to the chemical reaction and the absorption rate may be enhanced. The extent of this enhancement can be derived for most of the models for mass transfer in turbulent systems [113]. An interesting feature of those derivations is that the enhancement predicted by all the different models is virtually equal, and therefore the simplest model (the film model) [114] can be used.

Oxygen absorption into a fermentation broth is similar to the absorption of a gas into a liquid where it reacts with small particles suspended in it [115–118]. Since oxygen is consumed while it diffuses from the gas-liquid interface, the oxygen absorption rate may be enhanced. Indeed, Lee and Tsao [119] and

Tsao et al. [120] found that the absorption rate of oxygen in a surface-aerated stirred vessel was higher than expected from physical absorption. They hypothesized that accumulation of microorganisms near the gas-liquid interface, which they observed, could explain the phenomenon. On the other hand, Yagi and Yoshida [118] reported experimental evidence that the activity of microorganisms does not affect the oxygen transfer rate. They carried out their experiments in a sparged stirred fermenter.

Both of the foregoing observations were reconciled by a simple mathematical model which took into account the influence of the different hydrodynamics in the apparatuses used in the experiments [121]. The film model was adopted, the respiration rate r was taken as zero order in oxygen concentration, and three modes of distribution of microorganisms within the film were explored: (1) homogeneous distribution, (2) linear cell distribution as proposed by Tsao [122], and (3) an exponential cell distribution as proposed by Yagi and Yoshida [118]. The enhancement factor obtained had the following form:

$$E = 1 + \gamma_r \Pi \tag{90}$$

where

$$\gamma_r = \frac{Drb_L}{2k_L^2(C_0 - C_L)} \tag{91}$$

For model 1, homogeneous distribution of microorganisms,

$$\Pi = 1 \tag{92}$$

For model 2, linear distribution,

$$\Pi = \frac{1}{3} + \frac{2b_0}{3b_L} \tag{93}$$

and for model 3, exponential distribution,

$$\Pi = 2(B\delta)^{-2}\left[\left(\frac{b_0}{b_L}\right) - 1\right]\left\{B\delta + e^{-B\delta} + \left[\frac{b_L}{2(b_0 - b_L)}\right] - 1\right\} \tag{94}$$

Figure 24 shows the plot of E against γ_r as given by the model, for two values of the ratio of interface to bulk microorganism concentration. The model predicts that in equipment where the mass transfer coefficient k_L is small, as in surface-aerated vessels, the oxygen absorption rate may be enhanced due to the activity of the microorganisms. Distribution of microorganisms may exert some influence, especially if the concentration near the interface is much higher than in the bulk. On the other hand, when the mass transfer coefficient is relatively high, as in a conventional sparged or stirred fermenter or in a bubble column, no influence of the oxygen uptake by the microorganisms can be detected because the enhancement factor is practically unity.

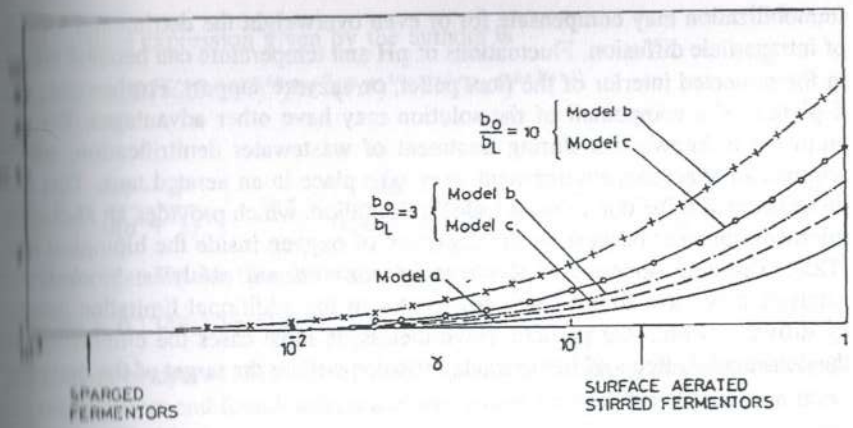


Figure 24 Enhancement of the absorption rate of oxygen due to biological consumption. (From Ref. 121.)

Although this is valid for most aerobic bioprocesses and therefore no enhancement is taken into account in the design of fermenters in general, the situation may change in the case of very viscous broths. In this case the mass transfer coefficient k_L will fall into the range of higher-than-unity enhancement factor E in Fig. 24. Ignoring the effect of the enhancement factor will lead to overdesign of the fermenter from the point of view of oxygen transfer. What happens in practice is that the process, which is oxygen limited in any case, will yield better results than expected.

G. Intraparticle Mass Transfer

In the case in which the microorganisms are not freely suspended in the liquid but are either aggregated in flocs or pellets or immobilized on a solid support (which is also the case for immobilized enzymes), an additional stage is added to the process of mass transfer. In addition to crossing the liquid boundary layer around the particle, the diffusing substrate has to reach from the external surface to the point where the biochemical transformation actually takes place. This means that for cells which are located in the center of the particle, the substrate must travel a long and tortuous way until it makes itself available.

This phenomenon has long been observed and analyzed [123] and the unwanted and costly effect of the diffusion limitation on the amount of biocatalyst required is well known (see Chapter 9). There is also another aspect of this phenomenon. Diffusion limitation can also be used as an artifact by the designer of a process [124]. Operation stability of enzymes as a consequence of

immobilization may compensate for or even overweight the detrimental aspects of intraparticle diffusion. Fluctuations of pH and temperature can become milder in the protected interior of the floc, pellet, or enzyme support. Furthermore, the depletion of a component of the solution may have other advantages. For example, it is known that during treatment of wastewater denitrification, which requires an anaerobic environment, may take place in an aerated tank. This can be considered to be due to mass transfer limitation, which provides an anaerobic microenvironment because of the depletion of oxygen inside the biological floc [125]. Thus, as opposed to the case of conventional catalysis, biochemical catalysis may have found some advantages in the additional limitation caused by diffusion within the particle. Nevertheless, in most cases the elimination of the detrimental effects of intraparticle diffusion will be the target of the designer.

V. HEAT TRANSFER

Because of the relatively low reaction rates of processes involving microorganisms and cells, it may in a very general way be said that heat effect problems related to local variations of temperature are not common in bioreactors. Even when polymeric products are released to the medium and very high viscosity is attained, heat transfer is not the controlling step, because such viscous media also hinder the mass transfer and the heat generation is consequently limited. The main point to focus on in those cases is therefore mass rather than heat transfer.

The case of reactions catalysed by immobilized enzymes, on the other hand, may require different considerations.

The heat transfer in stirred fermentors can be calculated using the equations available for chemical reactor design. The presence of bubbles in aerobic processes does not change very much the rate of heat transfer in those devices, where the flow is in most cases highly turbulent [125].

A. Heat Transfer in Bubble Columns

The heat transfer rate in bubble columns is much larger than that expected for single-phase flow [126]. This is caused by the bubble-driven turbulence and liquid recirculation, which are characteristic of the flow in bubble columns. Several correlations have been proposed for the prediction of the heat transfer coefficient in these reactors. Kawase and Moo-Young [127] presented an expression which fits satisfactorily most of the published data. The model is based on Levich's [128] three-zone concept and Kolmogoroff [129] isotropic turbulence theory and has no empirical adjustable parameters. The model can take into consideration non-Newtonian behavior of the liquid and predicts the enhancement of the heat transfer due to the shear-thinning effect of the fluid. The

dimensionless expression given by the authors is

$$\mathrm{Nu} = 0.075(10.3n^{-0.63})^{\beta} n^{1/3} (\mathrm{Pr}^*)^{1/3} \mathrm{Fr}^{-\beta} (\mathrm{Re}^*)^{\beta+3(n+1)} \tag{95}$$

with

$$\beta = \frac{4-n}{6(n+1)}, \qquad \mathrm{Pr}^* = \frac{\kappa D^{1-n}}{(k/C_p)\,(J_G)^{1-n}}, \qquad \mathrm{Re}^* = \frac{D^n\,(J_G)^{2-n}}{\kappa/p} \tag{96}$$

For Newtonian liquids this equation reduces to

$$\mathrm{Nu} = 0.134\mathrm{Pr}^{1/3}\mathrm{Fr}^{-1/4}\mathrm{Re}^{3/4} \tag{97}$$

The authors compared their model with data and correlations published by various investigators and found satisfactory agreement for both Newtonian and non-Newtonian liquids.

For the case of airlift reactors [130], the flow in the reactor may be similar to that in bubble columns, if the internal recirculation is high, or closer to the case of net two-phase flow in pipes, in which case the equations recommended for heat transfer in pipes can be used [131]. Blakebrough et al. [132] studied the heat transfer rates in an external-loop airlift fermentor. They reported an enhancement of this rate due to the presence of microorganisms in the case of laminar flow. In their studies, carried out with suspensions of *Aspergillus niger*, they concluded that this enhancement can be explained by two mechanisms: disturbances of the liquid layer near the surface at low microbial concentrations and a "scraped surface" type of heat transfer at high concentrations, or when the microorganism grows in the form of pellets.

VI. SHEAR IN BIOREACTORS

Reactor scale-up in a chemical process is basically focused on obtaining in the large-scale vessel the same average rates of production as in a smaller-scale experimental reactor. Reaching this objective is not a simple task, since the hydrodynamics of large vessels are often complicated and difficult to model. The importance of the hydrodynamics stems from the fact that in large vessels the transport of mass and heat depends on convective mechanisms and is usually associated with turbulent eddy flow.

Therefore, shearing flow is usually present in reactors. In most chemical processes, the shear rate is not important by itself but as a means of increasing the mass and heat transfer rates. In biochemical processes, on the other hand, the shear rate may be important by itself. This is certainly accepted for animal cells and can be extended to plant cell culture as well. The technical literature gives testimony that microbial cultures may also be influenced by the effects of shear.

It is customary to admit that excessive shear can damage suspended cells leading to loss of viability and even disruption in the case of the more fragile ones. In some cases, however, positive effects have been observed, within certain limits. Such positive effects may be due to enhancement of heat and mass transfer rates. It has been suggested, however [133], that shear by itself may sometimes have a beneficial effect on culture growth and metabolite production rates. In such cases, shear may become one of the variables in the kinetics of the process. Figure 25 shows schematically the interrelationship between fluid dynamics, heat or mass transfer, and kinetics. For a given bioreactor design, viscosity and power input will determine the flow patterns, which affect the reactor performance on both microscopic and macroscopic scales. Shear appears as one of the manifestations of the former, and it directly affects both heat and mass transfer. All of the transfer phenomena are finally integrated with the kinetics of the process to yield biomass growth and product formation. The dotted line in the figure indicates the additional connection between shear and kinetics already suggested [133].

Large vessels do not have uniform hydrodynamic conditions in the whole of their volume, and sometimes it makes sense to define regions within them

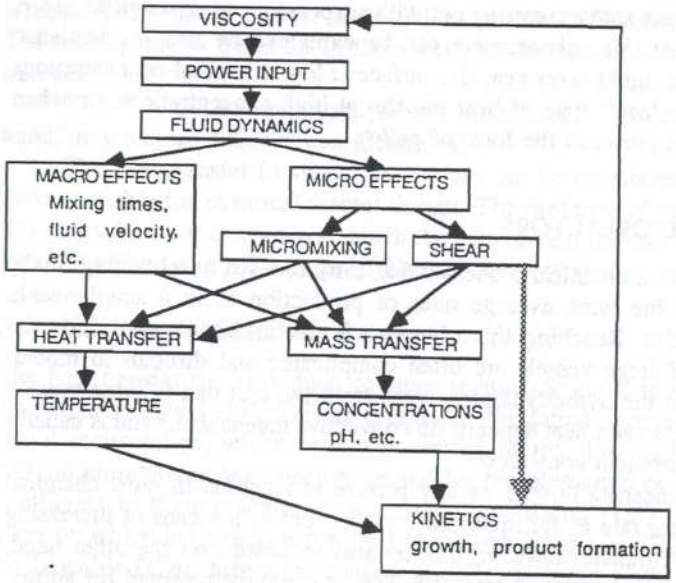


Figure 25 Schematic representation of the interrelationship between fluid dynamics, heat/mass transfer, and kinetics. The dotted line indicates the additional connection between shear and kinetics suggested in [133]. (From Ref. 133.)

according to velocity and turbulence. Each of these regions constitutes a different environment, which may have different substrate and product concentrations, different pH, different cell concentration, different temperatures, and different shear conditions. Once this is defined, the equations representing the system can be written, because the kinetics of the reactions and the transfer rates depend on these variables.

Recognition of these different environments in the fermentor in fact implies the statement of a structured model for the bioreactor. Here we do not refer to structured biokinetic models but to the structure of the bioreactor, from the point of view of hydrodynamics. This type of model can be very successful. See, for example, Bajpai and Reuss [134], Gibbs et al. [135], and Chapter 6 in this book. The inclusion of shear in the kinetic formulation is, however, limited by our lack of knowledge on the matter.

PROBLEMS

1. Roels [5] cites the following values for the kinetics of growth of *K. aerogenes*:

$$\mu_{max} = 0.85\ [h^{-1}]$$
$$Y_{XS} = 0.55$$
$$K_s = 9 \times 10^{-3}\ [kg\ m^{-3}]$$

Check if you could predict the measured value of K_s using Eq. (30).

2. The effects of temperature on the specific rate of growth of a microbial population and on the specific rate of product synthesis are well known. Figure 9 shows experimental data of μ (h^{-1}) for Saccharomyces. It appears that the equation used to fit the data, Eq. (64), gave a good fit. The same equation was able to fit the data on specific rate of product synthesis (Fig. 10). By using a simple curve fitting and simulation program (e.g., *Polymath*), determine whether Eq. (63) (which has also been proposed for this purpose) would be able to fit the experimental data as well as Eq. (64).

3. The mathematical modeling of recombinant microorganisms has become of prime importance in recent years. Section III.H shows in a very elementary way how the segregation of plasmids in a cell population will affect the overall population kinetics. This problem has been tackled mathematically by several groups in the last few years. Discuss how you would analyze this question mathematically and what is the effect of the overall kinetics of a broad vis-a-vis a narrow distribution of plasmid copy number in a population. Review issues of *Biotechnology and Bioengineering* of recent years to answer this question in more detail.

4. Show that Eqs. (90–93) can be obtained assuming that the oxygen concentration has no effect on oxygen uptake rate, that the film model is valid, and that biomass concentration is distributed as follows:

$$b = b_L \quad \text{and}$$

$$b = b_0 - [(b_0 - b_L)/\delta]x$$

where x is the depth of the liquid film and b the biomass concentration.

NOMENCLATURE

A	cross-sectional area, m^2; constant in Eqs. (63) and (64)
a_1	constant in Eq. (72)
a	interfacial area per unit volume, m^{-1}; stoichiometric coefficient in Eq. (1), mol NH_3 per SCF substrate
a_b	area of bubble, m^2
B	constant in Eq. (64)
Bo	Bodenstein number $(gD_c^2\rho_L/\sigma)$, dimensionless
b	stoichiometric coefficient in Eq. (1), mol O_2 per SCF substrate
C	concentration of oxygen, mol L^{-1}
C_b	bottom clearance, m
C_t	top clearance, m
C_p	specific heat capacity, J kg^{-1} K^{-1}
c	stoichiometric coefficient in Eq. (1), mol H_2O per SCF substrate
c_1	constant in Eq. (77)
D_c	column diameter, m
D_s	diameter of separator, m
D	diffusivity coefficient, m^2s^{-2}
d	stoichiometric coefficient in Eq. (1), mol CO_2 per SCF substrate
d_p	particle diameter, m
d_c	cell diameter, m
E	enhancement factor for gas absorption, dimensionless
E_a	energy of activation, kcal/mol
E_d	energy of activation for "thermal death," kcal/mol
Fr	Froude number $(J_G[D_c]^{-0.5})$, dimensionless
f	fraction of plasmid-containing cells in the total population
f_n	fraction of plasmid-containing cells after the nth generation
Ga	Galileo number $(gD_c^3\rho^2\mu^{-2})$, dimensionless
g	gravitational acceleration, ms^{-2}
h	heat transfer coefficient, J m^{-2} s^{-1} K^{-1}
H	height, m

J	superficial velocity, m s^{-1}
K_1	consistency index in power law model, Pa s^n
K_i	inhibition constant, kg m^{-3}
K_m	Michaelis–Menten constant, mol m^{-3}
K_s	Monod constant, kg m^{-3}
k	thermal conductivity, J m^{-1} s^{-1} K^{-1}; constant in Eq. (23)
k_L	gas–liquid mass transfer coefficient, m s^{-1}
k_s	liquid-solid mass transfer coefficient, m s^{-1}
L	ungased liquid height, m
M	bubble separation group $[D_s(4D_c)^{-1}]$
m	maintenance coefficient, SCF substrate per SCF biomass per hour
N	nitrogen in substrate, kg m^{-3}; cell number in Eq. (19)
N_s	flux of substrate, kg s^{-1} m^{-3}
Nu	Nusselt number, (hD_ck^{-1}), dimensionless
n	empirical constant in flow index in power law model, dimensionless; number of moles; number of generations; kinetic parameter
p	pressure, Pa
P	probability of plasmid-containing cells losing their plasmid after one division
P_1	power input, W
Pe	Peclet number, dimensionless
Pr	Prandtl number, dimensionless
R	residual
r	reaction rate, kg s^{-1} m^{-3}
Sc	Schmidt number $[\mu(\rho D_L)^{-1}]$, dimensionless
Sh	Sherwood number
S	substrate concentration, kg m^{-3}
T	temperature, K; dimensionless bursting period in Eqs. (87)–(88)
t	time, s
t_d	doubling time
t_e	exposure time, s
V	volume, m^3
V_{max}	maximum rate of an enzymatic reaction, mol s^{-1} m^{-3}
X	biomass concentration, kg m^{-3}
X'	concentration of plasmid-containing cells, kg m^{-3}
X''	concentration of plasmid free cells, kg m^{-3}
X_{dr}	bottom clearance ratio $(C_bD_{dr}^{-1})$
X_{max}	maximum biomass concentration, kg m^{-3}
X_0	initial biomass concentration, kg m^{-3}
Y	top clearance $(C_tD_{dr}^{-1})$
Y_{xs}	biomass yield on substrate, kg biomass per kg substrate

Y_b^c biomass yield on substrate, SCF biomass per SCF substrate
Y_p^c product yield on substrate, SCF product per SCF substrate
Y_{xs}^{max} maximal yield on substrate, SCF biomass per SCF substrate

Greek Symbols

α exponent in Eq. (72), ratio of growth rates of plasmid-free and plasmid containing cells in a culture; constant in Eq. (43)
β exponent in Eq. (72) dimensionless group, Eq. (96); constant in Eq. (45)
γ shear rate, s^{-1}
γ_r Hatta number, Eq. (91), dimensionless
δ film thickness, m
η energetic yield, dimensionless
ϵ gas holdup, dimensionless
ϵ' energy dissipation per unit mass, w kg^{-1}
κ fluid consistency index, Pa s^n
μ_L dynamic viscosity of liquid, Pa s
μ specific growth rate, h^{-1}
μ^+ specific growth rate of plasmid-containing cells, h^{-1}
μ^- specific growth rate of plasmid-free cells, h^{-1}
μ_{lim} specific growth rate under substrate limitation conditions, h^{-1}
μ_{max} maximal specific growth rate, h^{-1}
ν reductance degree (av. electrons)
π specific product formation rate, h^{-1}
ρ density, kg m^{-3}
ρ_c cell density, kg/m^3; specific substrate consumption rate, h^{-1}
σ surface tension, N m^{-1}
τ shear stress, Pa
ξ function defined in Eq. (74)
Π function defined in Eqs. (92)–(94)

Subscripts

a atmospheric
app apparent
b bottom, biomass
c cell
d downcomer
dr draft tube
G gas
h energy

i inlet
L liquid
i interface
p product
r riser
s substrate, stirrer
t top

ACKNOWLEDGMENTS

The authors wish to acknowledge SERC for support of part of this work through a Visiting Scientist Fellowship.

REFERENCES

1. J. G. Minkevich and V. K. Eroshin, *Folia Microbiol. 18*: 376–386 (1973).
2. W. J. Payne, *Annu. Rev. Microbiol. 24*: 17–43 (1970).
3. L. E. Erickson, I. G. Minkevich, and V. K. Eroshin, *Biotechnol. Bioeng. 20*: 1595–1621 (1978).
4. L. E. Erickson, S. E. Selga, and U. U. Viesturs, *Biotechnol. Bioeng. 20*: 1623–1638 (1978).
5. J. A. Roels, *Energetics and Kinetics in Biotechnology*, Elsevier Biomedical Press, New York, 1983.
6. J. A. Roels, *Biotechnol. Bioeng. 22*: 33–53 (1980).
7. T. Bauchop and S. R. Elsden, *J. Gen. Microbiol. 23*: 457–469 (1970).
8. A. H. Stouthamer and H. W. van Verseveld, Stoichiometry of microbial growth, *Comprehensive Biotechnology* (M. Moo-Young, ed.), Vol. 1, Pergamon Press, New York, (1985), pp. 215–238.
9. J. A. Asenjo and J. Suk, *J. Ferment. Technol. 64*: 271–278 (1986).
10. D. Herbert, *Recent Prog. Microbiol. 7*: 381 (1959).
11. S. J. Pirt, *Proc. R. Soc. London Ser. B 163*: 224–231 (1965).
12. J. A. Roels and N. W. F. Kossen, *Prog. Ind. Microbiol. 14*:95 (1978).
13. G. F. Andrews, *Biotechnol. Bioeng. 42*: 549–556 (1993).
14. F. Srienc, B. Arnold, and J. E. Bailey, *Biotechnol. Bioeng. 26*: 982 (1984).
15. R. Reuven, D. Mills, and J. C. Merchuk, Production of secondary metabolites from plant cells, Annual Congress of Chemical Engineers of Israel, Haifa, 1993.
16. M. L. Shuler and F. Kargi, *Bioprocess Engineering: Basic Concepts*, Prentice Hall, Englewood Cliffs, NJ, 1992.
17. M. Reuss and F. Wagner, *3rd Symp. Technol. Mikrobiol.*, (H. Dellweg ed.), Technische Universität Berlin, 1973, p. 89.
18. A. Rotem, J. C. Merchuk, and Sh. (Malis) Arad, *J. Chem. Technol. Biotechnol. 55*: 263–267 (1992).

19. A. Constantinides, J. L. Spencer, and E. L. Gaden, *Biotechnol. Bioeng. 12*: 1[illegible] (1970).
20. J. E. Bailey and D. F. Ollis, *Biochemical Engineering Kinetics*, McGraw-Hill, New York, 1986.
21. J. B. Hunter and J. A. Asenjo, *Biotechnol. Bioeng. 35*: 31–42 (1990).
22. M. L. Shuler and M. M. Domach, Mathematical Modeling of the growth of individual cells, *Foundations of Biochemical Engineering* (H. W. Blanch, E. T. Papoutsakis, and G. Stephanopoulos, eds.) ACS Symposium Series 207, ACS, Washington, DC, 1983, p. 93.
23. J. Monod, *Recherches sur la Croissance des Cultures Bactériennes*, Herrmann, Paris, 1942.
24. J. Monod, *Annu. Rev. Microbiol. 3*: 371–394 (1949).
25. T. Imanaka, T. Kaieda, K. Sato, and H. Taguchi, *J. Ferment. Technol. 50*: 6[illegible] (1972).
26. T. Imanaka, T. Kaieda, K. Sato, and H. Taguchi, *J. Ferment. Technol. 51*: 4[illegible] (1973).
27. J. A. Asenjo, J. L. Spencer, and V. Phuvan, *Ann. N.Y. Acad. Sci. 469*: 404–4[illegible] (1986).
28. J. A. Asenjo and C. Jew, *Ann. N.Y. Acad. Sci. 413*: 211 (1983).
29. J. A. Asenjo and J. Suk, *Biotechnol. Bioeng. Symp. 15*: 225–234 (1985).
30. R. Leudeking and E. L. Piret, *J. Biochem. Microbiol. Technol. Eng. 1*: 393 (1959).
31. A. Moser, Kinetics of batch fermentations, *Biotechnology*, Vol. 2 (H. J. Rehm and G. Reed, eds.), VCH, Weinheim, 1985, p. 245.
32. S. Aiba and M. Shoda, *J. Ferment. Technol. 47*: 790 (1969).
33. N. D. Jerusalimsky and N. M. Neronova, *Dokl. Akad. Nauk. SSR 161*: 1437 (1965).
34. T. K. Ghose and R. D. Tyagi, *Biotechnol. Bioeng. 21*: 1401 (1979).
35. F. H. Johnson, H. Eyring, and M. J. Polissar, *The Kinetic Basis of Molecular Biology*, Wiley, New York, 1954.
36. B. Atkinson and F. Mavituna, *Biochemical Engineering and Biotechnology Handbook*, Stockton Press, New York, 1991.
37. G. van Dedem and M. Moo-Young, *Biotechnol. Bioeng. 15*: 419–439 (1973).
38. G. Yagil and E. Yagil, *Biophys. J. 11*: 11–27 (1971).
39. K. Venkatasubramanian, Mathematical modelling of glucose isomerase induction and repression, Ph.D. dissertation, Rutgers University, Rutgers, NJ, 1977.
40. K. Toda, *Biotechnol. Bioeng. 18*: 1117–1124 (1976).
41. M. L. Shuler, S. Leung, and C. C. Dick, *Ann. N.Y. Acad. Sci. 326*: 7–16 (1979).
42. J. E. Bailey, J. Fazel-Madjlessi, D. N. McQuitty, and M. F. Gilbert, *Ann. N.Y. Acad. Sci. 326*: 7–16 (1979).
43. M. A. Hjortso and J. E. Bailey, *Math. Biosci. 60*: 235 (1982).
44. R. D. Kiss and Gr. Stephanopoulos, *Biotechnol. Bioeng. 39*: 565–574 (1992).
45. J. P. Delgado and J. C. Liao, *Biotechnol. Prog. 7*: 15–20 (1991).
46. M. M. Ataai and M. L. Shuler, *Biotechnol. Bioeng. 27*: 1051 (1985).
47. T. Imanaka and S. Aiba, *Ann. N.Y. Acad. Sci. 369*: 1–14 (1981).
48. D. F. Ollis and H. T. Chang, *Biotechnol. Bioeng. 24*: 2583–2586 (1982).
49. S. B. Lee and J. E. Bailey, *Biotechnol. Bioeng. 26*: 1372–1382 (1984).

50. J. H. Ponzo and W. A. Weigand, *Biotechnol. Bioeng. 38*: 1065–1081 (1991).
51. I. Harditjo, P. F. Greenfield, and P. L. Lee, *Biotechnol. Prog. 8*: 298–306 (1992).
52. D. D. Y. Ryu, J. Y. Kim, and S. B. Lee, Bioprocess kinetics and modelling of recombinant fermentation, *Biotechnology*, Vol. 4, *Measuring, Modelling and Control* (K. Schügerl, ed.), VCH Weinheim, 1991, pp. 485–505.
53. J. M. Lee, *Biochemical Engineering*, Prentice Hall, Englewood Cliffs, NJ, 1992.
54. P. R. Patnaik, *Biotechnol. Techniques 7*: 137–142 (1993).
55. A. P. Togna, J. Fu, and M. L. Shuler, *Biotechnol. Bioeng. 42*: 557–570 (1993).
56. S. B. Lee, D. D. Y. Ryu, R. Seigel, and S. H. Park, *Biotechnol. Bioeng. 31*: 805–830 (1988).
57. W. M. Miller, H. W. Blanch, C. R. Wilke, *Biotechnol. Bioeng. 32*: 947–965 (1988).
58. K. K. Frame and W. S. Hu, *Biotechnol. Bioeng. 38*: 1020–1028 (1991).
59. B. C. Batt and D. S. Kompala, *Biotechnol. Bioeng. 34*: 515–531 (1989).
60. G. W. Hiller, A. D. Aeschlimann, D. S. Clark, and H. W. Blanch, *Biotechnol. Bioeng. 38*: 733–741 (1991).
61. D. W. van Kervelen, *Proceedings of the First Symposium on Chemical Reaction Engineering* (D. W. van Krevelen, ed.), Pergamon Press, Oxford, 1957.
62. N. W. F. Kossen, Advanced Topics in Biochemical Engineering, Lectures at Ben-Gurion University of the Negev, Beer Sheeva, May 1984.
63. Y. Shi, D. D. Y. Ryu, and S. H. Park, *Biotechnol. Bioeng. 40*: 260 (1992).
64. C. W. Robinson and C. R. Wilke, *Biotechnol. Bioeng. 15*: 755 (1973).
65. K. van't Riet, *Ind. Eng. Chem. Proc. Des. Dev. 18*: 367 (1979).
66. K. van't Riet and J. Tramper, *Basic Bioreactor Design*, Marcel Decker, New York, 1991.
67. K. Akita and F. Yoshida, *Ind. Eng. Chem. Process Des. Dev. 12*: 76 (1973).
68. H. Hikita, S. Asai, K. Tanigawa, K. Segawa, and K. Kitao, *Chem. Eng. J. 20*: 59 (1980).
69. M. Cooke and J. C. Middleton, Mixing and mass transfer in filamentous fermentations, *Bioreactor Fluid Dynamics* (R. King, ed.), BHHRA & Elsevier, Barking, 1988, p. 37.
70. M. Reuss and R. Bajpai, *Biotechnology*, Vol. 4 (K. Schügerl, ed.), VCH, Weinheim, 1991, p. 299.
71. F. C. Bader, Improvements in multiple turbine mass transfer models, *Biotechnology Processes—Scale-up and Mixing* (Ch. S. Ho and J. Y. Oldshue, eds.), AICHE, New York, 1987, p. 96.
72. R. N. Baker, A. N. Emery, and A. W. Nineow, Mass transfer and power characteristics of a simulated filamentous fermentation broth exhibiting biological activity, *Bioreactor Fluid Dynamics* (R. King, ed.), Elsevier, Barking, 1988, p. 79.
73. A. Handa-Corrigan, A. N. Emery, and R. E. Spier, *Enzyme Microb. Technol. 11*: 230 (1989).
74. E. T. Papoutsakis, *Trends Biotechnol. 9*: 427 (1991).
75. J. C. Merchuk, Shear effects on animal cells, *Biologicals from Recombinant Microorganisms and Animal Cells* (M. D. White, S. Reuveni, and A. Shafferman, eds.), VCH, Weinheim, 1991, p. 199.
76. J. Tramper, D. Smit, J. Straatman, and J. M. Valk, *Bioprocess Eng. 3*: 37 (1988).

77. P. King, Fermentation special report, *Biotechnology 6*: 507 (1988).
78. S. Zjhang, A. Handa-Corrigan, and R. E. Spier, *Biotechnol. Bioeng. 40*: [illegible] (1992).
79. W. D. Deckwer, *Bubble Column Reactors*, John Wiley & Sons, New York, 1991.
80. J. C. Merchuk, *Trends Biotechnol. 8*: 66 (1990).
81. J. C. Merchuk, FENABIO-BIOLATINA 91, Air-Lift Reactor Design, São Paulo, Brasil, July 1991.
82. R. A. Bello, C. W. Robinson, and M. Moo Young, *Chem. Eng. Sci. 40*: 53 (1985).
83. M. Y. Chisti, K. Fujimoto, and M. Moo-Young, Hydrodynamics and oxygen mass transfer studies in bubble columns and air lift bioreactors, *Biotechnology Processes—Scale-up and Mixing* (Ch. S. Ho and J. Y. Oldshue, eds.), AICHE, New York, 1987, p. 72.
84. M. Popvic and C. W. Robinson, Estimation of some important design parameters for non-Newtonian fluids in pneumatically-agitated bioreactors, Proceedings of the 34th Canadian Chemical Engineering Conference, Quebec City, 1984, p. 258.
85. R. A. Bello, C. W. Robinson, and M. Moo-Young, M., *Biotech. Bioeng. 27*: 369 (1985).
86. K. Koide, K. Kurematsu, S. Iwamoto, Y. Iwata, and K. Horibe, *J. Chem. Eng. Japan 16*: 413 (1983).
87. M. Nishikawa, H. Kato, and K. Hashimoto, *Ind. Eng. Chem. Process Des. Dev. 16*: 133 (1977).
88. J. H. Henzler, *Chem. Ing. Tech. 52*: 643 (1980).
89. Y. Kawase and M. Moo-Young, *Chem. Eng. Commun. 40*, 67 (1986).
90. A. Schumpe and W. D. Deckwer, *Bioproc. Eng. 2*, 79 (1987).
91. A. Zaidi, H. Bourziza, and L. Echihabi, *Chem. Ing. Tech. 59*, 748–749 (1987).
92. S. A. El-Tamtamy, S. A. Khalil, A. A. Nour-El-Din, and A. Gaber, *Appl. Microbiol. Biotech. 19*: 376 (1984).
93. J. C. Merchuk and S. Ben-Zvi (Yona), *Chem. Eng. Sci. 47*: 3517 (1992).
94. A. Schumpe and W. D. Deckwer, *Ind. Eng. Chem. Process Des. Dev. 21*: 706 (1982).
95. D. W. Deckwer, K. Nguyen-Tien, A. Schumpe, and Y. Serpemen, *Biotech. Bioeng. 24*: 461 (1982).
96. S. P. Godbole, A. Schumpe, Y. T. Shah, and N. L. Carr, *AIChE J. 30*: 213 (1984).
97. M. Nakanoh and F. Yoshida, *Ind. Eng. Chem. Process Des. Dev. 19*: 190 (1980).
98. P. H. Calderbank and M. Moo-Young, *Chem. Eng. Sci. 16*: 39 (1961).
99. F. Kastanek, The volume mass transfer coefficient in a bubble bed column, *Collect. Czech. Chem. Com. 42*: 2491 (1977).
100. Y. Kawase, B. Halard, and M. Moo-Young, *Chem. Eng. Sci. 42*: 1609 (1987).
101. Y. Kawase, B. Halard, and M. Moo-Young, *Biotechnol. Bioeng. 39*: 1133 (1992).
102. Y. Kawase and J. J. Ulbretch, *Can. J. Chem. Eng. 61*: 791 (1983).
103. Y. Kawase and J. J. Ulbretch, *Physicochem. Hydrodyn. 4*: 351 (1983).
104. C. W. Robinson and C. R. Wilke, *Biotechnol. Bioeng. 15*: 755 (1973).
105. M. Schmitz, A. Strief, and P. M. Weinspach, *Chem. Ing. Tech. 57*: 984 (1985).
106. A. Benedek and W. J. Haideger, *Biotechnol. Bioeng. 13*: 663 (1971).
107. W. K. Koetsier and D. Thoenes, *Chem. Eng. J. 5*: 71 (1973).

108. L. L. van Dierendonck, Doctoral thesis, T. H. Twente, 1970.
109. K. Nakao, H. Takeuchi, H. Kataoka, T. Otake, and T. Miyauchi, *Ind. Eng. Chem. Process Des. Dev. 22*: 577 (1983).
110. F. Yoshida, personal communication, 1967.
111. A. Rotem, J. C. Merchuk, and Sh. (Malis) Arad, *J. Chem. Tech. Biotechnol. 55*: 363 (1992).
112. F. Sanger and W. D. Deckwer, *Chem. Eng. J. 22*: 179 (1981).
113. P. W. Danckwerts, *Gas Liquid Reactions*, McGraw-Hill, New York, 1970.
114. W. G. Withman, *Chem. Metal. Eng. 29*: 147 (1923).
115. P. A. Ramachandran and M. M. Sharma, *Chem. Eng. Sci. 24*: 1681 (1969).
116. G. T. N. Tsao, *Biotechnol. Bioeng. 10*: 765 (1968).
117. G. T. N. Tsao, *Chem. Eng. Sci. 27*: 1593 (1969).
118. H. Yagi and F. Yoshida, *J. Ferment. Technol. 52*: 905 (1974).
119. Y. Y. lee and G. T. Tsao, *Chem. Eng. Sci. 27*: 1601 (1972).
120. G. T. Tsao, A. Muherjee, and Y. Y. Lee, *Fermentation Technology Today*, Proc. IV IFS Kyoto, 1972, p. 65.
121. J. C. Merchuk, *Biotechnol. Bioeng. 19*: 1885 (1977).
122. G. T. N. Tsao, *Biotechnol. Bioeng. 11*: 1071 (1969).
123. R. Goldman, H. Kedem, and E. Katchalsky, *Applied Biochemistry and Bioengineering*, Vol. 1 (L. B. Wingard, E. Katchalsky-Katzir, and L. Goldsmith, eds), Academic Press, New York, 1976, p. 151.
124. M. Trevan, *Trends Biotechnol. 5*: 7 (1987).
125. H. G. Edney and M. F. Edwards, *Trans. I. Chem. E. 54*: 160 (1976).
126. Y. T. Shah, B. G. Kelhar, S. P. Godbole, and W. D. Deckwer, *AIChE J. 38*: 353 (1982).
127. Y. Kawase and M. Moo-Young, *Chem. Eng. Res. Des. 65*: 121 (1987).
128. V. G. Levich, *Physicochemical Hydrodynamics*, Prentice Hall, Englewood Cliffs, NJ, 1962.
129. A. N. Kolglmorov, *Dok. Akad. Nauk SSSR 66*: 825 (1949).
130. M. Siegel and J. C. Merchuk, *Can. J. Chem. Eng. 69*: 465 (1991).
131. S. P. Godboole and Y. T. Shah, Design and operation of bubble column reactors, *Encyclopedia of Fluid Mechanics* (N. P. Cheremisinoff, ed.), Gulf Publishing Co., 1986.
132. N. Blakebrough, I. A. Fatile, W. J. McManamey, and G. Walker, *Chem. Eng. Res. Des. 61*: 383 (1983).
133. J. C. Merchuk, *Adv. Biochem. Eng. Biotech. 44*: 65 (1991).
134. R. K. Bajpai and M. Reuss, *Can. J. Chem. Eng. 60*: 384 (1982).
135. S. J. Gibbs, D. F. Loy, K. A. Debelak, and R. N. Tanner, *Biotechnology Processes*, AIChE, New York, 1987.

6
Stirred Tank Bioreactors

Matthias Reuss
Universität Stuttgart
Stuttgart, Germany

1. INTRODUCTION

The stirred tank fermenter is the most important type of bioreactor for industrial production processes. The characteristics of a typical vessel are shown in Fig. 1. The agitator or agitators are required to perform a wide range of functions: adequate momentum, heat and mass transfer, and mixing as well as homogenization of suspensions. Optimization of the individual tasks would result in different design of the impellers. Because all these requirements must be accomplished at the same time, the agitators used in practice always reflect compromises. Conventional impellers used in fermentation are typically classified into axial and radial flow impellers. Examples of the two groups are illustrated in Fig. 2. Of the many impeller geometries, the six-blade disk impeller with gas sparging below the impeller is most often used in the standard configuration. However, an interest in using high-flow, low-power-number agitators such as Intermig (Fig. 3a), Lightnin A315 (Fig. 3b), Prochem Maxflow T (Fig. 3c), and Scaba 6SRGT (Fig. 3d) has been developed and improved performance of fermentation processes has been reported with such alternative designs [1–3].

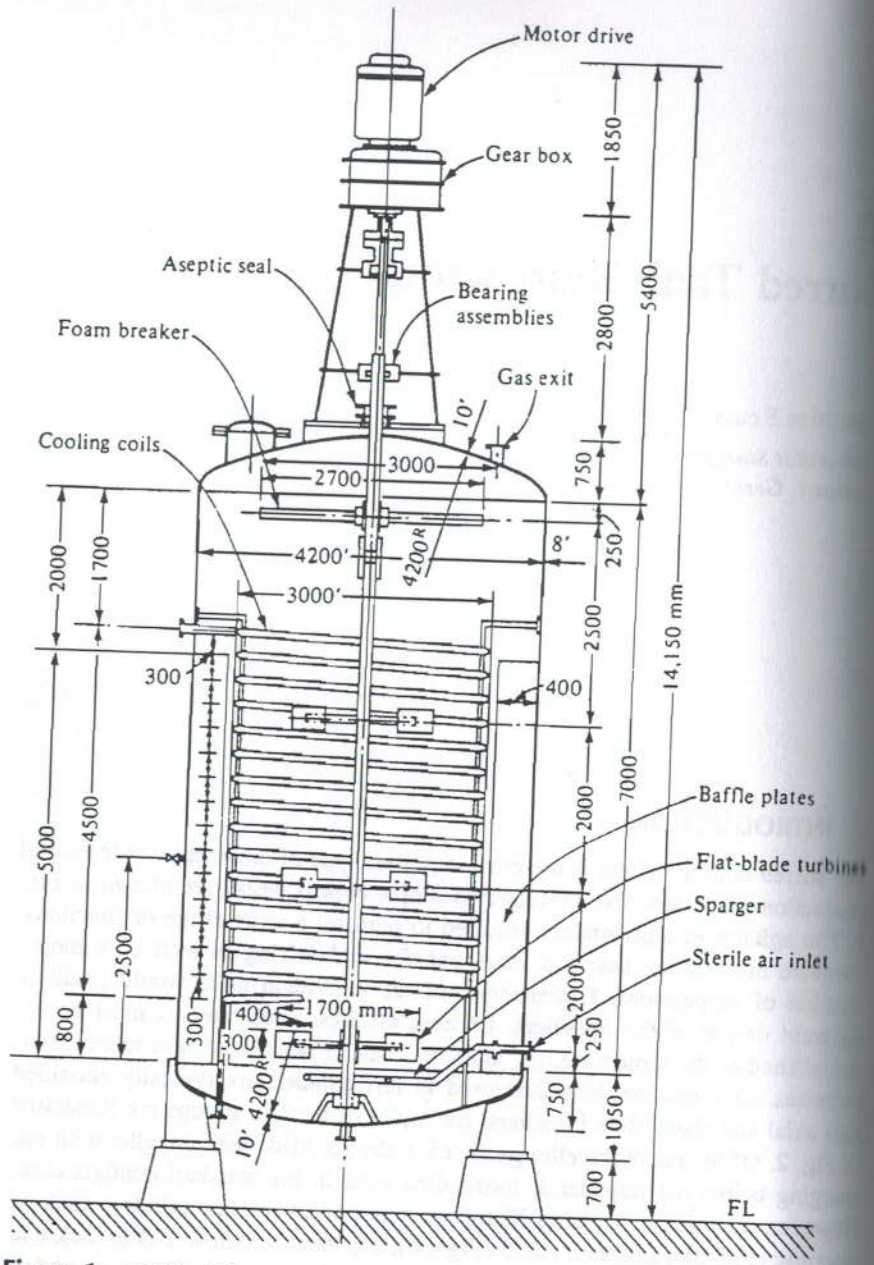


Figure 1 A 100-m³ fermentor used for penicillin production. (From Ref. 65.)

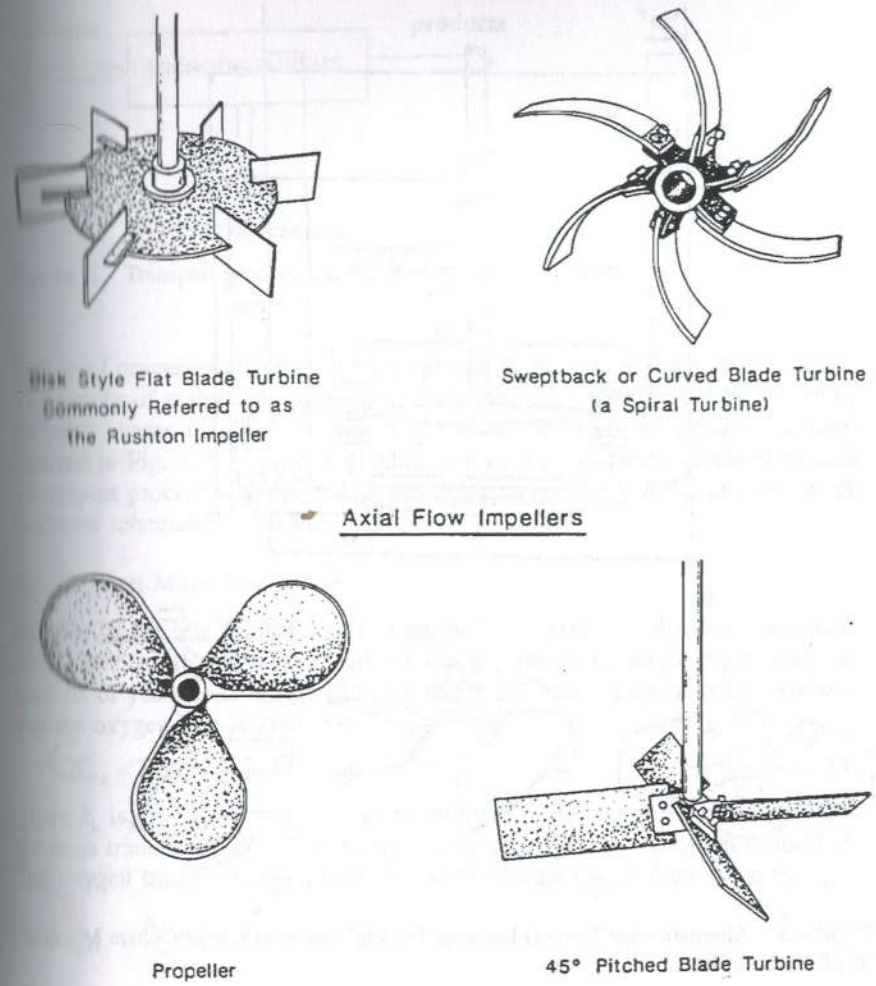


Figure 2 Turbulent impellers. (From Ref. 21, p. 7.)

Considering the large number of industrial fermentation processes with very specific and often unrelated demands, it is surprising to see that the standard stirred tank fermenter is used almost universally in the fermentation industry. Although part of the reasons for such constraint in the design might be related to widespread historical conventional wisdom, it also indicates that microbial production processes must have some common requirements. Some of these basic requirements are outlined in the following.

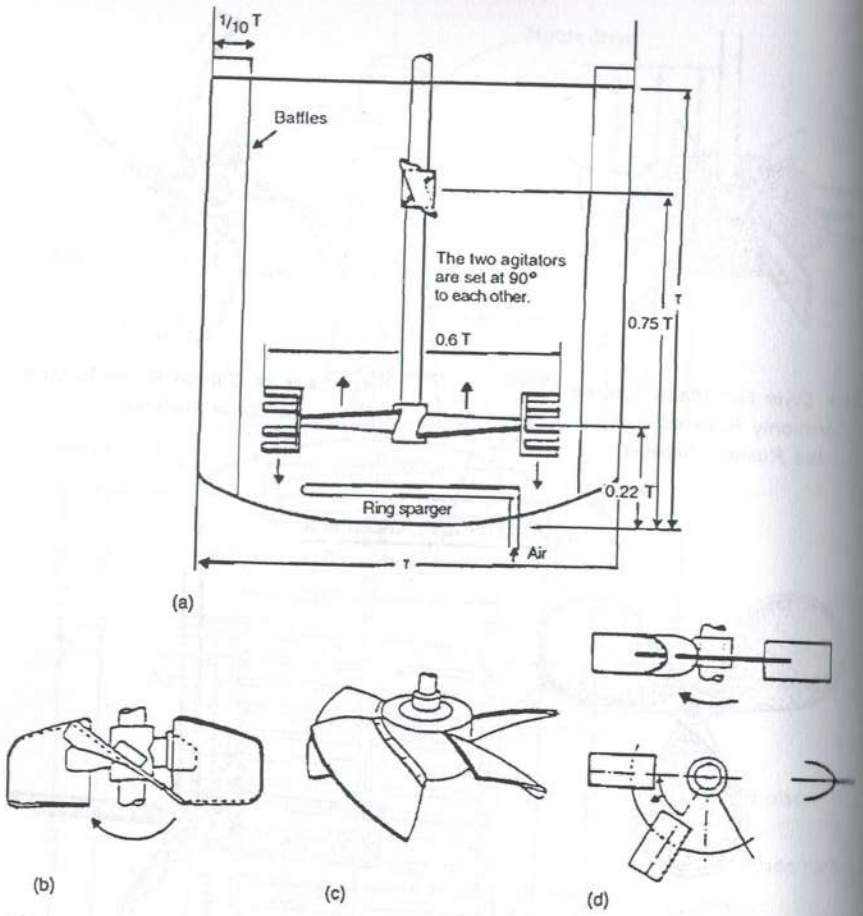


Figure 3 Alternate impellers: (a) Intermig, (b) Lightnin A 315, (c) Prochem Maxflow T, (d) Scaba 6SRGT.

II. IMPORTANT TRANSPORT PHENOMENA

The design of the reactor for a biotechnological production process, first of all, involves the basic task of guaranteeing the optimal environmental conditions for the required metabolic activity of the organisms (bacteria, yeasts, fungi, and plant and animal cells). From the projection of this task to the microscopic level (Fig. 4) it is easy to see that mastering the environment requires control of the transport processes of all components that take part in the process of biological material conversion. The relatively narrow range of optimal temperature for

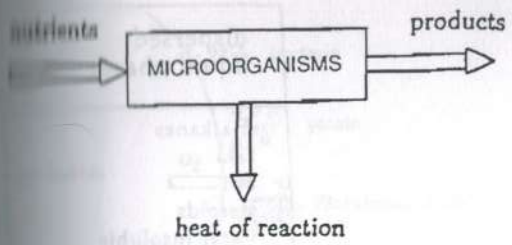


Figure 4 Transport processes at the level of single organisms.

biological processes also requires special actions to remove the heat of reaction. The supply of many important compounds is further complicated by the many different phases involved. A few examples of industrial importance are summarized in Fig. 5. It is easy to illustrate the kind of problems involved in such a transport process with the aid of gas-liquid oxygen transfer, a process which is shown schematically in Fig. 6.

A. Oxygen Mass Transfer

Because of the low solubility of oxygen in fermentation media, the mechanism of oxygen transfer from the bulk of the gas phase to single organisms like bacteria or yeast cells is controlled by the liquid-phase mass transfer resistance and the oxygen flux is given by

$$N_{O_2} = k_L A(c^* - c_L) \tag{1}$$

where k_L is the liquid mass transfer coefficient in m/s, A is the interfacial area for mass transfer in m^2, c^* is the equilibrium oxygen concentration in mol/m^3. The oxygen transfer rate per unit of reactor volume Q_{O_2} is then given by

$$Q_{O_2} = \frac{N_{O_2}}{V_L} = k_L a(c^* - c_L) \tag{2}$$

where V_L is the liquid volume and $a = A/V_L$ is the specific interfacial area. For practical purposes the mass transfer coefficient k_L and specific surface a are not separated and $k_L a$ is referred to as volumetric mass transfer coefficient.

The use of Eq. (2) will be illustrated in the following with the aid of a simple example, in which oxygen demand and growth rate are directly coupled. Consider the case that a microbial culture is characterized by its specific growth rate μ_X, biomass concentration c_X, and yield coefficient $Y_{O/X}$ in mol O_2/kg biomass. The oxygen demand of the culture is then given by

$$(Q_{O_2})_{demand} = \frac{\mu_X c_X}{Y_{O/X}} \tag{3}$$

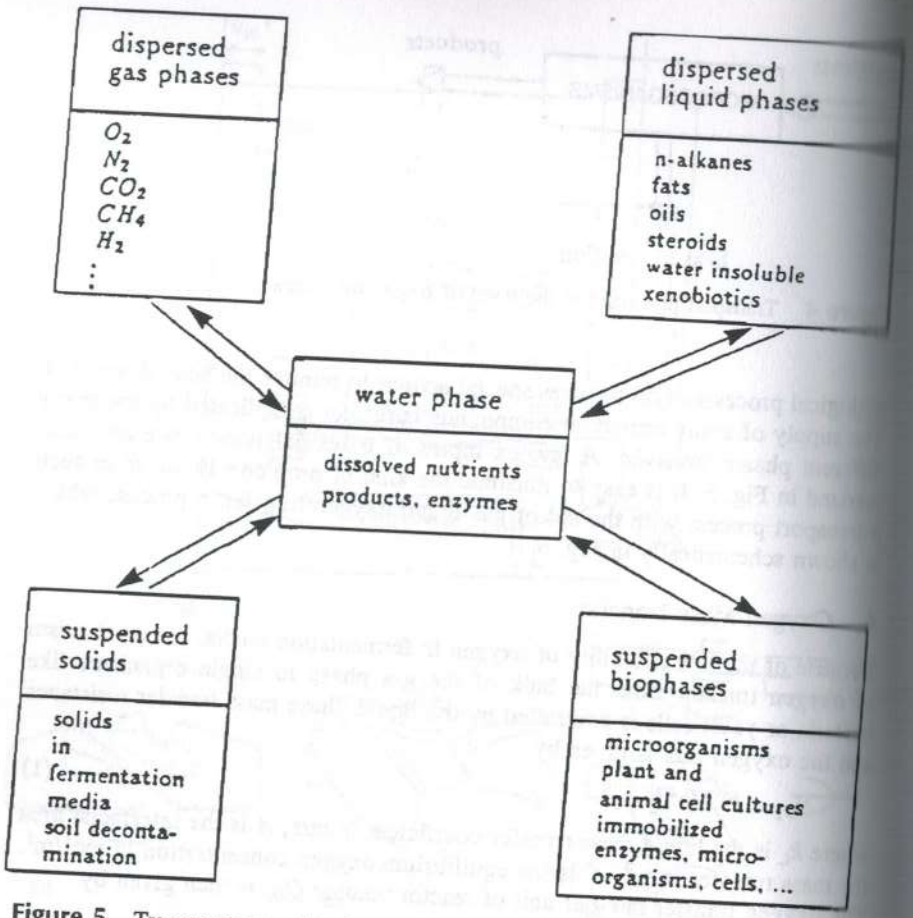


Figure 5 Transport across interfaces in bioreactors.

At steady state, the oxygen supply or absorption rate, Eq. (2), and demand must balance:

$$k_L a(c^* - c_L) = \frac{\mu_X c_X}{Y_{O/X}}$$

Let us next assume that the concentration of oxygen in the bulk of the liquid should be always greater than the critical value c_{crit}. Then

$$(k_L a)_{SP} = \frac{\mu_X c_X}{Y_{O/X}(c^* - c_{crit})} \tag{4}$$

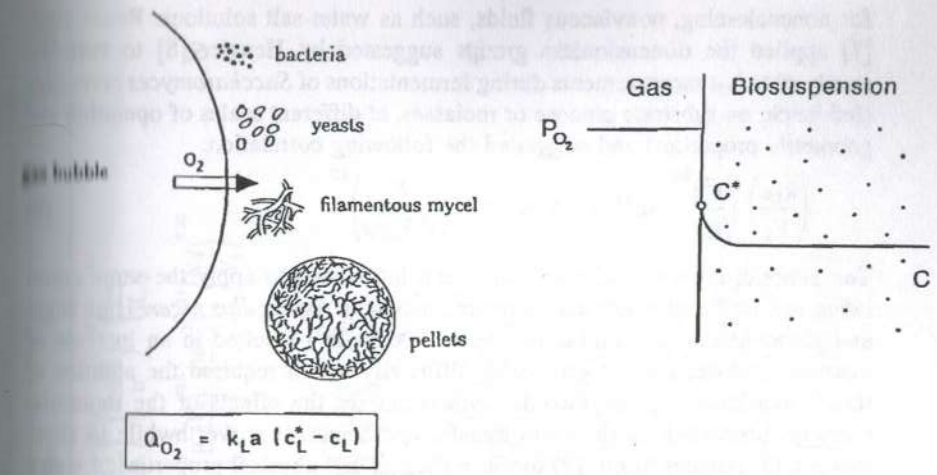


Figure 6 Oxygen supply of microorganisms.

Equation (4) sets the minimum value of $k_L a$ needed for process design. In order to make use of this estimation procedure an equation of the form

$$k_L a = f(\text{type of reactor, operation conditions, material properties})$$

is required.

The correlations suggested for stirred tank reactors are manifold and have been critically reviewed by Reuss [4]. Many of these correlations are corrupted by erroneous measurements and also most of the measurements even performed at only a very small scale. Van't Riet [5] reported in a review on this subject when writing the general form of $k_L a$ correlations as

$$k_L a = K \left(\frac{P}{V}\right)^{\alpha} V_S^{\beta} \tag{5}$$

that the exponents show a fairly great variation ($0.4 < \alpha < 1$ and $0 < \beta < 0.7$). The same authors [5,6] provided correlations for $k_L a$ data divided according to the nature of the solution:

$$k_L a = 2.6 \times 10^{-2} \left(\frac{P_G}{V_L}\right)^{0.4} V_S^{0.5} \tag{6}$$

for coalescing nonviscous fluids, for example, water, and

$$k_L a = 2.0 \times 10^{-3} \left(\frac{P_G}{V_L}\right)^{0.7} V_S^{0.2} \tag{7}$$

for noncoalescing, nonviscous fluids, such as water-salt solutions. Reuss et al. [7] applied the dimensionless groups suggested by Henzler [8] to correlate steady state k_La measurements during fermentations of *Saccharomyces cerevisiae* (fed batch, on substrate glucose or molasses, at different scales of operation and geometric properties) and suggested the following correlation:

$$\left(\frac{k_La}{V_S}\right)\left(\frac{\nu^2}{g}\right)^{1/3} Sc^{0.3} = 5.5 \times 10^{-4}\left(\frac{P}{V_L V_S \rho g}\right)^{0.7} \tag{8}$$

The Schmidt number ($Sc = \nu/D$) has been introduced to apply the same correlation to data for gluconic acid fermentations with *Aspergillus niger*. High sugar and gluconic acid concentrations (up to 300 kg/m^3) resulted in an increase of viscosity and decrease of molecular diffusivity which required the addition of this dimensionless group in order to account for the effects of the molecular transport properties on the mass transfer coefficient. It is worthwhile to stress that Eq. (8) reduces to Eq. (7) for $Sc = (Sc)_{water}$ and physical properties of water.

The situation is much more confusing in the case of the highly viscous non-Newtonian fluids. Table 1 [9] summarizes some of the correlations suggested in the literature. It should be emphasized that the validity and application of these correlations very much depend on the measurements of the flow behavior of the biosuspensions and also on the assumptions regarding the estimation of a representative shear rate for predicting the apparent viscosity.

As far as the oxygen transfer is concerned, there are certain production processes in which the situation is slightly more complicated because of further transfer resistances. An example of industrial importance—a pelletlike morphology of *A. niger*—is illustrated in Fig. 7. In the same figure concentration profiles for oxygen are shown indicating that further resistances due to diffusion limitations inside the pellets as well as external transport of oxygen from the bulk liquid to the surface of the pellet may contribute to the entire transport process of oxygen.

The same considerations are required for engineering evaluations of the various immobilized biosystems (immobilized enzymes and cells, biofilms, flocs, etc.), in which diffusion limitations for substrates and products are known to exist. The steady-state material balance for oxygen in a spherical particle is

$$D_{eff}\left(\frac{d^2c}{dr^2} + \frac{2}{r}\frac{dc}{dr}\right) = q(c) \tag{18}$$

where D_{eff} is the effective diffusion coefficient of oxygen in the pellet and $q(c)$ is the oxygen consumption rate, which may be presented by a simple Monod type of kinetics:

$$q(c) = q_{max}\frac{c}{K_M + c}$$

Table 1 Correlations of the Volumetric Mass Transfer Coefficient in Stirred Tank Bioreactors

Reference	Correlation	Equation
Perez and Sandall [10]	$\frac{k_La d_i^2}{D_L} = 21.2\left(\frac{d_i^2 N\rho_L}{\mu_{eff}}\right)^{1.22}\left(\frac{\mu_{eff}}{\rho_L D_L}\right)^{0.5}\left(\frac{\mu_{eff}V_S}{\sigma}\right)^{0.46}\left(\frac{\mu_G}{\mu_{eff}}\right)^{0.7}$	9
Yagi and Yoshida [11]	$\frac{k_La d_i^2}{D_L} = 0.06\left(\frac{d_i^2 N\rho_L}{\mu_{eff}}\right)^{1.5}\left(\frac{\mu_{eff}}{\rho_L D_L}\right)^{0.5}\left(\frac{\mu_{eff}V_S}{\sigma}\right)^{0.6}\left(\frac{dN^2}{g}\right)^{0.19}\left(\frac{Nd}{V_S}\right)^{0.32}$ $[1 + 2(\lambda N)^{0.5}]^{-0.67}$	10
Ranade and Ulbrecht [12]	$\frac{k_La d_i^2}{D_L} = 2.5 \times 10^{-4}\left(\frac{d_i^2 N\rho_L}{\mu_{eff}}\right)^{1.8}\left(\frac{\mu_{eff}}{\rho_L D_L}\right)^{0.5}\left(\frac{\mu_{eff}}{\mu_w}\right)^{1.39}$ $[1 + 100(\lambda N)]^{-0.67}$	11
Höcker et al. [13]	$\frac{k_La V}{V} = 0.105\left(\frac{P}{V\rho_L[g\mu_{eff}/\rho_L]^{2/3}}\right)^{0.59}\left(\frac{\mu_{eff}}{\rho_L D_L}\right)^{-0.3}$	12
Nishikawa et al. [14]	$\frac{k_La d_i^2}{D_L} = 0.115\left(\frac{d_i^2 N\rho_L}{\mu_{eff}}\right)^{1.5}\left(\frac{\mu_{eff}}{\rho_L D_L}\right)^{0.5}\left(\frac{\mu_{eff}V_S}{\sigma}\right)^{0.5}\left(\frac{d_i N^2}{g}\right)^{0.37}$ $\left(\frac{Nd_i}{V_S}\right)^{0.17}\left(\frac{D}{d_i}\right)^2\left(\frac{P_S/V}{N^3 d_i^5 \rho_L}\right)^{0.8}[1 + 2(\lambda N)^{0.5}]^{-0.67}$ $+ 0.112\left(\frac{P_G/V}{N^3 d_i^5 \rho_L + P_G/V}\right)\left(\frac{V_S}{(gD)^{0.5}}\right)\left(\frac{k(CF_S)^{n-1}}{\rho_L D_L}\right)^{0.5}$ $\left(\frac{gD^2\rho_L}{\sigma}\right)^{0.66}\left(\frac{d_i D^3 \rho_L^2}{k(CV_S)^{n-1}}\right)^{0.42}\left[1 + 0.18\left(\frac{u_b}{d_i}\right)^{0.45}\right]^{-1}$	13

Table 1 Continued

Reference	Correlation	Equation
Henzler [8]	$k_L a\left(\frac{\mu_{eff}}{g^2\rho_L}\right)^{1/3} = B_1\left(\frac{P/V}{\rho_L(g^4\mu_{eff}/\rho_L)^{1/3}}\right)^{\alpha}\left(\frac{V_S}{(g\mu_{eff}/\rho_L)^{1/3}}\right)^{\beta}$	14
Obernosterer and Henzler [15]	$\frac{k_L a}{V_S}\left(\frac{\mu_{eff}^2}{\rho_L^2 g}\right)^{1/3} = B_2\left(\frac{P/V}{\rho_L V_S g}\right)^{\alpha}$	15
Kawase and Moo-Young [16]	$k_L a = 0.675 D_L^{0.5}\frac{\rho_L^{3/5}(P/\rho_L V)^{(9+4n)/10(1+n)}}{(k/\rho_L)^{(1+n)/2}\,\sigma^{3/5}}\left(\frac{V_S}{u_b}\right)^{0.5}\left(\frac{\mu_{eff}}{\mu_W}\right)^{-0.25}$	16
Ogut and Hatch [17]	$\frac{k_L a}{N} = 0.0786\left(\frac{d_i^2 N\rho_L}{\mu_{eff}}\right)^{0.41}\left(\frac{\rho_L N^2 d_i^3}{\sigma}\right)^{0.13}\left(\frac{N d_i}{V_S}\right)^{0.69}$	17

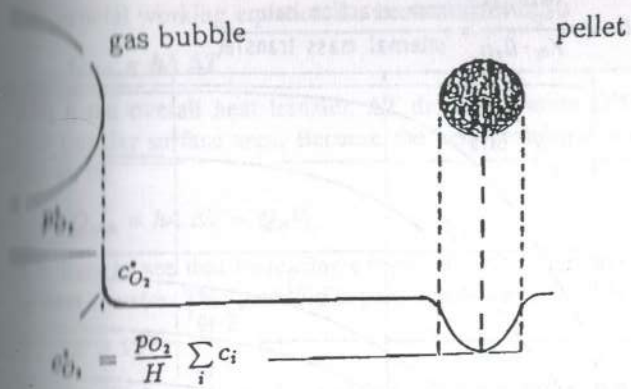


Figure 7 Oxygen transfer in presence of mycelial pellets.

The boundary conditions associated with Eq. (9) are

$$\left(\frac{dc}{dr}\right)_{r=0} = 0 \tag{19}$$

and

$$D_{eff}\left(\frac{dc}{dr}\right)_{r=R} = k_P(c_L - c_{r=R}) \tag{20}$$

Boundary condition Eq. (20) takes care of the mass transfer from the liquid bulk to the surface of the particle. The intensity of this transport is represented by the mass transfer coefficient k_p.

The results of numerical simulation of the differential equation (18) are shown in Fig. 8 in the form of a dimensionless oxygen consumption rate as a function of a Biot number for mass transfer with Damkoehler number as a parameter. These numbers are defined as

$$\mathrm{Bi} = \frac{\beta R^2}{D_{eff}} \qquad \mathrm{Da} = \frac{Q_{O_2}^{max} R^2}{K_M D_{eff}} \tag{21}$$

with

$$\frac{1}{\beta} = \frac{\epsilon_P}{k_L a} + \frac{1}{k_P a_P} \tag{22}$$

with ϵ_P volume fraction and a_P specific surface area, respectively. Equation (22) illustrates that the various external transport resistances have been summarized in an overall mass transfer coefficient β. Since the Biot number now includes

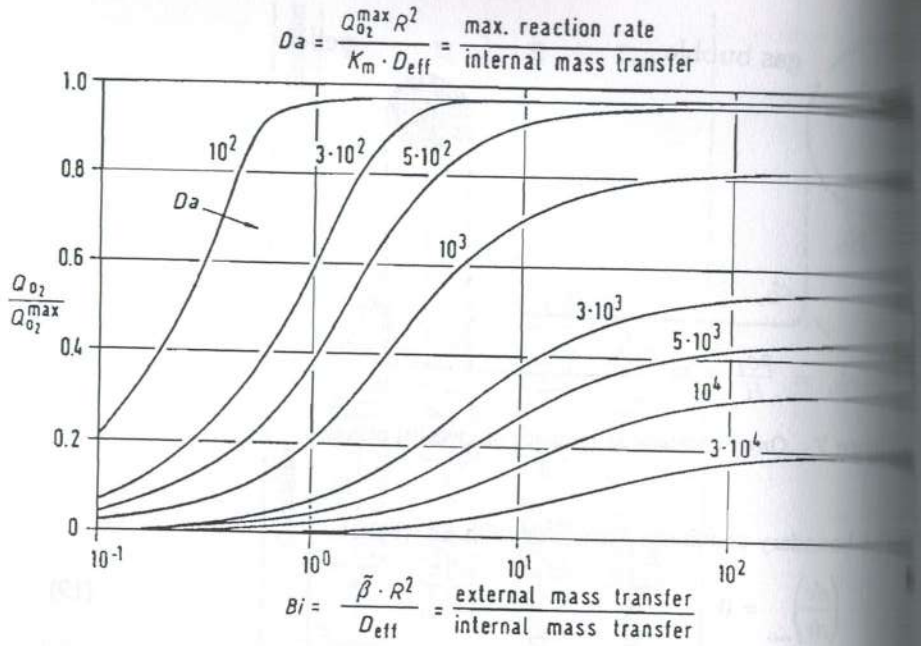


Figure 8 Effect of external and internal mass transfer limitations on effective oxygen consumption of spherical pellets.

the gas-liquid as well as liquid-solid mass transfer resistances, one can calculate an effective oxygen consumption rate for a given set of operation conditions in a bioreactor and a specified pellet diameter.

One can easily see that for a given Damkoehler number it is possible to increase the effective oxygen uptake rate by increasing external mass transfer coefficients. However, beyond a certain level a remaining limitation is observed. This level is approached if external mass transfer resistances can be neglected and reflects the interaction between internal diffusion and reaction.

B. Heat Transfer

When increasing the oxygen supply rate by increasing the k_La value or partial pressure of oxygen in the gas phase, one should always keep the metabolic heat generation in mind.

For strongly aerobic processes, the heat involved and the oxygen consumed are proportional regardless of whether a product is formed or not. Thus

$$Q_H = Y_{Q/O} Q_{O_2} \tag{23}$$

The general working equation for heat transfer is

$$Q_{exch} = hA\,\Delta T \tag{24}$$

with h the overall heat transfer, ΔT the temperature driving force, and A the heat transfer surface area. Because the heat generated must be equal to its removal, or

$$Q_{exch} = hA\,\Delta T = Q_H V_L \tag{25}$$

it is easy to see that increasing oxygen transfer rates may result in a limitation of heat transfer. This problem is particularly important during scale-up, because

$$A \sim V_L^{2/3} \tag{26}$$

Henzler [8] has reviewed a number of heat transfer data for unaerated stirred tanks and suggested the following correlation:

$$\frac{\alpha D_T}{\lambda_B} = 0.6 \left(\frac{N d_i^2}{\nu_B}\right)^{0.67} \left(\frac{C_{pb}\eta_b}{\lambda_b}\right)^{0.33} \tag{27}$$

The influence of aeration is complex, depends on stirrer speed, and might be related to flooding phenomena [19,20].

C. Power Consumption

The power input, key parameter for correlating the volumetric mass transfer coefficient k_La, is usually expressed in a power number.

$$Ne = \frac{P}{N^3 d_i^5 \rho} \tag{28}$$

which is equivalent to a drag coefficient of the impeller blades. Ne can be correlated with the Reynolds number

$$Re = \frac{N d_i^2 \rho}{\eta} \tag{29}$$

and approaches a constant value under turbulent flow conditions for most of the agitators used in fermentation.

The physical process of gas dispersion in the impeller region, as well as its interaction with the fluid dynamics in the tank, is not yet completely understood. It is therefore not surprising to see that an enormous number of equations for correlating these effects have been suggested. Most of the subject has been reviewed by Tatterson [21]. Earlier attempts are based on catchall correlations of the type

$$P_g/P_o = f(\text{operating conditions, geometric and fluid properties})$$

A typical example is the famous correlation of Michel and Miller [22], which has been critically examined by van't Riet and Tramper [6]. The same authors suggested the use of Hughmark's equation [23], which is given by

$$\frac{P_G}{P_O} = 0.1\left(\frac{\dot{V}_G}{NV_L}\right)^{-0.25}\left(\frac{N^2 d_i^4}{gH_L V_L^{2/3}}\right)^{-0.20} \tag{30}$$

and showed an average deviation between calculated and experimental of 11.7%.

The limits of using single correlations have been clearly demonstrated by more accurate investigations of the various flow regimes under gassed conditions [21,24]. From this work it seems that it is not possible to develop a single power curve correlation because the power characteristics are influenced by the complex interaction of gas cavity shapes at the impeller, geometric properties, the nature of recirculation, and fluid properties. The situation is all the more complex in the case of multiple impellers and highly viscous non-Newtonian fluids.

D. Use of Neural Networks

In trying to reflect critically the present state of the art in correlating the mass transfer coefficient as a function of the key variables power/volume and aeration, we are faced with a situation which is something of a dilemma. The literature is filled with myriad approaches to measurements and correlations, but very few sound arguments and knowledge exist about which equation to use. Even for the only apparently simple fluid, water, there is no guidance available as to which correlation serves best for a given situation. The complexity of two-phase flow can be used as an indicator that attempts to correlate the effects with simple catchall correlations based on a product of dimensionless groups raised to various powers are doomed to failure.

In the longer term it seems that more fundamental research on two-phase fluid dynamics, including measurements and modeling, is required to overcome these limits. An immediate way out of the dilemma, however, is to attempt to improve the correlation of data. The neural network approach could be an appropriate tool for this task. Before illustrating this approach with the aid of a simple example it should be emphasized that application of this technology should not result in a manifestation of ignorance. It is rather a recommendation for a compact representation of experimental observations with the aid of a tool which is more flexible than products of variables raised to various powers.

The illustrative example presented in the following is an attempt to use the neural network approach for an unique representation of diverse data and correlations for the gas-liquid mass transfer coefficient k_La in stirred tank reactors. The attempt has been restricted three different sources of k_La values in water but can easily be extended to more sources. As a matter of fact, the principle of training the network guarantees that the quality of the network

output improves with increasing number of data sets. For choosing appropriate equations and data it has been decided to prefer sources of data which are not filtered through the conventional correlation, Eq. (5).

The references considered are

1. Smith et al. [25]: the k_La data have been presented as a function of speed of agitation and aeration in three different vessels.
2. Chandrasekharan and Calderbank [26]: measurements of k_La in water and suggestion of the following correlation:

$$k_La = \left(\frac{C}{D_T^4}\right)\left(\frac{P_G}{V_L}\right)^A (\dot{V}_G)^{A/\sqrt{D_T}}$$

($C = 0.0248$ and $A = 0.551$ in SI units)

3. Nishikawa et al. [27]: suggestion of an approach for correlating k_La measurements in different vessels which is based on three different flow regimes depending on the ratio of power input via aeration and agitation.

The artificial neural network used is a feedforward net consisting of input and output layers and one hidden layer (Fig. 9). The network will be introduced here in a rather compact way. The reader interested in more details is referred to the extensive special literature [28–34]. Each neuron in the hidden layer then calculates a weighted sum of its inputs. This sum is applied as an argument to a nonlinear function, transforming the input into the output of the neuron. The function F used in this application is the most common sigmoidal function:

$$F = \frac{1}{1 + \exp(-S)} \tag{31}$$

$$S = \sum_{i=1}^{N} x_i W_i + w_b \tag{32}$$

where S is the weighted sum of inputs, w_i is the weight of the ith input, and w_b is the weight of bias. For determining the weights (learning of the neural network) the most general back-propagation algorithm was applied [35].

The structure of the neural network found to be appropriate for the present application has a 5-9-1 topology as illustrated in Fig. 8. It should be emphasized that the choice of input variables does not include any kind of dependent operating parameter, such as power under gassed conditions.

The results of training the network with the three sources of data are presented in Figs. 10, 11, and 12. It is interesting to recognize that one network with one set of parameters is able to fit all the different data. It should be

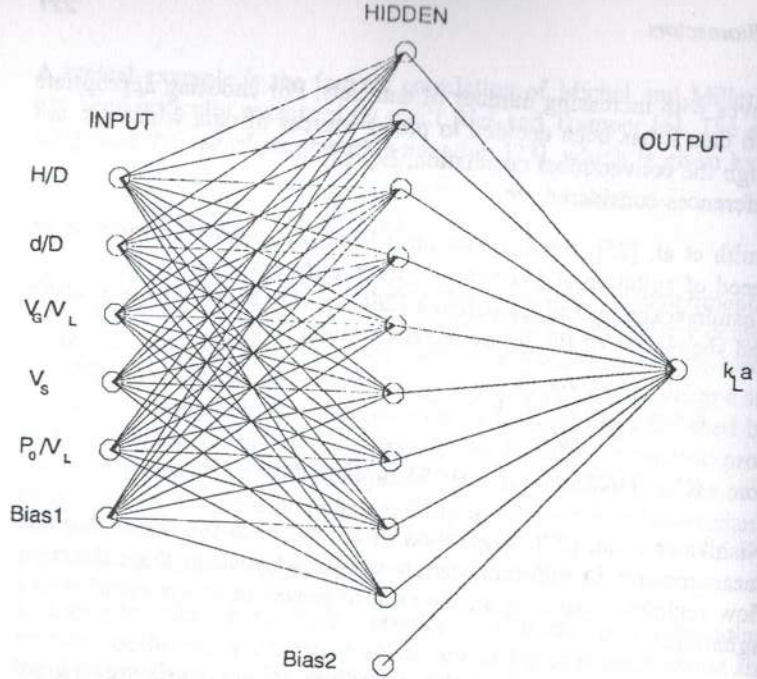


Figure 9 Two-layer neural network architecture of 5-9-1 topology for estimation of k_La values in stirred tank reactors.

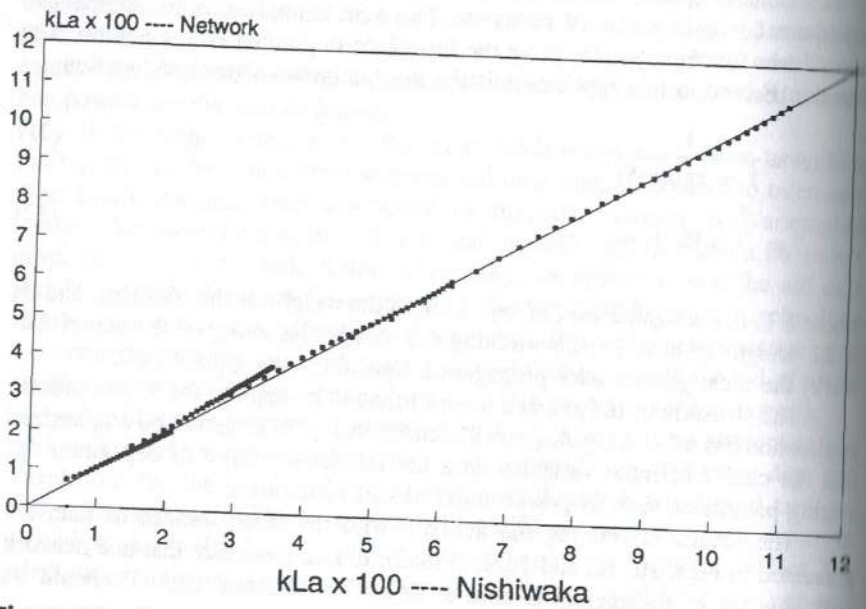


Figure 10 Comparison of k_La data from Nishiwaka et al. [27] and predictions with neural network.

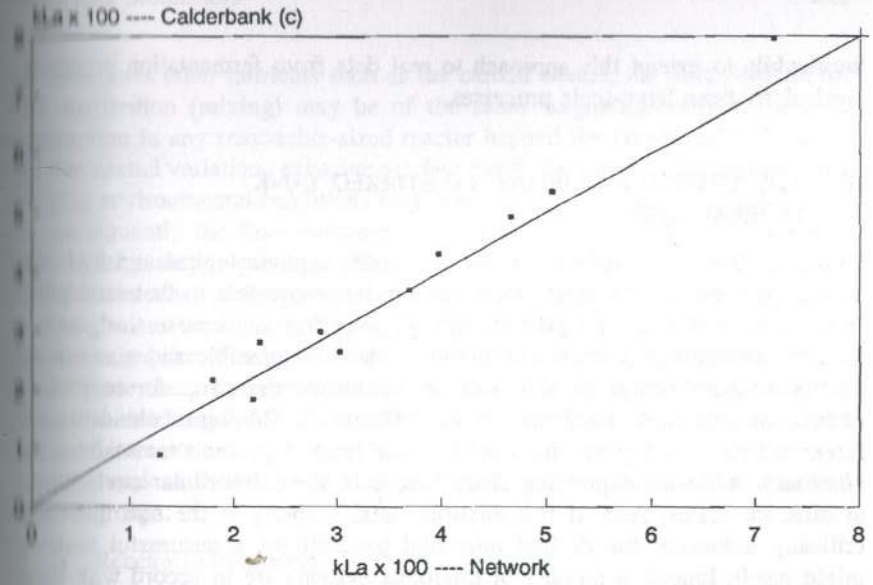


Figure 11 Comparison of k_La data from Chandrasekharan and Calderank [26] and predictions with neural network.

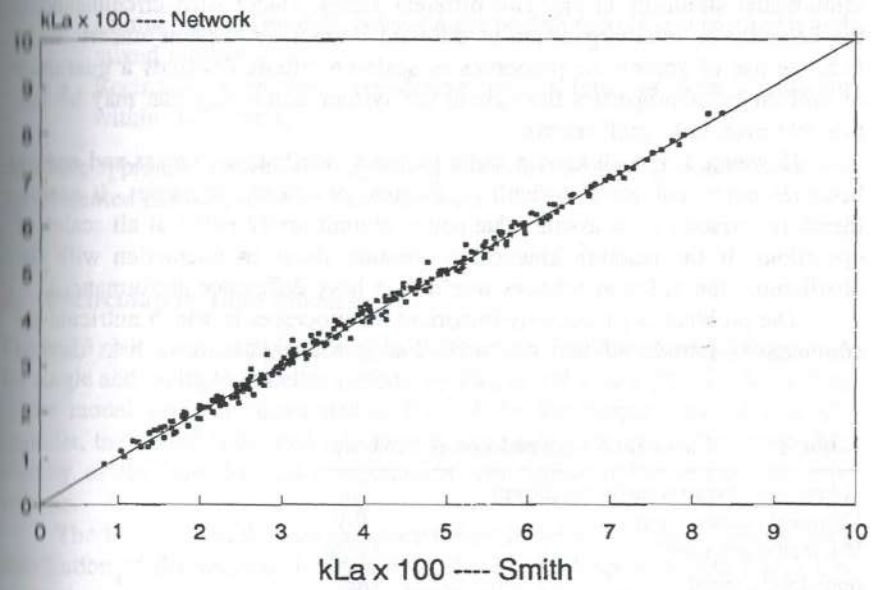


Figure 12 Comparison of k_La data from Smith et al. [25] and predictions with neural network.

worthwhile to extend this approach to real data from fermentation processes, particularly from large-scale processes.

III. STRUCTURED MODELING OF STIRRED TANK BIOREACTORS

During scale-up, an attempt is made to recreate a physiological and hydrodynamic environment in a large reactor as similar as possible to that established in bench-scale and/or pilot plant vessels. Engineering solutions to this problem include maintaining geometric similarities whenever possible and also criteria such as constant power per unit volume, volumetric mass transfer coefficient, circulation time, shear rate, and tip speed (Table 2). The logic behind the different criteria is that preserving each of these singly represents maintaining the constancy of the corresponding characteristic of the extracellular environment at different scales. And, if this environmental property is the one that most critically influences the desired microbial productivity, a successful scale-up might result. Indeed, a number of microbial systems are in accord with these techniques and permit a production-scale operation reasonably in agreement with that established in the laboratory. However, it is easy to see that these criteria are mutually exclusive and, therefore, do not allow an exact replication of environmental similarity at any two different scales. Under such circumstances, the behavior of microorganisms in different fermentors remains uncertain. In fact, the use of volumetric properties as scale-up criteria demands a guarantee of uniformity of properties throughout the system, something that may be impossible even in a small reactor.

However, it is well known that a uniform distribution of mass and energy becomes more and more difficult as the reactor volume increases. It seems, therefore, reasonable to assume that some nonuniformity exists at all scales of operations. If the reaction kinetics in question show an interaction with the distribution, the different reactors would then have difference performances.

The problem is particularly important for processes in which nutrients are continuously introduced into the broth. For specific nutrients such as oxygen

Table 2 Criteria to Be Maintained During Scale-Up

Volumetric oxygen transfer coefficient	k_La
Volumetric power input	P/V
Volumetric gas flow	$\dot{V}_G/V$
Impeller tip speed	nd_i
Agitation speed	n
Terminal mixing time	θ_∞

and sometimes other nutrients such as the carbon source, the time constant for their distribution (mixing) may be of the same magnitude as those of their consumption in any reasonable-sized reactor beyond the bench scale. If we accept that spatial variations exist, we are faced with the problem that dynamically changing environmental conditions may result in drastic changes in metabolism and consequently the final outcome of the process. Although well known in classical fermentation processes, these problems may have even more serious consequences when dealing with recombinant microorganisms, as during scale-up of high-density cultures. The long-term mathematical description of these phenomena requires flexible tools that can easily be adapted to different systems that integrate the process and the reactor. This, in turn, requires the design of conceptual instruments for structuring both the abiotic and biotic phases of the system of interest.

The various models suggested for stirred tank reactors may be classified into two groups:

- Reactor flow models
- Turbulence models

The most important tools for modeling situations of incomplete mixing based on reactor flow models are

- Compartmental models, consisting of perfect mixing compartments and mixed models
- Recirculation models, considering various types of fluid circulation within the reactor.

All these approaches have been applied to bioreactor modeling. A few examples are presented and discussed in the following.

A. Recirculation Time Models

Figure 13 shows an example of a recirculation time distribution model suggested for single and multiple impeller systems by Bajpai and Reuss [35,36]. According to the model structure, illustrated in Fig. 14 for the simpler case of a single impeller, the vessel is divided into two regions, a micromixer in the immediate vicinity of the impeller and a macromixer consisting of the rest of the tank volume.

The flow pattern through the macromixer is defined by the residence time distribution of the various elements in it; that is, the frequency with which the elements reenter the micromixer has a distribution. All the fluid elements passing through the micromixer lose their identity due to micromixing therein and enter the macromixer as a new, fresh born element.

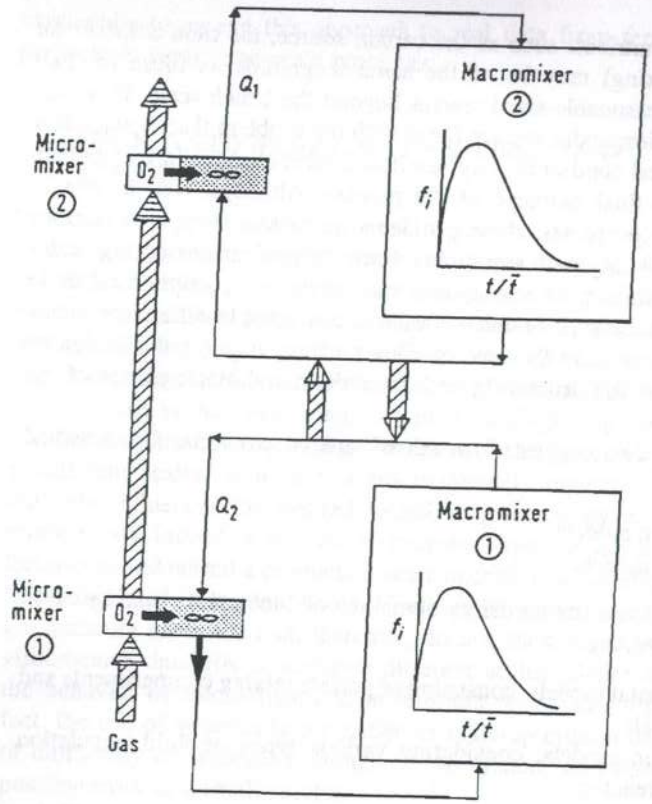


Figure 13 Two-environment model for mixing in a stirred bioreactor with two impellers.

Bajpai and Reuss [35] have used a Monte Carlo simulation method whereby the physical system is divided into a number of discrete elements. In each of these elements, the reaction process is simulated for a short period of time, at the end of which the system interactions are simulated. As a result of the interactions, the state and the number of elements may change. The simulation process is then repeated again for the next time period. For the case of complete segregation, the process may be represented schematically as in Fig. 15.

The volume of the macromixer is divided into N discrete elements, each having a unique age which is defined as the time spent in the macromixer since its entry there. If time is divided into segments of Δt, the volume of a freshly entered element (age zero) will be $Q\,\Delta t$, where Q is the recirculation rate in the system. At the same time, a recirculation stream of volume $Q\,\Delta t$ leaves the

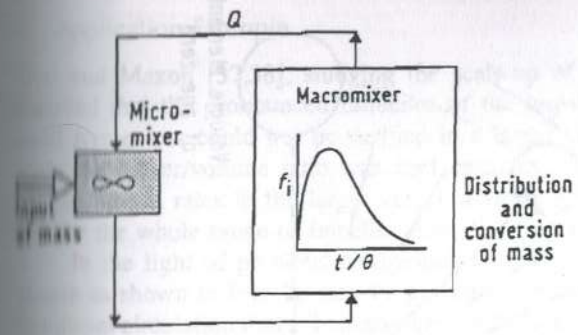


Figure 14 Two-environment model for mixing in a stirred bioreactor with a single impeller.

macromixer. All the existing elements in the macromixer contribute to the exiting stream according to the circulation time distribution. These contributions are denoted by dark areas in Fig. 15. As a result, volumes of the elements decrease. The contribution of the Nth element is exactly equal to its volume; in other words, this element leaves the macromixer. At this moment, the ages of all the elements are advances; i.e., the jth element is termed the $(j + 1)$th element. The newly entered element of volume $Q\,\Delta t$ becomes the first element. The composition of the exit stream is determined by the weighted average of the contributions of individual elements in the macromixer as in the following equation:

$$C_{exit} = \frac{\sum_{i=1}^{N} E_i C_i Q\,\Delta t}{\sum_{i=1}^{N} E_i Q\,\Delta t}$$

where E is the fraction of the circulation times between $(i - 1)\,\Delta t$ and $i\,\Delta t$. E_i is related to the circulation time distribution $f(t)$ by

$$E_i = \int_{(i-1)\Delta t}^{i\Delta t} f(t)\,dt$$

In case of simple impeller systems it has been shown that the log-normal distribution is the best fit of the experimental observations [36]. The volume elements in the macromixer are assumed completely segregated and the progress in each of these with age can be calculated with the help of known initial conditions from the time each entered the macromixer. Since each element has a unique age, reactions in each also proceed to an unique extent and each thus makes a different contribution to the recycle stream. For further details, the original paper [35] is recommended.

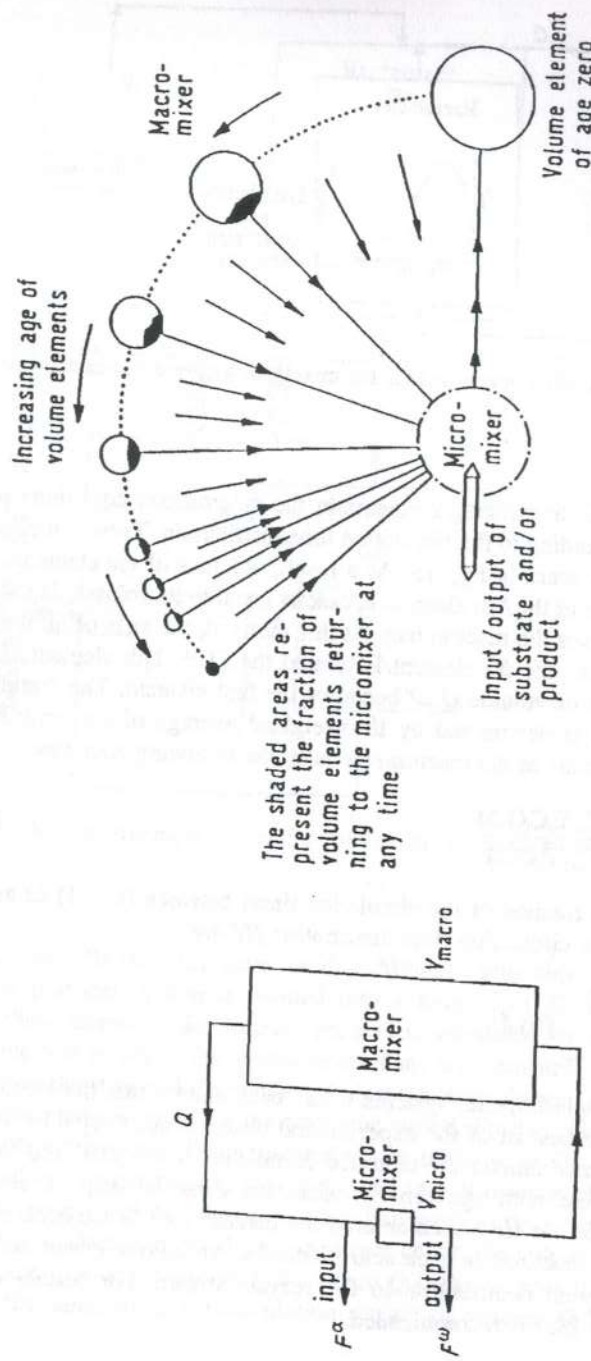


Figure 15 A schematic representation of the discrete simulation procedure.

I. Application Example

Steel and Maxon [37,38], studying the scale-up of novobiocin fermentation, observed that the pronounced influence of the impeller/tank diameter ratio in small fermentors could not be verified in a larger tank having 15 m^3 volume when the power/volume ratio was kept constant. Also, the measured specific oxygen uptake rates in the larger vessel were lower than those in the smaller unit for the whole range of impeller diameters (see Fig. 17).

In the light of previous discussions in this chapter, a phenomenological picture as shown in Fig. 16 may be proposed for any scale of operations with significant circulation times. In accordance with many experimental observations in highly viscous non-Newtonian systems, it may be assumed that the gas-liquid mass transfer takes place only in the impeller region. It is here that diffusion elements whose diameter is determined by the maximum energy dissipation rate [39] are loaded with oxygen. These loaded elements circulate through the system according to the circulation time distribution until their return to the impeller region. During these circulations, progressively decreasing turbulence is encountered as the distance from the impeller increases; hence, some flocculation may take place, as it is a fast process. Yet the nature of filaments would prevent their coalescence and for the interior of elements a case of complete segregation may be justified.

In Fig. 17, in which effective uptake rates are plotted against ratio of impeller to tank diameter, the simulation results are shown along with experimental observations of Steel and Maxon [37]. Because of the qualitative agreement between computed and measured results, it is justified to explain these pronounced scale-up effects on the basis of interaction of the different transport phenomena incorporated in the model.

Although the smaller impeller in the large tank will produce smaller diffusion elements because of higher local energy dissipation rates, these elements have to travel much longer on their circulation path because of less pumping capacity of the smaller impeller. At the same time the impellers in the large tank rotate with lower speed at same power input. This results in higher apparent viscosities and therefore lower k_La values. Finally, in the range of d_i/D_T shown for the 15-m^3 fermentor the influence of increased k_La for the smaller impeller is counterbalanced by increase by increase in circulation times. Thus, at this scale of operation, reducing d_i/D_T does not influence the overall uptake rate.

Further application of this model structure includes fed-batch cultivation of *Saccharomyces cerevisiae* [35,36].

II. Compartment Models

In what follows a short and concentrated overview of different compartment structures for stirred tank reactors will be presented. The discussion will be

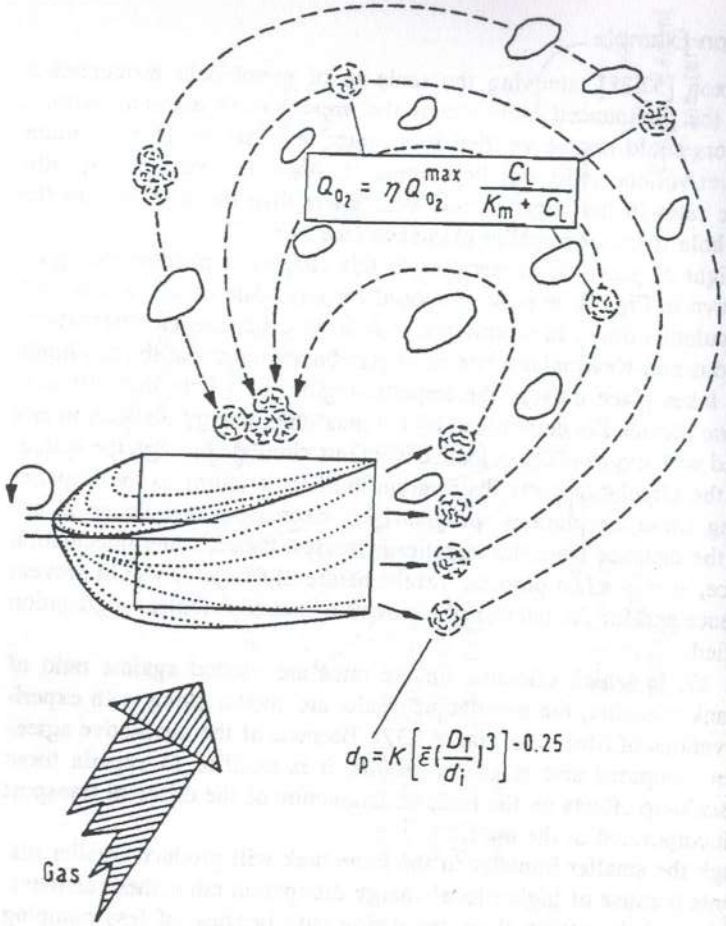


Figure 16 Phenomenological picture of mass and energy distribution for oxygen supply of highly viscous biosuspensions with oxygen limitations.

restricted to models which have been suggested for bioreactors in case of low viscous systems. For a more detailed description of the various models, including their application, the reader is referred to the original papers.

The two-compartment model of Sinclair and Brown [40] (Fig. 18) has been proposed for a continuous stirred tank reactor (CSTR) to explain the influence of the intensity of mixing. The solution of the material balance equations for the biomass and substrate illustrated the strong effect on the exit cell con-

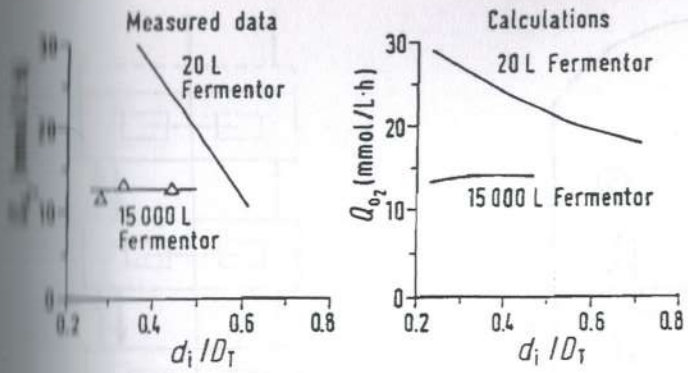


Figure 17 Comparison of experimental observations [37] and model predictions for oxygen supply in a fermentation broth of *Streptomyces niveus*.

centration in a CSTR. Figure 19 shows another example, the five-compartment model of Oosterhuis and Kossen [41]. The model consists of two impeller compartments and three compartments which characterize the flow conditions in the rest of the tank volume.

The multiturbine model of Bader [42,43] separates the reactor into a series of mixing zones (Fig. 20). Mixing of the liquid phase is presented by the liquid flow between the compartments and is related to the pumping capacity of the impellers.

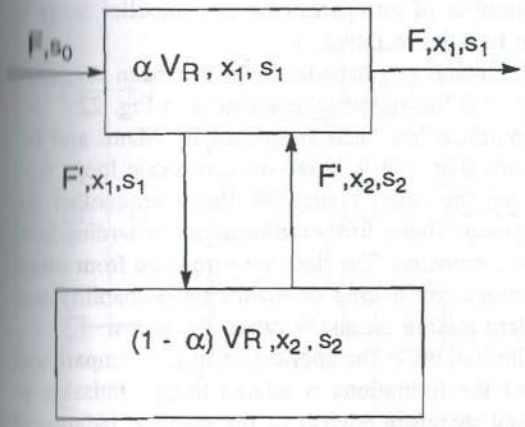


Figure 18 Two-region mixing model of Sinclair and Brown [40].

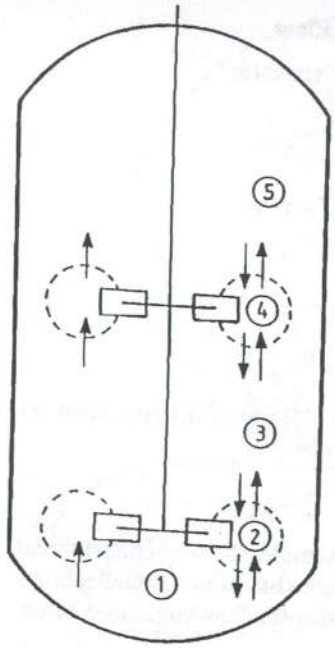


Figure 19 Five-compartment model of Oosterhuis and Kossen [41].

Figure 21 schematically illustrates the multicompartment model of Singh et al. [44–46]. The model consists of a series of well-mixed single-phase compartments with recycle. The number of compartments per impeller stage is assumed to be a function of the turbulence intensity.

An extended version of the model proposed by Singh has been suggested by Mayr et al. [47]. This structure is illustrated schematically in Fig. 22.

A completely different approach has been suggested by Mann and coworkers [48,49]. The cell network (Fig. 23) is based on concentric loops with additional exchange flow between the cells. Figure 24 illustrates another approach from the same research group. There, further information regarding local parametrization of circulation is considered. The data are estimated from measured radial and axial flow. Furthermore, a kind of switching probability was suggested to take care of turbulent mixing on and between the loops.

There are several serious limitations in the application of the compartment models discussed so far. One of the limitations is related to the omission of back-mixing of the gas phase and therefore neglect of the material balance of oxygen in the gas phase. Second, in many of the models the number of the

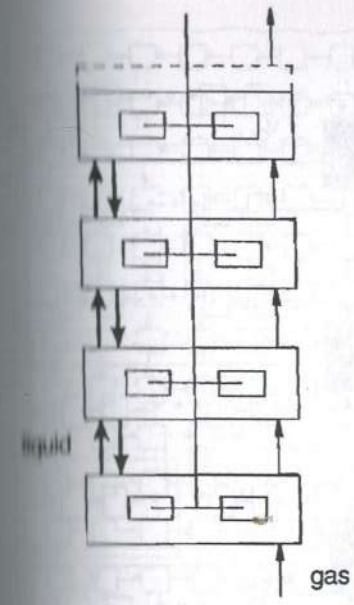


Figure 20 Mixing model for a multiturbine fermentor of Bader [42,43].

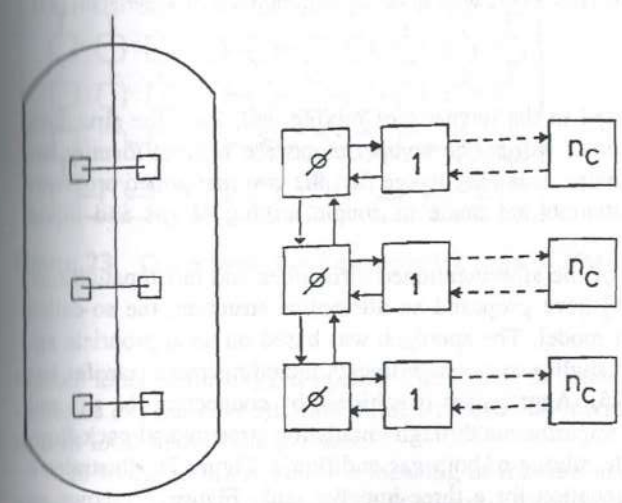


Figure 21 Recycle-backmix circulation model of Singh et al. [44–46].

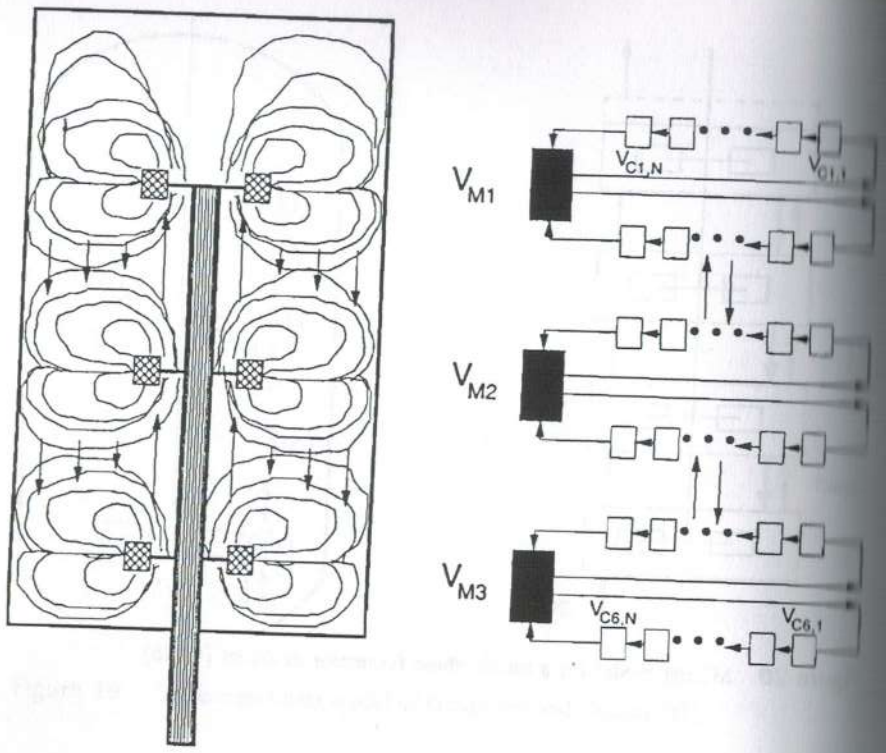


Figure 22 Model for the stirred tank with three Rushton turbines of Mayr et al. [47].

compartments is connected to the intensity of mixing and, thus, the structures are virtual in space. In other words, the compartments are without identity because of missing coordinates. It is easy to see that the two mentioned problems are closely related if attempts are made to couple mixing of gas and liquid phases.

To overcome part of the aforementioned difficulties and incompatibilities, Ragot and Reuss [50,36] have proposed an alternative structure, the so-called multiphase compartment model. The approach was based on an appropriate aggregation of well-mixed multiphase compartments including mass transfer between the phases (Fig. 25). Aggregation is achieved by connecting the gas and liquid fractions of the compartments through circulation streams and back-flow, thus trying to incorporate mixing of both gas and liquid. Figure 26 illustrates a typical example of aggregation for a three-impeller tank. Figure 27 shows an example of application for an oxygen sink estimated from simple Michaelis-

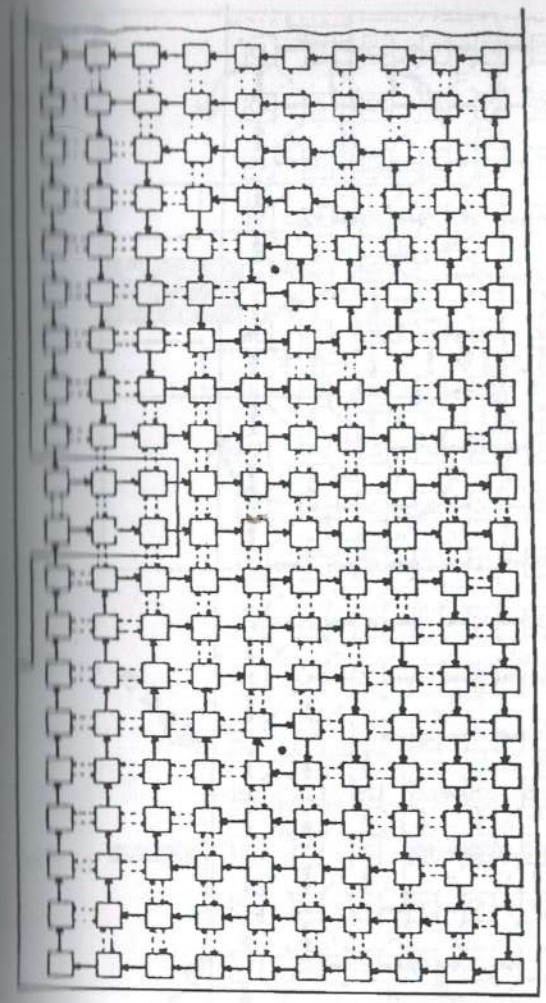


Figure 23 Cell network based on concentric loops of Mann and Mavros [48].

Menten kinetics for oxygen uptake. The results of model simulations are local values of oxygen concentration influenced by back-mixing of gas and liquid as well as local hydrostatic pressure.

In trying to understand the meaning of forward and backward flow from the physical point of view—which is essential for any further improvements of the model structure and meaningful parametrization—we again found some lim-

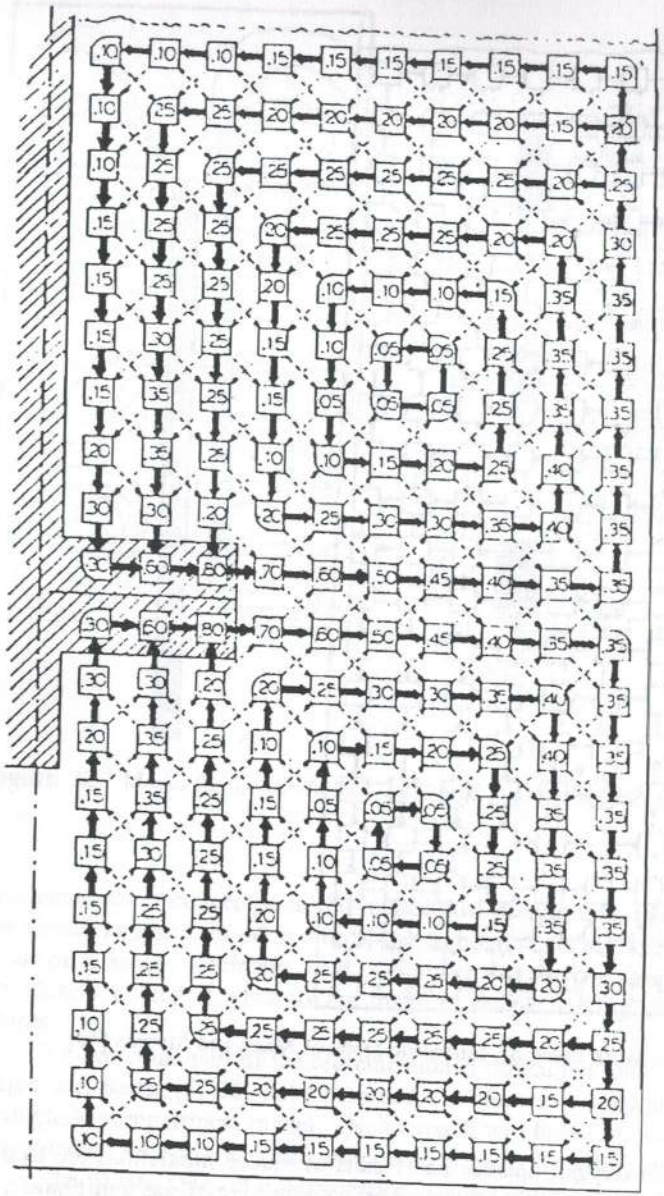
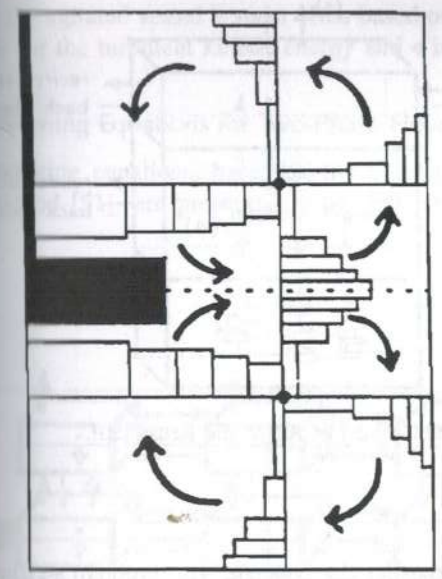
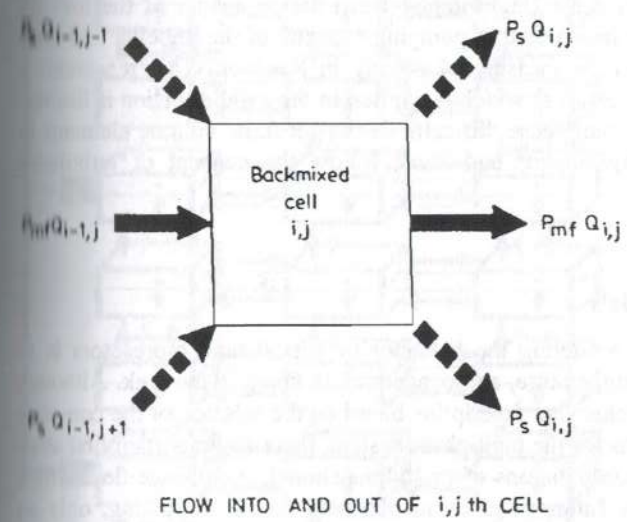


Figure 24 Cell network with local parametrization and switching probability of Mann and Knysh [49].





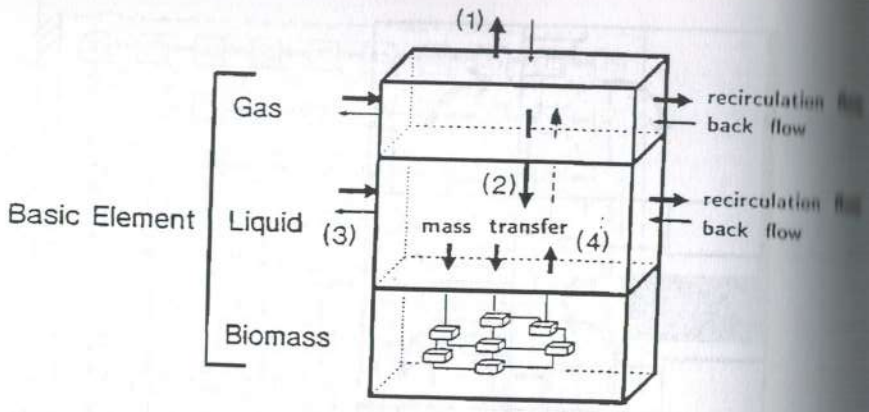


Figure 25 Multiphase-compartment model of Ragot and Reuss [50].

itations which forced us to reconsider the concept. The problem is illustrated schematically in Fig. 28. Only in cases in which the model structure consists of two symmetrical loops for a single impeller can the parameter of the forward and backward flow be interpreted as pumping capacity of the impeller and local dispersion coefficient as a measure of mixing in the loop. This represents a serious restriction as a result of which resolution in the axial direction is limited. The only way to overcome these difficulties is to put finite volume elements in the place of the compartments and, thus, follow the concept of turbulence modeling.

C. Turbulence Models

The ultimate goal of modeling the behavior of stirred tank bioreactors is to predict the velocity, temperature, and concentration fields in the tank. Although far from such a comprehensive description based on the solution of the complete set of balance equations for the multiphase system, there are important first steps regarding numerical computations of multidimensional, multiphase flow which show the direction for future research in this field. In the following, only an introduction to this topic is attempted. The interested reader must be referred to the extensive special literature.

Predicting the turbulent flow generated by a turbine impeller in a standard baffled vessel is a necessary first step in progressing toward the calculation of the coupled transport phenomena in the reactor. Computation of the turbulent

flow in the agitated vessel is most often based on the so-called k-ϵ model. Here k stands for the turbulent kinetic energy and ϵ is its dissipation rate.

1. Governing Equations for Two-Phase Flow

The governing equations, based on the Eulerian concept and called the two-fluid method [51], are presented in the following for the general situation of

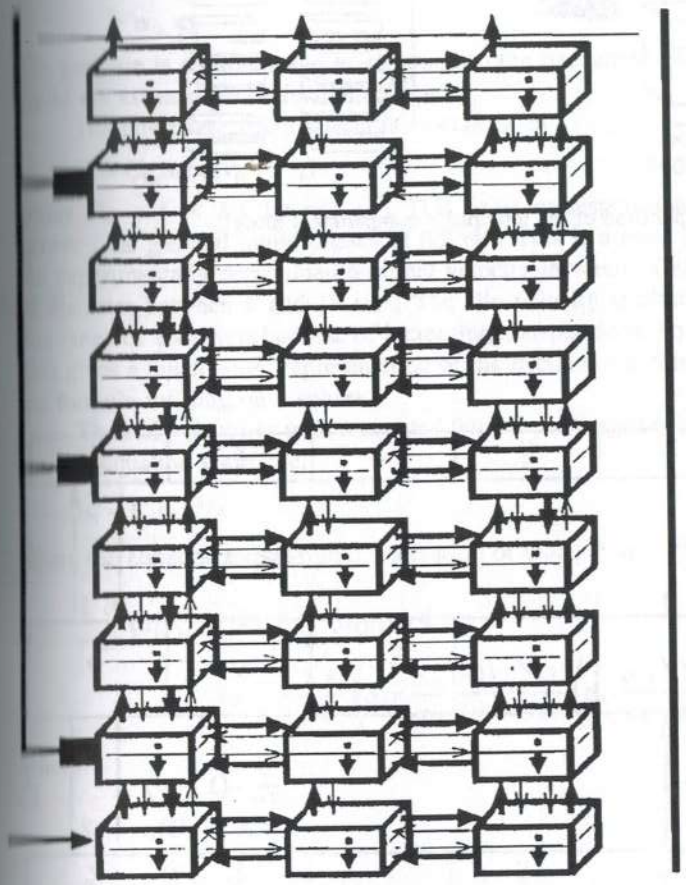


Figure 26 Typical example of the aggregation of multiphase compartments for a three-impeller tank.

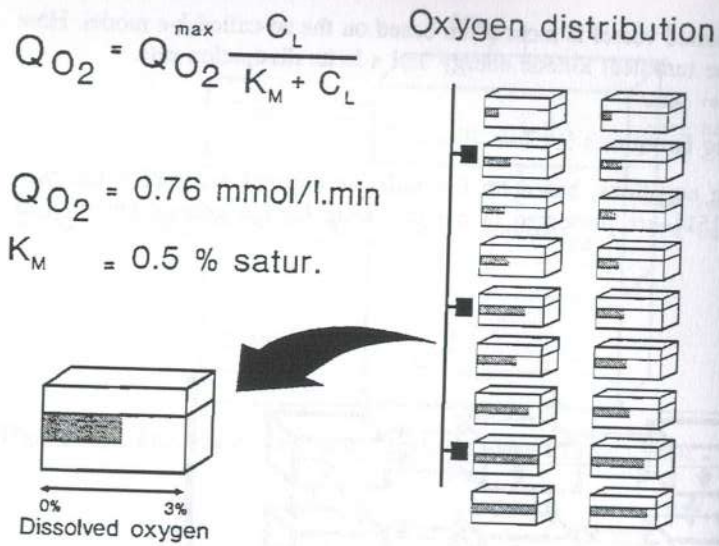


Figure 27 Application of the multiphase compartment model.

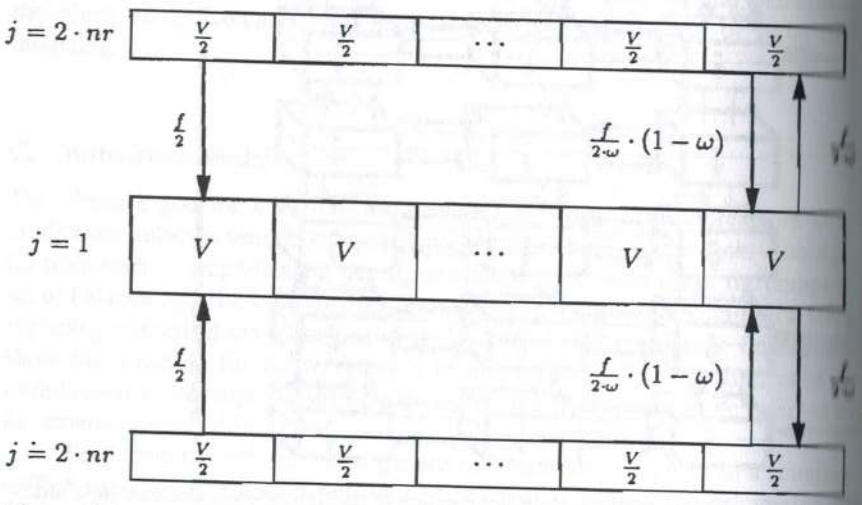


Figure 28 Limitations of the model structure of Ragot and Reuss [50].

two-phase flow (gas and liquid):

$$\nabla(\alpha_L v_L) = \nabla(D_t \nabla\alpha_L)$$

$$\nabla(\alpha_G v_G) = \nabla(D_t \nabla\alpha_G v^L)$$

$$\nabla(\alpha_L \rho_L v_L v_L) = \nabla(\mu_{eff} \nabla v_L) - \alpha_L \nabla p + F_f$$

$$\nabla(\alpha_G \rho_G v_G v_G) = \nabla(\mu_{eff} \nabla v_G) - \alpha_G \nabla p - F_f - \alpha_G \rho_L g$$

with

$$\mu_{eff} = \mu_l + \mu_t$$

and

$$\alpha_L + \alpha_G = 1$$

The pressure is considered to be common to the two phases, and both gas and liquid are assumed to be incompressible.

The interphase friction term is calculated as

$$F_f = C_{fip} \alpha_L \alpha_G (v_G - v_L) \tag{33}$$

where $C_{fip} = 5 \times 10^4$ kg m^{-3} s^{-1}. This value corresponds to a slip velocity between the gas and liquid phases of 0.2 m/s. This is a good approximation to the experimentally determined terminal velocity in water of single air bubbles of diameter between 1 and 10 mm. The slip velocity is almost constant over this range of diameters because of increasing nonsphericity. For this reason, Eq. (33) gives a much better representation of the friction over this range than does the formula for drag on a sphere.

The eddy viscosity μ_t is computed from the averaged velocity fields using the two-equation k-ϵ model:

$$\mu_t = C_\mu \rho_L k^2 / \epsilon$$

where the equation expressing conservation of either k or ϵ (Φ say) is

$$\frac{1}{r}\frac{\partial}{\partial r}(\alpha_L \rho_L r v_{r,L} \Phi) + \frac{\partial}{\partial z}(\alpha_L \rho_L v_{z,L} \Phi) - \frac{1}{r}\frac{\partial}{\partial r}\left(\alpha_L r \frac{\mu_{eff}}{\sigma_\Phi}\frac{\partial \Phi}{\partial r}\right) - \frac{\partial}{\partial z}\left(\alpha_L \frac{\mu_{eff}}{\sigma_\Phi}\frac{\partial \Phi}{\partial z}\right) = S_\Phi$$

where

$$S_k = \alpha_L (G_k - \rho_L \epsilon)$$

$$S_\epsilon = \alpha_L \frac{\epsilon}{k}(C_1 G_k - C_2 \rho_L \epsilon)$$

and

$$G_k = \mu_t \left\{ 2 \left[\left(\frac{\partial v_{z,L}}{\partial z} \right)^2 + \left(\frac{\partial v_{r,L}}{\partial r} \right)^2 + \left(\frac{v_{r,L}}{r} \right)^2 \right] + \left(\frac{\partial v_{z,L}}{\partial r} + \frac{\partial v_{r,L}}{\partial z} \right)^2 \right\}$$

The standard values $C_1 = 1.44$, $C_2 = 1.92$, $C_\mu = 0.09$, $\sigma_k = 1.0$, $\sigma_\epsilon = 1.3$ are used for the empirical constants. Finally, it is assumed that the turbulent diffusivity D_t is equal to the (kinematic) turbulent viscosity.

2. Prediction of Turbulence in Single-Phase Flow

Harvey and Greaves [52] predicted turbulent single-phase flow in an agitated vessel by application of the k-ϵ model. The computations, however, rely on a rather unrealistic boundary condition for the turbulence parameters in the impeller region. The adopted approach assumes that the gradients of mean velocity components determine the level of turbulence in the vicinity of the impeller. This inadequate oversimplification of the flow situation in the impeller region has been removed in the work of Placek et al. [53]. These authors extend the k-ϵ model through an incorporation of different turbulent energy scales. Thus, a multiple-scale model is created in which the energy spectrum of turbulence in the vessel is divided into the large-scale vortices produced by the impeller, the intermediate or transfer eddies, and the dissipation eddies. Furthermore, the boundary conditions in the impeller region rely on a discharge flow model suggested by Placek and Tavlarides [54], which includes the trailing vortices at the impeller blades observed by van't Riet and Smith [55].

An even more complex model of turbulence has been suggested by Yu et al. [56]. This nonisotropic turbulence model considers the rotation and curvature effect of turbulence and accounts for the three-dimensional effects through the nonisotropy of the viscosity.

The present situation in the modeling of turbulence in agitated vessels mirrors two different directions. One is characterized by the attempts to involve more and more complexity in the model structure and is, therefore, based on the assumption that isotropic k-ϵ models do not describe the three-dimensional turbulent flow. In contrast, the other type of model is still based on the standard k-ϵ model and tries to improve the boundary conditions in the impeller regions [57,58]. In other words, the modifications of the k-ϵ model are claimed to be unnecessary, provided the proper boundary conditions are applied.

3. Prediction of Turbulence in Two-Phase Flow

Issa and Gosman [59] tried to predict the three-dimensional turbulent two-phase flow in an agitated and aerated vessel. Again, the most critical point seems to be the boundary condition in the impeller region. The empirically described flow in this region makes the prediction of results uncertain. With the aid of a superposition of oxygen transfer from the gas bubbles into the liquid as well as a Monod-like expression for oxygen consumption, Träghard [60] presented the numerical solution of the equations of motion together with the k-ϵ turbulence model for the two-phase flow. As mentioned by the author, the treatment of the gas bubbles as well as the flow conditions in the impeller region must be considered as a weakness in this model. The most difficult problem and also the most important task for the future is the comprehensive mathematical treatment of the disruption and coalescence of the gas bubbles, including the existence of a size distribution. The other aspect, hitherto not thoroughly investigated, is the analysis of the relative motion of the gas bubbles in the turbulent flow.

Further simulations of two-phase turbulence in agitated vessels include the extension to the three-equation model of Placek [61] and simulations neglecting the turbulent dispersion of the gas phase [62]. In another approach [63] attempts are made to incorporate the phenomena of bubble breakup and bubble coalescence based on local turbulence intensity and local energy dissipation rate. Also included are predictions of local values of void fractions, bubble size, interfacial area, and mass transfer. It must be emphasized, however, that flow field computation in this work is based on neglecting the interphase-friction term.

It may be concluded that the investigation of turbulence in agitated and aerated vessels is only beginning. The task is not an easy one, and it seems too early to speculate on coupling the turbulent transport equations with the microbial reaction model, which must be structured in order to account for the behavior in a continuously changing environment. Nevertheless, results from models for turbulent two-phase flow, as far as the distribution of kinetic energy and dissipation in the tank is concerned, could be used immediately to improve confidence in the multiphase compartment models described in the previous section. This strategy would result in a simpler model structure for the abiotic phases with distributed parameters predicted from the two-phase turbulent model. This kind of model reduction is presently a prerequisite to coupling more complex metabolic models with the dynamic response of microorganisms to changing environmental conditions, and it differs from attempts to simulate the coupled system of momentum and mass balance equations.

D. Compartmentation with the Aid of Finite Volume Elements

The two-dimensional material balance equations for a component charcterized by its concentration c can be written as

$$\frac{\partial}{\partial t}(\rho c) + \frac{1}{r}\frac{\partial}{\partial r}(\rho\, r v_r c) + \frac{\partial}{\partial_z}(\rho\, v_z c)$$
$$= \frac{1}{r}\frac{\partial}{\partial r}\left(\rho r D_{\text{eff}} \frac{\partial c}{\partial r}\right) + \frac{\partial}{\partial z}\left(\mu\, D_{\text{eff}} \frac{\partial c}{\partial z}\right) + \text{reaction}$$

where

r and z = radial and axial coordinates, respectively

v_r and v_z = radial and axial components of the velocity vector

D_{eff} = eddy diffusivity or turbulent dispersion coefficient = ν_{eff}/Sc with ν_{eff} the turbulent effective viscosity and Sc the Schmidt number

Next, a finite volume is introduced as shown in Fig. 29 [63]. The finite volume element is located at point i, j in the coordinate system r, z. Integration of the material balance equation between position $i-1, j-1$ and i, j

$$\int_{z_{i-1}}^{z_i}\int_{r_{j-1}}^{r_j} \frac{\partial}{\partial t}(\rho c)\, r\, dr\, dz + \int_{z_{i-1}}^{z_i}\int_{r_{j-1}}^{r_j} \frac{1}{r}\frac{\partial}{\partial r}(\rho r v_r c)\, r\, dr\, dz$$

$$+ \int_{z_{i-1}}^{z_i}\int_{r_{j-1}}^{r_j} \frac{\partial}{\partial z}(\rho v_z c)\, r\, dr\, dz$$

$$= + \int_{z_{i-1}}^{z_i}\int_{r_{j-1}}^{r_j} \frac{1}{r}\frac{\partial}{\partial r}\left((\rho r D_{eff}\frac{\partial c}{\partial r}\right) r\, dr\, dz + \int_{z_{i-1}}^{z_i}\int_{r_{j-1}}^{r_j} \frac{\partial}{\partial z}\Big)\rho\, D_{eff}\frac{\partial c}{\partial z}\, r\, dr\, dz$$

$$+ \int_{z_{i-1}}^{z_i}\int_{r_{j-1}}^{r_j} \text{reaction}\; r\, dr\, dz$$

leads to a set of eight cell wall fluxes, which are summarized schematically in Fig. 30.

Thus, instead of two forward and two backward fluxes in the compartment model of Fig. 25, there are now four fluxes in each direction. The advantage, however, is that these fluxes are related to intrinsic fluid dynamic parameters. As an example and with the assumption ρ = constant, the flow fluxes leaving the cell are summarized in the following:

$$\dot{V}_{r,i,j} = 2\pi r_j (z_i - z_{i-1}) v_{r,i,j}$$

$$\dot{V}_{r,i,j} = 2\pi r_j (z_i - z_{i-1}) \frac{D_{eff,r,i,j}}{\delta r_j}$$

$$\dot{V}_{z,i,j} = \pi (r_j^2 - r_{j-1}^2) v_{z,i,j}$$

$$\dot{V}_{z,i,j} = \pi (r_j^2 - r_{i-1}^2) \frac{D_{eff,z,i,j}}{\delta z_i}$$

The values of the parameters (local values of v_r and v_z as well as local values of the turbulent dispersion coefficient D_{eff}) can be estimated from two different sources. Either simulation results from turbulence models are used or the data are predicted from experimental observations of local values of mean axial and radial velocities and eddy diffusities estimated from measured fluctuation velocities. The two routes are summarized schematically in Fig. 31.

In what follows an example of application of the experimental path is presented to illustrate the use of this approach, which is based on strict decoupling of the momentum and material balance equations.

To perform a quantitative analysis based on an intrinsic parametrization of the elements, the well-known experimental work of Nagata [64] was resorted to. From this work a consistent set of data regarding radial and axial velocity fields in

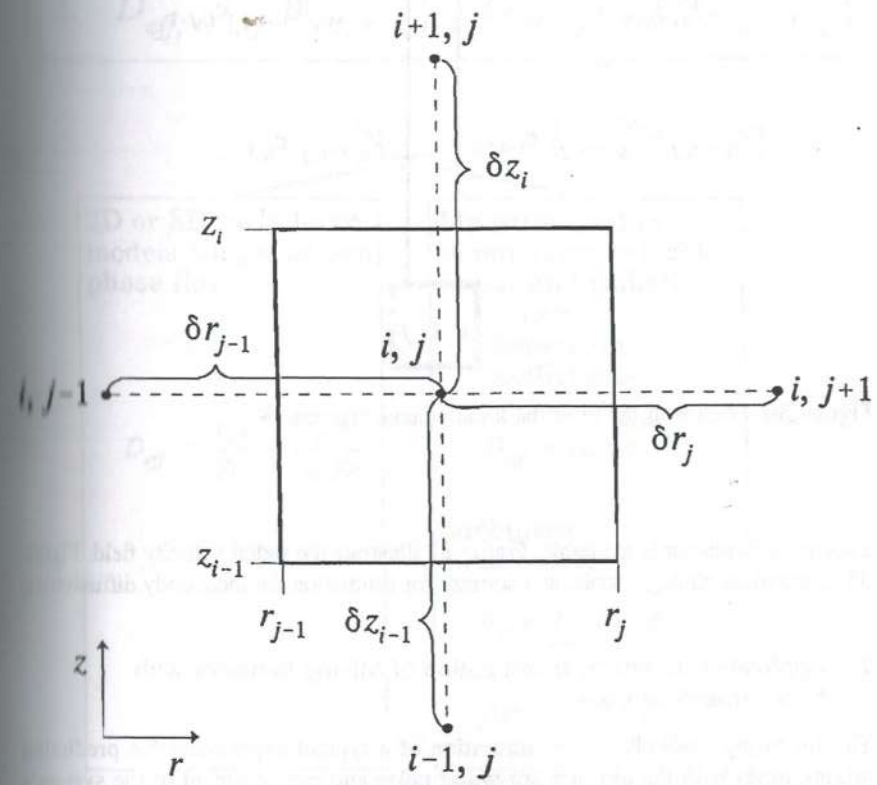


Figure 29 Finite volume domain.

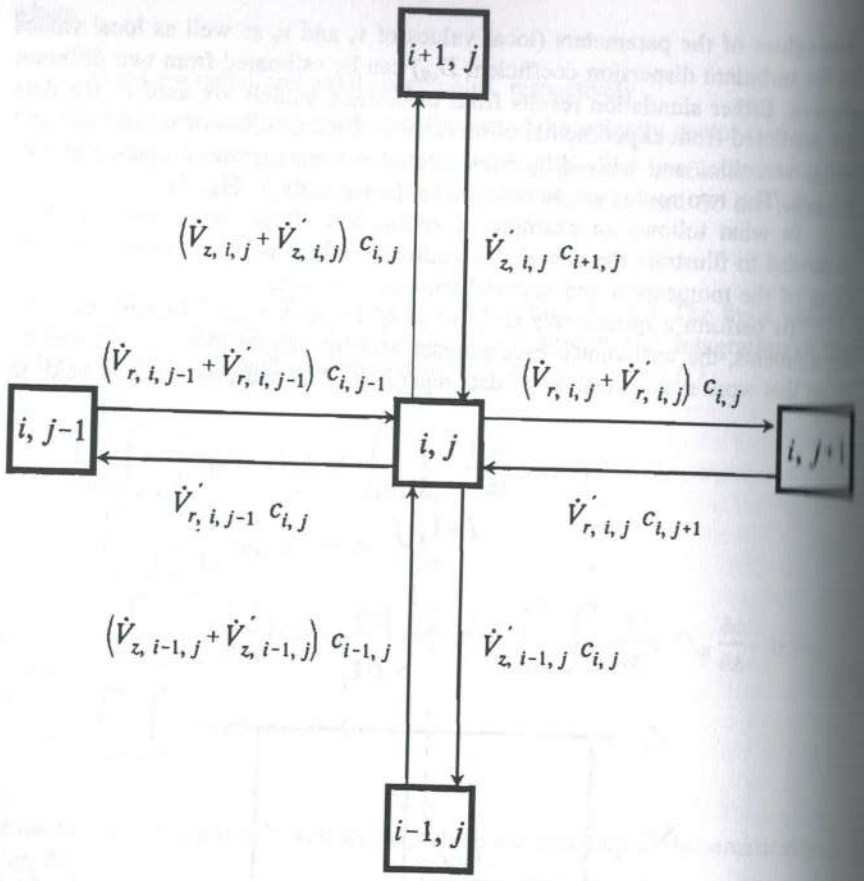


Figure 30 Cell wall fluxes of the finite volume element.

a stirred tank reactor is available. Figure 32 illustrate the radial velocity field. Figure 33 summarizes strategy, tools, and sources for estimation the local eddy diffusivities.

1. Application Example: Investigation of Mixing Behavior with Heat-Pulse Technique

The following example is the simulation of a typical experiment for predicting mixing times with the aid of a hot-water pulse and measurement of the system's response with a temperature sensor. The simulations have been performed for a

150 L stirred tank reactor using 864 compartments (finite volume elements). Hot water (400 ml, temperature 96°C) is pulsed from the top during a time span of 1 s. The simulation of this experiment requires the simultaneous solution of the energy balance equations for all the elements. Figure 34 illustrates the dynamic response or the dynamic change of the temperature field in the reactor.

The results of these simulations appear reasonable, they are physically understandable, and they are encouraging. Ongoing work is directed toward different applications related to bioprocesses including the necessary extension to gas-liquid systems.

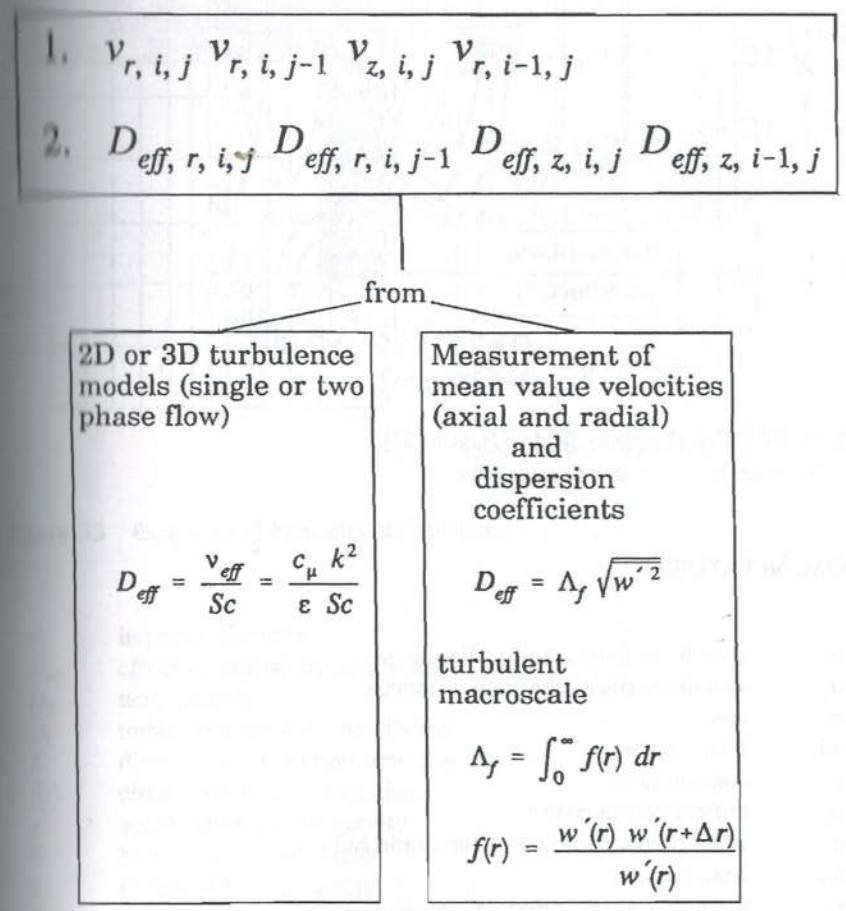


Figure 31

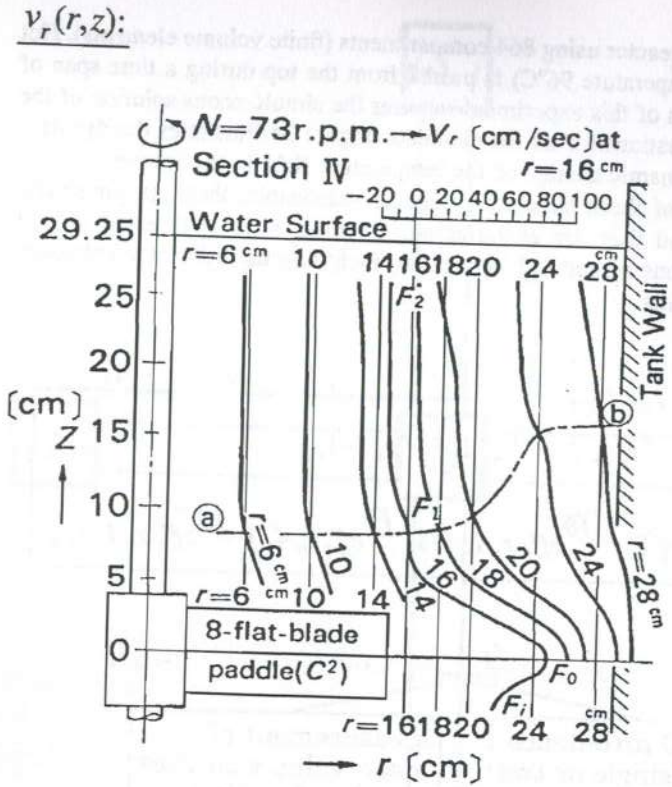
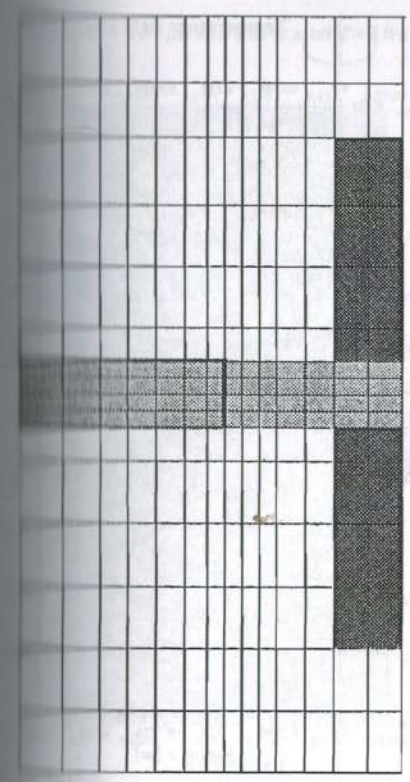


Figure 32 Radial velocity field of Nagata [64].

NOMENCLATURE

a	specific surface area gas-liquid
a_P	specific surface area liquid-biomass
A	area
Bi	Biot number
c	concentration
c_{crit}	critical concentration
c_L	oxygen concentration in the liquid bulk
c_{pb}	heat capacity
c_X	biomass concentration
c^*	concentration at gas-liquid interface



D_{eff} = const.

absolute level: Nagata (64)
constant dispersion coefficient is justified: see simulations of Placek (53),
3-equation-turbulence-model,
axisymmetric formulation

$D_{eff} = f(r, z)$

absolute level: Nagata (64)
relative local distribution: Placek (53)

$D_{eff} = f(r, z)$

absolute level: Nagata (64)
relative local distribution: Nagata (64)

Figure 33 Estimation of local eddy diffusivities.

d_i	impeller diameter
D_{eff}	effective diffusivity coefficient
D_T	tank diameter
D_t	turbulent dispersion coefficient
E_i	discretized circulation time distribution
$f(t)$	circulation time distribution
g	acceleration due to gravity
h	heat transfer coefficient
k	turbulent kinetic energy
k_L	mass transfer coefficient gas-liquid
k_P	mass transfer coefficient liquid-solid

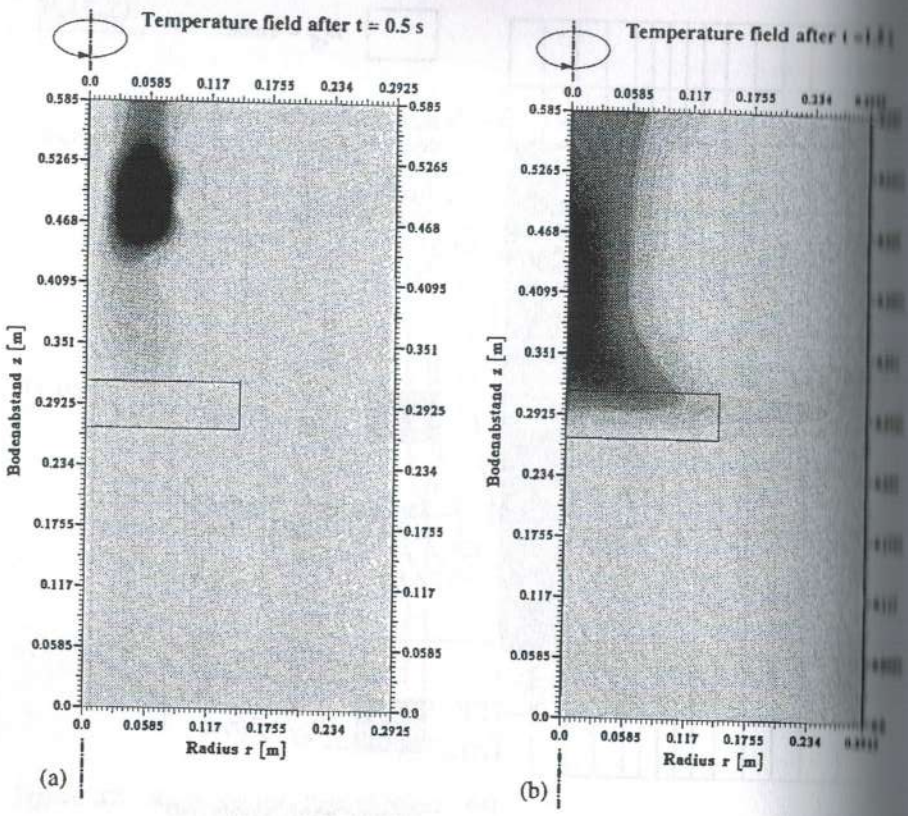
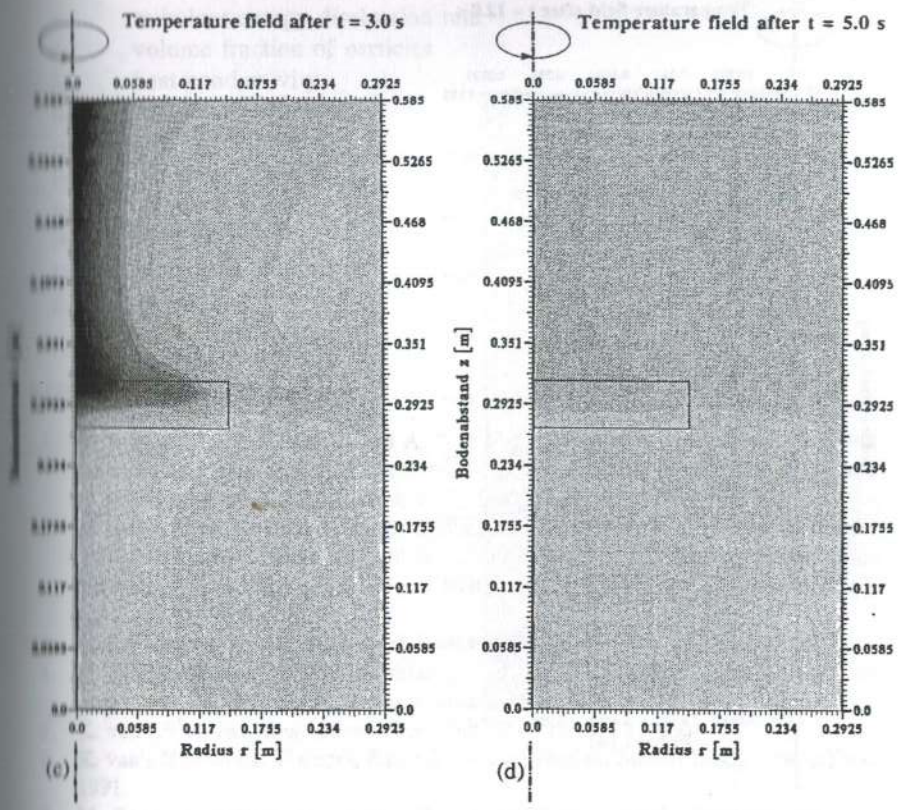


Figure 34 Response of temperature field after (a and b) 0.5 and 1.5 s, (c and d) 3.0 s and 5.0 s, and (e) 12 s.

K_M	Michaelis-Menten constant
k_La	volumetric mass transfer coefficient
N	speed of agitation
N_{O_2}	total oxygen flux
Ne	power (Newton) number
p	pressure
P	power input
P_G	power input at gassed conditions
P_0	power input at ungassed conditions
q	specific uptake rate



q_{max}	maximum specific uptake rate
Q	circulation rate
Q_{O_2}	oxygen uptake rate
$Q_{O_2}^{max}$	maximum oxygen uptake rate
Q_H	heat of reaction
r	radial coordinate
R	radius
Re	Reynolds number
Sc	Schmidt number
v_L	liquid velocity
v_G	gas velocity
V_G	gas flow rate

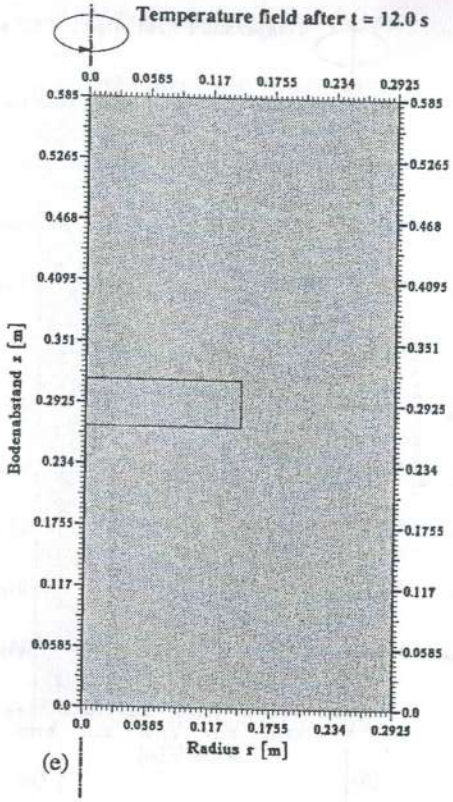


Figure 34 Continued

V_S	superficial gas flow velocity
V_L	liquid volume
$Y_{O/X}$	yield coefficient
$Y_{Q/O}$	yield coefficient
z	axial coordinate

Greek Symbols

α	heat transfer coefficient
α_G	gas holdup
α_L	liquid holdup
β	overall mass transfer coefficient

ε	turbulent energy dissipation rate
ε_p	volume fraction of particles
λ_B	heat conductivity
μ_X	specific growth rate
μ_{eff}	effective viscosity
μ_L, μ_l	dynamic viscosity
μ_t	turbulent viscosity
μ_b	bulk viscosity
ν_B	kinetmatic viscosity
ρ	density

REFERENCES

1. G. J. Balmer, I. P. T. Moore, and A. W. Nienow, Aerated and anaerated power and mass transfer characteristics, *Biotechnology Scale-up and Mixing* (E. S. Ho and J. Y. Oldshue, eds.), AIChE, New York, 1987, p. 116.
2. K. Gbewongyo, D. Dimasi, and B. C. Buckland, Characterization of oxygen transfer and power absorption of hydrofil impellers in viscous mycelial fermentats, *Biotechnology Processes, Scale-up and mixing* (C. S. Ho and I. Y. Oldshue, eds.), AIChE, New York, 1987, p. 128.
3. A. W. Nienow, *Trends Biotechnol.* 8: 224 (1990).
4. M. Reuss, Oxygen transfer and mixing: scale-up implications, *Biotechnology*, 2nd compl. rev. ed., Vol. 3 (G. Stephanopoulos, ed.), 1993, p. 186.
5. K. van't Riet, *Ind. Eng. Chem. Proc. Des. Dev.* 18: 357 (1979).
6. K. van't Riet and J. Tramper, *Basic Bioreactor Design*, Marcel Dekker, New York, 1991.
7. M. Reuss, S. Frölich, B. Kramer, K. Messerschmidt, and G. Pommerening, *Bioprocess Eng. 1*: 79 (1986).
8. H.-J. Henzler, *Chem. Ing. Tech. 54*: 461 (1982).
9. H. Herbst, A Schumpe, and W.-D. Deckwer, *Chem. Eng. Technol. 15*: 425 (1992).
10. J. F. Perez and O. C. Sandall, *AIChE J. 20*: 770 (1974).
11. H. Yagi and F. Yoshida, *Ind. Eng. Chem. Proc. Des. Dev. 14*: 488 (1975).
12. V. R. Ranade and J. J. Ulbrecht, *AIChE J. 24*: 796 (1978).
13. H. Höcker, G. Langer, and U. Werner, *Ger. Chem. Eng. 4*: 54 (1981).
14. M. Nishiwaka, M. Nakamura, and K. Hashimoto, *J. Chem. Eng. Japan 14*: 227 (1981).
15. G. Obernosterer and H.-J. Henzler, Einfluß viskoelastischer Eigenschaften von Flüssigkeiten auf den Stoff- und Wärmetransport, *Rheologie und mechanische Beanspruchung biologischer System*, VDI-GVC, Düsseldorf, 1987, p. 31.
16. Y. Kawase and M. Moo-Young, *Chem. Eng. Res. Des. 66*: 284 (1988).
17. A. Ogut and R. T. Hatch, *Can. J. Chem. Eng. 66*: 79 (1988).
18. B. Biron, I. W. Marison, and U. v. Stockar, *Biotechnol. Bioeng. 30*: 650 (1987).
19. K. O. Kipke, *Chem. Ing. Tech. 52*: 975 (1980).
20. A. Steiff, R. Poggemann, and P. M. Weinspach, *Chem. Ing. Tech. 52*: 492 (1980).

21. G. B. Tatterson, *Fluid Mixing and Gas Dispersion*, Mc-Graw Hill, New York, 1991.
22. B. J. Michel and S. A. Miller, *AIChE J. 8*: 262 (1962).
23. G. A. Hughmark, *Ind. Eng. Chem. Process Des. Dev. 19*: 638 (1980).
24. M. M. C. G. Warmoeskerken and J. M. Smith, The dispersion of gases in liquids with turbines, Proceedings, 5th European Mixing Conference, BHRA Fluid Engineering, Cranfield, 1985, p. 115.
25. J. M. Smith, K. van't Riet, and J. C. Middleton, Scale-up of agitated gas-liquid reactors for mass transfer, Proceedings, 2nd European Conference on Mixing, BHRA Fluid Engineering, Paper F4, 1977.
26. K. Chandrasekharan and P. H. Calderbank, *Chem. Eng. Sci. 36*: 819 (1981).
27. M. Nishikawa, M. Nakamura, H. Yagi, and K. Hashimoto, *J. Chem. Eng. Japan 14*: 219 (1981).
28. J. C. Hoskins and D. M. Himmelblau, *Comput. Chem. Eng. 12*: 881 (1988).
29. S. Chen, S. A. Billings, and P. M. Grant, *Int. J. Control 51*: 1191 (1990).
30. J. Leonhard and M. A. Kramer, *Comput Chem. Eng. 14*: 337 (1990).
31. J. Thibault, V. van Brensegem, and N. Cheruy, *Biotechnol. Bioeng. 36*: 1041 (1990).
32. P. Linko and Y. Zhu, *J. Biotechnol. 21*: 253 (1991).
33. P. Linko and Y. Zhu, *Kemia-Kemi 19*: 215 (1992).
34. S. P. Chitra, *Chem. Eng. Prog. April*: 44 (1993).
35. R. K. Bajpai and M. Reuss, *Can. J. Chem. Eng. 60*: 384 (1982).
36. M. Reuss and R. K. Bajpai, Stirred tank models, *Biotechnology*, 2nd compl. rev. ed., Vol. 4 (K Schügerl, ed.), 1991, p. 299.
37. R. Steel and W. D. Maxon, *Biotechnol. Bioeng. 4*: 231 (1962).
38. R. Steel and W. D. Maxon, *Biotechnol. Bioeng. 8*: 97 (1966).
39. M. Reuss, R. K. Bajpai, and W. Berke, *J. Chem. Technol. Biotechnol. 32*: 81 (1982).
40. C. G. Sinclair and D. E. Brown, *Biotechnol. Bioeng. 12*: 1001 (1970).
41. N. M. G. Oosterhuis and N. W. F. Kossen, *Biotechnol. Bioeng. 26*: 546 (1984).
42. F. G. Bader, Improvements in multiturbine mass transfer models, *Biotechnology Processes, Scale-up and Mixing* (C. S. Ho and J. Y. Oldshue, eds.), AIChE, New York, 1987, p. 96.
43. F. G. Bader, *Biotechnol. Bioeng. 30*: 37 (1987).
44. V. Singh, W. Hensler, and R. Fuchs, Bioreactor Fluid Dynamics, April 15–17, Cambridge, Paper 18, 1986, p. 231.
45. V. Singh, R. Fuchs, and A. Constantinides, A new method for fermentor scale-up incorporating both mixing and mass transfer effects. I. Theoretical basis, *Biotechnology Processes, Scale-up and Mixing* (C. S. Ho and J. Y. Oldshue, eds.), AIChE, New York, 1987, p. 200.
46. V. Singh, R. Fuchs, and A. Constantinides, Bioreactor Fluid Dynamics, Proceedings, 2nd International Conference, Sept. 21–23, Cranfield, UK, 1988, p. 95.
47. B. Mayr, P. Horvat, E. Nagy, and A. Moser, *Bioprocess Eng. 9*: 1 (1993).
48. R. Mann and P. Mavros, 4th European Conference on Mixing, April 27–29, BHRA, Fluid Engineering, Cranfield, UK, Paper B3, 1982.
49. R. Mann and P. Knysh, *Inst. Chem. Eng. Symp. Ser. 89*: 127 (1984).
50. F. Ragot and M. Reuss, A multi-phase compartment model for stirred bioreactors incorporating mass transfer and mixing, *Biochemical Engineering—Stuttgart* (M. Reuss, H. Chmiel, E.-D. Gilles, and H.-J. Knackmuss, eds.), Gustav-Fischer Verlag, Stuttgart, 1991, p. 184.

51. D. B. Spalding, Computer simulation of two-phase flow with special reference to nuclear reactor systems, *Computational Techniques in Heat Transfer* (R. W. Lewis, K. Morgan, I. A. Johnson, and W. R. Smith, eds.), Pineridge Press, 1985, p. 1.
52. P. S. Harvey and M. Greaves, *Trans. Inst. Chem. Eng. 60*: 195 (1982).
53. J. Placek, L. L. Tavlarides, G. W. Smith, and I. Fort, *AIChE J. 32*: 1771 (1986).
54. J. Placek and L. L. Tavlarides, *AIChE J. 31*: 1113 (1985).
55. K. van't Riet and J. M. Smith, *Chem. Eng. Sci. 30*: 1093 (1975).
56. S. Y. Ju, T. M. Mulvahill, and R. W. Pike, *Can. J. Chem. Eng. 68*: 3 (1990).
57. V. V. Ranade and J. B. Joshi, *Trans. Inst. Chem. Eng. 68*: 34 (1990).
58. S. M. Kresta and P. E. Wood, *AIChE J. 37*: 448 (1991).
59. R. I. Issa and A. D. Gosman, The Computation of three-dimensional turbulent two-phase flows in mixer vessel, *Numerical Methods in Laminar and Turbulent Flow*, Pineridge Press, Swansea, 1981, p. 829.
60. C. Träghard, A hydrodynamic model for the simulation of anaerated agitated fed-batch fermentor, Proc. 2nd Int. Conf. Bioreactor Fluid Dynamics, Sept. 21–23, Cambridge, UK 1988, p. 117.
61. A. D. Gosman, C. Lekakou, S. Politis, R. I. Issa, and M. K. Looney, *AIChE J. 38*: 1946 (1992).
62. A. Bakker and H. E. Akker, A computational study on dispersing gas in a stirred reactor, Proceedings, 7th European Congress on Mixing, 1991, p. 189.
63. S. V. Patankar, *Numerical Heat Transfer and Fluid Flow*, McGraw-Hill, New York, 1980.
64. S. Nagata, *Mixing: Principles and Application*, Wiley, New York, 1975.
65. S. Aiba, A. E. Humphrey, and N. F. Millis, *Biochemical Engineering*, 2nd ed., University of Tokyo Press, Tokyo, 1973, p. 304.

7
Pneumatically Agitated Bioreactors

Karl Schügerl and Andreas Lübbert

Universität Hannover
Hannover, Germany

I. INTRODUCTION

Stirred tanks became the most popular bioreactors after the manufacturing companies succeeded in maintaining aseptic conditions with suitable stirrer shaft bush bearings. These reactors have important advantages for industrial production: easy control of the gas dispersion and medium mixing by stirrer speed, efficient gas dispersion by stirrers, and use in highly viscous media. Since the regulation of the aeration rate is used mainly for the oxygen supply but not for the power input, in foaming systems low aeration rates are admissible.

Because of the relatively low oxygen transfer efficiency of stirred tank reactors with respect to power input, a rather high specific power input is necessary for a sufficient oxygen transfer rate, which is independent of the size of the reactor. Therefore, the high degree of heat produced by the stirring in large (>500 m^3) stirred tank reactors is difficult or impossible to reduce in the reactor. Furthermore, at low stirrer speeds, the gas dispersion and mixing efficiency of stirrers are extremely low. Therefore, it is technically and economically inappropriate to use stirred tank reactors for low-performance processes (e.g., biological wastewater treatment) which are carried out in large reactors at low

specific power input. In some cases, biological factors (e.g., shear sensitivity of cells) require reactors other than stirred tanks.

In nonmechanically agitated bioreactors, the power can be supplied by the kinetic energy of the liquid circulated by a pump in a loop or by compressed gas expansion. In both, the agitation is caused by the bubble movement, i.e., pneumatically.

Several nonmechanically agitated reactors have been recommended in patents and scientific literature. However, only tower jet loop reactors, plunging jet reactors, tower jet reactors, and tower reactors with power supplied by compressed gas expansion alone with or without a loop reached the industrial scale. The plunging jet reactors are used mainly in the biological treatment of effluents; only a few applications are known in baker's yeast production. Very large scale tower jet reactors and reactors with static gas distributors are employed in effluent treatment and in the cultivation of microorganisms.

In the following, only tower jet reactors and reactors with static gas distributors are considered. Both of them can be operated as bubble columns or with an internal or external loop. Three books [1–3], several reviews [4–14], and chapters in books [15–17] deal with these reactors. Most of these consider the performance of pneumatically agitated bioreactors with model systems or with low-viscosity cultivation media, which can be treated analogously to model media. A few papers deal with the application of these reactors for the production of primary and secondary metabolites by bacteria (e.g., *Streptomyces*), yeasts, or fungi in highly viscous culture media, where the rheology of the medium varies during the production due to the change of the medium viscosity and cell morphology [68,95,99,101,116,126]. With increasing viscosity and reactor volume the nonuniformity of the medium composition in the reactors gradually becomes dominant. It is no longer possible to apply lumped parameter or axial dispersion models for the liquid phase; structured reactor models are needed. However, because of the lack of experimental data, no structured reactor models exist at present for the description of cultivation processes in large reactors. Therefore, in this review, only lumped parameter and simple axial dispersion models are considered.

II. BASIC PRINCIPLES AND FLOW MODELS

Bubbles produced by gas dispersion rise because of their low density in liquids at rest or in liquids flowing upward or downward at low velocity. This is the case in bubble columns used in biotechnological practice. At high liquid velocity, the bubbles are dragged with the liquid flowing upward (in the riser) or downward (in the downcomer). These flow regimes occur in airlift tower loop reactors at high liquid circulation rates.

There is a continuous transition between the fluid dynamics in bubble columns and airlift tower loop reactors. In tower reactors with an outer loop

and a phase separation section, only the liquid phase is recirculated. At high aeration rates, a gas-phase circulation prevails as well. In tower reactors with a draft tube and at low aeration rates, different flow regimes exist, which differ significantly from those in bubble columns. With increasing superficial gas velocity, however, the fluid dynamic performance of tower reactors with an outer and inner loop, respectively, gradually approaches that of bubble columns. These reactor types, therefore, must be considered together.

The flow regimes in bubble columns depend on the gas velocity and column diameter [18]. At low superficial gas velocity, a homogeneous (bubbly) flow (Fig. 1A) of narrow size and velocity distributions prevails in columns of broad diameter range. Downflow bubble columns always operate in the bubble flow regime. By increasing the superficial gas velocity above the flooding point (≈ 5 cm s^{-1}), large bubbles formed by coalescence are stabilized by the wall and fill the entire column diameter in laboratory columns (diameter <10 cm), and the homogeneous flow changes into slug flow (Fig. 1C). In large columns (diameter >20 cm), large bubbles formed by coalescence and bubble clusters move predominantly at the centerline of the column and rise in a swarm of small bubbles with high velocity. The bubble size and velocity distribution are broad in this heterogeneous churn-turbulent flow (Fig. 1B), which is typical in industrial reactors. These three regimes are separated by a broad transition regime (Fig. 2). With increasing viscosity, the slug flow regime boundary shifts to higher tower diameters and lower superficial gas velocities.

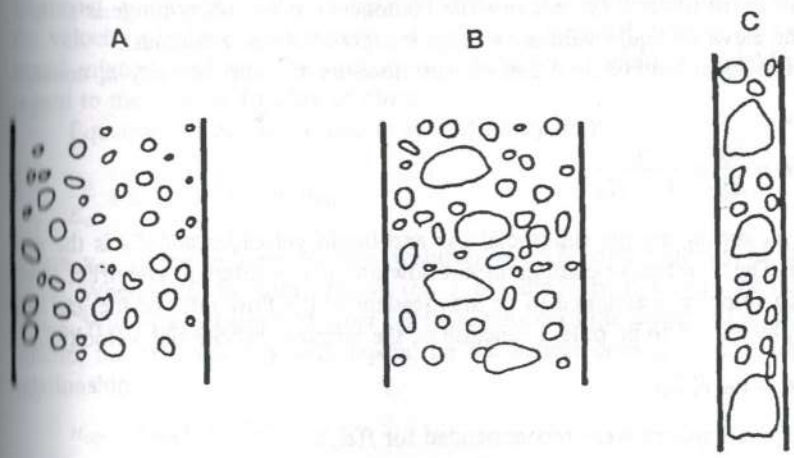


Figure 1 Flow regimes: (A) homogeneous bubbly flow; (B) heterogeneous churn-turbulent flow; (C) slug flow.

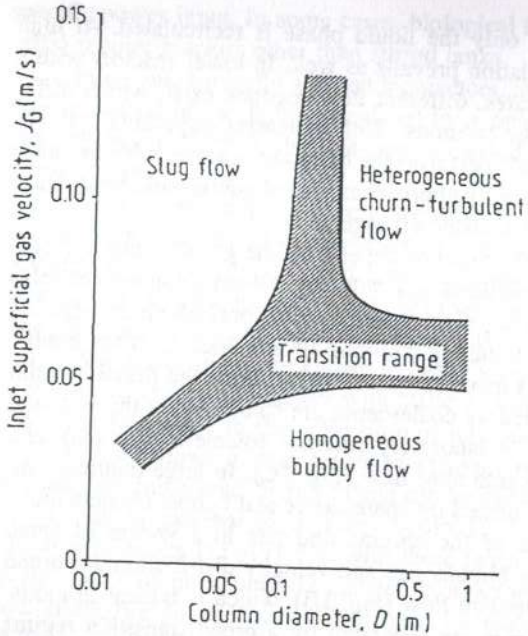


Figure 2 Transition flow regimes in bubble columns. (From Ref. 14.)

In airlift tower loop reactors, the homogeneous boundary range is shifted with the elevated liquid velocity to higher superficial gas velocities.

The ideal bubbly flow can be described by the slip velocity u_s concept [19]:

$$u_s = \frac{u_G}{E_G} \pm \frac{u_L}{1 - E_G} \tag{1}$$

where u_G and u_L are the superficial gas and liquid velocities and E_G is the gas holdup. The + refers to countercurrent flow and the − refers to cocurrent flow. The slip velocity is assumed to be independent of the flow rates of the gas and liquid phases and to be only a function of the terminal bubble rise velocity u_{BT}:

$$u_s = u_{BT} f(E_G) \tag{2}$$

Several relationships were recommended for $f(E_G)$:

$f(E) = 1$	Turner [20]	(3a)
$(1 - E_G)^{-1}$	Davidson and Harrison [21]	(3b)
$(1 - E_G)^{n-1}$	Wallis [22]: $n = 2$ for small bubbles	(3c)
	and $n = 0$ for large bubbles;	(3d)
	Richardson and Zaki [23]: n depends on $\mathrm{Re} = u_G d_C \nu_L^{-1}$)	
$(1 - E_G)(1 - E_G^{5/3})$	Marucci [24]	(3e)

which give nearly the same value for low E_G values but at a high gas holdup differ considerably [25].

Wallis [22] introduced the drift flux concept, which relates the phase flow velocities with the gas holdup. Drift flux F_{DF} is defined as the volumetric flux of gas relative to the surface moving at an average velocity:

$$F_{DF} = u_S E_G (1 - E_G) \tag{4}$$

With the combination of Eqs. (2) and (4)

$$F_{DF} = u_{BT} E_G (1 - E_G) f(E_G) = u_G (1 - E_G) \pm u_L E_G \tag{5}$$

is obtained. This concept was used successfully by Weiland [26] for airlift tower reactors with an outer loop, by Freedman and Davidson [27] for airlift tower reactors with a draft tube, and by Herbrechtsmeier and Steiner [28] for downflow bubble columns.

Zuber and Findlay [29] developed a model which applies for both bubble and churn-turbulent flow:

$$\frac{w_{S1}}{E_1} = w_1 = c_0 w_M + w_{1D} \tag{6}$$

where w_{S1} is the superficial velocity of phase 1, E_1 the holdup of phase 1, w_1 the velocity of phase 1, c_0 a constant, w_M the superficial velocity of the gas-liquid mixture, and w_{1D} the average drift velocity of phase 1 weighted with regard to the volume fraction of phase 1.

Equation (6) can be written in the following way:

$$\frac{u_G}{E_G} = C_0 (u_G + u_L) + u_{GD} \tag{7}$$

where u_{GD} is the volume fraction weighted-average drift velocity of the gas phase. When u_G/E_G is plotted versus $(u_G + u_L)$, the slope is C_0 and the intercept is u_{GD}. When the presence of other bubbles influences the motion of a particular bubble, the drift velocity will depend on the bubble density. In this case, the relationship

$$u_{GD} = u_{BT} (1 - E_G)^k \tag{8}$$

was recommended by Zuber and Findlay [29]. Based on the measurement of Hills [30] in a bubble column 15 cm in diameter and 10.5 m in height with

0.07–3.5 m s^{-1} gas velocities and up to 2.7 m s^{-1} water velocities, the relationship for interstitial velocity u_{Gi}

$$u_{Gi} = \frac{u_G}{E_G} = 0.24 + 1.35(u_G + u_L)^{0.93} \tag{9}$$

was obtained for $u_L > 0.3$ m s^{-1}.

Several models were recommended for the description of the flow pattern in the liquid phase. One of the most popular models was developed by Miyauchi and co-workers [31,32] based on the force balance:

$$-\frac{1}{r}\frac{d}{dr}(rT) = \frac{dP}{dz} + (1 - E_G)R_L g \tag{10}$$

where r is the radial distance from the column axis, T the shear stress defined through the turbulent kinematic viscosity, P the pressure at the column top, z the dimensionless axial coordinate, R_L the liquid density, and g the gravitational constant. By assuming a parabolic upward liquid velocity profile in the column center, a downward liquid velocity close to the wall, and a zero liquid velocity at the wall, they developed a complete solution and identified the unknown parameters based on experimental velocity profiles [32]. Riquarts [33,34] simplified the relationship by neglecting the effect of wall friction on the pressure gradient and assumed that the velocity gradient is zero in the centerline of the column.

$$u_{Gi}(\phi) = u_{Gio} - \frac{E_G g d_c^2}{32\nu_t}[1 - (1 - \phi^2]^2] \tag{11}$$

where $\phi = r/R_o$, with r the radial coordinate, R_o is the column radius, u_{Gio} is u_{Gi} at the center of the column, d_C is the column diameter, and ν_t is the turbulent kinematic viscosity. u_{Lio} is given by

$$u_{Lio} = \left(\frac{1 - 0.75E_G}{1 - E_G}\right)\frac{E_G g d_C^2}{48\nu_t} \tag{12}$$

Riquarts [33,34] and Shah and Deckwer [11] described the velocity profile by regression polynomials as

$$\frac{u_{Li}}{u_{Lio}} = a_0 + a_1\phi + a_2\phi^2 + a_3\phi^3 + a_4\phi^4 \tag{13}$$

where $\phi = r/R_o$.

With the coefficients of Eq. (13)—$a_0 = 1$, $a_1 = 0$ $a_2 = -0.5$, $a_3 = 0$, $a_4 = -3$ for $0 < \phi < 0.7$, and $a_0 = -234.397$, $a_1 = 1166.379$, $a_2 = -2142.483$, $a_3 = 1723.390$, $a_4 = -513.702$ for $0.7 < \phi < 0.9$—good agreement was found with experimental data evaluated in water-air bubble columns.

Freedman and Davidson [27] developed a "gulf stream" model based on pressure balance. However, Whalley and Davidson [35] and Joshi and Sharma [36–39] showed that the energy balance method is better suited for modeling than the pressure balance method. Joshi and Sharma [39] pointed out that for high bubble columns the energy balance method yields too high a velocity in comparison with experiments. Therefore, they developed a multiple-cell circulation model. By choosing the height of each circulation cell, so that the liquid-phase vorticity is at a minimum, the height of the circulation cells equals the column diameter. For the average liquid circulation velocity u_C

$$u_C = 1.31\left[gd_C\left(u_G \pm \frac{E_G u_L}{1 - E_g} - E_G u_{BT}\right)\right]^{1/3} \tag{14}$$

was obtained. Again, the minus and plus signs represent the cocurrent and countercurrent flow patterns, respectively. For the liquid-phase dispersion coefficient D_L

$$D_L = 0.31 d_C u_C \tag{15}$$

was obtained by means of this model [39], which is in close agreement with experimental results.

According to Riquarts [34], D_L can be calculated by means of the bubble diameter d_B and terminal bubble velocity u_{BT}:

$$D_L = 0.5 u_{BT} d_B \tag{16}$$

If one assumes that the dispersion is caused mainly by the movement of large bubbles with the terminal velocity

$$u_{BT} = 0.71(d_B g)^{0.5} \tag{17}$$

and the number of bubbles of diameter d_B present in the longitudinal section of d_C is n_B, one obtains for D_L

$$D_L = 0.0685 d_C(d_C g)^{0.5}\left(\frac{u^3_G}{\nu_L g}\right)^{1/8} \tag{18}$$

if one designates $n_B = 4$. Both the liquid dispersion coefficient D_L and the turbulent viscosity ν_t are related to the centerline liquid velocity u_{Lio}:

$$D_L = 0.322 u_{Lio} d_c \quad \text{and} \quad \nu_t = 0.0526 u_{Lio} d_c \tag{19}$$

Zehner [40,41] and Zehner and Schuh [42] extended the recirculation model of Joshi and Sharma to a three-dimensional model. They derived

$$u_c = \left(\frac{1}{f}\frac{\Delta R}{R_L} g d_C u_{Go}\right)^3 \tag{20}$$

for the mean liquid velocity in the recirculation path, u_c, where f is the drag

coefficient, ΔR the difference between liquid and gas densities, R_L the liquid density, and j_{Go} the superficial gas velocity in the top of the column. For the dispersion coefficient in the liquid phase D_L

$$D_L = \frac{u_C d_c}{2} \tag{21}$$

was obtained, which shows good agreement with the experimentally determined values when $f = 2.5$ is used. In a similar way, the mixing coefficient in the gas phase D_G

$$D_G = \frac{u_{Go} h_G}{2E_G} \frac{u_C}{u_{SB}} \tag{22}$$

was evaluated.

In Eq. (22), u_{SB} is the swarm velocity of the bubbles and h_G the mixing height of the bubble swarm, which is proportional to $d_C(u_C/u_{SB})^2$.

For the gas dispersion coefficient

$$D_G = \frac{u_{Go} d_C}{2} \left(\frac{u_C}{u_{SB}}\right)^3 = \frac{1}{2f} \frac{\Delta R}{R_L} \frac{g d_c^2 u_{Go}^2}{u_{SB}^3} \tag{23}$$

was obtained. With $f = 2.5$ and $u_{SB} = 23.5$ cm s^{-1}, good agreement was found with the experiments.

As can be seen from these models, the vortex velocity u_C has a significant effect on the performance of the bubble column reactors. However, in airlift tower loop reactors with a low aeration rate, the liquid velocity profile is uniform in the riser. Consequently, the vortex velocity u_C can be neglected, and thus the axial dispersion in the liquid and gas phases of airlift tower loop reactors is considerably lower than in bubble columns. With increasing aeration rate, however, a radial liquid velocity profile is formed gradually, the vortex velocity increases, the intensity of mixing is enhanced, and the difference between bubble column and airlift tower loop reactor gradually disappears.

The following types of airlift tower reactors are used in practice: reactors with gas distributor and internal (draft tube) or external loop (Fig. 3) and reactors with nozzle gas distributor and internal or external loop (Fig. 4). Different types of nozzles are used in industrial practice (Fig. 5). Liquid and gas are fed separately but mixed immediately at the nozzle tips (Fig. 5A). In most two-phase nozzles the liquid is fed through the internal and the gas through the annular nozzle (Fig. 5B and C), and in radial flow nozzles the gas and liquid are fed separately and mixed by a conical deflector (Fig. 5D). Figure 5B shows an injector nozzle with an impulse exchange tube, and Fig. 5C an ejector nozzle with a mixing tube.

In airlift tower reactors with an external loop and horizontal connection between the riser and downcomer at the top of the column, the gas is separated

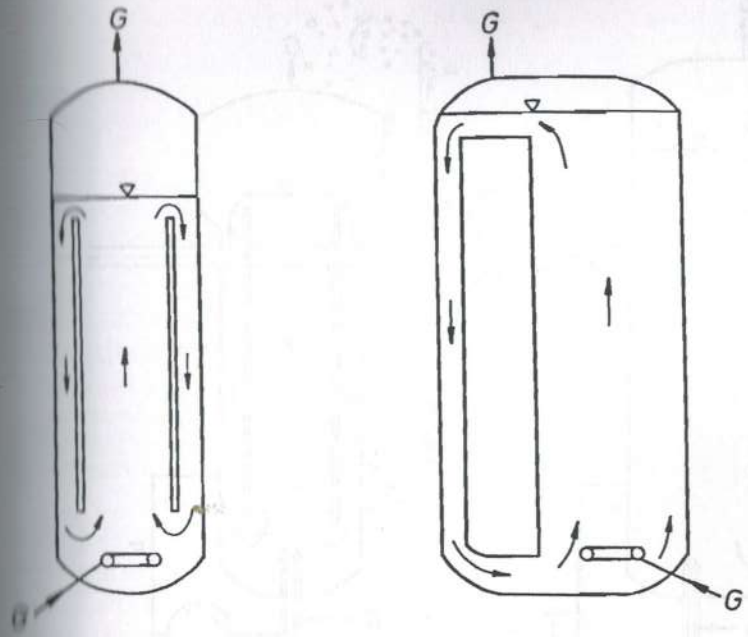


Figure 3 Airlift tower reactor with gas distributor and internal loop (draft tube) (left) and external loop (right).

from the liquid. Thus, at least at low and intermediate aeration rates, only liquid is recirculating. In airlift tower reactors with a draft tube, three states can be distinguished:

1. At low specific power input: liquid circulation with no gas circulation
2. With increasing specific power input: liquid circulation with partial gas circulation
3. At high specific power input: liquid circulation with constant gas circulation.

In practice, only the third state plays a role.

Chakravarty et al. [43] used the energy balance approach to develop a model for fully developed liquid and gas circulation in airlift tower loop reactors with energy input by compressed gas expansion alone. They obtained the following expression for the balance:

$$a u_{Li}^3 + b u_{Li}^2 + c u_{Li} + d = 0 \tag{24}$$

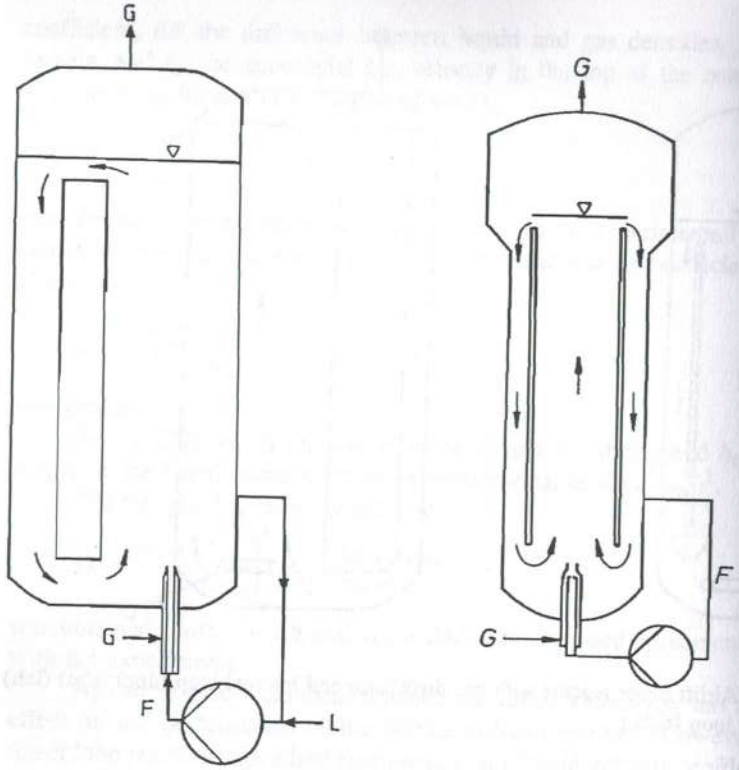


Figure 4 Airlift reactor with nozzle for gas dispersion and internal loop (draft tube) (right) and external loop (left).

where u_{Li} is the interstitial liquid velocity and

$$a = (K + MH_o) + \frac{E_{Gr}}{E_{Gd}}$$

$$b = \frac{u_G}{E_{Gr}} - u_{BT}\frac{A_d E_{Gd}}{A_r E_{Gr}}$$

$$c = 2gH_o\frac{E_{Gr} - E_{Gd}}{1 - E_{Gr}}$$

$$d = \frac{2gH_o}{1 - E_{Gr}}\left[2u_{BT}\frac{A_d E_{Gd}}{A_r} - u_G(1 - E_{Gr})\right]$$

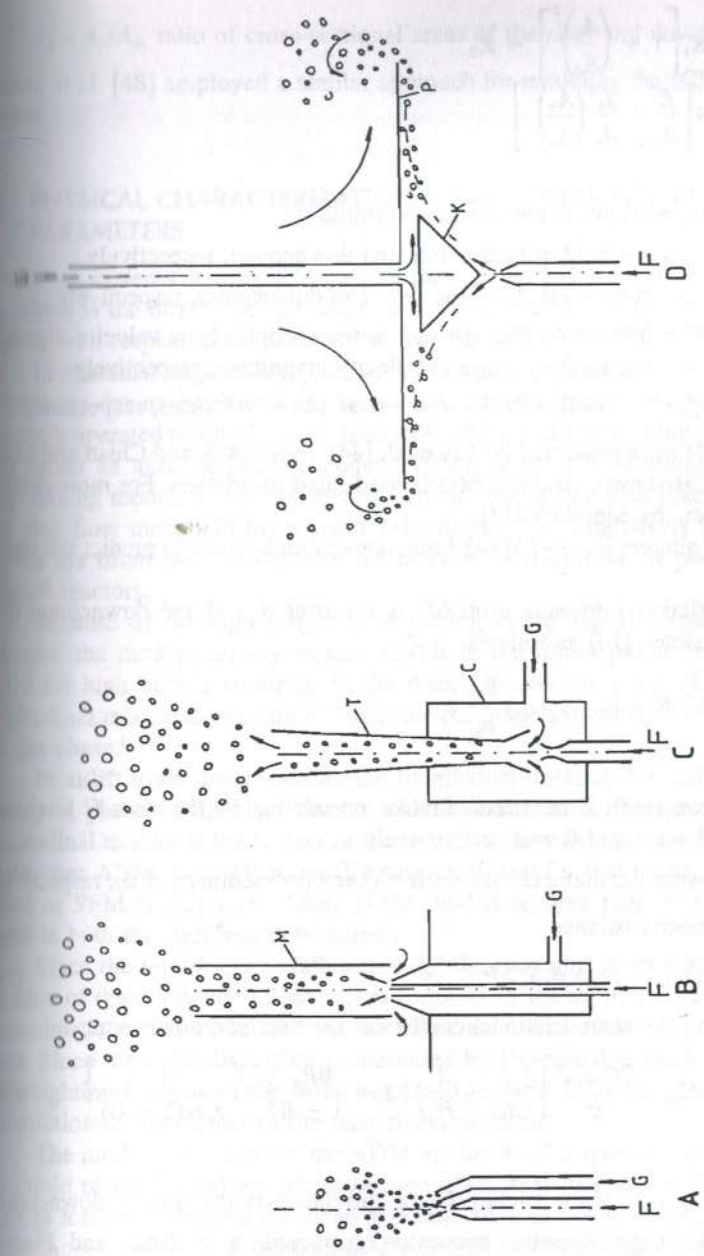


Figure 5 Different types of nozzles for gas dispersion [7]: (A) gas and liquid are fed separately (BASF); (B) two-phase nozzle injector nozzle with impulse exchange tube (BASF); (C) two-phase ejector nozzle (Bayer); (D) radial flow nozzle (Hoechst). F, liquid; G, gas; M, momentum exchange tube; T, nozzle; C, mixing chamber for gas feed; K, cone for gas dispersion; P, bubble distributor baffle.

$$K = K_1\left[1 + \left(\frac{A_r}{A_d}\right)^2\right] + K_2$$

$$M = 4\left[\frac{f_r}{d_r} + \frac{f_d}{d_d}\left(\frac{A_r}{A_d}\right)^2\right]$$

where

H_o = height of the nonaerated liquid

E_{Gr}, E_{Gd} = gas holdup in the riser and downcomer, respectively

A_r, A_d = cross sections of the riser and downcomer, respectively

K_1, K_2 = friction coefficients due to the change of the velocity direction and to the change of velocity magnitude, respectively

f_r, f_d = the friction factor in the riser adn downcomer, respectively

Other models were presented by Lee et al. [44], Jones [45], and Chisti and Moo-Young [46]. However, all these models used fitted parameters. For more details, see the review by Merchuk [14].

Other authors [8,47–53] used the momentum balance to predict the liquid velocity.

The frictional pressure drop ΔP_i in the riser (i = r) and downcomer (i = d) can be defined [14] as follows:

$$P_i = 2C_{fi}R_L u_L(u_{Li} + u_{Gi})\frac{H_o}{d_{hi}} \tag{25}$$

where

C_{fi} = constant of the friction factor correlation in the riser (i = r) and downcomer (i = d), respectively

d_{hi} = hydraulic diameter of riser (i = r) and downcomer (i = d), respectively

With the pressure balance

$$H_o R_L g(E_{Gr} - E_{Gd}) = \Sigma\,\Delta P_i \tag{26}$$

and assuming the same frictional coefficient for riser and downcomer

$$E_{Gr} - E_{Gd} = \frac{2C_L u_{Ld}^2}{g}\left[\left(\frac{1}{d_{hd}} + \frac{1}{H_o}\right)\left(1 + \frac{\beta R_R}{1-\beta}\right) + \frac{1}{d_{hr}r_R(1-\beta)}\right] \tag{27}$$

where

C_L = constant of the friction factor correlation in the riser + downcomer

β = flowing volumetric concentration according to Zuber and Findlay [29]

R_R = recycle ratio

$r_R = A_r/A_d$, ratio of cross-sectional areas of the riser and downcomer

Kubota et al. [48] employed a similar approach for modeling the ICI deep shaft reactor.

III. PHYSICAL CHARACTERIZATION OF REACTORS WITH INTEGRAL PARAMETERS

Two-phase systems are characterized by the relative gas content or gas holdup E_G, which is the difference betweeen the aerated volume V and nonaerated volume V_L with respect to the aerated volume: $E_G = (V - V_L)V^{-1}$.

In chemical engineering practice, the weighting function is used to characterize the dynamic behavior of the reactors. The weighting function of a continuously operated reactor is often described with the dispersed plug flow model, also called an axial dispersion model (ADM), or with its limiting states, the ideal mixing model, which prevails in a continuous stirred tank reactor (CSTR) and plug flow model (PFM) without axial dispersion, respectively [55]. These models are often used to describe the physical performance of pneumatically agitated reactors.

Because of the high longitudinal mixing in the liquid phase of bubble columns, the models usually assume CSTR in the liquid phase for short and ADM for high bubble columns. In the discontinuous gas phase (bubbles) the longitudinal mixing is negligible. Therefore the models usually assume PFM for the gas phase.

In airlift tower loop reactors the longitudinal mixing is usually lower in the liquid phase of the riser than in bubble columns. In the downcomer the longitudinal mixing is lower than in the riser. For the mixing in the liquid phase of the riser ADM or CSTR is usually employed, and for that in the downcomer ADM or PFM is employed. Most of the models assume plug flow in the gas phase in both the riser and downcomer.

Since the liquid is circulating through the riser and downcomer, the convolution of their weighting functions is formed. As shown by Riquarts [34], the deterministic vortex model and the stochastic axial dispersion model are equivalent. Since the axial dispersion is measured by the signal-response technique, the weighting function of the reactor is usually described by an ADM or by the convolution of the ADMs of the riser and downcomer.

The model parameters of the ADM are the axial dispersion coefficients of the liquid phase D_L and gas phase D_G, the circulation time of the liquid t_L and gas t_G in loop reactors, and the mean residence time of the gas phase τ_G and that of the liquid phase τ_L in continuously operated reactors. The axial dispersion coefficients D_L and D_G are related to the mixing times in the liquid t_{ML} and the gas phases t_{MG}. These model parameters are usually determined by signal-

response techniques [56]. The two phases are coupled by the mass transfer of O_2 and CO_2 between them. However, most models take only the O_2 mass transfer into consideration.

Mass transfer between the phases is usually described by the volumetric mass transfer coefficients k_La, defined according Eq. (28):

$$\mathrm{OTR} = k_La(C_L^* - C_L) = k_Ga(p_{O2} - p_{O2}^*)\ (\mathrm{kg\ m^{-3}\ h^{-1}}) \tag{28}$$

where

k_L = liquid side mass transfer coefficient

k_G = gas-side mass transfer coefficient, $\mathrm{mh^{-1}}$

a = specific gas/liquid interfacial area with respect to the volume of the liquid phase V_L, $\mathrm{m^{-1}}$

C_L, C_L^* = dissolved oxygen concentration in the liquid bulk and at the interface, respectively, $\mathrm{kg\ m^{-3}}$

p_{O2}, p_{O2}^* = partial pressure of oxygen in the gas phase bulk and at the interface

The evaluation of k_La is performed by means of the oxygen balance during the cultivation by measuring C_L and p_{O2} and assuming the equilibrium at the interface to be $p_{O2}^* = \mathrm{He}\ C_L^*$, where He is the Henry coefficient, and neglecting the mass transfer resistance in the gas film: $p_{O2} = p_{O2}^*$.

Since the intensity of the mixing and OTR in the continuous liquid phase are extremely low in nonagitated reactors, they are enhanced by putting power P into the liquid. This power is related to the liquid volume V_L, which is called specific power input: PV_L^{-1} ($\mathrm{kW\ m^{-3}}$). The efficiency of the oxygen transfer rate with respect to the specific power input $E_P = \mathrm{OTR}\ VP^{-1}$ ($\mathrm{kg\ O_2\ m^{-3}}$ liquid volume) is an important measure of the reactor performance.

Heat is produced in the reactor by power input and cell growth and is removed partly by evaporating the liquid in the gas flow and partly by cooling the reactor. The determination of the overall heat transfer coefficient k_h is carried out during the cultivation by means of the heat balance.

IV. RELATIONSHIPS BETWEEN REACTOR PARAMETERS

The most important relationships are between the relative gas content E_G and oxygen transfer rate OTR, respectively, and the specific power input PV_L^{-1}. In tower jet reactors, different types of nozzles are used for gas dispersion (Fig. 4). Their type and geometric scale have an important influence on the gas holdup E_G, specific gas-liquid interfacial area a, and volumetric mass transfer coefficient k_La. According to Schügerl et al. [4], the gas holdup is only a function of the

superficial gas velocity u_G, acceleration of gravity g, and the Sauter diameter of the bubbles d_s in bubble column reactors:

$$E_G = \mathrm{Fr}^{1.19} \tag{29}$$

where $\mathrm{Fr} = u_G(gd_s)^{-0.5}$ is the modified Froude number. This relationship holds true for different types of nozzles, static gas distributors, and aqueous liquids—model and culture media with different compositions in the validity range:

$$0.05 < E_G < 0.60$$

$$0.02 < \mathrm{Fr} < 1.0$$

Since d_s is usually unknown, in practice, relationships such as Eq. (30) are preferred:

$$E_G = u_G^n \tag{30}$$

where n depends on the flow regime, gas distributor, and medium composition. In the homogeneous flow regime, n varies between 0.7 and 1.0; for alcohol and salt solutions $E_G \propto u_G^{1.19}$. In the heterogeneous regime n is between 0.4 and 0.7 with an average of 0.6 [1]. Some authors tried to find a general relationship. Hikita et al. [57] recommend the following relationship:

$$E_G = 0.672b\left(\frac{u_G\mu_L}{\sigma}\right)^{0.578}\left(\frac{\mu_L^4 g}{R_L\sigma^3}\right)\left(\frac{R_G}{R_L}\right)^{0.062}\left(\frac{\mu_G}{\mu_L}\right)^{0.107} \tag{31}$$

where

R_L, R_G = viscosities of liquid and gas phases, respectively

σ = surface tension

R_L, R_G = densities of liquid and gas phases, respectively

$b = 1$ for nonelectrolytes

$b = 1.1$ for $I > 1$ g-ion $\mathrm{l^{-1}}$

$b = 10^{0.04141}$ for $I < 1$ g-ion $\mathrm{l^{-1}}$

I = ionic strength

This relationship was evaluated in laboratory bubble columns (d_C = 10 cm) with a single-hole gas distributor (d_O = 1.1 cm) in the range u_G between 4.2 and 38 $\mathrm{cm\ s^{-1}}$ with sugar solutions, aqueous methanol and n-butanol solutions, salt solutions, and organic solvents as well as with different gases in the following validity range:

For nonelectrolytes: R_L = 0.79–1.24 g $\mathrm{l^{-1}}$, R_G = (0.0837–1.84) × 10^{-3} g $\mathrm{l^{-1}}$, μ_L = 0.66–17.8 mPa s, μ_G = (0.8–1.81) × 10^{-2} mPa s, σ = 30–76 mN $\mathrm{m^{-1}}$

For electrolyte solutions: $R_L = 1.01–1.17$ g cm^{-3}, $\mu_L = 0.9–1.87$ mPa s, $\sigma = 71.9–79.6$ mN m^{-1}

Since the gas distributor was a single-hole plate with a 1.1-cm bore diameter, the primary bubble size was larger than the equilibrium bubble size. The latter is quickly attained in coalescence-promoting and -repressing media; therefore, Eq. (31) holds true for bubble columns with equilibrium bubble sizes independent of the gas distributor.

In large bubble columns, also with coalescence-repressing media and primary bubble sizes which are smaller than the equilibrium ones (e.g., with nozzles or porous plate gas distributors), the equilibrium bubble size dominates.

Hikita et al. [58] recommended a relationship for volumetric mass transfer coefficient k_La as follows:

$$k_La = \frac{14.9gb}{u_G}\left(\frac{u_G\mu_L}{\sigma}\right)^{1.76}\left(\frac{\mu_L^4 g}{R_L\sigma^3}\right)^{-0.284}\left(\frac{\mu_G}{\mu_L}\right)^{0.243}\left(\frac{\mu_L}{R_L D_m}\right)^{-0.604} \tag{32}$$

This relationship was evaluated in the same reactor (also for $d_c = 19$ cm) as Eq. (31) with the same liquid and gas phases. The validity ranges are the same. For nonelectrolytes, $b = 1$. For electrolyte solutions, $b = 10^{0.0681}$ for $I < 1$ val L^{-1}, $b = 1.114 \times 10^{0.0211}$ for $I > 1$ val L^{-1}. However, it is questionable whether Eqs. (31) and (32) evaluated in a laboratory column can be employed for industrial equipment.

Zlokarnik [59–63] developed his relationships by making measurements in pilot plant bubble columns with nozzle aerators and testing them on industrial apparatus. He found for a coalescence-suppressing medium (sulfite solution)

$$k_La\left(\frac{\nu}{g^2}\right)^{0.33} = 2.75 \times 10^{-4}\left[\frac{P_L V_L^{-1}}{R_L(\nu_L g^4)^{0.33}}\right]^{0.8} \tag{33a}$$

with validity range

$$0.05 < \frac{P_L V_L^{-1}}{R_L(\nu_L g^4)^{0.333}} < 1.0$$

$$2 \times 10^{-5} < k_La\left(\frac{\nu_L}{g^2}\right)^{0.33} < 2 \times 10^{-4}$$

and for a coalescence-promoting medium

$$k_La\left(\frac{q_G}{V_L}\right)^{-1} = 1.5 \times 10^{-2}\left[\frac{P_L q_G^{-1}}{R_L(g\nu_L)^{0.666}}\right]^{0.5} \tag{33b}$$

with validity range

$$4 \times 10^4 < \frac{P_L q_G^{-1}}{R_L(g\nu_L)^{0.666}} < 10^6$$

$$3 < k_La\left(\frac{q_G}{V_L}\right)^{-1} < 10$$

where q_G is gas throughput.

For the industrial reactors he used a somewhat modified relationship between a characteristic sorption number Y and a characteristic power number X:

$$Y = aX^b \tag{34}$$

where

$$Y = \frac{k_La}{u_G}\left(\frac{\nu_L^2}{g}\right)^{0.333}$$

$$X = \frac{P_L q_G^{-1}}{R_L(\nu_L g)^{0.666}}$$

Constants depend on the medium composition and nozzle type:

For injector 8/14 (Fig. 5C): $a \times 10^5 = 1.0$, $b = 0.2$ (water), $a \times 10^5 = 1.9$, $b = 0.2$ (3 g NaCl L^{-1}) $a \times 10^5 = 1.3$, $b = 0.25$ (5 g NaCl L^{-1}), $a \times 10^5 = 1.2$, $b = 0.5$ (10 g NaCl L^{-}), $a \times 10^5 = 0.15$, $b = 0.5$ (15–20 g NaCl L^{-1})

For slot nozzle (Figs. 5C and 17): $a \times 10^6 = 2.4$, $b = 0.33$ (water), $a \times 10^6 = 2.2$, $b = 0.37$ (3 g NaCl L^{-1}), $a \times 10^6 = 1.65$, $b = 0.39$ (5 g NaCl L^{-1}), $a \times 10^7 = 1.25$, $b = 0.34$ (10 g NaCl L^{-1})

He also defined the efficiency of the OTR with reference to the power input

$$E_{O2} = \frac{\text{OTR } V_L}{P_L + P_G}\ [\text{kg O}_2\ (\text{kWh})^{-1}] \tag{35}$$

where P_L and P_G are the power input by liquid kinetic energy and gas expansion, respectively.

A 9.5-m-high airlift tower reactor with an external loop was used in the investigations by Weiland [26] and Weiland and Onken [64,65]. They recommended the following relationships for the gas holdup:

$$E_G = 55.8u_G^{0.725} \tag{36a}$$

with porous plate, with validity range

$$I < 0.1 \text{ g L}^{-1}$$

$$0.01 \le E_G \le 0.12$$

$$0.005 < u_G < 0.14 \text{ m s}^{-1}$$

and

$$E_G = 62.3 u_G^{0.803} \tag{36b}$$

with a perforated plate as gas distributor, with validity range

$$0.08 \le E_G \le 0.12$$

$$0.005 \le u_G \le 0.16 \text{ m s}^{-1}$$

For the liquid circulation rate u_L

$$u_L = 1.15 u_G^{0.41} \tag{36c}$$

was obtained. Validity range:

$$0.002 \le u_L \le 0.5 \text{ m s}^{-1}$$

$$0.005 \le u_G \le 0.15 \text{ m s}^{-1}$$

Popovic and Robinson [66] recommended the following relationship for the volumetric mass transfer coefficient in bubble columns and tower reactors with an external loop with Newtonian (sugar solution) and non-Newtonian (CMC solution) media:

$$k_L a = 0.5 \times 10^{-2} u_{Gr}^{0.52} D_m^{0.5} R_L^{1.03} \left[1 + \left(\frac{A_d}{A_r}\right)\right]^{-0.85} (\mu_{eff})^{-0.89} \times \sigma^{-0.75} \tag{37}$$

where

u_{Gr} = superficial gas velocity in the riser

A_r, A_d = cross sections of riser and downcomer, respectively

μ_{eff} = effective dynamic viscosity of the medium

σ_L = interfacial tension

Validity range:

$$0.03 \le u_{Gr} \le 0.26 \text{ m s}^{-1}$$

$$0 \le A_d/A_r \le 0.444$$

$$0.02 \le \mu_{eff} \le 0.5 \text{ Pas}$$

$$0.33 \le D_m \times 10^9 \le 2.52 \text{ m}^2 \text{ s}^{-1}$$

$$1.003 \le R_L \le 1.240 \text{ kg m}^{-3}$$

$$59 \le \sigma_L \times 10^3 \le 79 \text{ N m}^{-1}$$

Schumpe et al. [67] proposed the following relationship for the gas holdup in highly viscous media based on their measurements with glycerol, CMC, PAA, and xanthan gum:

$$E_G = 0.36\left(\frac{g d_c^2 R_L}{\sigma}\right)^{-0.15}\left(\frac{g d_c^3 R_L^2}{\mu_{eff}^2}\right)^{0.09}\left(\frac{u_G}{\sqrt{g d_c}}\right)^{0.53} \tag{38}$$

According to Moo-Young et al. [68], this relationship describes well the gas holdup in mycelial cultivation media.

Schumpe et al. [67] also proposed a relationship for the volumetric mass transfer coefficient based on the same measurements:

$$\frac{k_L a d_c^2}{D_m} = 0.021\left(\frac{\mu_{eff}}{R_L D_m}\right)^{0.50}\left(\frac{g d_c^2 R_L}{\sigma}\right)^{0.21}\left(\frac{g d_c^3 R_L^2}{\mu_{eff}^2}\right)^{0.60}\left(\frac{u_G}{\sqrt{g d_c}}\right)^{0.49} \tag{39}$$

Validity ranges for Eqs. (38) and (39):

$$5.4 \times 10^3 < \text{Sh} < 1.8 \times 10^6$$

$$2.2 \times 10^3 < \text{Sc} < 2.3 \times 10^5$$

$$4.9 \times 10^2 < \text{Bo} < 1.2 \times 10^4$$

$$1.3 \times 10^5 < \text{Ga} < 1.1 \times 10^{10}$$

$$1.4 \times 10^{-2} < \text{Fr} < 2.5 \times 10^{-1}$$

where

$$\text{Sh} = \frac{k_L a d_c^2}{D_m} \qquad \text{Sc} = \frac{\mu_{eff}}{R_L D_m} \qquad \text{Bo} = \frac{g d_c^2 R_L}{\sigma}$$

$$\text{Ga} = \frac{g d_c^3 R_L^2}{\mu_{eff}^2} \qquad \text{Fr} = \frac{u_G}{\sqrt{g d_c}}$$

and Sh is the Sherwood number, Sc the Schmidt number, Bo the Bond number, Ga the Galilei number, and Fr the Froude number.

Equation (39) also describes well the experimental results for mycelial cultivation [68].

V. DETERMINATION OF FLOW PATTERNS AND DISTRIBUTION OF LOCAL PARAMETERS

In large reactors, lumped parameter models are not suitable to describe the bioreactor behavior. Structured models are needed to describe the nonuniformity of the energy dissipation rate, concentrations, and temperature in these reactors. Unfortunately, we do not as yet have enough information for these models.

In the past 10 years, however, several new techniques have been developed for local and global characterization and multiphase reactors (see review articles

of Drahos and Cermak [69], Joshi et al. [70], and Lübbert [56]). Only advanced measuring techniques are considered here, which can be used in pneumatically agitated reactors during cell cultivation. We can characterize the lumped and local properties of the liquid and gas phases.

A. Characterization of the Continuous Liquid Phase

The main transport agent in bioreactors is the continuous liquid phase. It transports the nutrients to the cells and carries away the metabolites and heat. Hence, the fluid motions are of primary importance in bioreactors.

In bubble columns and airlift tower loop reactors with static aerators all the enegy needed to maintain the liquid flow is introduced by means of the dispersed gas phase. The specific power input with respect to the weight of the liquid P_P/M_L is a function of the superficial gas velocity u_G and the acceleration due to gravity g:

$$\frac{P_P}{M_L} = u_G g \tag{40}$$

The energy introduced into the pneumatically agitated bioreactors is channeled into different modes of fluid motion. A main categorization is the quasi-deterministic mean flow of the liquid and the gas phase and the superimposed random motions of the fluid phase. The main global flow is usually a circulatory flow also in continuously operated reactors. Characterization of these main motions requires special measuring techniques. The random motion, on the other hand, is quite distinct on different scales of observation. On the intermediate scale, random circulatory (eddy) motions, usually called turbulence, are preferred because they are very effective in mixing on these scales. The smaller the scale, the more important the viscous forces become, and the final small-scale limit is purely characterized by viscous losses, i.e., the direct transfer of the available kinetic energy into the smallest-scale motion, the molecular motion, known as heat motion.

Circulation and mixing times are usually measured by means of tracer experiments. Although conceptually simple, tracer experiments are difficult to implement in biotechnological practice, because it is by no means easy to find a suitable tracer together with an appropriate detector [71]. The common approaches found in the chemical engineering literature are mostly suited for measurements in model liquids and are not appropriate for cultivation media. They should not impair the cell growth and product formation and the fluid dynamic behavior of the liquid [72]. In model media, salt or dye is used. Figure 6 shows the concentration variation of the tracer response (salt solution) to an impulse-shaped input in an 80-L airlift tower loop reactor with a draft tube. The persistence of the concentration maxima indicates the low mixing intensity in these

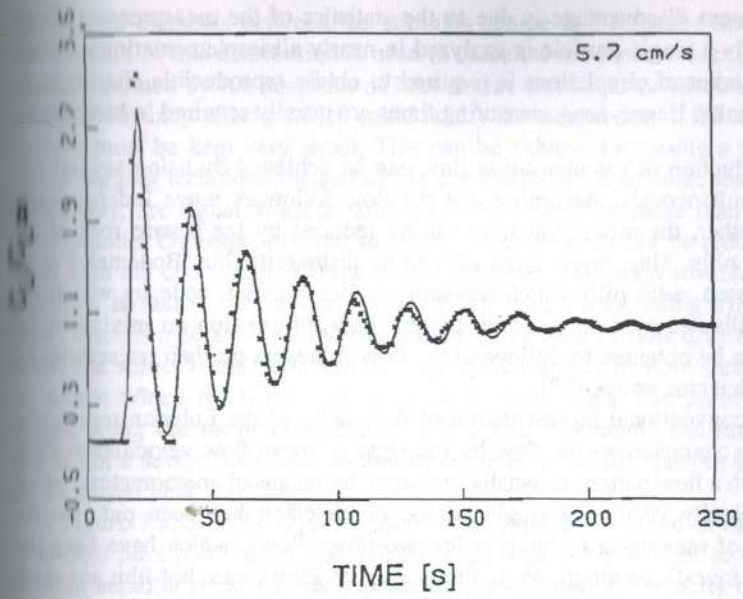


Figure 6 Response curve to the pulse-shaped tracer input in the batch liquid phase in an 80-L airlift tower loop reactor at a superficial gas velocity u_G = 5.7 cm/s. (From Ref. 119.)

reactors. At a higher aeration rate and in cultivation media, the mixing is more intense but still less than in mechanically well-agitated reactors.

Where no suitable fluid tracers are available, solid-phase tracers can be the matter of choice. Several approaches using so-called flow followers have been reported in the literature [73]. Flow followers are macroscopic particles on the scale of some millimeters; for this reason, they are called pills. Their density is adjusted to neutral buoyancy in the aerated liquid phase of the bioreactor. In this way, they circulate with the mean liquid flow through the reactor. Usually one monitors the pill when it crosses some control area. In an airloop reactor, for example, it may be registered every time it enters the downcomer.

Monitoring is performed using magnetic pills and an induction coil [74], a small radio transmitter and receiver [75], or radiactive pills and a scintillation counter detector. They have the advantage that, in contrast to liquid tracers, it is easy to build flow follower pills which do not interfere with the culture in the bioreactor. There are, however, some disadvantages:

The most severe disadvantage is the uncertainty in the assumption of the degree to which the flow follower really follows the fluid motion.

The next disadvantage is due to the statistics of the measurement. Since only a single particle is analyzed in nearly all implementations, a large number of circulations is required to obtain reproducible characteristic results. Hence, long measuring times are usually required in bioreactors.

A reduction of the measuring time can be achieved by using several followers simultaneously. Assuming that the flow followers move independently of one another, the measuring time can be reduced by the square root of the number of pills. This requires the pills to be distinguishable. Bodemeier et al. [76] discussed radio pills which transmit an identification code by which the different pills can be distinguished. Langrangian information on mixing in the reactors can be obtained by following the flow followers on their trajectories by means of detector arrays [77].

The conventional representation of flow fields is the Eulerian representation, which characterizes the flow by the array of mean flow velocities at fixed places. Such a flow pattern is usually measured by means of anemometers, which provide velocity vectors at single points. Much effort has been put into the adaptation of measuring techniques for two-phase flows, which have been developed to investigate single-phase flows, such as Pitot tubes, hot-film anemometers, laser Doppler anemometers, fan anemometers, electrochemical probes, etc.

In two-phase gas-liquid flows, however, locally highly resolving techniques with extremely small measuring volumes are used to measure velocity fluctuations of fairly high frequencies, but their signals contain invalid information every time a bubble passes the measuring volume. Many investigators tried to analyze the signal numerically to eliminate the parts of the signal where the liquid velocity is undefined. This cannot be done unambiguously and is, furthermore, very time-consuming. With increasing gas holdup the quality of the signal quickly deteriorates. The alternative is to increase the effective measuring volume.

By using a local tracer technique the time of flow of the fluid elements between the tracer source and the detector is measured. Then, the measuring volume, over which the liquid velocity is averaged, is the whole area through which the tracer proceeds from the source to the detector. Clearly the larger measuring volume decreases the spatial and time resolution of this technique, compared with laser Doppler anemometry, for example. On the other hand, these techniques offer some advantages which compensate for these deficiencies. An example is the heat pulse technique.

The heat pulse method can be regarded as a local stimulus-response technique. At a given point within the flow, fluid elements are tagged by small enhancements of their temperature, and some centimeters downstream along the flow trajectory, the temperature response is monitored by a sensitive sensor. Hence the primary measurement quantity is the time-of-flow distribution of the

tagged fluid elements between the stimulus source and response detector. The first moment of this distribution is used to calculate the mean velocity along the direction defined by the positions of heat source and response detector. In order to avoid buoyancy effects, which could falsify the measurement, the temperature increase must be kept very small. This can be achieved by using a pseudostochastic tagging technique instead of the conventional Dirac pulse test function. In this way, the signal-to-noise ratio can be enhanced by more than one order of magnitude. Consequently, the strength of the stimulus can be reduced, compared with the conventional Dirac pulse technique. The main advantage of this technique is that it can be used in nearly all systems including systems with highly viscous media. One disadvantage is that the time-of-flow distribution and, hence, the velocity can be measured only if the number of flow elements from the source which reach the detector is sufficiently high. The probes must be aligned along the mean flow direction to obtain reasonable measuring times. This is not a severe restriction in bubble column and airlift reactors but may be one in stirred tank reactors.

Figure 7 shows the liquid velocity distributions measured in the rise of a 23-m-high pilot plant airlift tower loop reactor for baker's yeast production at different aeration rates. At low superficial gas velocity, the velocity is uniform. With increasing aeration rate, the velocity distribution becomes more and more nonuniform and gradually approaches the velocity profile which prevails in a bubble column reactor. At the same time, the intensity of mixing increases.

The intensity of local mixing can be evaluated for the standard deviation of the time-of-flow distribution. If the time-of-flow distributions are measured at several distances downstream from the source, it is possible to determine the speed with which the fluid elements are dispersed and the local mixing mechanism can be evaluated.

In airlift reactors, the bubble-wake mechanism, i.e., the transport of the bubble wake relative to the bulk, was shown to be the dominating mechanism along the column axis, whereas in stirred tank reactors turbulent mixing prevails [78,79].

B. Characterization of the Discontinuous Gas Phase

In aerobic biological processes, bubbles serve as the source of oxygen and, at the same time, act as agitators. According to Eq. (28), OTR depends on the specific gas–liquid interfacial area a, which is a function of the Sauter bubble diameter d_S and gas holdup E_G:

$$a = \frac{6E_G}{(1 - E_G)d_S} \tag{41}$$

If the bubbles are small ($d_S < 1$ mm), their residence time in the liquid is much

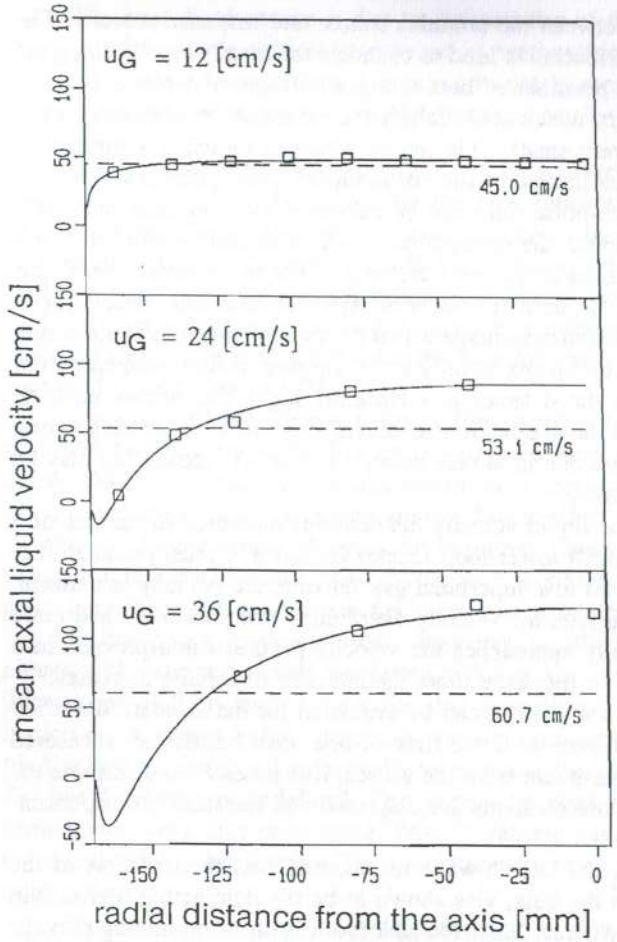


Figure 7 Liquid (tap water) velocity distribution in the riser of a 23-m-high pilot plant airlift tower loop reactor at different superficial gas velocities. (From Ref. 122.)

longer than the time necessary to exhaust their oxygen content. If they are large, their residence time is much shorter than the exhaust time and the oxygen content of the air is not used efficiently; furthermore, the specific gas-liquid interfacial area a is low. Small bubbles influence the mixing intensity in pneumatically agitated reactors only slightly. Large bubbles are needed for high mixing intensity. Bubbles of intermediate size are a good compromise.

The gas phase, continuously fed into bioreactors, is characterized by the gas residence time distributions. In airlift tower loop reactors, not only the liquid phase but also a part of the gas phase is recirculated through the downcomer. Analogous to the characterization of liquid phase properties, signal-response techniques are used to gain more information on the gas-phase flow behavior. To improve the signal-to-noise ratio, a pseudostochastical test signal is frequently used for the measurements.

Figure 8 shows the distribution of the gas residence time measured in tap water in a 4-m^3 pilot plant airlift tower loop reactor. The low intensity of mixing in the gas phase is obvious. A comparison of the gas circulation time t_{GC}, evaluated from the gas distribution of the residence time with the liquid circulation time t_{LC}, indicates that $t_{GC} > t_{LC}$. That is, the higher velocity of the bubbles relative to the liquid caused by the buoyancy forces in the riser is overcompensated by their lower velocity relative to the liquid in the downcomer. Since the

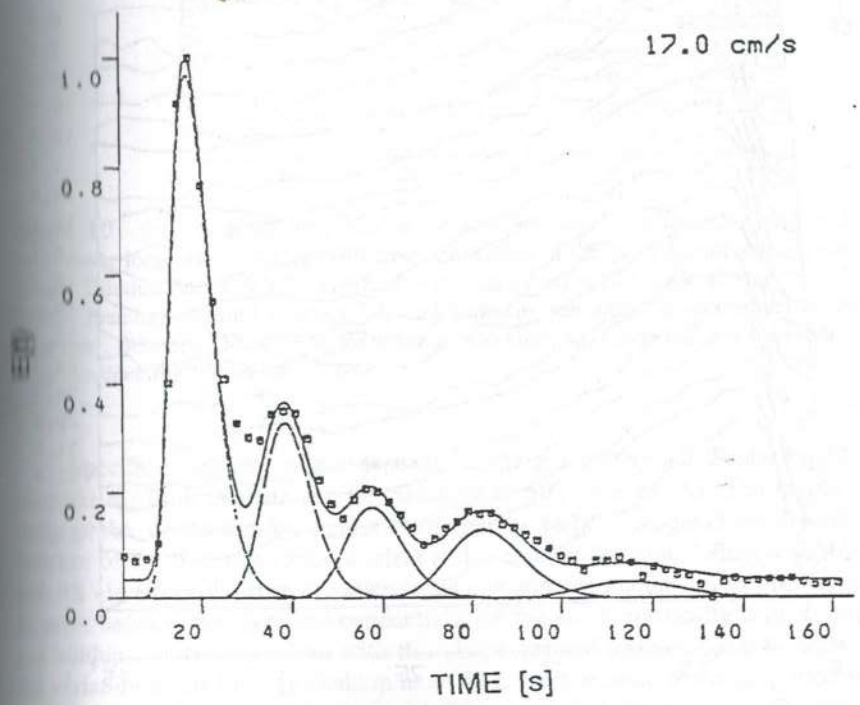


Figure 8 Distribution of gas residence times in a 9-m-high, 4-m^3 volume pilot plant airlift tower loop reactor at superficial gas velocity u_G = 17.0 cm/s in tap water. (From Ref. 119.)

axial mixing in the reactor is much higher in cultivation medium, a determination of the gas circulation time therein by means of the gas residence time distribution is not possible.

In Fig. 9, the gas residence time in a 9-m-high, 4-m^3 volume pilot plant airlift tower loop reactor is shown during batch cultivation of baker's yeast. It is obvious that no dramatic change occurs as long as the medium properties do not change. Foam formation and addition of antifoam agent, however, dramatically influence the gas residence time distribution (Fig. 10).

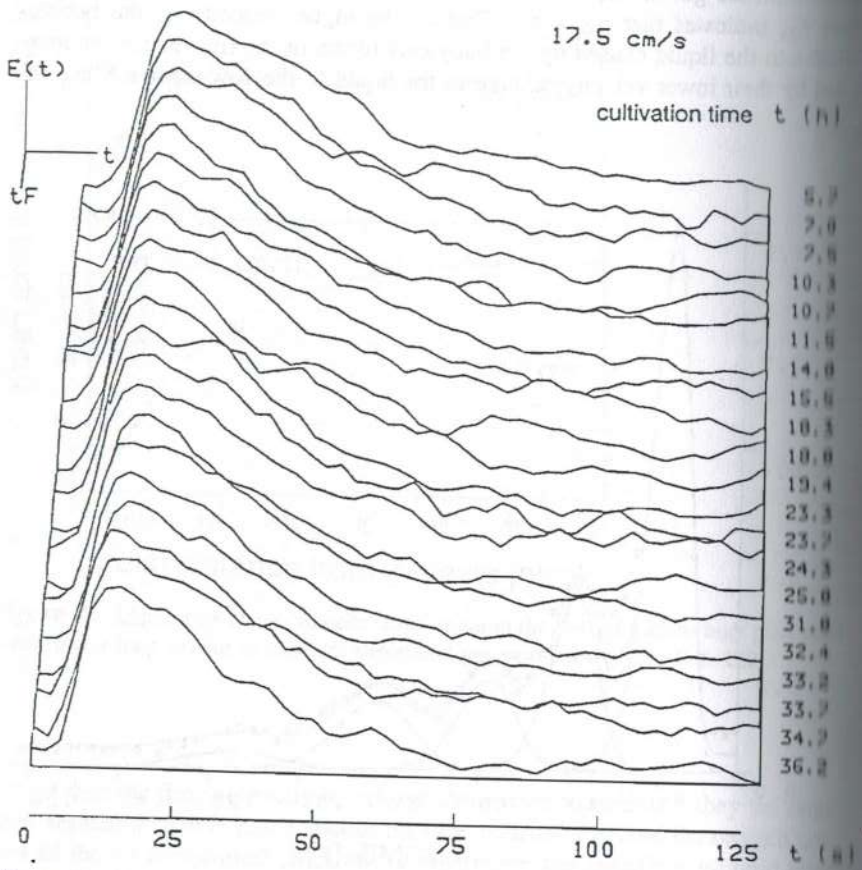


Figure 9 Gas residence time distribution in a 9-m-high, 4-m^3 volume pilot plant airlift tower loop reactor during batch cultivation of the yeast *Saccharomyces cerevisiae*. (From Ref. 120.)

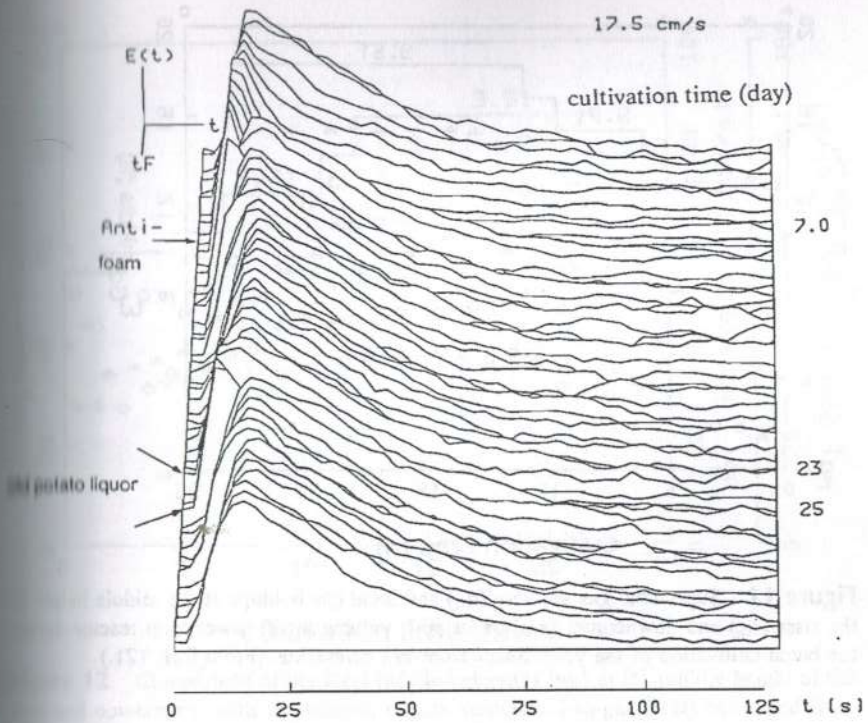


Figure 10 Gas residence time distribution in a 9-m-high, 4-m^3 volume pilot plant airlift tower loop reactor during continuous cultivation of the yeast *Saccharomyces cerevisiae*. Dilution rate 0.19 h^{-1}, superficial gas velocity u_G = 17.5 cm/s. During the cultivation foaming occurred at about 7 h, and antifoam was added to themedium at the same time. Between 23 and 25 h, the medium was changed, which reduced the productivity considerably. (From Ref. 126.)

Specific interfacial area a depends on the gas holdup and Sauter bubble diameter d_S. Both of them can be measured locally with an electrical conductivity probe in electrically conductive solutions [80]. A single-channel probe consists of an electrode (thin Pt wire) and a counterelectrode. When a bubble hits the electrode, the current between the electrode and counterelectrode drops to zero, because the electrical conductivity of the air is practically zero. Local gas holdup can be determined with this single-channel probe. Figure 11 shows the variation of the local gas holdup in an 80-L volume airlift tower loop reactor during the cultivation of baker's yeast. With increasing aeration rate, the local gas holdup ϵ_{Gr} in the riser increases, but the local gas holdup ϵ_{Gd} in the downcomer is not influenced.

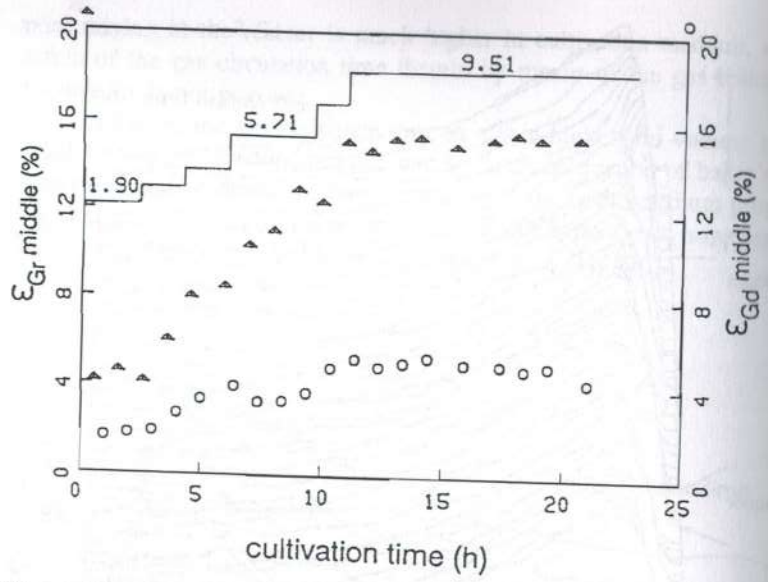


Figure 11 Superficial gas velocity (u_G) and local gas holdups at the middle height in the riser (ϵ_{Gr}) and downcomer (ϵ_{Gd}) of an 80-L volume airlift tower loop reactor during the batch cultivation of the yeast *Saccharomyces cerevisiae*. (From Ref. 121.)

When using a two-channel probe consisting of two electrodes and a counterelectrode, the bubble velocity and bubble piercing length characteristic for the bubble size can be evaluated. With this method, only bubbles larger than 1 mm can be observed. Smaller bubbles do not hit the electrode, but they are deflected from it. In Fig. 12, locally measured bubble velocities, and in Fig. 13, local mean diameters of bubbles D_{bl} calculated from the piercing length, are plotted; they were determined in the riser and downcomer of an 80-L airlift tower loop reactor during the cultivation of baker's yeast. The local bubble velocities (w_{bl}) in the riser are compared with the integral mean gas-phase velocity calculated from the residence time distribution (w_{bl}-gas-RTD) and with the local bubble velocities in the downcomer. In spite of the higher liquid velocity in the downcomer, the mean bubble velocity is lower here than in the riser, probably due to the buoyancy forces. With increasing aeration rate, bubble velocity and diameter in the riser increase, but the bubble diameter remains constant in the downcomer.

Measurements of D_{bl} along the axis of a 9-m-high airlift tower loop reactor during the cultivation of baker's yeast indicate that except close to the aerator, where the bubbles are larger, the uniform bubble size prevails in the rest of the

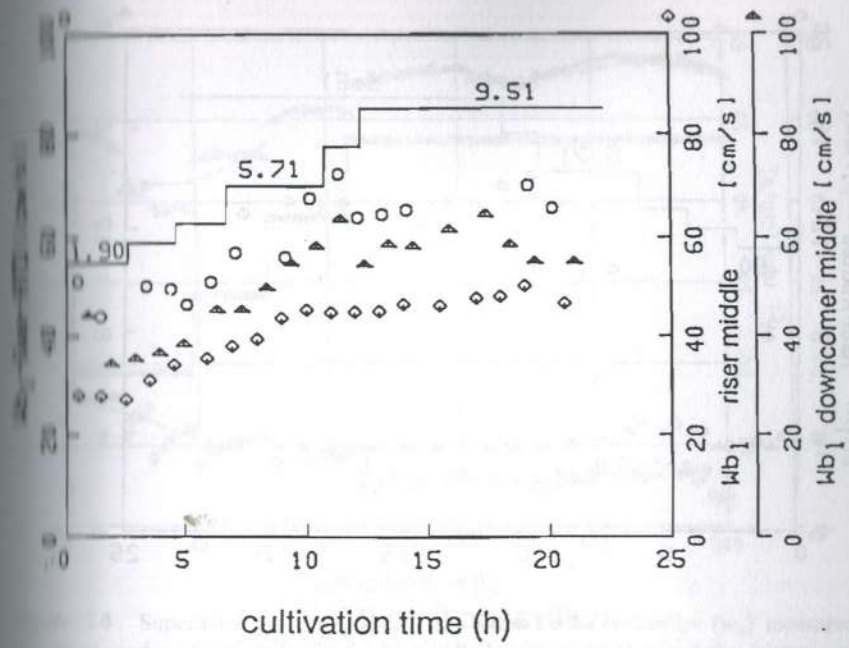


Figure 12 Comparison of the local bubble velocities (w_{bl}) at the middle height of the riser and downcomer with the integral bubble velocities (w_{bl}-gas-RTD) calculated from the mean gas residence time in the riser of an 80-L volume airlift tower loop reactor during the batch cultivation of the yeast *Saccharomyces cerevisiae*. (From Ref. 121.)

reactor. The dynamic equilibrium bubble size is smaller than the primary bubble size. Therefore, the gas distributor has no influence on the bubble size in the reactor.

A comparison of the bubble size measured in 80-L and 4-m^3 pilot plant airlift tower loop reactors indicates that in the pilot plant reactor the bubble size is smaller than in the laboratory reactor, as expected, because of the higher superficial gas velocity u_G, specific power input [according to Eq. (40)], and energy dissipation rate in the larger reactor.

When using a four-channel probe, consisting of four electrodes and a counterelectrode, the velocity vector of the bubbles can be determined [80]. This makes it possible to measure the bubble flow pattern in multiphase reactors.

In an in situ technique, which avoids direct contact between the probe and the bubble, the property that the bubbles can perfectly reflect the ultrasound is utilized. The ultrasound Doppler anemometry relies on the high reflectance of ultrasound power at liquid-gas interfaces. Since the bubbles usually move by

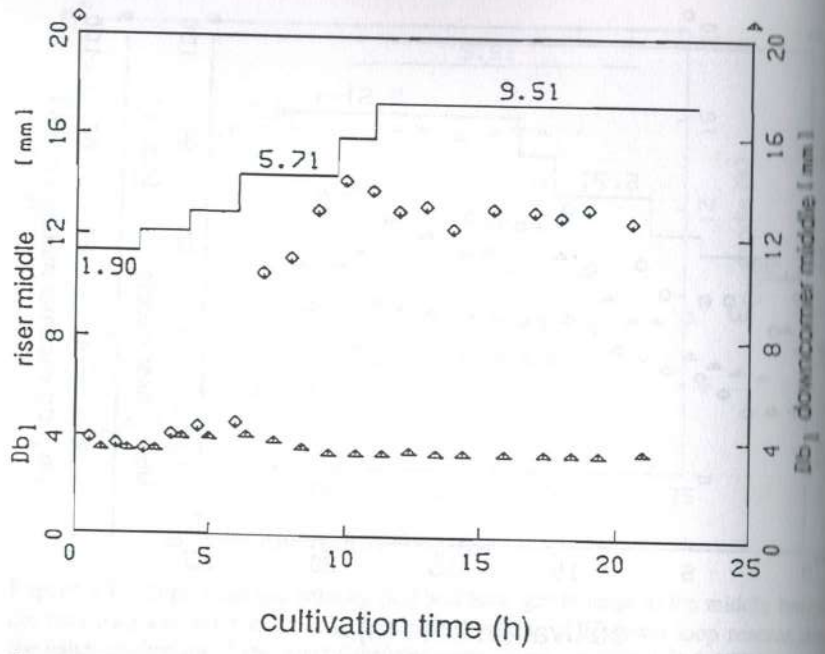


Figure 13 Superficial gas velocity (u_G), local bubble diameter (D_{bl}) calculated from the pierced length measured at the middle height in the riser and downcomer of an 80-L volume airlift tower loop reactor during the batch cultivation of the yeast *Saccharomyces cerevisiae*. (From Ref. 121.)

means of the ultrasound transmitter, the reflected ultrasound is shifted in frequency according to the Doppler effect. This frequency shift is proportional to the velocity of the bubble [56,81]. The same small piezoceramic transducer elements can be used as transmitters as well as receivers for the ultrasound in such measurements. In Fig. 14, the bubble velocities are shown in the riser and downcomer of the 80-L airlift tower loop reactor measured during sugar alcohol production by the yeast *Moniella tomentosa* var. *pollinis* with the ultrasound Doppler anomometer [82]. The bubble velocity increases immediately after an antifoam agent is added to the cultivation medium measured by the ultrasound Doppler anemometer during the cultivation of a recombinant *Escherichia coli* JM 103 strain in the 80-L airlift tower loop reactor to produce a fusion protein (Fig. 15). This is caused by the bubble size increase by coalescence. The antifoam obviously disappears gradually from the medium, since the bubble velocities attain their original value after about 60 minutes (Fig. 15).

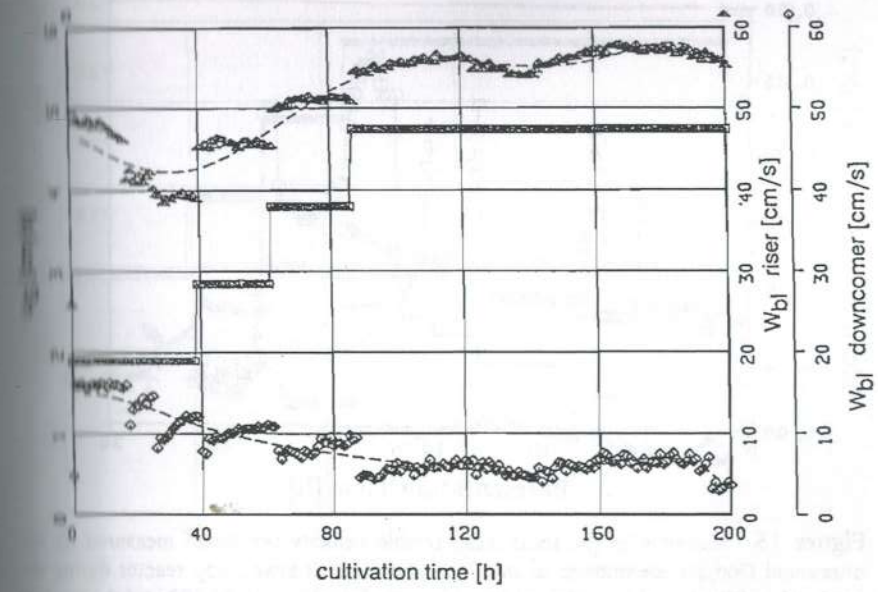


Figure 14 Superficial gas velocity (u_G) and local bubble velocities (w_{bl}) measured with ultrasound anemometer in situ in the middle height of the riser and the downcomer of an 80-L volume airlift tower loop reactor during the batch cultivation of the yeast *Moniella tometosa* var. *pollinis* and the production of sugar alcohols. (From Ref. 82.)

Whereas in bubble velocity measurements the frequency shift of the received signals is analyzed, the intensity of the received signal is used to determine the specific interfacial area of the reflecting bubbles [81]. Figure 16 shows the specific gas-liquid interfacial area measured during the sugar alcohol production by the yeast *Moniellea tomentose* var. *pollinis* in an 80-L airlift tower loop reactor by the ultrasound signal intensity variation [82].

C. Mass Exchange Between the Gas and Liquid Phases

During the cultivation of microorganisms the integral volumetric mass transfer coefficient k_La of oxygen and CO_2 can be evaluated by measuring the dissolved oxygen and CO_2 concentration and the O_2 and CO_2 concentration, respectively, in the off-gas and by calculating the O_2 and CO_2 mass balance, respectively. To measure the local volumetric mass transfer coefficients of oxygen, a new fluorometric device is used which detects the fluorescence of a special dye dissolved in the liquid phase of the dispersion. This fluorescence is quenched by the dissolved oxygen. Hence, the dissolved oxygen can be detected locally with time

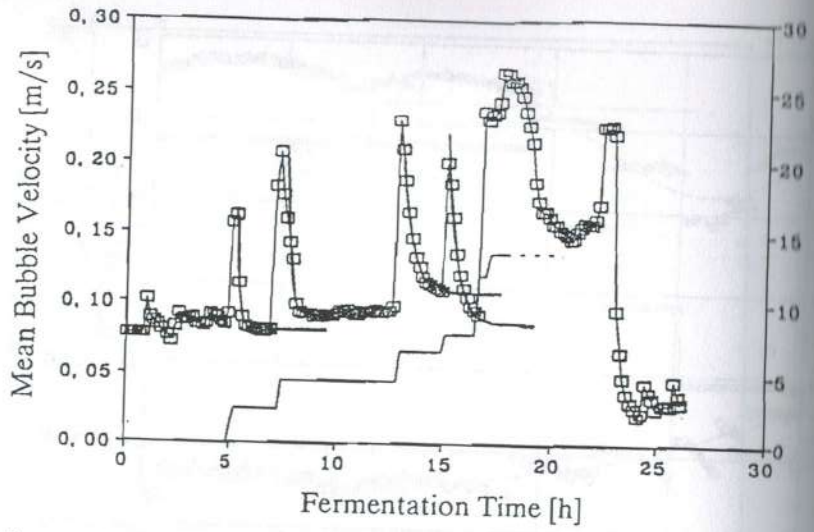


Figure 15 Variation of the local mean bubble velocity (symbols) measured by the ultrasound Doppler anenometer in an 80-L volume airlift tower loop reactor during the fed-batch cultivation of recombinant bacterium *Escherichia coli* JM 103 and the production of the fusion protein *Eco*RI::SPA. After the addition of antifoam agent to the medium, the bubble velocity immediately increased due to bubble coalescence and within about 120 minutes it decreased to its original value [123,124].

resolutions of less than 100 ms—much faster than required in efficient mixers. The technique can easily be implemented in bioreactors by using fiber optics. Schmidt et al. [79] used such a measuring technique to determine the local mass transfer properties of different parts of an airlift tower loop reactor and could show that they contribute differently to the overall mass transfer. In particular, the important role of the surface aeration at the riser-downcomer connection could be proved. The dye they used was also applied for local k_La measurements during yeast cultivation.

The information gained by these local measurements is needed to develop structured models for large reactors and to improve the reactor construction and performance. Further improvement of the measuring methods and more investigations are needed to have sufficient data for this task.

VI. BIOPROCESS PERFORMANCE

The most important property of bioreactors is the bioprocess performance: the productivity Pr and the yield coefficient Y. In the case of single-cell protein

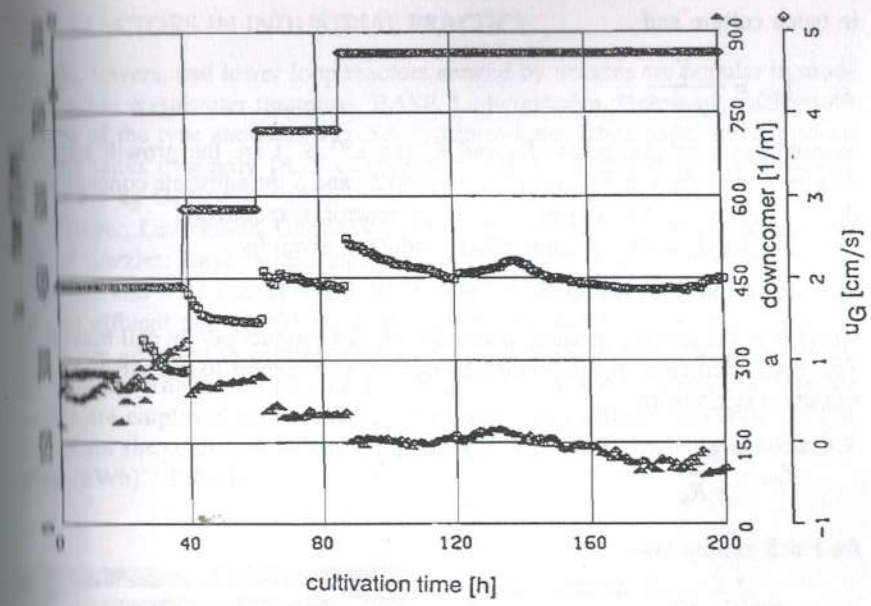


Figure 16 Superficial gas velocity (u_G) and specific interfacial area (a) measured by means of the reflected ultrasound intensity in the middle height of an 80-L volume airlift tower loop reactor during the batch cultivation of the yeast *Moniella tometosa* var. *polinis* and the production. (From Ref. 82.)

(e.g., baker's yeast) the productivity is defined in a batch reactor as

$$Pr_X = \mu_X X \text{ [kg cell mass L}^{-1}\text{ h}^{-1}\text{]}$$

and in a continuous reactor as

$$Pr_X = DX$$

where μ_X is the specific growth rate (h^{-1}), X the cell mass concentration (kg L^{-1}), D the dilution rate = $q_L V_L^{-1}$ (h^{-1}), and q_L the medium throughput (1 h^{-1}) in the reactor of volume V_L [1, 3].

The yield coefficient of the produced cell mass with respect to the substrate consumed is given by

$$Y_{X/S} = \frac{R_X}{-R_S} \tag{42a}$$

in batch culture and

$$Y_{X/S} = \frac{X}{S_O - S} \tag{42b}$$

in continuous culture, where R_X and R_S (kg L^{-1} h^{-1}) are the growth rate and substrate consumption rate, respectively, and S_O and S the substrate concentration (kg L^{-1}) in the feed and at the exit of the reactor, respectively.

The productivity of some other product is given by

$$Pr_P = X\pi \text{ (kg product } L^{-1}\ h^{-1})$$

where π is the specific product formation rate (kg product per kg cell mass h^{-1}). The yield coefficient of the product formation with respect to the substrate consumption is given by

$$Y_{P/S} = \frac{R_P}{-R_S} \tag{43a}$$

for batch culture and

$$Y_{P/S} = \frac{P}{S_O - S} \tag{43b}$$

for continuous culture, where R_P is the product formation rate.

If R_{O2} is the oxygen consumption rate, the following relationships hold true:

$$Y_{X/O2} = \frac{R_X}{-R_{O2}} \tag{44}$$

In the steady state, R_{O2} = OTR, because the difference between the dissolved oxygen concentrations in feed and culture medium at the reactor exit is irrelevant for the oxygen balance in a high-performance bioreactor.

The specific productivities regarding the power input $P_P = P_L + P_G$ are given:

$$E_{X/PP} = \frac{Pr_X}{P_P V_L^{-1}} \quad \text{[kg cell mass (kWh)}^{-1}\text{]} \tag{45a}$$

$$E_{P/PP} = \frac{Pr_P}{P_P V_L^{-1}} \quad \text{[kg product (kWh)}^{-1}\text{]} \tag{45b}$$

In the case of high-added-value products (e.g., proteins), the product quality and the by-products (which influence the downstream processing) also play an important role. However, they are not considered here.

VII. REACTORS IN INDUSTRIAL PRACTICE

Vessels, towers, and tower loop reactors aerated by nozzles are popular in modern aerobic wastewater treatment. BASF, Ludwigshafen, Germany, employs 40 nozzles of the type shown in Fig. 5A in 125-m-long, 20-m-wide, and 5-m-deep aeration tanks to supply 18.75 t O_2 per day. The ORT efficiency of the nozzles is 1.5–1.6 kg O_2 (kWh)$^{-1}$ [83].

Bayer, Leverkusen, Germany aerobic effluent treatment plants employ two type of nozzles: Bayer's injector 8/14 type, shown in Fig. 5C, and slot nozzle (Figs. 5C and 17). The TOWER BIOLOGY at Leverkusen Bürrig is operated with an effluent rate of 90,000 m^3 per day with a BOD_5 of 95 t per day. Four towers are arranged in parallel, each of them 26 m in diameter, 30 m in height, and with an effluent capacity of 13,600 m^3. In each tower, about 200 injector nozzles are employed for the coalescence-suppressing effluent and about 53 slot nozzles for the coalescence-promoting effluent. The OTR efficiency is about 3.7 kg O_2 (kWh)$^{-1}$ [60,61].

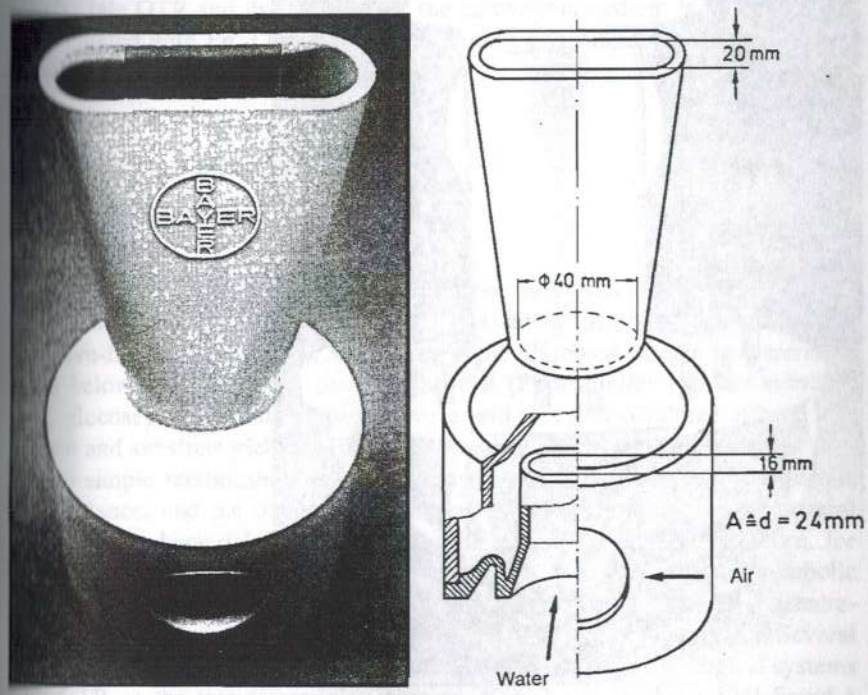


Figure 17 Slot nozzle for gas dispersion used in aerobic effluent treatment (Bayer Co.).

Hoechst (Höchst, Germany) employs radial flow nozzles of the type shown in Figs. 5D and 18 with draft tubes for aerobic effluent treatment. Two BIOHOCHREAKTORs are applied, each having 24 radial nozzles in a tower of 32-m bottom diameter, 46-m head diameter, 20-m height, and 15,000 m^3 capacity. The radial nozzles have an OTR efficiency of 3.8 kg O_2 $(kWh)^{-1}$ [84].

ICI (Billingham, UK) employed a pressure cycle tower reactor with a non-coaxial internal loop of 2100 m^3 capacity, in which *Methylophilus methylotrophus* was cultivated with an annual capacity of 50,000 tons of Pruteen protein. The high oxygen demand of 10 kg O_2 m^{-3} h^{-1} was maintained with a high specific power input of 6.6 kW m^{-3}, which resulted in a rather low OTR efficiency of 1.5 kg O_2 $(kWh)^{-1}$ [3].

The same principle was used by ICI in the deep shaft reactor with different (up to 300 m) depths for effluent treatment. The liquid moves downward in one shaft and upward in the other. At steady state, the downward flow is aerated.

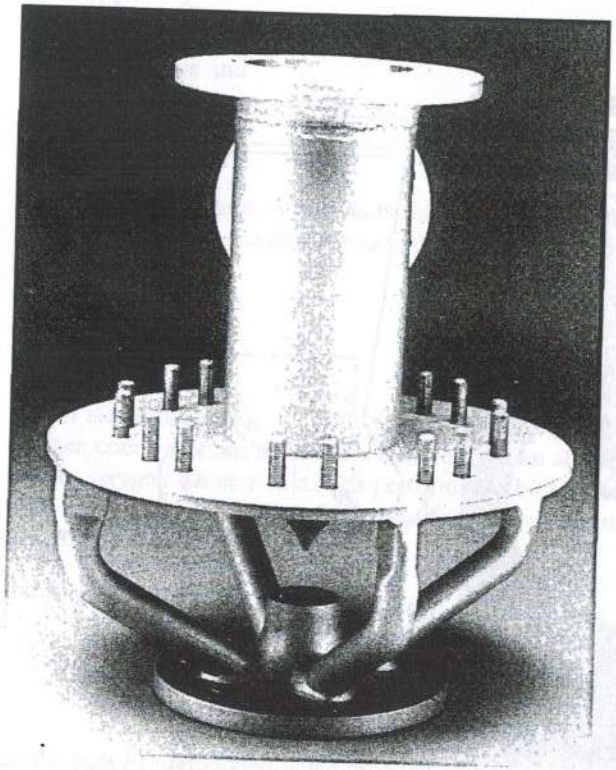


Figure 18 Radial flow nozzle for gas dispersion used in aerobic effluent treatment (Hoechst Co.).

Because of the low specific power input used in these reactors, the OTR efficiency is fairly high (6 kg O_2 $(kWh)^{-1}$ [85,86,47]).

Hoechst also employed a pilot plant tower axial loop reactor with a draft tube for the production of bacterium protein by means of *Methylomonas clara*. The 1000 tons per day plant was 20 m high and had a top diameter of 2.2 m, narrowing to 1.6 m at the bottom, and a volume of 40 m^3. It was operated with a perforated ring to maintain an OTR of 6 kg m^{-3} h^{-} with a specific power input of 2.5 kW m^{-3} and an OTR efficiency of 2.45 kg O_2 $(kWh)^{-1}$ [3].

The designs of the TOWER BIOLOGY of Bayer and the BIOHOCHREACTOR of Hoechst for aerobic treatment of effluents were based on systematic investigations in pilot plant reactors, calculating the axial dissolved oxygen profiles in reactors of different geometry and minimizing the specific power input.

VIII. DESIGN EQUATIONS

The designs of bubble column and airlift tower loop reactors for bacterium and yeast cultivations are relatively easy, if the key design parameter is the oxygen transfer rate OTR and the viscosity of the cultivation medium is low. OTR can be calculated with Eq. (28) for oxygen-limiting cultivations ($C_L = 0$), if k_La is known:

$$\mathrm{OTR} = k_L a C_L^* \tag{28a}$$

and the cell mass productivity

$$\mathrm{Pr}_X = k_L a C_L^* Y_{X/O2} \tag{46}$$

for constant yield coefficient $Y_{X/O2}$.

In several yeasts (e.g., *Saccharomyces cerevisiae*), however, the cell growth is impaired by the change of the oxidative metabolic pathway to the oxidative-fermentative one, or vice versa, if the dissolved oxygen concentration drops below a critical level or rises above it (Pasteur effect) or the substrate (e.g., glucose) concentration rises above a critical value (Crabtree effect). The oxygen and substrate yield coefficients of the cell growth change in these cases, and no simple relationships exist between the cell mass balance, the substrate mass balance, and the oxygen mass balance. This also holds true for several bacteria (e.g., bacterial Crabtree effect with *Escherichia coli*). Therefore, for reactor testing, special strains were chosen which do not change their metabolic pathway at low dissolved oxygen concentrations or at high substrate concentrations. Such a microorganism is the yeast *Trichorosporum cutaneum*. Several authors [87–94] used this yeast for reactor characterization of biological systems with OTR as the rate-determining process. Yonsel and Deckwer [94] found a simple relationship between the *T. cutaneum* cell productivity Pr_X and OTR based on measurements in airlift tower loop reactors of 0.03 m^3 ($H/d_C = 2$ and

10) with 1.2 m^3 working volumes and bubble columns of 0.03 m^3 volume (H/d_C = 10) at low glucose concentrations:

$$Pr_X = 2.2OTR \tag{47}$$

Validity range:

$$0.5 < OTR < 2.8 \text{ kg } O_2 \text{ m}^{-3} \text{ h}^{-1}$$

$$0.8 < Pr_X < 6.0 \text{ kg cell mass m}^{-3} \text{ h}^{-1}$$

The same relationship also holds true for stirred tank reactors up to 1.5 m^3.

The relationship between the specific power input P_G/V_L and OTR is closely linear if the pressure drop through the gas distributor is negligible compared with the hydraulic pressure drop [94]. In the case of variable yield coefficients $Y_{X/O2}$ and $Y_{X/S}$, their dependence on the process variables is required. In highly viscous non-Newtonian cultivation media, but with constant morphology, e.g., xanthan gum production with the bacterium *Xanthomonas campestris*, Eq. (39) can be used to calculate k_La in airlift tower loop reactors [68]. In order to calculate the cell growth rate R_X, product formation rate R_P, and substrate consumption rate R_S, the following relations can be used [96]:

$$R_P = a_1R_X + b_1X$$

$$R_S - = a_2R_X - b_2X$$

$$R_{O2} -= a_3R_X - b_3X$$

The constants a_1, b_1, a_2, b_2, a_3, and b_3 must be determined experimentally (e.g., Pinches and Pallent [97]). The prdouct quality depends on the process variables [98] as well.

Suh et al. [99] applied the xanthan production model with *X. campestris*, developed by Peters et al. [100] for stirred tank reactors, and the relationship of Suh et al. [101], which is a slight modification of Eq. (39) for viscoelastic cultivation media:

$$Sh = 0.018Sc^{0.5}Bo^{0.2}Ga^{0.62}Fr^{0.51}(1 + 0.12Wi)^{-1} \tag{48}$$

where Wi is the Weissberg number (ratio of first normal stress difference to shear stress) with the validity range:

$$6.26 \times 10^3 \leq Sh \leq 2.31 \times 10^6$$

$$1.72 \times 10^3 \leq Sc \leq 2.27 \times 10^5$$

$$4.91 \times 10^2 \leq Bo \leq 1.40 \times 10^4$$

$$2.05 \times 10^5 \leq Ga \leq 1.07 \times 10^{10}$$

$$0.14 \leq Fr \leq 0.40$$

$$0 \leq Wi \leq 41.4,$$

to describe the xanthan production in a 0.05-m^3 bubble column reactor and in a 1.2-m^3 airlift tower reactor. In the small bubble column, the model described well the time course of the cell and product concentrations as well as the molecular weight of the polysaccharide, even when complex medium was employed. In the airlift reactor, however, the xanthan production rate and the xanthan yields with respect to oxygen and glucose were lower than expected at the overall oxygen transfer rate. The poor performance of the airlift was traced back to the lack of any oxygen supply in the downcomer. However, this difference in OTR between the riser and downcomer cannot be included in a lumped parameter model. In this airlift reactor of volume 1.2 m^3, a structured reactor model must be applied to describe the reactor performance.

This holds generally true for high tower reactors. Reactor models with lumped parameters cannot be used. In some cases, unstructured models with distributed parameters can be employed. The dissolved oxygen concentration is usually calculated along the tower, e.g., by means of a dispersion model by employing a double Monod model:

$$\frac{R_X}{X} = \frac{S}{S + K_S} \frac{O_2}{O_2 + K_{O2}} \mu_{max} \tag{49}$$

and assuming constant cell concentrations and yield coefficients $Y_{X/S}$ and $Y_{X/O2}$ in the entire tower. In the case of tower loop reactors, both the riser and downcomer are described with axial dispersion models but with different model parameter sets. Such models were used to describe, e.g., the cultivation of *Hansenula polymorpha* in a tower loop reacator [102,103,105,12] and the production of *Saccharomyces cerevisiae* in a combined tower loop–bubble column pilot plant reactor [106].

The reactor design is more difficult if fungi and streptomycetes, respectively, are employed for the production of secondary metabolites, which usually exhibit two distinct phases: a growth phase and a production phase. The biological parameters of the cells, the properties of the media (medium composition and rheology), and the cell morphology can be completely different. This strong variation of physical and biological parameters has significant influence on the process performance. One often needs a structured cell model as well as a structured reactor model to describe the reactor and process performances, systematic experimental investigations in laboratory reactors for the identification of the biological model parameters, and experimental investigations on a pilot plant scale for the identification of the reactor parameters.

Several research groups investigated the morphology of *Penicillium chrysogenum* [107–112], the morphology of *Cephalosporium acremonium* [113,114], and *Streptomyces aureofaciens* [112].

It has been shown that the reactor and process performance depends on the morphology of the cells. For example, the volumetric mass transfer coeffi-

cient increases by a factor of 3 to 4 at the same specific power input, if the highly viscous filamentous mycel changes into a pellet suspension of low viscosity. At the same time, the productivity may diminish to half its value, provided the mean pellet size increases from 0.5 mm to 1.0 mm [109,115,116].

On the other hand, the high concentration of peanut flour and the increasing concentration of the filamentous mycels with long hyphae during the growth phase cause an increase of the medium viscosity, a reduction of the OTR, and interruption of the biosynthesis with an intermediate product. This is due to a strong diminution of the medium recirculation rate in the airlift tower loop reactor. With the gradual consumption of the peanut flour and change of the long hyphae to spherical arthrospores, the medium viscosity decreases, the OTR increases, and the biosynthesis progresses to the final product [117, 118].

IX. CLOSURE

The improved measuring techniques for characterization of hydrodynamics of the multiphase systems, the morphology and rheology of the cultivation broth, and the behavior of the microorganisms will make it possible in the future to evaluate the interrelationship between them. Advanced mathematical methods will also permit the modeling and optimization of large bubble columns and airlift-tower loop bioreactors with locally segregated media.

NOMENCLATURE

a	specific gas-liquid interfacial area with respect to the volume of the liquid phase
A_r, A_d	cross sections of the riser and downcomer
Bo	Bond number
C_L	constant of the friction factor correlation in the riser + downcomer
C_L, C_L^*	dissolved oxygen concentrations in the bulk liquid and at the interface
d_c	column diameter
d_B	bubble diameter
d_{hi}	hydraulic diameter of the riser (i = r), downcomer (i = d)
d_S	Sauter diameter of the bubbles
D	dilution rate
D_G	gas-phase dispersion coefficient
D_L	liquid-phase dispersion coefficient
D_m	molecular diffusivity

E_P	efficiency of oxygen transfer with regard to the specific power input
E_1	holdup of phase 1
E_G, E_L	gas and liquid holdups
E_{Gr}, E_{Gd}	gas holdups in the riser and downcomer
E_{O2}	efficiency of OTR with reference to the power input
$E_{X/PP}$	specific productivity of cell mass with respect to power input
$E_{P/PP}$	specific productivity of product with respect to power input
f	drag coefficient
f_r, f_d	friction factors in the riser and downcomer
F_{GP}	drift flux
Fr	Froude number
g	gravitational constant
Ga	Galileo number
h_G	mixing height of the bubble swarm
He	Henry coefficient
H_o	height of nonaerated liquid
I	ion strength
k_L	liquid side mass transfer coefficient
k_La	volumetric mass transfer coefficient
k_G	gas side mass transfer coefficient
K_1, K_2	friction coefficients due to change of the velocity direction and change of velocity magnitude
K_S	Monod constant for substrate consumption
K_{O2}	Monod constant for oxygen consumption
M_L	weight of the liquid
n_B	number of bubbles
OTR	oxygen transfer rate
pO_2, pO_2^*	partial pressure of oxygen in the gas-phase bulk and at the interface
P	pressure
P	product concentration
P, P_P	total power input
P_L, P_G	power input with liquid kinetic energy and with gas expansion
DP	frictional pressure drop
q_G	gas throughout
q_L	medium throughput
r	radial distance from the column axis/radial coordinate
$r_R = A_r/A_d$	ratio of cross-sectional areas of riser and downcomer
Pr_P	productivity of product

Pr_X	productivity of cell mass
ΔR	difference of liquid and gas densities
R_G	gas density
R_L	liquid density
R_P	product formation rate
R_R	recycle ratio
R_o	column radius
$-R_{O2}$	oxygen consumption rate
$-R_S$	substrate consumption rate
R_X	growth rate
S	substrate concentration at the exit
S_o	substrate concentration in the feed
Sc	Schmidt number
Sh	Sherwood number
T	shear stress
u_{BT}	terminal bubble rise velocity
u_c	average liquid circulation velocity
u_G, u_L	superficial gas and liquid velocities
u_{GD}	volume fraction weighted-average drift velocity of the gas phase
u_{Gi}	interstitial velocity of gas
u_{Gio}	u_{Gi} in the column axis
u_{Go}	superficial gas velocity in the top of the column
u_{Li}	volume fraction weighted-average drift velocity of the liquid phase
u_{Lio}	u_{Li} at the column axis
u_{SB}	swarm velocity of bubbles
w_1	velocity of phase 1
w_{1D}	average drift velocity of phase 1
ν_L	kinematic viscosity of the liquid
ν_t	turbulent kinematic viscosity
V	aerated volume of the liquid
V_L	nonaerated volume of the liquid
w_M	superficial velocity of gas–liquid mixture
w_{S1}	superficial velocity of phase 1
X	cell mass concentration
$Y_{P/S}$	yield coefficient of product formation with respect to substrate consumption
$Y_{X/S}$	yield coefficient of growth with respect to substrate consumption
$Y_{X/O2}$	yield coefficient of growth with respect to oxygen consumption
z	dimensionless axial coordinate

Greek Symbols

β	flowing volumetric concentration according to Zuber and Findlay
μ_L, μ_G	dynamic viscosities of liquid and gas phases
μ_X	specific growth rate
π	specific product formation rate with respect to cell mass
σ	surface tension

Indices

r, d	riser and downcomer
i	i = r, d in ALR

REFERENCES

1. W. D. Deckwer, *Reaktionstechnik in Blasensäulen* (Reaction engineering in bubble columns), Otto Salle Verlag, Frankfurt (M) and Verlag Sauerländer, Aarau, 1985; *Bubble Column Reactors*, John Wiley & Sons, Chishester, 1991.
2. M. Y. Chisti, *Airlift Bioreactors*, Elsevier Science Publishing, Amsterdam, 1989.
3. K. Schügerl, *Bioreaction Engineering*, Vol. 2, *Characteristic Features of Bioreactors*, John Wiley & Sons, Chichester, 1991.
4. K. Schügerl, J. Lücke, and U. Oels, *Adv. Biochem. Eng. 7*: 1 (1977).
5. K. Schügerl, J. Lücke, J. Lehmann, and F. Wagner, *Adv. Biochem. Eng. 8*: 63 (1978).
6. K. Schügerl, *Adv. Biochem. Eng. 22*: 94 (1982).
7. K. Schügerl, Nonmechanical agitated bioreactor systems, *Principles of Biotechnology Engineering Considerations* (C. L. Cooney and A. E. Humphrey, eds.), Vol. 2 of *Comprehensive Biotechnology* (M. Moo-Young, ed.), Pergamon Press, Oxford, 1985, p. 99.
8. H. Blenke, *Adv. Biochem. Eng. 13*: 121 (1979).
9. H. Blenke, Biochemical loop reactors, *Fundamentals of Biochemical Engineering* (H. Brauer, ed.), Vol. 2 of *Biotechnology* (H. J. Rehm and G. Reed, eds.), VCH, Weinheim, 1985 p. 465.
10. Y. T. Shah, B. G. Kelkar, S. P. Godbole, and W. D. Deckwer, *AIChE J. 28*: 353 (1982).
11. Y. T. Shah and W. D. Deckwer, Hydrodynamics of bubble column, *Handbook of Fluids in Motion* (N. P. Cheremisinoff and R. Gupta, eds.), Ann Arbor Science, Butterworth Co., 1983, p. 583.
12. R. Luttmann, A. Munack, and M. Thoma, *Adv. Biochem. Eng. 32*: 95 (1985).
13. W. D. Deckwer, Bubble column bioreactors, *Fundamentals of Biochemical Engineering* (H. Brauer, ed.), Vol. 2 of *Biotechnology* (H. J. Rehm and G. Reed, eds.), VCH, Weinheim, 1985, p. 445.

14. J. C. Merchuk, Tower reactor models, *Measuring, Modelling and Control* (K. Schügerl, ed.), Vol. 4 of *Biotechnology* end ed. (H. J. Rehm and G. Reed, eds.), VCH, Weinheim, 1991, p. 349.
15. *Bioreactor Fluid Dynamics*, BHRA, 1986.
16. *Bioreactor Fluid Dynamics* (R. King, ed), BHRA, 1988.
17. C. S. Ho and J. Y. Oldshue, eds., *Biotechnology Processes. Scale-up and Mixing* Am. Chem. Eng., New York, 1987.
18. G. W. Givier and K. Aziz, *The Flow of Complex Mixtures in Pipes*, Van Nostrand Reinhold, New York, 1972.
19. L. Lapidus and J. C. Elgin, *AIChE J. 3*: 63 (1957).
20. J. C. R. Turner, *Chem. Eng. Sci. 21*: 971 (1966).
21. J. F. Davidson and D. Harrison, *Chem. Eng. Sci. 21*: 731 (1966).
22. G. B. Wallis, *One Dimensional Two-Phase Flow*, McGraw-Hill, New York, 1969.
23. J. F. Richardson and W. N. Zaki, *Trans. Inst. Chem. Eng. 32*: 35 (1954).
24. G. Marucci, *Ind. Eng. Chem. Fundam. 4*: 224 (1965).
25. R. Clift, J. R. Grace, and M. E. Weber, *Bubbles, Drops and Particles*, Academic Press, New York, 1978.
26. P. Weiland, Doctoral Thesis, University Dortmund, Germany, 1978.
27. W. Freedman and J. F. Davidson, *Trans. Inst. Chem. Eng. 47*: T251 (1969).
28. P. Herbrechtsmeier and R. Steiner, *Chem. Ing. Techn. 52*: 468 (1980).
29. M. Zuber and J. A. Findlay, *Trans. ASME J. Heat Transfer 87c*: 453 (1965).
30. J. H. Hills, *Trans. Inst. Chem. Eng. 52*: 1 (1974).
31. T. Miyauchi and C. N. Shyu, *Kagaku Kogaku 34*: 958 (1970).
32. K. Ueyama and T. Miyauchi, *AIChE J. 25*: 258 (1979).
33. H. P. Riquarts, *Ger. Chem. Eng. 2*: 268 (1979).
34. H. P. Riquarts, *Ger. Chem. Eng. 4*: 18 (1981).
35. P. B. Whalley and J. F. Davidson, *Inst. Chem. Eng. Symp. Ser. 38*: 55 (1974).
36. J. B. Joshi and M. M. Sharma, *Trans. Inst. Chem. Eng. 54*: 42 (1976).
37. J. B. Joshi and M. M. Sharma, *Can. J. Chem. Eng. 56*: 116 (1978).
38. J. B. Joshi and M. M. Sharma, *Can. J. Chem. Eng. 57*: 375 (1979).
39. J. B. Joshi and M. M. Sharma, *Trans. Inst. Chem. Eng. 57*: 244 (1979).
40. P. Zehner, *Verfahrenstechnik 16*: 347 (1982).
41. P. Zehner, *Verfahrenstechnik 16*: 514 (1982).
42. P. Zehner and G. Schuh, *Ger. Chem. Eng. 8*: 282 (1985).
43. M. Chakravarty, H. D. Singh, J. N. Baruah, and M. S. Iyengar, *Ind. Chem. Eng 16*: 17 (1974).
44. C. H. Lee, L. A. Glasgow, L. E. Erickson, and S. A. Patel, Liquid circulation in airlift fermentors, 8th AICHE Annual Meeting, Miami Beach, November 2–7, paper 8.d, 1986.
45. A. G. Jones, *Chem. Eng. Sci. 40*: 499 (1985).
46. M. Y. Chisti and M. Moo-Young, *J. Chem. Techn. Biotechnol. 42*: 211 (1988).
47. Y. C. Hsu and M. P. Dudukovich, *Chem. Eng. Sci. 35*: 135 (1980).
48. H. Kubota, Y. Hosono, and K. Fujie, *J. Chem. Eng. Japan 11*: 318 (1978).
49. R. A. Bello, Ph. D. thesis, University of Waterloo, Ontario, Canada, 1981.
50. K. Koide, S. Iwamoto, Y. Takashaka, S. Matsura, E. Takasashi, M. Kimura, and H. Kubota, *J. Chem. Eng. Japan 17*: 611 (1984).

51. K. Koide, M. Kimura, H. Nitta, and H. Kawabata, *J. Chem. Eng. Japan 21*: 393 (1988).
52. J. C. Merchuk and Y. Stein, *AIChE J. 27*: 377 (1981).
53. J. C. Merchuk and Y. Stein, *Biotechnol. Bioeng. 23*: 1309 (1981).
54. P. Verlaan, J. Tramper, K. van't Riet, and K. Ch. A. M. Luyben, *Chem. Eng. J. 33*: B43 (1986).
55. O. Levenspiel, *Chemical Reaction Engineering*, John Wiley & Sons, New York, 1962.
56. A. Lübbert, Characterization of bioreactors, *Measuring, Modelling and Control in Biotechnology* (K. Schügerl, ed.), Vol. 4 of *Biotechnology*, 2nd ed. (H. J. Rehm and G. Reed, eds.), VCH, Weinheim, 1991, p. 107.
57. H. Hikita, S. Asai, K. Tanigawa, K. Segawa, and M. Kitato, *Chem. Eng. J. 20*: 59 (1980).
58. H. Hikita, A. Asai, K. Tanigawa, K. Segawa, and M. Kitao, *Chem. Eng. J. 22*: 61 (1981).
59. M. Zlokarnik, *Adv. Biochem. Eng. 8*: 133 (1977).
60. M. Zlokarnik, *Chem. Eng. Sci. 34*: 1265 (1979).
61. M. Zlokarnik, *Adv. Biochem. Eng. 11*: 157 (1979).
62. M. Zlokarnik, *Verfahrenstechnik 13*: 601 (1979).
63. M. Zlokarnik, *Acta Biotechnol. 1*: 311 (1981).
64. P. Weiland and U. Onken, *Chem. Ing. Techn. 52*: 986 (1980).
65. P. Weiland and U. Onken, *Chem. Ing. Techn. 52*: 264 (1980).
66. M. K. Popovic and C. W. Robinson, *AIChE J. 35*: 393 (1989).
67. A. Schumpe, C. Singh, and W. D. Deckwer, *Chem. Ing. Techn. 57*: 988 (1985).
68. M. Moo-Young, B. Halard, D. G. Allen, R. Burrell, and Y. Kawase, *Biotechnol. Bioeng. 30*: 746 (1987).
69. J. Drahos and J. Cermak, *Chem. Eng. Process. 26*: 147–164 (1989).
70. J. B. Joshi, T. A. Patil, V. V. Ranade, and Y. T. Shah, *Rev. Chem. Eng. 6*: (2/3) (1990).
71. A. Prokop, L. E. Erickson, J. Fernandez, and A. E. Humphrey, *Biotechnol. Bioeng. 11*: 945–966 (1969).
72. D. E. Swaine and A. J. Daugulis, *Biotechnol. Prog. 4*: 134–148 (1988).
73. J. Bryant, *Adv. Biochem. Eng. 5*: 101–123 (1977).
74. H. Funahashio, H. Harada, H. Tagushi, and T. Yoshida, *J. Chem. Eng. Japan 20*: 277–283 (1987).
75. H. Blenke, *BTF-Biotech-Forum 5*: 6–23 (1988).
76. S. Bodemeyer, W. Claas, and A. Lübbert, *AIChE Symp. Ser. 286 88*: 109–113 (1992).
77. N. Devanthan, D. Moslemian, and M. P. Dudocovic, *Chem. Eng. Sci. 45*: 2285–2291 (1990).
78. A. Lübbert and B. Larson, *Chem. Eng. Sci. 45*: 3047–3053 (1990).
79. J. Schmidt, R. Nassar, and A. Lübbert, *Chem. Eng. Sci. 47*: 3363–3370 (1992).
80. J. Steinemann and R. Buchholz, *Part. Charact. 1*: 102–107 (1984).
81. S. Bröring, J. Fischer, T. Koert, S. Sollinger, and A. Lübbert, *Can. J. Chem. Eng. 69*: 1247–1256 (1991).

82. T. Korte, *Fortschritt-Berichte VDI 8*: 110 (1986).
83. H. H. Daucher, *Chem. Ing. Techn. 54*: 169 (1982).
84. G. Müller and G. Sell, *Chem. Ing. Techn. 56*: 399 (1984).
85. D. A. Hines, M. Bailey, J. C. Ousby, and F. C. Roesler, *Inst. Chem. Eng. Symp. Ser. 41*: D1 (1975).
86. D. H. Bolton, D. A. Hines, and J. P. Bouchard, 31st Annual Purdue Industrial Waste Conference, Lafayette, Indiana, 1976, p. 344.
87. D. Karrer, Dissertation 6254, ETH Zürich, 1978.
88. O. Käppeli and A. Fiechter, *Biotech. Bioeng. 22*: 1509 (1980).
89. K. W. Gschwend, Dissertation 6975, ETH Zürich, 1982.
90. I. Adler and A. Fiechter, *Chem. Ing. Techn. 55*: 322 (1983).
91. I. Adler and A. Fiechter, *Bioprocess Eng. 1*: 51 (1986).
92. A. Jamarillo, I. Adler, and A. Fiechter, *Chem. Ing. Techn. 58*: 914 (1986).
93. A. Moser and W. Kung, International Conference on Bioreactor Fluid Dynamics, Cambridge, April 15–17, 1986.
94. S. Yonsel and W. D. Deckwer, *BioEngineering 3*: 12 (1990).
95. D. Grant Allen and C. W. Robinson, *Biotechnol. Bioeng. 34*: 731–740 (1989).
96. R. Luedeking and E. L. Piret, *J. Biochem. Microbiol. Technol. Eng. 1*: 393 (1959).
97. A. Pinches and L. J. Pallent, *Biotechnol. Bioeng. 28*: 1484 (1986).
98. I. S. Suh, H. Herbst, A. Schumpe, and W. D. Deckwer, *Biotechnol. Lett. 12*: 201 (1990).
99. I. S. Suh, A. Schumpe, and W. D. Deckwer, *Biotechnol. Bioeng. 39*: 85–94 (1992).
100. H. U. Peters, I. S. Suh, A. Schumpe, and W. D. Deckwer, *Can. J. Chem. Eng. 70*: 742–750 (1992).
101. I. S. Suh, A. Schumpe, W. D. Deckwer, and W. M. Kulicke, *Can. J. Chem. Eng. 69*: 506 (1991).
102. R. Luttmann, M. Thoma, H. Buchholz, W. Scheiding, and K. Schügerl, *Eur. J. Appl. Microbiol. Biotechnol. 13*: 90 (1981).
103. R. Luttmann, M. Thoma, H. Buchholz, W. Scheiding, and K. Schügerl, *Eur. J. Appl. Microbiol. Biotechnol. 13*: 145 (1981).
104. R. Luttmann, H. Buchholz, W. Zakrzewski, and K. Schügerl, *Biotechnol. Bioeng. 24*: 817 (1982).
105. R. Luttmann, M. Thoma, H. Buchholz, J. Lehmann, and K. Schügerl, *Biotechnol. Bioeng. 24*: 1851 (1982).
106. G. Strauss, Dissertation, University Hannover, 1990.
107. B. Metz, N. W. Kossen, and J. C. van Suijdam, *Adv. Biochem. Eng. 11*: 103 (1979).
108. C. T. Calam, *Process Development in Antibiotic Fermentations*, Cambridge University Press, Cambridge, 1987.
109. B. König, K. Schügerl, and C. Seewald, *Biotechnol. Bioeng. 24*: 259 (1982).
110. M. D. Smith and C. S. Ho, *J. Biotechnol. 2*: 347 (1985).
111. R. Wittler, H. Baumgartl, D. W. Lübbers, and K. Schügerl, *Biotechnol. Bioeng. 28*: 1024 (1986).
112. K. Schügerl, *J. Biotechnol. 13*: 251 (1990).
113. M. Matsumura, T. Imanaka, T. Yoshida, and H. Taguchi, *J. Ferment. Technol. Japan 58*: 197 (1980).

114. T. Bayer, W. Zhou, K. Holzhauer, and K. Schügerl, K. *Appl. Microbiol. Biotechnol. 30*: 26 (1989).
115. J. Möller, J. Niehoff, M. Dors, R. Hiddessen, and K. Schügerl *J. Biotechnol. 25*: 245–259 (1992).
116. J. Möller, J. Niehoff, S. Hotop, M. Dors, and K. Schügerl, *Appl. Microbiol. Biotechnol. 37*: 157–163 (1992).
117. W. Zhou, K. Holzhauer-Rieger, M. Dors, and K. Schügerl, *J. Biotechnol. 23*: 315–329 (1992).
118. W. Zhou, K. Holzhauer-Rieger, T. Bayer, and K. Schügerl, *J. Biotechnol., 28*:165–177 (1993).
119. S. Fröhlich, M. Lotz, T. Korte, A. Lübbert, K. Schügerl, and M. Seekamp, *Biotechnol. Bioeng. 38*: 43–55 (1991).
120. S. Fröhlich, M. Lotz, T. Korte, A. Lübbert, K. Schügerl, and M. Seekamp, *Biotechnol. Bioeng. 37*: 910–917 (1991).
121. S. Fröhlich, M. Lotz, B. Larson, A. Lübbert, K. Schügerl, and M. Seekamp, *Biotechnol. Bioeng. 38*: 56–64 (1991).
122. A. Lübbert, *Chem. Ing. Techn. 63*: 6–16 (1991).
123. H. M. Rüffer, Dissertation, University Hannover, 1990.
124. L. Brandes, Dissertation, University Hannover, 1991.
125. S. Fröhlich, Dissertation, University Hannover, 1986.
126. Y. Kawase and M. Moo-Young, *Biotechnol. Bioeng. 37*: 960–966 (1991).

8
Membrane Reactors

Peter M. Salmon

Merck & Company, Inc.
Rahway, New Jersey

Channing R. Robertson

Stanford University
Stanford, California

I. INTRODUCTION

Artificial membranes are routinely used during all stages of a biochemical process, from initial medium preparation to final purification. In this chapter, we examine the use of membranes as an integral part of reactors employed with either enzymes or cells. To limit the scope of the discussion, however, we do not describe the application of separate membrane modules to process feed or recycle streams for conventional reactors.

A *membrane reactor* is a flow reactor within which membranes are used to separate cells or enzymes from the feed or product streams. The diversity of the applications described in the literature is sufficiently great that a more precise definition is difficult. A common characteristic of most membrane reactors, however, is that feed streams are delivered continuously. Products may also be removed continuously, but in some applications they must be harvested intermittently or at the end of the run.

Polymeric microfiltration or ultrafiltration membranes are most commonly employed for the construction of membrane reactors [1], although other types of membranes have been used, including ceramic [2], silicone rubber [3], and ion exchange membranes [4]. Microfiltration membranes have pore sizes between 0.1 and 5 μm and can be used to confine cells within a reactor while imposing little restriction on the passage of soluble nutrients and products. The selection of pore size is generally based on the size of the cells (which may vary from less than 1 μm for some bacteria to 100 μm for certain plant cells). Ultrafiltration membranes typically have pore sizes between 20 and 1000 Å and are used to retain or exclude macromolecules with molecular weights from 10^4 to 10^6. Polymeric reverse osmosis membranes, with effective pore sizes less than 20 Å, have also been employed to selectively concentrate small molecules (with molecular weights less than about 200). Membranes made from a variety of materials are available, including cellulose acetate and nitrate, polyvinylidene difluoride, polysulfone, polypropylene, polytetrafluoroethylene (PTFE), and polyacrylonitrile. The permeability of a membrane is dependent not only on the pore size and structure but also on characteristics such as hydrophobicity and charge. In particular, hydrophobic polypropylene and PTFE membranes have been used to supply and remove gases selectively, while retaining aqueous species within the reactor.

The structure of artificial membranes is generally referred to either as isotropic or asymmetric. Isotropic membranes have a uniform structure and are typically 10–30 μm in thickness. Asymmetric membranes comprise a highly porous spongy support layer, often 50–500 μm thick [5], with a thin layer about 0.5 μm in depth that provides the permselective properties of the membrane. An asymmetric membrane provides less resistance to mass transfer than a comparable isotropic membrane but is more prone to mechanical damage.

Membranes can be obtained in hollow-fiber or flat-sheet form. The most commonly used geometry for membrane reactors is the hollow fiber [1,6]. A bundle of hollow fibers is potted into a cylindrical shell with epoxy or polyurethane resin. The reactors may be spun during the potting operation to ensure that the resin completely seals both ends of the reactor shell. A simple laboratory reactor is shown schematically in Fig. 1. Commercial units may contain thousands of hollow fibers, providing large surface areas (up to about 1 m^2) for mass transfer, cell adhesion, and so on. Flat-sheet membranes require support screens and are generally installed in a plate-and-frame system with spacers and gaskets [7]. These reactors are more complicated and expensive than hollow-fiber units with the same membrane surface area. They may, however, be more appropriate when the medium contains particulates [5]. Moreover, reactors with multiple sets of membranes used to selectively supply, retain, and remove different species may be more easily constructed using the flat sheet geometry [8].

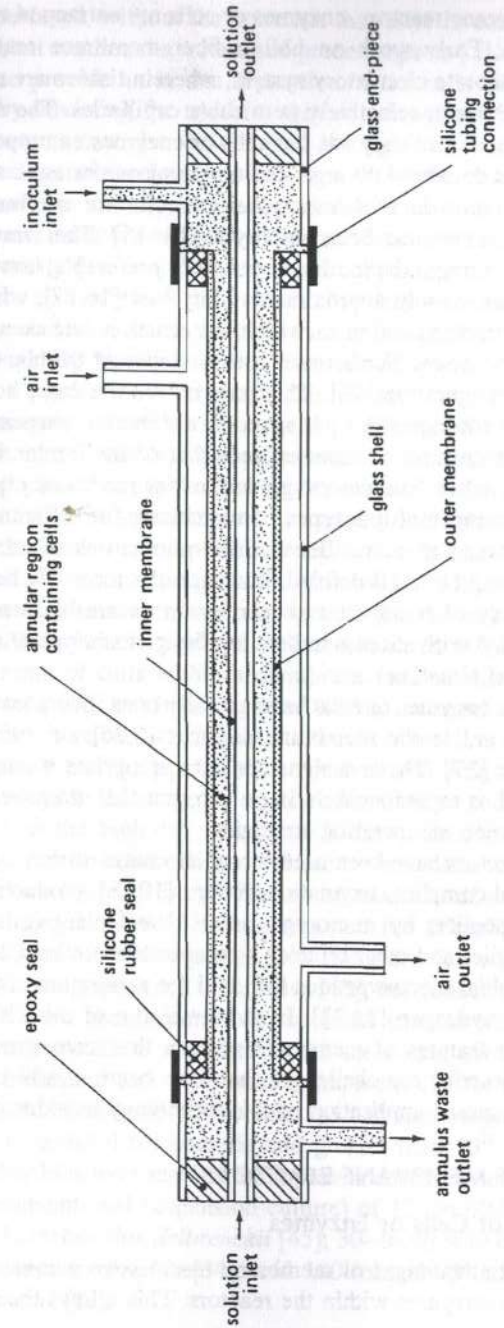


Figure 1 Axial section through a dual-membrane hollow-fiber reactor for entrapment of microbial cells.

In most membrane reactors, enzymes or cells are entrapped without chemical immobilization. Early work on hollow-fiber membrane reactors [9] was modeled on the vertebrate circulatory system, wherein tissues are maintained by nutrients provided through selectively permeable capillaries. The most common reactor configuration accordingly has the cells or enzymes entrapped in the shell space, with nutrients delivered through the lumina of one or more sets of hollow fibers [10–12]. Reactors in which enzymes or cells are confined within the lumina of the fibers have also been employed [13–15]. The arrangement with the substrate passed through the lumina is generally preferable, however, because the flow pattern more closely approximates plug flow [16,17], which is advantageous for the common situation in which the reaction rate decreases as substrate conversion increases. Furthermore, mass transport within the reactor is improved in this configuration [18]. This may not be the case, however, if the substrate is directed through the shell space in a direction perpendicular to the fibers and if the extraluminal volume exceeds that of the lumina by a factor of 4 or more [15]. There are few general guidelines for more complicated hollow-fiber reactors containing multiple types of membrane for different purposes or for flat-sheet membrane reactors. Because the geometries of most membrane reactors are comparatively well defined, however, the tools of chemical reactor design and the theory of transport and reaction in permeable media [19] have routinely been applied with success to develop designs and operating procedures for a wide variety of situations.

In some cases, enzymes or cells have actually been incorporated into membranes [20–25], bound to the membrane surfaces [2,26], or immobilized in a matrix of some type [27]. These designs are not appropriate if enzymes or cells need to be removed or replenished during a run, but they do provide additional flexibility in the choice of operating strategies.

Membrane reactors have been used for an enormous variety of applications, including simple and complex enzymatic reactions [10,28], production of primary and secondary metabolites by microorganisms [1] and plant cells [7], and the generation of antibodies and other proteins by mammalian cells [6,29]. Other uses have included microbial bioconversions [30] and the production of methane from carbon dioxide and hydrogen [31,32]. In the remainder of this chapter, we first discuss some of the features of membrane reactors that have attracted so much research and then describe the challenges that have been, or still must be, overcome to allow widespread application of this technology in industry.

II. FEATURES OF MEMBRANE REACTORS

A. Confinement of Cells or Enzymes

The most important advantages of membrane reactors are a consequence of the retention of cells or enzymes within the reactors. This allows the reactors to be

continuously perfused, without the concerns that arise in conventional continuous reactors about washout as a result of process upsets [1]. In many cases, the membranes also provide an in situ separation of the cells or enzymes from the product, thereby obviating the need for this step downstream.

These features can also be obtained through other immobilization techniques [33], but membrane entrapment is perhaps the most gentle approach available because no chemical agents or harsh conditions are employed. For example, enzyme immobilization by other methods generally results in a 10–90% loss in activity [34], and attachment of enzymes to a surface in particular can cause steric and rotational hindrances that decrease both productivity and specificity, especially for macromolecular substrates [35]. In contrast, the work of several groups [10,36,37] indicates that membrane entrapment of enzymes does not have a significant influence on the intrinsic kinetics. By careful application of mathematical models to analyze the influence of mass transfer (which can mask the reaction kinetics), the performance of the membrane reactors could be accurately predicted using the kinetics measured for the free enzyme in solution. The gentle nature of membrane entrapment is particularly significant for mammalian cells, which are especially sensitive to damage because they lack the resilient cell wall of microorganisms. Moreover, the high membrane surface area in hollow-fiber reactors is advantageous for the culture of anchorage-dependent cell lines (which require a surface on which to grow).

The retention of cells within a membrane reactor allows a high biomass density to be achieved. In simple agitated tanks, mammalian cell densities of $1–2 \times 10^6$ cells/ml are typical [27]. In hollow-fiber reactors, on the other hand, cells can be grown to fill essentially all the available volume [38], and mammalian cell densities in the range 10^8 to 10^9 cells/ml have been reported [39,40]. In consequence of the high cell density, a 50-fold increase in volumetric productivity compared to conventional stirred vessels has been observed for membrane reactors used for the production of monoclonal antibodies [41].

Corresponding results have been reported for microbial processes. Biomass concentrations somewhat greater than 100 g dry cell wt/L can be obtained in agitated tanks using fed-batch techniques [42], but concentrations of over 500 g dry cell wt/L have been reported for bacterial cells in hollow-fiber reactors [1,43]. These concentrations are higher than the solids content of normal microorganisms, indicating that membrane entrapment can lead to significant dewatering of cells. Inloes and co-workers [11,44] have reported cell densities greater than 10^{12} cells/ml for bacteria and greater than 10^{10} cells/ml for yeast. The high cell densities have resulted in improvements in volumetric productivity (compared to conventional suspension culture) of 12- to 40-fold for lactic acid production by *Lactobacillus delbreuckii* [45], 30- to 40-fold for ethanol production by *Kluyveromyces fragilis* [34], and 100-fold for β-lactamase production by *Escherichia coli* [11].

Membrane entrapment may be particularly helpful if aggregation is an advantage, as suggested for plant cells [46]. Plant cell productivity may be dependent on aggregate size, which can be better controlled in a membrane reactor than in suspension culture [7]. The effect of aggregate size could be a result of the depletion of substrates or the accumulation of intermediate metabolites or hormones within aggregates, or could be due to as yet poorly understood cell-to-cell interactions.

Membrane reactors have also been used for the coentrapment of different enzymes, cofactors, or microbes. A common application has been to bind cofactors such as NAD to polymers such as polyethylene glycol so that they can be retained in a reactor by ultrafiltration membranes [47–52]. The coentrapment of several enzymes has also been successfully employed to perform more complex enzymatic reactions [28,52]. In another set of interesting experiments [53], penicillin acylase has been coentrapped with soluble linear-chain polymers that stabilized the enzyme (perhaps due to a "cage" effect exerted on the protein by the polymeric network formed).

B. Selective Supply and Removal of Different Chemical Species

1. Permselectivity to Substrates and Products

One of the most attractive features of membrane reactors is the ability to harness the extensive technology originally developed for the use of membranes in separation processes. Membranes that are selectively permeable to a wide variety of different species are available and can be used to facilitate the removal of inhibitory metabolites and the recovery of unstable products before degradation. For example, Shiomi and co-workers [54] were able to effect some separation between mono- and disaccharides in an enzyme reactor employed for the hydrolysis of sucrose. Vasudevan and co-workers [55] used a reverse osmosis membrane to remove inhibitory ethanol selectively while retaining glucose in an entrapped-yeast membrane reactor. Another interesting area of research involves the use of ion exchange membranes to selectively remove or supply ionic species. These membranes have been employed in microbial fuel cells [4,56], and have possibilities for industrial application.

Macromolecular products can be concentrated by an appropriate choice of membrane pore size. This is a common practice in animal cell culture and results in an enriched product, with a concomitant reduction in isolation costs [57–59]. The concentrated product solution is harvested from the cell-containing region of the reactor intermittently or at the end of the run. Belfort [60] presents data showing the concentrations of monoclonal antibody produced by hybridomas to be as high as 4 mg/ml in hollow-fiber reactors, compared to less than 0.1 mg/ml for conventional suspension culture. Caution should be used in comparing these results because the cell lines and media differ. Nevertheless, they do provide an indication of the increased product concentration available through the use of membrane reactors. Despite the potential benefits of product retention in membrane reactors, the possibility of proteolytic degradation should be considered [61], particularly because proteases may be concentrated together with the product. In some cases, it may prove necessary to remove product frequently (or even continuously) to ensure that product degradation is limited.

In many instances it is also desirable to retain macromolecular nutrients and intermediates within a reactor. The provision of serum is a major cost factor in mammalian cell culture [62], and it is generally beneficial to employ ultrafiltration membranes to retain serum proteins (such as growth factors) in the cell-containing compartment of a reactor. Assuming that an equivalent serum concentration is required as in other cell culture techniques, the high cell density in membrane reactors means that less serum must be used [40]. In some cases, the serum proteins are supplemented during the run [63], but in others it has proved possible to include an adequate supply when the reactor is initially inoculated [64]. In conventional cell culture, the concentration of unwanted proteins may be orders of magnitude greater than that of the product, and consequently the ability to employ a small quantity of serum can significantly facilitate product recovery and purification.

2. Distinct Channels for Different Purposes

Separate Channels for Gases and Aqueous Species. The original membrane reactors employed a single set of hollow fibers, through which oxygenated medium was passed. The limitations of this approach soon became apparent, particularly in reactors used to entrap microbial cells for which the respiration rate is high. The solubility of oxygen in aqueous media is sufficiently low that it is not practicable to employ membrane reactors for aerobic microbial cultures unless oxygen can be supplied in gaseous form. Smith and co-workers [65] addressed this problem by extending the analogy between hollow-fiber reactors and the vertebrate circulatory system in which two phases, oxygen-rich erythrocytes and nutrient-rich plasma, pass alternately through capillaries. These researchers developed a reactor in which oxygen-rich gas bubbles and slugs of nutrient-rich medium flowed alternately through the lumina of the fibers to support β-lactamase production by *Bacillus licheniformis* entrapped within the shell space. Although this reactor provided a volumetric protein productivity at least an order of magnitude larger than that in a reactor supplied solely with oxygen-saturated aqueous medium, the approach is difficult to extend to reactors containing hundreds or thousands of fibers because of flow heterogeneities.

A more promising approach is the use of a spatially distinct arrangement of channels to transport different streams [3]. The wide variety of geometric configurations available provides great flexibility in the design of membrane reactors to supply and remove various species through different conduits. In

situations in which enzymes or cells can be confined to the spongy wall structure of asymmetric hollow fibers, it is possible to use the space exterior to the fibers for oxygen supply. Here the reactor comprises three regions: the fiber lumina for transport of aqueous nutrients and products, the fiber walls for retention of enzymes or cells, and the reactor shell for supply and removal of gases. In most situations, however, the membrane walls provide insufficient volume and containment, and several sets of membranes with different properties are used to create the three-compartment topography. The simplest cases have involved the supply of oxygen and glucose through separate sets of membranes in enzyme reactors for the production of gluconic acid [66–68]. The approach has also been applied to bioconversions [30] and the production (using both bacteria and fungi) of primary and secondary metabolites [3,43,69–71] and proteins [72]. The configurations employed have varied from bundles of hydrophilic fibers (for aqueous streams) and hydrophobic fibers (for gaseous streams) [70] to assemblages of concentric silicone rubber and microfiltration membranes [3]. Most of the reactors described in the literature have employed hollow fibers, but flat-sheet membranes have also been used [72]. The efficacy of these configurations has been demonstrated by both mathematical modeling and experiments, which have generally shown that it is not the supply of oxygen or nutrients but mechanical difficulties (discussed in Section III.B) that have limited the applicability of reactors used for microbial processes [3,68].

Reactors with multiple sets of membranes have also been used for mammalian cells [73–76], and a device incorporating hollow fibers for medium perfusion and flat-sheet membranes for oxygenation is available commercially from Endotronics, Inc. (Minneapolis, MN). Another example is the Static Maintenance Reactor originally developed by Monsanto Co. (St. Louis, MO) [62], in which a set of porous tubes is employed to supply nutrients and remove products by perfusing the matrix wherein cells are entrapped. A separate set of gas-permeable conduits is used to supply oxygen and remove carbon dioxide.

Separate Channels for Different Medium Components. In some instances it is also advantageous to incorporate separate channels for different aqueous streams. For example, Scholz and Hu [27] have developed hollow-fiber and flat-sheet membrane reactors in which cells are entrapped within a gel that contracts to provide separate channels for low-molecular-weight nutrients and metabolites and for high-molecular-weight components (such as products and serum proteins). This permits reactor operation with a long residence time for the latter channel (thereby conserving serum and concentrating products) but still provides continuous product removal with in situ separation of the stream from the cells.

Brown and co-workers [63] have described a system developed by Bio-Response, Inc. (Hayward, CA) in which microfiltration membranes (with pore size between 0.1 and 0.2 μm) are used to separate cells from an aqueous stream

used to supply oxygen and remove products. Ultrafiltration membranes are used to allow low-molecular-weight metabolites such as lactate and ammonia (but not the product and lymph proteins) to be removed in a separate stream. Protein-supplemented medium is supplied directly to the cell-containing compartment (which contains a packing to provide adequate surface area if an anchorage-dependent cell line is being cultivated).

Nonaqueous Liquids The use of membranes to contact aqueous and organic streams has several applications in membrane reactor technology. Flat-sheet membrane reactors have been used to contact oleic and linoleic acids with enzymes in aqueous solution for the production of glycerides [77,78]. These reactors avoid many of the complications associated with the conventional emulsion system, including high-speed agitation for emulsification, the use of surfactants, and centrifugation to recover the product. Vaidya and co-workers [79] have reviewed the use of membrane bioreactors for reactions involving aqueous and organic phases.

Organic solvents have also been used to extract products from microbial bioreactors. For example, Frank and Sirkar [80] perfused microporous hollow fibers with dibutyl phthalate to remove ethanol from a membrane reactor containing *Saccharomyces cerevisiae*. Similar reactors using flat-sheet membranes with tributyl phosphate as an extractant have been developed by Shuler and co-workers [5,8,81] to reduce product inhibition and thereby increase productivity.

C. Protection of Enzymes and Cells from the Environment

Mechanical stresses can have significant effects on the activity of mycelial microorganisms [82], plant cells [83], and especially animal cells (in consequence of their lack of a rigid cell wall) [84]. The issue has been reviewed in detail by Prokop and Bajpai [85]. There is considerable controversy about the causes of the effects observed and in particular about the relative roles of collisions, shear due to mechanical agitation, and the rapid acceleration that entrained cells experience when bubbles burst [86]. The balance of evidence indicates that enzymes and simple prokaryotes (excluding the actinomycetes) are not sensitive to the shear stresses generated in typical reactors [85]. An additional cause of inactivation, however, is the denaturation of proteins and other biological macromolecules at the air-liquid interfaces commonly encountered in agitated vessels. This is a particular concern in situations in which foam is present. In recycle reactors, which are often considered as a means of generating a high biomass density in a conventional reactor, a further source of trauma is the pump employed to move the cells through the external circuit [87].

All of these problems can be eliminated in a membrane reactor because the enzymes or cells are sequestered in a relatively quiescent region wherein they are protected from mechanical damage and generally not in direct contact

with air. Indeed, it is this feature of membrane reactors that has provided the primary motivation for their adoption by some researchers [75]. In a typical study, Käsehagen and co-workers [59] examined the extracellular levels of the normally intracellular enzymes lactate dehydrogenase and glutamate-oxaloacetate transaminase as indicators of cell damage and obtained significantly lower values for membrane-entrapped hybridomas than for those grown in stirred vessels.

In some cases, membrane reactors can be used with relatively crude raw materials because micelles, particulates, and other unwanted components can be excluded from the compartment containing the enzymes and cells. For example, Prenosil and Hediger [36] used a pilot-scale enzyme reactor for the hydrolysis of lactose and demonstrated that it was capable of handling untreated whey or skimmed milk. Similarly, unneeded protein in the culture medium for animal cells can be excluded from the products concentrated in the cell-containing region [6].

There have been suggestions that membrane reactors provide in situ protection against contamination by bacteria, mycoplasmas, and even viruses [60,88]. This has not been adequately demonstrated, however, and the connections necessary for the delivery of inoculum, nutrients, and gases to the reactor require that significant attention be given to aseptic operations. A related idea is that membrane reactors provide containment of recombinant organisms and opportunistic pathogens, and in this regard downstream processing can be simplified.

D. Ability to Change Media Rapidly

There are numerous biological processes in which one or more changes in the medium are required. Of particular importance in the pharmaceutical industry are bioconversions and resting-cell systems in which the biosynthetic apparatus of microorganisms is exploited in the absence of cell growth [89]. A typical laboratory procedure for a microbial bioconversion, for example, involves fermentation, centrifugation or filtration to remove the growth medium, followed by washing of the cells and resuspension in buffer with the compound to be converted. Similarly, plant cells often require different media for growth and product synthesis, and the removal of products may require solutions containing permeabilizing agents (such as dimethyl sulfoxide) or a change in the pH or ionic strength [46,90]. Processes of this nature are difficult to scale up using conventional equipment. Perfused membrane reactors, on the other hand, allow rapid changes in the medium because the hydraulic residence time is typically low, and are therefore readily adapted for these applications.

In some cases, a change from a growth medium to a maintenance or production medium is necessary to prevent membrane damage in consequence of sustained cell growth. This issue is discussed further in Section III.B.

E. Long-Term Production Without Growth

A central idea in the development of membrane reactors has been that cells should be maintained for an extended period, and perfused to continuously generate product. The analogy with the use of fixed catalysts in the chemical industry has often been presented [11,27]. The Static Maintenance Reactor discussed in Section II.B is based explicitly on this concept [62]. Animal cells are cultured in a separate vessel and then loaded into the membrane reactor, where they are maintained on a medium that supports protein production but limits growth. These reactors can be run continuously for up to 4 months and have been used for the commercial manufacture of proteins.

Altshuler and co-workers [12] have shown that the maximum productivity of hybridomas grown in a hollow-fiber reactor occurs after the cell mass reaches a maximum, indicating that monoclonal antibody production can be uncoupled (at least partially) from growth. Hollow-fiber reactors have been used by a number of researchers to generate proteins from animal cells for periods of several months [91,92].

Plant cells are also well suited to this type of process. The cells undergo gradual changes in their genetic characteristics, which can be minimized by limiting growth [90]. High-density culture seems to reduce growth beyond its normal slow rate and therefore renders the use of membrane reactors particularly attractive [46].

Attempts to employ membrane-entrapped microbial cells for long-term product synthesis have, however, encountered difficulties related to membrane damage in consequence of continued growth [1]. This issue and other challenges that need to be addressed before membrane reactors become more generally useful in industry are discussed in Section III.

III. CHALLENGES

A. Mass Transport Effects

Among the most significant and widely studied challenges facing the application of membrane reactors is the influence of mass transport effects on reactor operation. In this section, we first examine the transport of cells, enzymes, and other macromolecules and then discuss the supply of low-molecular-weight substrates and the removal of metabolites.

1. Transport of Cells and Proteins

Distribution of Cells and Enzymes. Cells and enzymes entrapped within membrane reactors are subject to diffusion and convection that can render their distribution heterogeneous. In this regard, it should be noted that membranes are generally permeable to aqueous solutions, and in consequence flow is rarely

restricted to the channels to which medium is supplied. For example, consider a simple reactor with a sealed shell containing enzymes or cells and with a single set of hollow-fiber membranes through which the aqueous substrate solution is passed. Because the shell is sealed there can be no net radial flow, but the axial hydrostatic pressure gradient within each lumen will cause some fluid to flow from the upstream ends of the fibers, through the shell space, and back into the downstream ends of the fiber lumina. The streamlines for such a flow field are shown in Fig. 2. The resulting toroidal fluid movement is known as Starling flow in recognition of Ernest Starling, an English physiologist who in 1896 proposed that fluid movement of this nature occurred in the interstitial space between capillaries in the human body [93].

Waterland et al. [10] and Salmon [68] both measured the distribution of enzymes entrapped in hollow-fiber reactors and discovered the downstream concentrations to be much greater than those upstream. In an attempt to describe this phenomenon quantitatively, Waterland developed a simple model of convection and diffusion, which unfortunately overpredicted the extent of heterogeneity of the enzyme distribution. As shown in Fig. 3, Salmon obtained significantly improved agreement with the measurements using a theory that accounted for the variation in the transmembrane osmotic pressure differential caused by the nonuniform protein distribution. Piret and Cooney [94] have subsequently shown, using colored azoalbumin, that gravitational effects also influence the distribution of proteins. The density of the concentrated protein solution at the downstream end of a reactor may be about 1% greater than that of the medium as a whole, and this is sufficient for sedimentation effects to be significant.

In addition, Piret and Cooney experimentally determined the axial distribution of hybridomas in hollow-fiber reactors and found significant downstream polarization. This could have been caused by convective transport of the inoculum, but because net cell growth had apparently ended in these reactors, Piret and Cooney suggest that the phenomenon was caused by the concentration of growth factors downstream. By periodically reversing the direction of flow through the reactors, the distributions of proteins and cell growth were rendered more uniform, and in consequence the antibody production rate was more than doubled. Conversely, by maintaining flow in a single direction before and during harvest, the concentration of the proteins recovered could be maximized [94]. The flow-reversal strategy has been employed in the commercially available Celltronics reactor system (New Brunswick Scientific, Edison, NJ), which has been used successfully for the production of monoclonal antibodies for periods of several months [95].

For enzyme reactors, another approach to overcoming convective and gravitational polarization is to immobilize the proteins chemically. Some of the features of membrane reactors described in Section II are evidently lost through

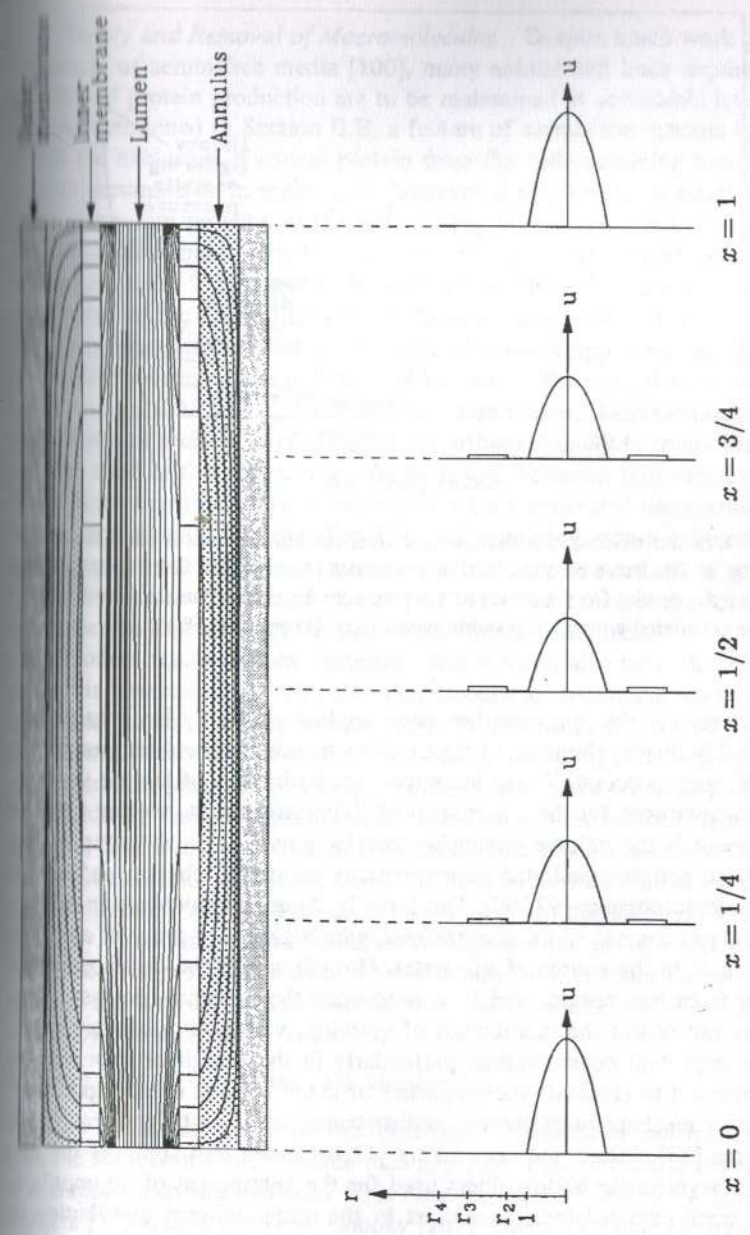


Figure 2 Streamlines and axial velocity profiles of the flow field in a hollow-fiber reactor such as that depicted in Fig. 1. Notation: r is the radial coordinate; x is the axial coordinate; r_2, r_3, and r_4 are the radii of the fiber, cell region, and outer membrane relative to the lumen radius; u is the axial flow velocity. (From Ref. 115.)

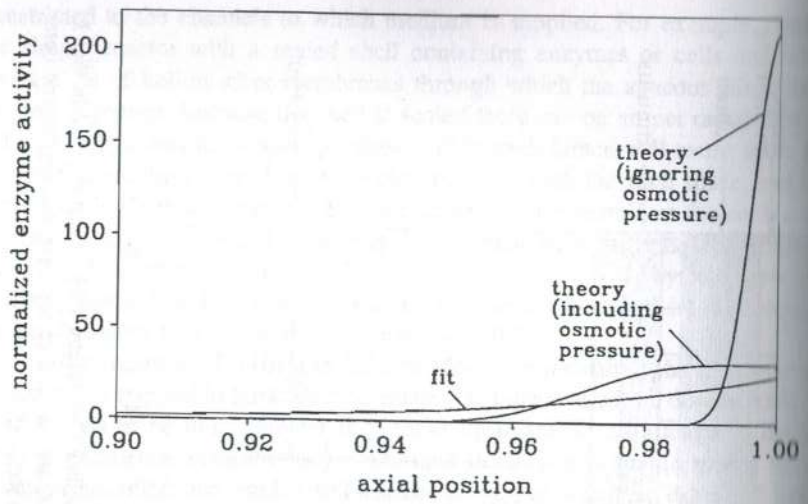


Figure 3 Glucose oxidase distribution in a dual-membrane reactor such as that depicted in Fig. 1. The curve labelled "fit" represents a least-squares fit of an exponential expression to the results from a series of enzyme activity measurements. The "theory" curves were calculated without adjustable parameters. (From Ref. 68.)

this procedure, but the approach has been applied profitably by Prenosil and Hediger [96] in the development of the capillary membrane fixed enzyme reactor (CAMFER) used successfully for the pilot-scale hydrolysis of lactose in whey.

In reactors used for the entrapment of living organisms, another cause of cell movement is the volume expansion due to growth. The phenomenon has been modeled and demonstrated experimentally using ^{35}S labeling and liquid emulsion autoradiography [97–99]. This issue is of particular concern in reactors used for the entrapment of microorganisms, which generally grow in a narrow region adjacent to the source of substrates. Growth causes the displacement of cells away from this region, and in consequence the eventual biomass distribution may not reflect the distribution of growing, viable, or productive cells. This is an important consideration, particularly in the design of experimental reactors intended to elucidate the properties of the entrapped microorganisms.

Another mechanism of biomass redistribution is the motility of many microorganisms [33]. Inloes and co-workers [11,44] noted that some of the macrovoids in asymmetric hollow-fibers used for the entrapment of nonmotile *S. cerevisiae* were unpopulated, in contrast to the more uniform distribution of biomass observed in similar reactors used with motile *E. coli*. This may have been a consequence of the motility of the bacteria, and the issue is worthy of additional study.

Supply and Removal of Macromolecules. Despite much work on the development of serum-free media [100], many animal cell lines require serum if growth and protein production are to be maintained at acceptable levels in culture. As mentioned in Section II.B, a feature of membrane reactors is that they permit the exclusion of excess protein from the cell-containing region wherein product accumulates. In some cases, however, it may prove necessary to provide additional serum proteins to the cells during reactor operation. One method is to add supplementary serum directly to the cell compartment [95], but some researchers have found this to be ineffective, perhaps because of an uneven distribution of the serum to the cells. Altshuler and co-workers [12] noted that the monoclonal antibody yields from membrane-entrapped murine hybridomas progressively increased in hollow-fiber reactors with nominal molecular weight cutoff ratings of 10×10^3, 50×10^3, and 100×10^3. These results indicate the significance of the supply of high-molecular-weight medium components for the cell line used in this study. It should be noted, however, that various cell lines have different requirements, and many have been cultivated successfully without serum supplementation [64]. The issue of supplying serum uniformly to the heterogeneous cell mass in membrane reactors may be resolved to some extent by the flow-reversal approach discussed earlier and by other reactor configurations wherein flow through the cell compartment is induced.

Product removal from membrane reactors can also be a challenge. If the product is intracellular or otherwise cell-associated, membrane reactors are generally inappropriate, unless secretion can be induced in some way [46]. If this process inactivates the cells, then the potential advantage of long-term continuous operation (discussed in Section II.E) is lost. Even secreted products may be difficult to remove from a dense membrane-entrapped cell aggregate. For example, Smith and co-workers [65] found that β-lactamase production in a hollow-fiber reactor used to entrap *B. licheniformis* was limited by the rate of protein diffusion through the cell mass. A common approach is to harvest periodically by ultrafiltration of medium through the cell mass, thereby exploiting convection for product removal. Even this may be insufficient if tissue-like aggregates are formed, as observed by Ryll and co-workers [74] in experiments with baby hamster kidney cells.

2. Transport of Substrates and Metabolites

The role of limitations in the transport rates of substrates and metabolites has been the subject of more research than any other topic related to the application of entrapped enzymes and cells. This issue has been reviewed by Karel and co-workers [33] and by Piret and Cooney [101]. Direct evidence for transport limitations has frequently been obtained through the use of microscopy. For example, examination of sections from hollow-fiber reactors used for mammalian cells frequently shows necrotic regions 100–200 μm from the fibers [64,102],

and these have been presumed to be a consequence of oxygen or substrate limitations. Piret and Cooney [39] have measured the solute concentration variations in the axial and radial dimensions of hollow-fiber reactors used for mammalian cell culture and have established that the transport of oxygen is generally the limiting factor, rather than nutrient deprivation or inhibition by metabolites such as lactate or ammonia.

Transport effects are generally even more pronounced for microbial cells, whose metabolism rates are typically several orders of magnitude greater than those of animal cells in culture. Karel and co-workers [103] have presented micrographs that show *E. coli* cells more than about 10 μm from the fiber lumina to be abnormally small and elongated. As already mentioned, the distribution of cell mass does not necessarily reflect the distribution of cell growth because of biomass transport phenomena. Pulse labeling with radioactive tracers coupled with autoradiography can, however, be used to identify regions within which protein synthesis occurred at the time of each pulse. This technique has been employed with hollow-fiber reactors containing various bacteria to reveal growth zones 5–50 μm in depth under conditions of oxygen or glucose limitation [97,98] or product inhibition [99]. Transport limitations can also be demonstrated by identifying products characteristic of metabolism under conditions in which the component of interest is depleted—for example, Paterson and co-workers [104] employed acetic acid as an indicator of oxygen limitation for *E. coli* in a hollow-fiber reactor.

In this section, we describe efforts that have been made to understand and address mass transport effects in membrane reactors. Because of the available flexibility in the quantity, location, and properties of the membranes employed, the range of possibilities for engineering to overcome transport limitations is enormous. For this reason, only a sample can be presented here.

Flow Distribution. An uneven flow distribution among the various channels in a membrane reactor can have significant effects. For example, Inloes and co-workers [44] discovered that certain hollow fibers in a study with *S. cerevisiae* were almost devoid of cells. This observation was attributed to a nonuniform flow distribution, perhaps caused by the presence of gas bubbles in some of the fiber lumina. There has been some theoretical work on this issue [105,106] and several groups have performed residence-time distribution experiments using tracers such as blue dextran [17,38,106–108]. In studies with hollow-fiber cartridges, Engasser and co-workers [107] found that the accessible volume increased as the axial pressure differential was increased, presumably because of the displacement of gas. The variation in the flow rate through perfused fibers was, however, small. Nuclear magnetic resonance imaging has been applied to examine the flow distribution in membrane reactors and is likely to become an important tool for the analysis of reactor performance [109,110].

Ku and co-workers [38] found that flow maldistribution of both medium and air in a pressure-pulsed hollow-fiber reactor (described below) restricted the scale-up potential of the device. For this reason, they developed a cross-flow reactor wherein medium is passed over a shallow bed of hollow fibers, through the lumina of which air is provided. Anchorage-dependent cells grow on the surface of the fibers and are supplied with nutrients from the surrounding medium. The bed is kept shallow (six or fewer fibers deep) to ensure that the environment is uniform. A similar reactor configuration has been developed by Tharakan and Chau [111].

Solute Transport by Diffusion. In the simplest membrane reactor configurations, diffusion is the only mechanism of solute transport in the enzyme- or cell-containing compartment. Reactor performance in this case can be modeled by formulating differential equations that balance diffusion and consumption or production at each location. The equations can be solved analytically or numerically with appropriate boundary conditions specified to match the imposed operating conditions. The extensive published research in this area has been ably reviewed by Karel and co-workers [33] and by Piret and Cooney [101]. The results are generally expressed in terms of an effectiveness factor η, defined as the ratio of the actual rate of substrate consumption (or product formation) to the rate that would pertain in the absence of transport limitations. An alternative approach is to calculate a length ratio z_k/z, where z_k is the reaction-limited reactor length, calculated assuming there are no resistances to mass transport, that provides the same conversion as the actual reactor of length z [112–115]. This ratio provides a direct indication of the influence of transport limitations on the size of a reactor for a given application—for example, if the value of z_k/z is 1/2, the reactor length is exactly twice that of a reactor with the same productivity, but in which there are no transport limitations. In a hollow-fiber reactor without axial gradients, η is equal to z_k/z. This situation is often achieved in practice, at least approximately, by incorporating the membrane reactor in a recycle circuit so that the conversion per pass is small. This is particularly desirable when living organisms are employed because the environment within the cell-containing compartment is more uniform.

Figure 4 is a typical plot of the variation of z_k/z with dimensionless reaction rate, Da_D. The length ratio is unity for small Da_D and decreases in proportion to Da_D^{-2} as $Da_D \rightarrow \infty$ (when transport limitations prevail). Such a plot can be prepared for the geometry, reaction kinetics, and transport properties of interest and used to select operating conditions for the reactor. For reactors employed for enzymatic reactions or the production of metabolites by microorganisms, a rule of thumb is that the target value of z_k/z should be somewhat less than unity but generally greater than 0.1. If $z_k/z = 1$, then the reactor volume is not being fully utilized because more catalyst (enzymes or cells) can be prof-

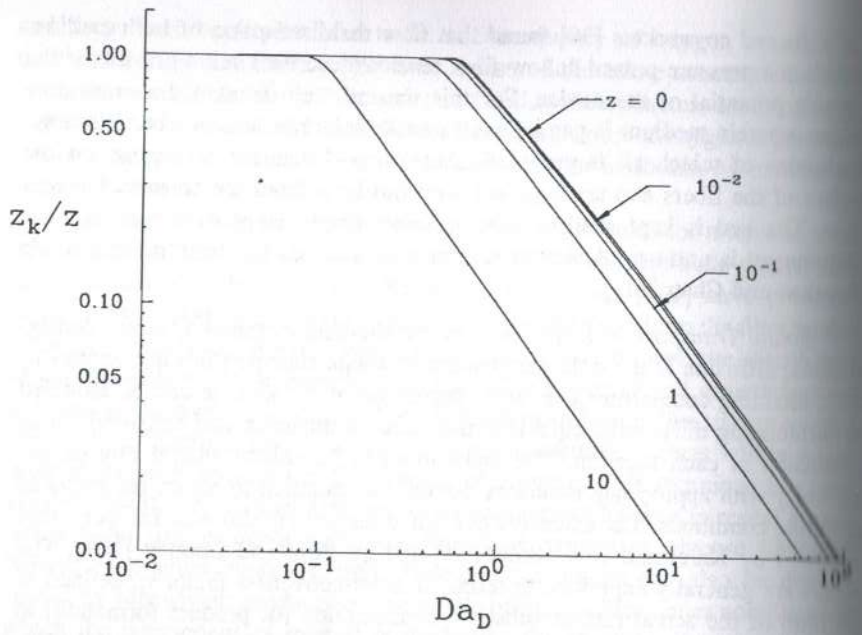


Figure 4 A typical plot of the length ratio z_k/z versus Da_D for a hollow-fiber reactor with a single limiting reactant and without convection through the entrapped enzymes or cells. The kinetics are of the Michaelis-Menten form: $R = c(\vartheta + 1)/(\vartheta + c)$, with $\vartheta = 10^{-3}$. Notation: Da_D is a Damköhler number (a dimensionless reaction rate constant); R is the relative reaction rate, c is the solute concentration, ϑ is the Michaelis constant, z is the reactor length. (From Ref. 115.)

itably introduced to increase productivity. Conversely, if $z_k/z << 1$, then reactor performance is limited almost entirely by solute transport, and the provision of additional catalyst is of little consequence. This guideline should clearly be used only when other considerations do not dictate an alternative strategy. In particular, reactors used for the production of proteins (particularly by animal cells) should generally be operated with $z_k/z \approx 1$ because variations in the environment within the cell-containing compartment can have subtle but potentially significant effects on the product stream generated. This issue is especially important when proteins are being produced for clinical use as therapeutic agents or vaccines.

The use of reaction-diffusion theory is contingent on the availability of values for the transport properties of the solutes of interest (generally O_2 for aerobic systems) and of the kinetics and stoichiometry of substrate consumption and product formation. Libicki and co-workers [116] investigated the transport

properties for nonpolar solutes in aggregates of microbial cells. The magnitude of the effective diffusive permeability was found to decrease with increasing cell density to a value for close-packed cells about 25% that in the external aqueous medium. The evaluation of kinetics and stoichiometry is potentially more difficult, but for enzymes and bacterial cells we have successfully predicted the reactor performance by assuming that the parameters measured in solution or suspension also pertain in membrane reactors, provided that variations in the local environment are adequately taken into account [e.g., 97–99,117]. A potential complication in the theory arises when individual clusters or aggregates of biomass within the cell-containing compartment of a reactor become sufficiently large that local transport limitations (over the length scale of a cluster) become significant [116]. This issue has been examined by Salmon and Robertson [68,118] and is generally a concern only with microbial clusters of diameter greater than about 25 μm.

Reactor design for aerobic culture is generally determined by the transport rates of oxygen, primarily in consequence of the low aqueous solubility of this species (~0.2 mmol/L/atm). There have been some attempts to improve this by employing oxygen carriers such as perfluorocarbons, hemoglobin, and myoglobin [119] or through the use of hyperbaric oxygen [120]. These techniques can alleviate oxygen transport limitations, but their use must be balanced against their potential inhibitory effects on viability and growth [121,122].

Convective Transport. An alternative approach to alleviating transport limitations is to exploit convection as well as diffusion. In this regard, it is worth recalling that in consequence of Starling flow (discussed earlier in the context of cell and protein transport) there is generally some convective transport through the compartment containing cells or enzymes. Libicki and co-workers [116] measured the transport rate of a nonpolar tracer through the cell-containing annulus of hollow-fiber reactors such as that depicted in Fig. 1. The dependence of the tracer flux on the flow rate of aqueous medium through the fiber lumina was much greater than predicted by a model in which transport through the cell compartment was assumed to occur by diffusion alone. To ensure that this effect was not caused by experimental artifacts, the experiment was repeated with a reactor in which the porous membranes were replaced with silicone rubber tubules that are impervious to liquid flow. In this case the results were consistent with the simple theory. Incorporation of Starling flow in the model allowed accurate simulation of the tracer flux variation with solution flow rate for the original experiments, as depicted in Fig. 5. Application of the model with the operating parameters employed with other hollow-fiber reactors in our laboratory indicates, however, that Starling flow does not generally have a significant influence on substrate and product transport [68]. Piret and Cooney [101] have independently confirmed this conclusion for oxygen transport in typical hollow-

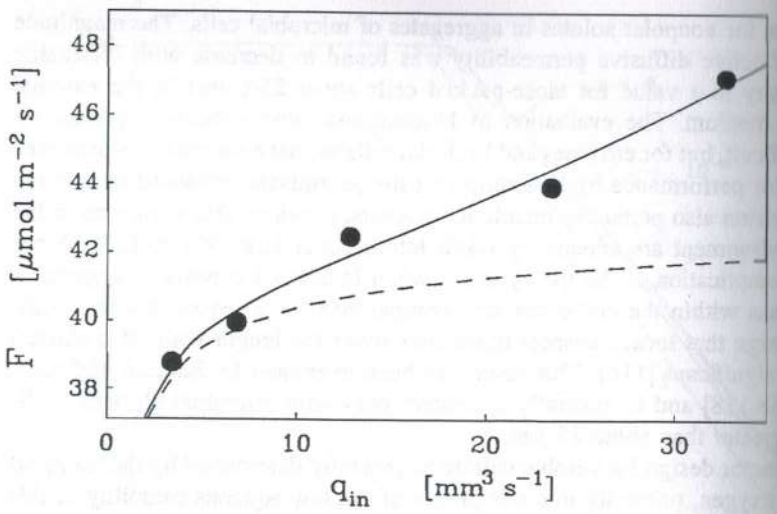


Figure 5 Tracer flux, F, from the exterior of outer membrane into the fluid flowing through the lumen, versus the influent flow rate, q_{in}, for a typical experiment using a reactor such as that depicted in Fig. 1. The points represent experimental data obtained by Libicki and co-workers [116]. The solid line represents a simulation using a model that incorporates Starling flow. The dashed line shows the simulation results obtained if Starling flow is neglected. (From Ref. 68.)

fiber reactors used for cell culture. On the other hand, Paterson and co-workers [123] incorporated hollow-fiber reactors in a recycle loop and deliberately enhanced the effect of Starling flow by using high flow rates through the fiber lumina. Simulation of these conditions with the mathematical model reveals that extraluminal flow would indeed have had a marked influence on the performance of these reactors [68].

Another common method for the use of convective solute transport is to pass the substrate solution through a membrane within which catalysts are incorporated [25,55] or through a set of membranes between which the enzymes or cells are entrapped [7,55]. In a similar approach known as backflushing, at least part of the substrate solution is delivered into the compartment that contains the enzymes or cells and thence ultrafiltered across the confining membrane into the channels used to remove the product stream [96,124–126]. Operational difficulties due to membrane fouling may be encountered using these techniques, unless the feed solution is of ultrafiltrate quality. An alternative is to use some tangential flow, although in this case attention must be given in the reactor design to ensure that the enzymes or cells are not washed out [2].

Convection through the cell-containing region has also been induced by incorporating two sets of hollow fibers between which a pressure differential is maintained [127]. This approach further extends the analogy mentioned earlier between hollow-fiber reactors and the vertebrate circulatory system, with the high- and low-pressure fibers corresponding in turn to the arteries and veins. A limitation of this configuration is that the arrangement of the two sets of fibers must be carefully controlled to homogeneously supply the cells with nutrients and remove products.

The flat-bed and radial-flow hollow-fiber reactors developed by Ku and co-workers [38] and by Tharakan and Chau [111] also employ convection through the cell-containing region. Medium is supplied directly to the cell compartment rather than being directed through membrane-confined channels, and in consequence the magnitude of the flow rate must be restricted to avoid cell damage or detachment. This flow rate restriction in turn limits the reactor depth that can be employed without substrate concentration gradients. These reactor configurations are in general appropriate only for anchorage-dependent cell lines that adhere to the fibers.

An interesting and widely investigated method for exploiting convective solute transport in membrane reactors is that of pulsed flow. The concept is to induce flow in alternating directions between two compartments, or into and out of an expansion chamber. Enzyme reactors using this approach have been developed by several research groups [128–133], and improvements in conversion of over 100% compared to that obtained without the transmembrane flow have been reported [132]. Kim and Chang [131] have shown that the conversion is improved as the flow amplitude increases but is comparatively insensitive to the period with which the flow direction is alternated. The flow amplitude is in practice limited by the mechanical properties of the membrane—enzyme leakage may be encountered if the transmembrane flow rate is too great [129,133]. The cycle period is limited by fouling, which progressively reduces the membrane permeability until flow reversal becomes mandatory [129].

Hollow-fiber reactors with pulsed or periodic flow have also been employed for microbial and animal cell culture [81,134]. Ku and co-workers [38] developed a reactor in which hollow fibers were used to supply air and carbon dioxide. Medium was supplied directly to the extraluminal region wherein cells were cultured. The intraluminal pressure was cycled to ultrafilter some medium periodically from the cell compartment into the fiber lumens. As already mentioned, this configuration was abandoned because the distribution of gas and liquid through the reactor was found to be inhomogeneous.

A configuration similar to that described for enzyme reactors has been developed by Endotronics, Inc. (Minneapolis, MN). The Acusyst-P system incorporates an external expansion chamber that permits cyclic flow across the hollow fibers [134]. Ultrasonic sensors are used to monitor level in the expansion

chamber and the flow direction is reversed by adjusting the pressure of the feed stream. Although a primary motivation for the development of this configuration was a desire to minimize nutrient and metabolite concentration gradients, Fiest and Cooney [39] have noted that the main benefits of cyclic flow may be related to the distribution of protein growth factors. In an example application of the Acusyst-P, Liu and co-workers [135] employed a system with 7.2 m^2 of hollow-fiber area to generate 30 g of protein in 80 days from Hep G2 cells. Microscopy after 90 days of culture revealed some cellular degeneration and necrosis, suggesting that transport limitations remained despite convection through the cell-containing regions.

Stirred Membrane Reactors. Perhaps the most extreme approach toward inducing convection through the cell compartment of a membrane reactor is to employ mechanical agitation. Such a reactor configuration represents a hybrid between a traditional stirred tank and a membrane reactor such as those discussed above, and in consequence it shares some of the advantages and disadvantages of each. In an interesting series of papers, the performance of stirred membrane reactors has been compared with that of conventional hollow-fiber reactors [74,76]. The stirred reactors contained two sets of polypropylene fibers coiled around an agitator—one set of fibers was used for the supply and removal of gases, and the other (having been wet with ethanol) was used for nutrient supply and product removal. The hollow-fiber reactors were supplied with medium that had been oxygenated in a separate aerator. In experiments using BHK cells to produce interleukin-2 and Epstein-Barr virus (EBV)-transformed B lymphocytes to produce antibodies, the product yield achieved per liter of medium using the stirred membrane reactors was two- to threefold greater than that for the conventional hollow-fiber reactors (even though the volumetric productivity of the latter was two to eight times higher in consequence of a greater cell density). These results were attributed to transport limitations within the dense cell mass in the hollow-fiber reactors. In other experiments with hybridomas, which are anchorage-independent and did not form tissue-like aggregates, the performance of the hollow-fiber reactors was apparently more favorable [74].

A similar reactor configuration has been reported by Graf and Schügerl [136], who employed silicon rubber tubing for gas supply and hydrophilized PTFE membranes for medium perfusion in a stirred reactor for monoclonal antibody production using hybridomas. This system also included an in situ microfiltration membrane for sampling and is a fine example of the range of potential applications for membrane technology with otherwise traditional reactors.

Stirred membrane reactors for microbial fermentations have been investigated by Kim and co-workers [137], and other applications have been summarized by Cheryan and Mehaia [34]. A more common approach with microorganisms, however, has been the use of an external recycle circuit, with a

membrane module to separate cells from the effluent stream [138]. Such a setup may be somewhat simpler to assemble than a reactor with internal membranes, but lacks many of the features described in Section II. As mentioned in the introduction, discussion of recycle systems is beyond the scope of this chapter.

Direct Contact with Gas Phase. In some instances, enzymes or cells have been placed in direct contact with the gas phase to eliminate the difficulties that arise from the low aqueous solubility of oxygen and other gases. Although such an approach could lead to denaturation of proteins and other biological macromolecules, the improved mass transport thus obtained can significantly improve reactor performance. For example, Salmon [68] examined a hollow-fiber reactor for the enzymatic oxidation of glucose to gluconic acid. Enzyme solution was loaded into the macroporous walls of asymmetric hollow fibers. Air was then applied to the shell space to maintain a pressure 10 kPa greater than that of the glucose solution delivered to the fiber lumina. In this fashion, the majority of liquid in the macroporous fiber walls was displaced by ultrafiltration into the fiber lumina, while the enzyme was retained by the thin skin of the membranes. The measured conversion was found to be consistent with a model in which the enzyme was assumed to be located entirely at the thin skin and about 40% greater than that predicted if the enzyme had been homogeneously distributed throughout the macroporous region of the fibers.

A more sophisticated example is the reactor developed by Efthymiou and Shuler [81], wherein the pressure across a membrane-entrapped cell layer is cycled so that the organisms are in contact alternately with aqueous medium and air. This technique combines the benefits of both convection and direct contact with the gas phase and can in principle eliminate mass transport limitations for both aqueous and gaseous nutrients or products. In an application employing *S. cerevisiae*, the use of the pressure cycling technique increased biomass production by more than 100% compared to a similar reactor operated in a more conventional manner without periodic displacement of the liquid from the cell compartment [81].

In studies of CH_4 production from CO_2 and H_2, Nagai and co-workers [31,32] have developed membrane reactors intended to overcome limitations imposed by the poor aqueous solubility of hydrogen. *Methanobacterium thermoautotrophicum* cells are filtered onto membrane surfaces and directly contacted with a CO_2 and H_2 gas mixture. Nutrients are provided by diffusion from an aqueous medium delivered to the reverse side of the membranes. This rather unusual example illustrates the breadth of the possibilities that membrane reactors provide.

II. Interactions Among Cells or Enzymes and Membranes

1. Compatibility of Enzymes or Cells with Membranes

There have been many reports of interactions between enzymes or cells and the membranes (and other components) used in reactor construction. Some of these

are related to hydrophobicity and surface charge, but others are likely caused by specific aspects of the surface chemistry [33]. Cheryan and Mehaia [34] summarized observations of the influence of various membranes on entrapped enzymes and found no general rules to guide the choice of materials. Some enzymes, such as β-galactosidase, α-galactosidase, and glucose isomerase, were found to be inactivated by polysulfone membranes but not by acrylic membranes. On the other hand, proteases were not affected by polysulfone membranes. Jones and co-workers [37] determined that polysulfone membranes adsorbed lactases and that this was detrimental to various lactases from yeast but not to the mold lactase used. The activity, but not stability, of the yeast lactases could be improved by pretreatment of the membranes with milk proteins.

Animal cells are particularly sensitive to the chemical nature of the surfaces with which they are in contact. The properties of various cell lines may differ in this regard [139,140], and the choice of materials for a given application should be evaluated experimentally. The surface properties are of particular importance for the attachment and propagation of anchorage-dependent cell lines. Treatment of the membranes with materials that enhance attachment, such as poly-D-lysine and collagen, has proved beneficial [29]. An important practical issue is that surface properties may change with age, particularly if heat-sterilized, and care should therefore be taken if reactor components are to be reused [141].

2. Interactions Between Cells

The role of cell-to-cell interactions is the least understood aspect of entrapped cell processes and one that presents a particular challenge for future research. Anchorage-dependent animal cells are typically considered subject to contact inhibition, with the result that net cell growth and proliferation cease once a confluent monolayer has formed. When maintained in an appropriate chemical and physical environment, however, the cells can often grow to form a multilayered cell mass [40,111,142]. Effects other than growth inhibition have also been observed in dense cell culture. For example, Käsehagen and co-workers [59] found in a study with high-density cultures of hybridomas (which are not anchorage dependent) that the metabolic activity and specific antibody productivity (with regard to viable cells) were each greater than at low cell density. Many of the observed phenomena may be a consequence of the depletion or accumulation of various substances (such as metabolic intermediates or hormones) within cell aggregates [33]. The observation that plant cells differentiate preferentially in the center of aggregates might also be explained in this manner [83].

There have also been many reports of modifications of the properties of microorganisms upon entrapment. Study of this issue is especially troublesome because of the spatial heterogeneities caused by mass transport limitations in dense cell cultures. In particular, it is difficult to distinguish the consequences

of physical cell-to-cell contact from the effects of variations in the chemical composition of the immediate environment surrounding the cells. In some cases, the solute concentration variations alone are sufficient to explain the properties of entrapped microorganisms. For example, in a series of experiments with membrane-entrapped *Escherichia coli* and *Pseudomonas putida*, the observed growth rates and yields have been shown to be consistent with those obtained in conventional suspension culture, provided that the influence of diffusional limitations on the transport of substrates and products is taken into account [97–99,117,143,144].

Karel and Robertson [98] have proposed that the bacterial response to the physical stresses induced by growth in a confined volume might be similar to the response to osmotic stress. Bacteria employ active transport to sustain an internal hydrostatic (turgor) pressure of as much as 5 atmospheres [145], and it is reasonable to suppose that this capability could be used in an attempt to counteract a physical constraint on cell volume expansion. Consequently, the observable response to volume limitation might include a decreased growth rate or yield as metabolic energy is diverted to active transport and an eventual decreased rate of metabolism as intracellular solutes reach inhibitory levels.

3. Mechanical Damage to Membranes

Cell growth and carbon dioxide accumulation can generate sufficient pressure to cause physical damage to membranes. Stewart [146] made direct measurements of the pressure exerted by *E. coli* entrapped in a membrane reactor and obtained values greater than 3 atmospheres. Asymmetric membranes are particularly prone to disruption, and even isotropic hollow fibers can collapse under the pressure exerted by the surrounding biomass. Moreover, cells have been reported to grow through pores smaller than themselves [3]. These difficulties are typically not a concern with animal cells but have been encountered with a wide variety of bacterial, fungal, and plant cells [1,3,11,43,44,65,69,70].

One approach to resolving this problem is simply to employ membranes of greater strength, such as those constructed using ceramic materials. Another technique is to deprive the cells of a nutrient required for growth but not for product synthesis. In general, attempts to implement such a strategy for unagitated reactors have been only partially successful [45,69,147]. For example, Inloes and co-workers [147] grew *S. cerevisiae* in a hollow-fiber reactor and then changed to a nitrogen-deficient medium to curtail growth. Unfortunately, ethanol production with this medium was reduced to 10% of the previous rate, and periodic perfusion with the complete medium only partially restored productivity. An inherent problem with deliberate nutrient limitation in unagitated reactors is that the extent of deprivation is almost certainly not uniform in consequence of transport limitations. Greater success has been achieved with this strategy when an agitated membrane reactor is used [137].

Another proposed solution is the use of specific inhibitors of DNA synthesis or temperature-sensitive mutants that are blocked in DNA replication at high temperature [11]. Inhibitors that have been used (with varying degrees of success) in different immobilized-cell systems to limit growth include azide, arsenate, dinitrophenol, hydroxyurea, and novobiocin [33]. Substrate inhibition at high glucose concentrations [148], product inhibition by organic acids [117], and operation at reduced temperature [144] have also been investigated. Some of these approaches may be useful for a particular situation, but none provides a universally applicable method to limit growth while maintaining productivity.

The problem of excess microbial growth can also be resolved by bleeding biomass out of the cell compartment [149–151]. This is typically achieved by ultrafiltration of medium across the membranes and through the cell mass, either continuously or intermittently. This technique is also commonly employed to limit cell density and maintain viability in hollow-fiber reactors used for anchorage-independent animal cells [63].

C. Monitoring and Control

The monitoring and control of membrane reactors is the final challenge that we discuss in this chapter. This issue is of particular consequence in the highly regulated pharmaceutical industry because of the requirements for formal process specification and validation. In this context, validation is considered to include both justification of the process specification and a demonstration that the process is consistently run according to that specification [152]. As a result of these requirements, extensive monitoring and control are essential during both process development and manufacturing.

In this regard, membrane reactors present a particular challenge because there are generally local variations in both the physicochemical environment and the activity of entrapped enzymes or cells [61]. This drawback is to some extent balanced by the ability to perfuse the reactors continuously, which may result in a process that is temporally more constant (albeit spatially more heterogeneous) than conventional batch and fed-batch processes. The difficulties associated with process monitoring are primarily associated with reactors containing living cells (rather than those used for enzymes) and accordingly we restrict discussion to these applications.

Sophisticated systems for the monitoring and control of substrate and metabolite concentrations have been developed by the suppliers of commercially available hollow-fiber reactors [6,63,95,134]. The techniques used by Endotronics, Inc. (Minneapolis, MN) have been described in some detail by Hirschel and Gruenberg [134]. The pH and dissolved oxygen tension are monitored by on-line probes installed in the reactor influent and effluent lines. Glucose, lactate, glutamine, ammonia, and product assays are performed off line, and the results

are entered into the control computer, which integrates these data with the on-line measurements. The pH and the concentrations of oxygen, glucose, and lactate are regulated by a hierarchy of control actions, including gas blending, medium dilution, and the addition of glucose and base solutions.

Whereas it is comparatively straightforward to monitor and control fluid streams in this manner, measurement of the quantity, distribution, and state of the biomass is difficult. Measurements of the effluent concentrations of normally intracellular enzymes such as lactate dehydrogenase are routinely employed to assess cell lysis, but examination of the biomass itself is a challenge. If non-adherent cells are employed, it may be possible to sample the cell compartment directly [12] or to sacrifice one section of the reactor for analysis periodically [39]. It is generally not possible, however, to obtain a representative sample unless the cell compartment is stirred [74] or the cells are recycled through an external circuit [151]. In consequence, indirect monitoring techniques are usually employed. The most common approach is to monitor the production of lactate or the depletion of oxygen or glucose [57,139,153]. These techniques are particularly prone to error because the yield coefficients are not constant. For example, Chresand and co-workers [153] used glucose utilization to estimate biomass content in hollow-fiber reactors employed for the culture of Ehrlich ascites tumor cells. The cell density estimated in this manner using data from experiments with spinner flasks was about half that predicted on the basis of oxygen limitation, suggesting that the value of the yield coefficient was inappropriate.

The monitoring of sulfur incorporation may provide improved estimates because the yield coefficient is essentially constant for a wide range of growth conditions, at least for bacteria such as *E. coli* and *P. putida* [65,97–99]. Karel and Robertson [97] have used pulse labeling techniques with radioactive tracers such as $^{35}SO_4^{2-}$ to estimate cell growth and degradation from the tracer uptake and release kinetics, respectively. Residence time distribution studies using either radiolabeled or colored tracers can also be employed to determine the volume accessible to various species, which can be used to estimate the reactor biomass content. These tracer techniques are clearly inappropriate in manufacturing but are valuable during process development.

Measurement of conductivity or permittivity provides another set of non-destructive techniques for estimating cell density [154] and could be applied in manufacturing if reactors were built to incorporate the necessary electrodes. Widespread application of this approach is contingent on a demonstration of the reliability of the technique applied to various types of cells forming potentially heterogeneous cell aggregates.

The most powerful technique for monitoring the cell compartment within a membrane reactor is nuclear magnetic resonance (NMR) spectroscopy. Hollow-fiber reactors can be inserted directly into the probe of an NMR spectrometer

and perfused with nutrient medium. ^{31}P NMR spectroscopy allows online monitoring of pH, cellular energy levels, and a wide variety of metabolites [143,144]. Other applications of NMR include the measurement of intracellular ion activities, enzyme kinetics, and the metabolism of labeled tracers [155]. NMR spectroscopy has the potential to become a powerful tool for process development, although its expense and complexity preclude its use for the foreseeable future in a manufacturing environment.

IV. CONCLUSIONS

In this chapter we have described some of the features of membrane reactors that make their inclusion in the design of a biochemical process particularly attractive. We have also discussed some of the problems that will provide a challenge during process development and scale-up. Membrane reactors for enzymatic reactions have been used at the production scale in industry [156], and their prospects are promising because the equipment can be employed repeatedly for long-term operation. The use of membranes is particularly attractive for two-phase systems because many of the difficulties associated with emulsification and phase separation can be avoided.

On the other hand, equipment costs are comparatively high for membrane reactors employed with living cells because repeated use is rarely possible. As a result, industrial application of membrane reactors for the production of relatively low-value products using microorganisms is unlikely. This dim prognosis applies to both primary and secondary metabolites from bacteria, yeasts, or molds. A field in which exceptions may arise is that of microbial bioconversions, particularly under circumstances where an organic solvent is employed because of poor aqueous solubility of the substrate.

The use of membrane reactors for animal cell culture is somewhat more promising because the equipment cost may be offset to some extent by the impact on downstream processing of reduced serum use and in situ concentration of the product. Major drawbacks, however, include the possibility of spatial variations in the environment (which may influence product quality) and the difficulties associated with process monitoring. For these reasons, culture in agitated vessels is likely to remain the strategy of choice at the production scale, although membrane reactors may find application for situations wherein features such as protein retention, high surface area, and long-term culture maintenance and protein production are especially desirable.

PROBLEMS

1. Is it possible for a hollow-fiber enzyme reactor to maintain a nearly constant conversion, while simultaneously losing catalytic activity due to enzyme inactivation? Explain your answer. Discuss how hollow-fiber reactor design would differ for a stable enzyme with a half-life of 100 days and for one with a comparatively short half-life of 5 days.

2. Hybridomas grown in a hollow-fiber reactor typically achieve a cell density of 2×10^8 cells/ml and have an oxygen uptake rate of 0.1 pmol/cell/h [101]. Close-packed bacteria achieve a cell concentration of 300 g dry wt/L. The respiration rates of various bacteria vary between 1 and 600 mmol/g dry wt/h [46]. Assume that the effective diffusive permeability of oxygen in a dense layer of cells is one-fourth that in water. Consider a simple hollow-fiber reactor through the lumina of which we pass an aqueous medium that has been aerated in an external oxygenator. What is the depth of the region of entrapped cells that could be sustained by the oxygen in the medium? How would the results change if air is supplied through separate channels? Explain the assumptions that you make. Discuss your answer in the context of the design of different membrane reactors for the growth of bacteria and animal cells.

3. Consider a reactor containing a set of hollow fibers such as that depicted in Fig. 1. Each fiber has length L = 100 mm, lumen radius ρ_1 = 0.5 mm, and outer radius ρ_2 = 0.75 mm, and is surrounded by a region of radius ρ_3 = 1.25 mm wherein an enzyme solution is entrapped. An aqueous feed solution containing a single limiting substrate with concentration s_{in} = 1 mM is delivered to the fiber lumina. The plot presented in Fig. 4 is obtained from a mathematical analysis of reaction and diffusion in this reactor. The parameters in the plot are $Da_D = (\rho_3^2 - \rho_2^2)e\mathcal{R}(s_{in})/\mathcal{D}s_{in}$ and $z = \pi L\mathcal{D}/q_{in}$, where e is the entrapped enzyme concentration (g/L), q_{in} is the feed flow rate per fiber (mm³/s), $\mathcal{D} = 10^{-9}$ m²/s is the diffusivity of the substrate in the aqueous feed solution, and $\mathcal{R}(s_{in}) = 10^{-3}$ mol/g/s is the specific enzymatic reaction rate at the influent substrate concentration.

Assuming that the enzyme is stable, discuss the specification of the enzyme concentration, e, to be used in this reactor. What issues need to be considered? How would the substrate conversion change if the enzyme concentration was increased or decreased 10-fold from the value that you recommend? Discuss the assumptions that you make. How would your recommendation change if the half-life of the enzyme was comparatively short?

To achieve 90% conversion of substrate in a single pass, what feed flow rate per fiber, q_{in}, should be employed (using the enzyme concentration you recommended above)? An alternative would be to incorporate the reactor in a recycle circuit with an overall conversion of 90% (but with a smaller conversion per pass through the reactor). Discuss this strategy compared to the use of a single pass. Which approach would you recommend?

REFERENCES

1. T. B. Vick Roy, H. W. Blanch, and C. R. Wilke, *Trends Biotechnol. 1*: 135 (1983).
2. M. Nakajima, A. Watanabe, N. Jimbo, K. Nishizawa, and S. Nakao, *Biotechnol. Bioeng. 33*: 856 (1989).

3. C. R. Robertson and I. H. Kim, *Biotechnol. Bioeng. 27*: 1012 (1985).
4. H. P. Bennetto, J. L. Stirling, K. Tanaka, and C. A. Vega, *Biotechnol. Bioeng. 25*: 559 (1983).
5. M. L. Shuler, *Methods Enzymol. 135*: 372 (1987).
6. J. Hopkinson, *Biotechnology 3*: 225 (1985).
7. M. L. Shuler, *Membrane Separations in Biotechnology* (W. C. McGregor, ed.), Marcel Dekker, New York, 1986, p. 303.
8. T. Cho and M. L. Shuler, *Biotechnol. Prog. 2*: 53 (1986).
9. R. A. Knazek, P. M. Gullino, P. O. Kohler, and R. L. Dedrick, *Science 178*: 65 (1972).
10. L. R. Waterland, C. R. Robertson, and A. S. Michaels, *Chem. Eng. Commun. 1*: 37 (1975).
11. D. S. Inloes, W. J. Smith, D. P. Taylor, S. N. Cohen, A. S. Michaels, and C. R. Robertson, *Biotechnol. Bioeng. 25*: 2653 (1983).
12. G. L. Altshuler, D. M. Dziewulski, J. A. Sowek, and G. Belfort, *Biotechnol. Bioeng. 28*: 646 (1986).
13. P. R. Rony, *Biotechnol. Bioeng. 13*: 431 (1971).
14. C. Georgakis, P. C.-H. Chan, and R. Aris, *Biotechnol. Bioeng. 17*: 99 (1975).
15. E. Adema and A. J. Sinskey, *Biotechnol. Prog. 3*: 74 (1987).
16. K. Kawakami, T. Hamada, and K. Kusunoki, *Enzyme Microb. Technol. 2*: 293 (1980).
17. H. Kataoka, T. Saigusa, S. Mukataka, and J. Takahashi, *J. Ferment. Technol. 58*: 431 (1980).
18. L. R. Waterland, Enzyme Immobilization Using Asymmetric Hollow Fiber Membranes, Ph.D. dissertation, Stanford University, 1975.
19. R. Aris, *The Mathematical Theory of Diffusion and Reaction in Permeable Catalysts.* Vol. I. *The Theory of the Steady State*, Clarendon Press, Oxford, 1975.
20. E. Drioli, G. Iorio, A. Gambacorta, and B. Nicolaus, *J. Membr. Sci. 11*: 365 (1982).
21. G. Catapano, G. Iorio, E. Drioli, and M. Filosa, *J. Membr. Sci. 35*: 325 (1988).
22. W. R. Vieth, S. S. Wang, and R. Saini, *Biotechnol. Bioeng. 15*: 565 (1973).
23. M. de Rosa, A. Gambacorta, E. Esposito, E. Drioli, and S. Gaeta, *Biochimie 62*: 517 (1980).
24. E. Drioli, G. Iorio, R. Molinari, M. de Rosa, A. Gambacorta, and E. Esposito, *Biotechnol. Bioeng. 23*: 221 (1981).
25. E. Drioli, G. Iorio, M. de Rosa, A. Gambacorta, and B. Nicolaus, *J. Membr. Sci. 11*: 365 (1982).
26. H. P. Gregor and P. W. Rauf, *Biotechnol. Bioeng. 17*: 445 (1975).
27. M. Scholz and W.-S. Hu, *Cytotechnology 4*: 127 (1990).
28. H. Ishikawa, S. Takase, T. Tanaka, and H. Hikita, *Biotechnol. Bioeng. 34*: 369 (1989).
29. J. P. Tharakan, S. L. Gallagher, and P. C. Chau, *Advances in Biotechnological Processes.* Vol. 7. *Upstream Processes: Equipment and Techniques* (A. Mizrahi, ed.), Alan R. Liss, New York, 1988, p. 153.
30. Y. B. Hwang, B. H. Chung, H. N. Chang, and M. H. Han, *Bioprocess Eng. 3*: 159 (1988).

31. H. S. Jee, T. Yano, N. Nishio, and S. Nagai, *J. Ferment. Technol. 65*: 413 (1987).
32. T. Yano, K. Aoki, and S. Nagai, *J. Ferment. Biotechnol. 71*: 203 (1991).
33. S. F. Karel, S. B. Libicki, and C. R. Robertson, *Chem. Eng. Sci. 40*: 1321 (1985).
34. M. Cheryan and M. A. Mehaia, *Membrane Separations in Biotechnology* (W. C. McGregor, ed.), Marcel Dekker, New York, 1986, p. 255.
35. M. D. Lilly and P. Dunnill, *Methods Enzymol. 44*: 717 (1976).
36. J. E. Prenosil and T. Hediger, *Desalination 53*: 265 (1985).
37. C. K. S. Jones, R. Y. K. Yang, and E. T. White, *AIChE J. 34*: 293 (1988).
38. K. Ku, M. J. Kuo, J. Delente, B. S. Wildi, and J. Feder, *Biotechnol. Bioeng. 23*: 79 (1981).
39. J. M. Piret and C. L. Cooney, *Biotechnol. Adv. 8*: 763 (1990).
40. M. A. Tyo and R. E. Spier, *Enzyme Microb. Technol. 9*: 514 (1987).
41. P. Calabresi, K. L. McCarthy, D. L. Dexter, F. J. Cummings, and B. Rotman, *Proc. Am. Assoc. Cancer Res. 2*: 302 (1981).
42. D. Riesenberg, *Curr. Opin. Biotechnol. 2*: 380 (1991).
43. B. H. Chung, H. N. Chang, and I. H. Kim, *Enzyme Microb. Technol. 9*: 345 (1987).
44. D. S. Inloes, D. P. Taylor, S. N. Cohen, A. S. Michaels, and C. R. Robertson, *Appl. Environ. Microbiol. 46*: 264 (1983).
45. T. B. Vick Roy, H. W. Blanch, and C. R. Wilke, *Biotechnol. Lett. 4*: 483 (1982).
46. G. F. Payne, M. L. Shuler, and P. Brodelius, *Large Scale Cell Culture Technology* (B. K. Lydersen, ed.), Hanser Publishers, Munich, 1987, p. 193.
47. Y. Yamazaki, H. Maeda, and H. Suzuki, *Biotechnol. Bioeng. 18*: 1761 (1976).
48. R. Wichmann, C. Wandrey, A. F. Bückmann, and M. R. Kula, *Biotechnol. Bioeng. 18*: 1761 (1976).
49. Y. Morikawa, I. Karube, and S. Suzuki, *Biochim. Biophys. Acta 523*: 263 (1978).
50. A. F. Bückmann, M.-R. Kula, R. Wichmann, and C. Wandrey, *J. Appl. Biochem. 3*: 301 (1981).
51. W. Hummel, H. Schütte, E. Schmidt, C. Wandrey, and M.-R. Kula, *Appl. Microbiol. Biotechnol. 26*: 409 (1987).
52. W. Berke, H.-J. Schüz, C. Wandrey, M. Morr, G. Denda, and M.-R. Kula, *Biotechnol. Bioeng. 32*: 130 (1988).
53. G. Greco, Jr., F. Veronese, R. Largajolli, and L. Gianfreda, *Eur. J. Appl. Microbiol. Biotechnol. 18*: 333 (1983).
54. T. Shiomi, M. Tohyama, M. Satoh, M. Miya, and K. Imai, *Biotechnol. Bioeng. 32*: 664 (1988).
55. M. Vasudevan, T. Matsuura, G. K. Chotani, and W. R. Vieth, *Sep. Sci. Technol. 22*: 1651 (1987).
56. H. P. Bennetto, G. M. Delaney, J. R. Mason, S. D. Roller, J. L. Stirling, and C. F. Thurston, *Biotechnol. Lett. 7*: 699 (1986).
57. W.-S. Hu and T. C. Dodge, *Biotechnol. Prog. 1*: 209 (1985).
58. A. H. Heifetz, J. A. Braatz, R. A. Wolfe, R. M. Barry, D. A. Miller, and B. A. Solomon, *Biotechniques 7*: 192 (1989).
59. C. Käsehagen, F. Linz, G. Kretzmer, T. Scheper, and K. Schügerl, *Enzyme Microb. Technol. 13*: 873 (1991).
60. G. Belfort, *Biotechnol. Bioeng. 33*: 1047 (1989).

61. R. van Erp, M. Adorf, A. P. G. van Sommeren, O. T. Schönherr, and T. C. J. Gribnau, *J. Biotechnol. 20*: 235 (1991).
62. W. R. Tolbert, C. Lewis, Jr., P. J. White, and J. Feder, *Large-Scale Mammalian Cell Culture* (J. Feder and W. R. Tolbert, eds.), Academic Press, London, 1985, p. 97.
63. P. C. Brown, M. A. C. Costello, R. Oakley, and J. L. Lewis, *Large-Scale Mammalian Cell Culture* (J. Feder and W. R. Tolbert, eds.), Academic Press, London, 1985, p. 59.
64. J. Hopkinson, *Large-Scale Mammalian Cell Culture* (J. Feder and W. R. Tolbert, eds.), Academic Press, London, 1985, p. 151.
65. W. J. Smith, P. M. Salmon, and C. R. Robertson, 196th ACS National Meeting, Los Angeles, 1988.
66. M. Hashimoto, T. Tsukamoto, S. Morita, and J. Okada, *Chem. Pharm. Bull. 31*: 1 (1983).
67. H. N. Chang, Y. S. Kyung, and B. H. Chung, *Biotechnol. Bioeng. 29*: 552 (1987).
68. P. M. Salmon, Mass Transport Phenomena in Reactors Containing Immobilized Cells or Enzymes, Ph.D. dissertation, Stanford University, 1989.
69. B. H. Chung and H. N. Chang, *Biotechnol. Bioeng. 32*: 205 (1988).
70. B. H. Chung and H. N. Chang, *J. Ferment. Biotechnol. 69*: 175 (1990).
71. H. N. Chang, B. H. Chung, and I. H. Kim, *ACS Symp. Ser. 314*: 32 (1986).
72. I. A. Belushkina, R. J. Manolov, E. D. Vorobiev, and A. M. Bezborodov, *Folia Microbiol. 36*: 75 (1991).
73. V. Jäger, H. Büntemeyer, and J. Lehmann, *Proceedings of 4th European Congress on Biotechnology* (O. M. Neijssel, R. R. van der Meer, and K. Ch. A. M. Luyben, eds.), Elsevier Science, Amsterdam, 1987, p. 581.
74. T. Ryll, M. Lucki-Lange, V. Jäger, and R. Wagner, *J. Biotechnol. 14*: 377 (1990).
75. R. Wagner and J. Lehmann, *Trends Biotechnol. 6*: 101 (1988).
76. J. Lehmann, H. Büntemeyer, and V. Jäger, *Food Biotechnol. 4*: 423 (1990).
77. T. Yamane, M. M. Hoq, S. Shimizu, S. Ishida, and T. Funada, *Ann. N.Y. Acad. Sci. 434*: 558 (1984).
78. M. M. Hoq, H. Tagami, T. Yamane, and S. Shimizu, *Agric. Biol. Chem. 49*: 333 (1985).
79. A. M. Vaidya, G. Bell, and P. J. Halling, *J. Membr. Sci. 71*: 139 (1992).
80. G. T. Frank and K. K. Sirkar, *Biotechnol. Bioeng. Symp. 17*: 303 (1986).
81. G. S. Efthymiou and M. L. Shuler, *Biotechnol. Prog. 3*: 259 (1987).
82. M. Reuß, *Chem. Eng. Technol. 11*: 178 (1988).
83. M. L. Shuler, *Ann. N.Y. Acad. Sci. 369*: 65 (1981).
84. R. Cherry and E. T. Papoutsakis, *Bioprocess Eng. 1*: 29 (1986).
85. A. Prokop and R. K. Bajpai, *Adv. Appl. Microbiol. 37*: 165 (1992).
86. K. T. Kunas and E. T. Papoutsakis, *Biotechnol. Bioeng. 36*: 476 (1990).
87. V. L. Yarovenko, *Adv. Biochem. Eng. 9*: 1 (1978).
88. H. Kurosawa, H. Märkl, C. Niebuhr-Redder, and M. Matsumura, *J. Ferment. Biotechnol. 72*: 41 (1991).
89. J. B. K. Nielsen and L. Kaplan, *J. Antibiot. 42*: 944 (1989).
90. H. Pedersen, C. K. Chin, and K. Venkatasubramanian, *ACS Symp. Ser. 389*: 193 (1989).

91. G. S. David, R. A. Reisfeld, and T. H. Chino, *J. Natl. Cancer Inst. 60*: 303 (1978).
92. T. L. Evans and R. A. Miller, *Biotechniques 6*: 762 (1988).
93. E. H. Starling, *J. Physiol. (Lond.) 19*: 312 (1896).
94. J. M. Piret and C. L. Cooney, *Biotechnol. Bioeng. 36*: 902 (1990).
95. A. Handa-Corrigan, S. Nikolay, D. Jeffery, B. Heffernan, and A. Young, *Enzyme Microb. Technol. 14*: 58 (1992).
96. J. E. Prenosil and T. Hediger, *Biotechnol. Bioeng. 31*: 913 (1988).
97. S. F. Karel and C. R. Robertson, *Biotechnol. Bioeng. 34*: 320 (1989).
98. S. F. Karel and C. R. Robertson, *Biotechnol. Bioeng. 34*: 337 (1989).
99. P. S. Stewart, S. F. Karel, and C. R. Robertson, *Biotechnol. Bioeng. 37*: 824 (1991).
100. D. W. Barnes, D. A. Sirbasku, and G. Sato, *Methods for Preparation of Media, Supplements, and Substrata for Serum-Free Animal Cell Culture*, Alan R. Liss, New York, 1984.
101. J. M. Piret and C. L. Cooney, *Biotechnol. Bioeng. 37*: 80 (1991).
102. R. A. Knazek, *Fed. Proc. 3*: 1978 (1974).
103. S. F. Karel, C. A. Briasco, and C. R. Robertson, *Ann. N.Y. Acad. Sci. 506*: 84 (1987).
104. S. L. Paterson, A. G. Fane, C. J. D. Fell, and P. L. Rogers, *Biotechnol. Lett. 8*: 561 (1986).
105. J. P. Tharakan and P. C. Chau, *Biotechnol. Bioeng. 28*: 1064 (1986).
106. J. K. Park and H. N. Chang, *AIChE J. 32*: 1937 (1986).
107. J. M. Engasser, J. Caumon, and A. Marc, *Chem. Eng. Sci. 35*: 99 (1980).
108. A. Marc, J. Caumon, and J. M. Engasser, *Adv. Biotechnol. 1*: 705 (1981).
109. B. J. Pangrle, E. G. Walsh, S. Moore, and D. Dibiasio, *Biotechnol. Tech. 3*: 67 (1989).
110. B. E. Hammer, C. A. Heath, S. D. Mirer, and G. Belfort, *Biotechnology 8*: 327 (1990).
111. J. P. Tharakan and P. C. Chau, *Biotechnol. Bioeng. 28*: 329 (1986).
112. C. Horvath, L. H. Shendalman, and R. H. Light, *Chem. Eng. Sci. 28*: 375 (1973).
113. P. M. Salmon and C. R. Robertson, *Chem. Eng. J. 35*: B1 (1987).
114. P. M. Salmon and C. R. Robertson, *Chem. Eng. J. 38*: 57 (1988).
115. P. M. Salmon, S. B. Libicki, and C. R. Robertson, *Chem. Eng. Commun. 66*: 221 (1988).
116. S. B. Libicki, P. M. Salmon, and C. R. Robertson, *Biotechnol. Bioeng. 32*: 68 (1988).
117. P. S. Stewart and C. R. Robertson, *Appl. Environ. Microbiol. 54*: 2464 (1988).
118. P. M. Salmon and C. R. Robertson, *J. Theor. Biol. 125*: 325 (1987).
119. P. Aldercreutz and B. Mattiasson, *Eur. J. Appl. Microbiol. Biotechnol. 16*: 165 (1982).
120. M. Fujimura, J. Kato, T. Tosa, and I. Chibata, *Appl. Microbiol. Biotechnol. 19*: 79 (1984).
121. A. R. Oller, C. W. Buser, M. A. Tyo, and W. G. Thilly, *J. Cell. Sci. 94*: 43 (1989).
122. A. K. Balkin, D. B. P. Goodman, H. Rasmussen, and V. J. Cristofalo, *J. Cell. Physiol. 78*: 390 (1978).
123. S. L. Paterson, A. G. Fane, C. J. D. Fell, U. H. Chun, and P. L. Rogers, *Biocatalysis 1*: 217 (1988).

124. B. R. Breslau and B. H. Kilcullen, *Enzyme Eng. 3*: 179 (1978).
125. C. K. S. Jones, E. T. White, and R. Y. K. Yang, *Ann. N.Y. Acad. Sci. 434*: 119 (1984).
126. E. A. Linton, C. J. Knowles, A. J. Bunch, and G. Higton, *Bioreactors and fine transformations* (G. W. Moody and P. B. Baker, eds.), Elsevier, Barking, UK, 1987, p. 299.
127. R. A. Knazek, P. M. Gullino, and D. S. Frankel, U.S. Patent No. 4,206,015, 1980.
128. S. Furusaki, T. Kojima, and T. Miyauchi, *J. Chem. Eng. Jpn. 10*: 233 (1977).
129. S. Furusaki and T. Miyauchi, *J. Chem. Eng. Jpn. 14*: 479 (1981).
130. I. H. Kim and H. N. Chang, *AIChE J. 29*: 645 (1983).
131. I. H. Kim and H. N. Chang, *AIChE J. 29*: 910 (1983).
132. T. H. Park, I. H. Kim, and H. N. Chang, *Biotechnol. Bioeng. 27*: 1185 (1985).
133. S. R. Reiken and D. M. Briedis, *Biotechnol. Bioeng. 35*: 260 (1990).
134. M. D. Hirschel and M. L. Gruenberg, *Large Scale Cell Culture Technology* (B. K. Lydersen, ed.), Hanser Publishers, Munich, 1987, p. 113.
135. J. J. Liu, B.-H. Chen, T.-F. Tsai, Y.-J. Wu, V. F. Pang, A. Hsieh, J.-H. Hsieh, and T. H. Chang, *Cytotechnology 5*: 129 (1991).
136. H. Graf and K. Schügerl, *Appl. Microbiol. Biotechnol. 35*: 165 (1991).
137. E. K. Kim, J. R. Ambriano, and R. S. Roberts, *Biotechnol. Bioeng. 30*: 805 (1987).
138. M. C. Porter and A. S. Michaels, *Chem. Technol. 1*: 59 (1970).
139. J. Feder, *Methods Enzymol. 135*: 393 (1987).
140. J. Van Brunt, *Biotechnology 4*: 505 (1986).
141. J. D. Legan and J. D. Owens, *Lett. Appl. Microbiol. 5*: 75 (1987).
142. P. F. Kruse and E. Miedema, *J. Cell Biol. 27*: 273 (1965).
143. C. A. Briasco, D. A. Ross, and C. R. Robertson, *Biotechnol. Bioeng. 36*: 879 (1990).
144. C. A. Briasco, C. R. Robertson, and S. F. Karel, *Biotechnol. Bioeng. 36*: 881 (1990).
145. A. L. Koch, *J. Bacteriol. 159*: 919 (1984).
146. P. S. Stewart, Physiology of Immobilized *Escherichia coli*, Ph.D. dissertation, Stanford University, 1988.
147. D. S. Inloes, A. S. Michaels, C. R. Robertson, and A. Matin, *Appl. Microbiol. Biotechnol. 23*: 85 (1981).
148. M. Wada, J. Kato, and I. Chibata, *Appl. Microbiol. Biotechnol. 11*: 67 (1981).
149. M. A. Mehaia and M. Cheryan, *Appl. Microbiol. Biotechnol. 20*: 100 (1984).
150. M. A. Mehaia and M. Cheryan, *Enzyme Microb. Technol. 6*: 117 (1984).
151. T. H. Park and I. H. Kim, *Appl. Microbiol. Biotechnol. 22*: 190 (1985).
152. P. M. Salmon and B. C. Buckland, *Proceedings of the 5th International Conference on Computer Applications in Fermentation Technology and 2nd IFAC Symposium on Modeling and Control of Biotechnical Processes* (M. N. Karim, ed.), Pergamon Press, Oxford, 1992, p. 1.
153. T. J. Chresand, R. J. Gillies, and B. E. Dale, *Biotechnol. Bioeng. 32*: 983 (1988).
154. T. Blute, R. J. Gillies, and B. E. Dale, *Biotechnol. Prog. 4*: 203 (1988).
155. R. J. Gillies, N. E. MacKenzie, and B. E. Dale, *Biotechnology 7*: 50 (1989).
156. C. Wandrey, *Proceedings of the International Conference on the Commercial Applications and Implications of Biotechnology*, Online Conferences Ltd., London, 1983, p. 577.

9

Immobilized Microorganism Bioreactors

Hideki Fukuda*

Kaneka Corporation
Kobe, Hyogo, Japan

I. INTRODUCTION

Over the past two decades there have been rapid developments in the use of enzymes as catalysts for industrial, analytical, and medical purposes, leading to the appearance of a new field of research known as enzyme technology. In order to make their use more convenient, enzymes have been immobilized in such a way that they resemble ordinary solid-phase catalysts used conventionally in synthetic chemical reactions. To be immobilized, enzymes have to be either extracted from the cell (for intracellular enzymes) or recovered from the culture broth (for extracellular enzymes); an additional purification operation is also generally required for the practical use of these enzymes. Enzymes recovered through such operations are generally unstable and expensive, and consequently there has been considerable interest in the direct use of immobilized cells as a means of catalysis. Studies of immobilized cells were initiated by immobilizing

Current affiliation: Kobe University, Kobe, Hyogo, Japan.

single enzymes, i.e., dead cells, for simple reactions; however, recently much attention has been directed toward the use of immobilized viable cells with multienzymes for more complicated or conjugated reactions. Thus, immobilized cells have been employed for the production of useful compounds by various types of bioreactions, such as transformation, synthesis, and degradation. Current representative applications of immobilized cells in industry are listed in Table 1.

The several advantages of immobilized cells include the following: (1) application to a multistep enzyme reaction may be possible, (2) the enzyme activity yield on immobilization is high, (3) operational stability is generally high, (4) operations for enzyme extraction and/or purification are unnecessary, (5) high cell densities can be employed, (6) cell densities and enzyme activities can be expected to be maintained over a long period of operation, (7) products can easily be recovered from immobilized cells, and (8) immobilized cells appear to be less susceptible to microbial contamination. At the same time, the following potential disadvantages associated with immobilized cells should be pointed out: (1) unwanted side reactions may be catalyzed by numerous catalytically active enzymes, (2) contamination by cells leaking from carriers may occur, and (3) the permeability of the substrate and product through the cell membrane may be limited. In spite of these drawbacks, immobilized cell systems have been extensively studied because of their attractive features and potential.

To adopt immobilized cell systems successfully on an industrial scale, an appropriate bioreactor design which can provide a suitable environment for immobilized cells under given requirements is necessary. The choice of a particular design for a given process depends on the requirements for mass transfer, the growth behavior of the cells, and the structural properties of the aggregate. Prolonged viability maintenance, reduced mass transfer limitations, and stabilization of immobilized cells are, therefore, essential for immobilized cell bioreactors. A great deal of effort has been expended in developing novel bioreactor designs, either by theoretical analysis or through experimental studies of mass transfer, intrinsic kinetics and hydrodynamics, etc. Several reactor configurations have been used successfully with immobilized cells, and many more have been proposed.

In the present chapter, several categories of immobilized cell technology and reactor configuration are discussed, and the concept of mass transfer based on external liquid-film and intraparticle diffusion resistances is considered.

Table 1 Current Application of Immobilized Microbial Cells in Industry

Application	Immobilized cells	Operating since
Enzyme		
Production of L-aspartic acid	*Escherichia coli* (aspartase)	1973
Production of L-malic acid	*Brevibacterium amoniagenes* (fumarase)	1974
Production of L-alanine	*Pseudomonas dacuhae* (L-aspartate β-decarboxylase)	1982
Viable cells		
Production of ethanol (pilot plant)	*Saccharomyces* sp.	1982
Production of acrylamide	*Corynebacterium* sp. (nitrilase)	1985

II. METHODS FOR THE IMMOBILIZATION OF MICROBIAL CELLS

Numerous cell immobilization methods have been demonstrated, and these have been reviewed by many authors [1–11]. In principle, the methods can be divided into two major categories based on active and passive techniques [12]. Figure 1 illustrates this classification system and lists the various individual methods that are discussed in detail in subsequent sections. Active immobilization can be accomplished by various chemical agents, whereas passive immobilization occurs when films or flocs of cells form naturally around or within support material provided for that purpose or when cell adhesion to a solid surface occurs through electrostatic interaction.

In general, it is rather difficult to determine precisely the optimal cell immobilization method for a particular purpose, although the relationship between the efficiency of the reaction and the physicochemical properties in each method is becoming increasingly clear. In practice, the most suitable method is generally achieved on a trial-and-error basis.

A. Active Immobilization

In this category, cross-linking of cells to each other, covalent bonding to surfaces, and entrapment within gels can be included. However, in the first two methods, cross-linking reagents such as glutaraldehyde [13,14] or toluene diisocyanate [13] and coupling agents such as polyisocyanate [15] or carbodiimide are used, resulting in the loss of both enzyme activity and viability because of the toxicity of these agents. With the entrapment method, no binding between cells and the support material should occur, and preparations should provide a high level of activity retention. Using nontoxic materials such as calcium alginate or κ-carrageenan, this method has been applied successfully in many cases.

1. Covalent Bonding

Covalent coupling is the direct linkage of cells to an activated support. Each of the various coupling agents, such as polyisocyanate or carbodiimide, places a

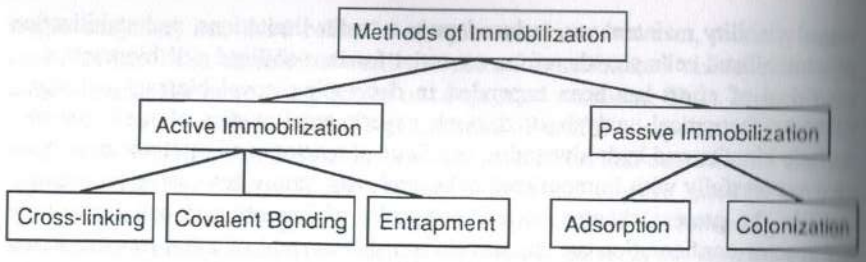


Figure 1 Classification of cell immobilization methods.

specific group on the support surface, and these groups later react with reactive groups on the cell surface. The main advantages of this technique are that it offers a system free of the diffusion limitations present in entrapment processes and cells which are bound to a uniform surface by a bond that is stable for long periods so that cell leakage is minimized. The chief disadvantage is that the coupling reagents can severely damage both the activity and viability of the cells.

There have been several reports on the use of this method in cell immobilization, with either inorganic [16,17] or organic [18,19] supports. In the case of organic supports, carboiimide reagents have been used; *Micrococcus luteu* ATCC 9341 were covalently linked to carboxymethylcellulose, activated with carboiimide [2] in a two-step process that avoided exposure of the cells to carboiimide. Thus, the urocanic acid–forming activity was still retained after cell viability had been lost.

2. Entrapment in Gels

The entrapping method is based on the occlusion of cells within the lattice of a polymer matrix tightly enough to prevent their release, while allowing the penetration of substrates and diffusion of products. This method differs from the chemical coupling methods in that the biocatalyst itself does not bind to the gel matrix. Thus, it can be most generally applied to entrap any kind of biocatalyst, e.g., enzymes, whole cells, and organelles, whether animal or plant cells, with little destruction of their biological activity as compared with the chemical coupling methods.

As shown in Table 2, various synthetic polymers, polysaccharides, and proteins can be used in entrapment. Among these, the most commonly used methods are described below.

Polyacrylamide. The first gel used to entrap living microbial cells was polyacrylamide; this method is based on the free radical polymerization of acrylamide in an aqueous solution.

Table 2 Various Entrapping Materials for Microbial Cells

Synthetic polymers	Polyacrylamide
	Polymethacrylamide
	Photo-cross-linkable resin prepolymer
	Urethane prepolymer
	Polyethyleneglycol
	Polyvinyl alcohol
Polysaccharides	Alginate
	κ-Carrageenan
	Agar
	Chitosan
Proteins	Collagen
	Gelatin

In the gel entrapment method for whole cells, the polymerization of acrylamide is conducted in an aqueous solution containing the whole cells and a cross-linking agent (usually *N,N'*-methylenebisacrylamide, BIS). Polymerization is commonly carried out in the absence of oxygen and at a lower temperature in order to avoid cell damage during this operation. A very wide variation in gel composition, e.g., pore size and the fragility of the gel, can be achieved by varying the overall monomer composition and the ratio of acrylamide to cross-linking agent.

However, the acrylamide monomer, the cross-linking agent, and the initiator are toxic to microbial cells, and significant losses of cell activity and viability may consequently occur during entrapment. Thus, microbial cells should be exposed to these reagents for the shortest time possible.

The first application of immobilized cells using polyacrylamide gel was described by Chibata et al. in 1974 [13] for the production of aspartic acid using whole cells of *Escherichia coli* containing aspartase. Subsequently, they developed a similar process to make L-malic acid [20].

In 1985, a novel production process for acrylamide with immobilized cells of *Corynebacterium* sp. containing nitrilase was developed by Nitto Chemical Co. [21]. This was the first process to produce a commodity chemical using an immobilized biocatalyst. In comparison with the conventional synthetic process, which involves the hydration of nitriles with sulfuric acid or the use of copper salts as a catalyst, the following advantages are evident:

A high conversion yield is obtained (extremely low formation of by-products); a recovery process for nitriles is thus not necessary.
Hydration can be carried out under low-pressure, low-temperature conditions; it is therefore an easy and safe industrial operation.
The process investment and operating costs are low.

Photo-Cross-Linkable Resin Prepolymers and Urethane Prepolymers. Fukui and co-workers developed methods using photo-cross-linkable resin prepolymers and urethane prepolymers to make gel-entrapped biocatalysts of the desired hydrophobicity that are applicable to the transformation of hydrophobic substances in an organic solvent system.

The activity in converting 3β-hydroxy-Δ5-steroids to corresponding 3-keto-Δ4-steroids in a water-insoluble organic solvent system using cells of *Nocardia rhodocrous* immobilized with photo-cross-linkable resin prepolymers or urethane prepolymers either hydrophilic or hydrophobic in character has been studied [22]. Although both hydrophilic and hydrophobic gel-entrapped cells could transform dehydroepiandrosterone to 4-androstene-3,17-dione, the latter showed significantly higher activity than the former. The activity of the gel-entrapped cells was found to correspond closely to the partition coefficients of substrates between the gels and the external solvent.

In synthesis of the water-insoluble adenine arabinoside from uracil arabinoside and adenine, cells of *Enterobactor aerogenes* entrapped with ENT-4000 or a urethane prepolymer, PU-6, had a high and stable transglycosylation activity. The immobilized cells with these prepolymers exhibited a markedly improved operational stability, retaining their initial level of activity during repeated use for at least 35 days at 60°C in 40% dimethyl sulfoxide [23].

Other examples of this method include conversion of cortexolone to hydrocortison using entrapped *Curvularia lunata* [24,25], and regio-specific interesterification of triglyceride in *n*-hexane solvent using entrapped *Rhizopus delemar* [26].

Thus, the advantages of the entrapment of microbial cells with photo-crosslinkable resin prepolymers and urethane prepolymers lie not only in the simplicity and convenience of these methods, but also in the protective effect of gels against denaturation by an organic solvent and in an improvement in the operational stability of the activity.

Calcium Alginate. Alginate, which is extracted from seaweed and is a linear block copolymer of D-manuronic and L-guluronic acids, can be gelled by multivalent ions, usually calcium and aluminum.

The process of immobilization includes the preparation of a solution of sodium alginate, the addition of the cell mass, and the dispersion of this mixture into a counter-ion solution, which results in the formation of a uniform, spherical, highly microporous structure that retains cells, organelles, and enzyme molecules larger than pores. The pores are large and continuous, such that substrate molecules may diffuse throughout the beads.

The mechanical properties of alginate gels are related to the distribution of the D-mannuronic and L-guluronic residues and to the molecular weight and

degree of dispersion within the pellets. The pellets show excellent resistance to hydrostatic pressures, as was reported by Cheetham [27] when measuring low intrinsic pressure drops of pellets in packed bed columns.

However, the employment of this polymer has been limited due to the instability of the calcium alginate gel upon contact with various complexing anions, such as phosphate, citrate, EDTA, and lactate. Several reports have described methods for improving the physical and chemical stability of alginate gel. These include increasing the alginate and calcium ion concentration [28], inducing gelation with cations having higher affinity for alginate [29], drying the entrapped preparation [30], and treatment with glutaraldehyde and hexamethylenediamine [31]; by using such methods it was possible to produce beads with enhanced mechanical stability. Birnbaum et al. [32] investigated three methods which involve covalent cross-linking of preparations with a polyamine, polyethyleneimine, using glutaraldehyde, or preactivating the alginate solution with periodate or a water-soluble carbodiimide followed by treatment with polyethyleneimine. They concluded that oxidation of the alginate solution is probably the best method, as it is simple and significantly cheaper than treatment with carbodiimide.

Tanaka et al. [33] developed a double gel layer method for prevention of cell leakage from the gel matrix. Cells entrapped in alginate fibers with double gel layers using a double nozzle are restricted to the inner layer only, while the outer layer helps to prevent cell leakage. Normal ethanol production was maintained for 300 h without cell leakage from the polymeric matrix.

Coimmobilization systems for bimicroorganisms and for microorganism and substrate using alginate gel beads have been investigated. A desirable coimmobilized mixed culture system, in which aerobic *Aspergillus awamori* grew on and near the oxygen-rich surface of gel beads while anaerobic *Zymomonas mobilis* grew mainly in the central part of the beads, has been developed, and the ethanol productivity from starch was greatly enhanced by this system [34]. Kaul et al. [35] coimmobilized steroids with *Arthrobactor simplex* in calcium alginate gel. This method may be applied to cases in which contact between the biocatalyst and organic solvent is unacceptable.

To improve the overall efficiency of immobilized cells within alginate gel, several production methods for microgel beads have been proposed. Klein et al. [36] describe a simple means of producing alginate beads with diameters of 0.5–3.0 mm, using a tube through which the sodium alginate is passed and a slightly larger coaxial tube. The size of the droplets of alginate is controlled by blowing them off the outlet by passing compressed air through the outer tube. Ogbonna et al. [37] also describe a method for the immobilization of viable cells in microgel beads (200–1200 μm in diameter) by atomization of a sodium alginate–cell mixture with a rotating disk atomizer. By immobilizing viable baker's yeast cells in beads 500 μm in diameter, the effectiveness factor for oxygen consumption

was six times higher than for cells immobilized in beads 3.2 mm in diameter. Hulst et al. [38] developed a new technique based on the breakup of a jet of biocatalyst-presupport mixture in uniform droplets by means of mechanical vibration. The principle of the technique is as follows. The alginate-biocatalyst mixture is forced through the exit of a resonance nozzle at such a speed that a jet is formed. When the signal of a sine wave generator is transferred to the liquid in the nozzle, it causes an instability in the jet. Within a certain range of frequences, this disturbance breaks the jet into drops of uniform size. The production capacity using a nozzle of 1.1 mm in diameter is two orders of magnitude larger than that of the conventional dropping method, and the bead size varies between 1 and 2 mm, with a standard deviation of 3–5% for yeast immobilization.

An industrial process using viable *Saccharomyces* yeast cells entrapped in calcium alginate for ethanol production was reported by Oda et al. [39]. A pilot plant comprised of two reactor channels was designed; one channel consisted of two columns (each 1000 L in size) in series, while the other had three columns (800 L × 1600 L × 2). The plant was investigated over a long period, and the process could be positively evaluated for the following reasons:

The productivity of alcohol was more than 20 times higher than in a conventional batchwise fermentor.

The plant could be operated for more than 4 months without sterilization and seed fermentation processes.

A higher alcohol concentration of more than 10% v/v could be attained with a more alcohol-tolerant strain.

The conversion yield was about 95% against the theoretical yield.

κ-*Carrageenan.* The employment of a very useful polymer, κ-carrageenan, as a matrix for entrapping microbial cells was reported by Chibata and co-workers [40,41]. κ-Carrageenan is a copolymer of β-D-galactose sulfate and 3,6-anhydro-α-D-galactose, which is a readily available polysaccharide isolated from seaweed and a nontoxic compound widely used as a food additive.

Gel entrapment of whole cells based on this matrix has been accomplished by mixing a cell suspension at 37–50°C with a solution of κ-carrageenan dissolved in physiological saline at the same temperature. Gelation occurs by cooling or by contact with an aqueous solution containing metal ions, ammonium ion, amines, amino acid derivatives, or water-miscible organic solvents.

The advantages of immobilization using κ-carrageenan are that it can be performed under very mild conditions without the use of chemicals that could destroy the enzyme activity of microbial cells. In fact, it may enhance the enzyme activity and viability of the cells. In addition, as this method can be used to make immobilized preparations of various shapes, a suitable reactor for a given application can be designed. A great number of examples of the use of κ-carrageenan immobilization are reported, including an industrial process for the production of amino acids. Since the gel-entrapped cells in this matrix have operational stabilities superior to those obtained with polyacrylamide, in 1977 Tanabe Seiyaku Co., Japan, changed from the conventional polyacrylamide method to the use of κ-carrageenan for the industrial production of L-aspartic acid and L-malic acid using *Escherichia coli* and *Brevibacterium flavum*, respectively. The productivities of L-aspartic acid using immobilized cells hardened with glutaraldehyde and hexamethylenediamine [41] and L-malic acid using immobilized cells with polyethyleneimine [42] increased by as much as 15 and 7.5 times, respectively, in comparison with the conventional method. Subsequently, in 1982, the company successfully industrialized a continuous production process for L-alanine using a sequential reactor system of a column of immobilized *E. coli* followed by a closed column of immobilized *Pseudomonas dacunhae* [43].

B. Passive Immobilization

The methods described up to now are all active and cells are immobilized by chemical or physical means. Passive immobilization techniques, i.e., adsorption and colonization of porous materials, which provide more "natural" methods of immobilization, are also available.

1. Adsorption

Adsorption of cells to solid surfaces is dependent on the characteristics of the environment. Because the adsorption phenomenon is mainly based on electrostatic interactions between the cell surface and the support material, the actual zeta potential on both of them plays an important role in cell-support interaction. Among the support materials used in this method, mainly ion exchange organic materials, both natural substances such as cellulose and dextran and synthetic resins can be employed.

One of the first attempts to immobilize cells using anion exchange was performed by Hattori and Furusaka [44,45]; *Azotobactor agile*, adsorbed on Dowex resin, showed higher glucose oxidase enzyme activity and disappearance of a lag time in succinate oxidation compared with free cells.

Daugulis et al. [46] demonstrated that one particular type of strongly basic anionic exchange resin, XE352, was superior to seven chemically similar resins for the adsorption of *Saccharomyces cerevisiae* producing ethanol. However, when using *Zymomonas mobilis* immobilized on anionic exchange resins, long-term operation was inhibited by filamentous-form cells of *Z. mobilis* produced during continuous operation [47].

Seyhan and Kirwan [48] showed that immobilization was accomplished by adsorption onto an anionic exchange cellulose (Cellex E, made up of

epichlorohydrin-methanolamine-cellulose) with loading as high as 10^{11} cells/g resin. Thus, immobilized *Azotobactor vinelandii* retained their activity for over 100 h in a continuous-flow reactor. Johnson and Ciegler [49] adsorbed various types of spores of *Aspergillus* and *Penicillium* which exhibited invertase activity onto several ion exchange celluloses.

2. Colonization

A technique using porous biomass support particles (BSPs) was developed by Atkinson et al. [50]. The BSPs are essentially an interconnecting void within an open network of matrix support material. Unlike techniques involving active immobilization, the use of BSPs does not require the growth of cells prior to immobilization. The inert particles are simply placed in the fermentor before sterilization, and the fermentor is inoculated in the normal way. Cells become immobilized within the BSPs as a natural consequence of growth during an initial batch period. This technique has been applied successfully to a wide variety of microbial, animal, and plant cell systems, as shown in Table 3.

Since the development of stainless steel particles was described by Atkinson et al. [50], various types of particles such as polyester, polyether, polyurethane and silicone foams, polyvinyl formal resin, and porous cellulose have been employed for cell immobilization.

Black et al. [12] applied spheres of stainless steel knitted mesh and cubes of reticulated polyester foam particles to yeast cells for continuous ethanol production. With fluidized bed and circulating bed fermentors, the production rates were higher than those with a continuous stirred tank reactor (CSTR) [68], with the flocs that formed within particles being robust enough to allow a sufficient degree of particle movement. Webb et al. [55] and Armentia and Webb [62] also reported that in continuous operation the specific cellulase productivity of immobilized *Trichoderma viride* within stainless steel particles and the volumetric ferric iron productivity of immobilized *Thiobacillus ferrooxidans* within polyurethane foam particles were greatly enhanced compared with the conventional method.

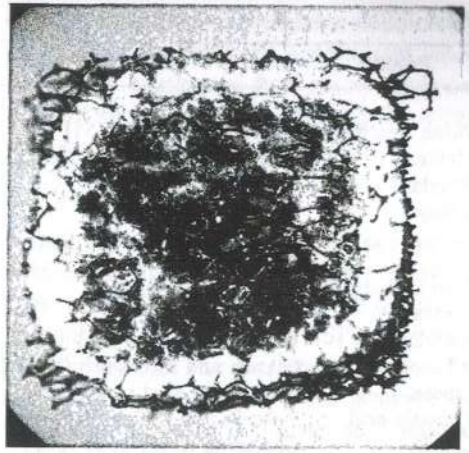
Repeated batch cultivation on a "draw and fill" basis using cells immobilized within various types of particles has been carried out by several researchers. Oriel [53] achieved a five-fold increase in amylase production using silicone foam, highly inert and hydrophobic, as a support for immobilized recombinant *E. coli*. Fukuda and Morikawa [56] reported that the fermentor productivity of γ-linolenic acid using *Mucor ambiguus* immobilized within polyurethane foam particles was over 80% higher than in batch culture. A significant enhancement in productivity using polyurethane foam particles has been also reported in other cases, e.g., lignin peroxidase [59] and penicillin [60] production.

Fukuda and co-workers [57,69,70], in a series of articles, reported a promising process for the industrial interestification of fats and oils. Unlike the conventional methods using extracellular lipase enzyme prepared by recovery from the culture broth, acetone-dried cells of *Rhizopus chinensis* immobilized within polyurethane foam particles were used directly as a lipase catalyst. Figure 2 shows cross-sectional micrographs of a 6-mm cubic particle, dried under a vacuum, taken with optical (Fig. 2a) and scanning electron microscopes (Fig. 2b) [57]. As can be seen in these illustrations, the cells formed a dense film near the surface of the particle, and the adhesion of cells to the support matrix seems to have been strong. Nakashima et al. [57,69] found that the specific intracellular lipase activity of dried *Rh. chinensis* within particles increased sevenfold compared with that obtained from freely suspended cells and also that when the specific feed rate of substrate was controlled at the optimal value the lipase activity within particles could be enhanced 1.3–1.5 times that obtained in a batch

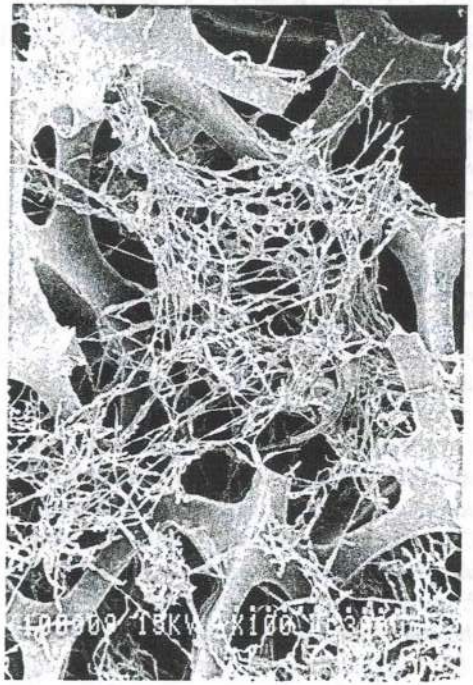
Table 3 Some Reported Applications of BSPs

Organism	BSPs[a]	Product	Reference
Microbial cell			
Mixed culture	PUF	Waste treatment	Cooper et al., 1986 [51]
Methanogen sp.	PUF	Methane	Fynn and Whitemore, 1982 [52]
Escherichia coli	SF	Amylase	Oriel, 1988 [53]
Saccharomyces cerevisiae and *Saccharomyces uvarum*	SS and PEF	Ethanol	Black et al., 1984 [12]
Clostridium acetobutylicum	NS	Aceton, butanol, ethanol	Park et al., 1989 [54]
Trichoderma viride	SS	Cellulase	Webb et al., 1986 [55]
Mucor ambiguus	PUF	γ-Linolenic acid	Fukuda and Morikawa, 1987 [56]
Rhizopus chinensis	PUF	Lipase	Nakashima et al., 1988 [57]
Rhizopus arrhizus	PUF	Fumaric acid	Kautola and Linko, 1989 [58]
Phanerochaete chrysoporium	PUF	Lignin peroxidase	Capdevila et al., 1989 [59]
Penicillium chrysogenum	PUF	Penicillin	Kobayashi et al., 1990 [60]
Botryococcus braunii	PUF	Hydrocarbon	Bailliez et al., 1988 [61]
Thiobacillus ferroxidans	PUF	Ferrous iron oxidation	Armentia and Webb, 1992 [62]
Animal cell			
Mouse myeloma	PVF	Immunoglobulin G	Yamaji et al., 1989 [63]
Hepatocyte	PVF	—	Yanagi et al., 1992 [64]
CHO-KI and Vero	PUF	—	Matsushita et al., 1990 [65]
Plant cell			
Capsicum frutescens	PUF	Capsaicin	Lindsey et al., 1983 [66]
Humulus lupulus	PUF	Hop flavors	Rhodes and Kirsop, 1982 [67]

[a] PUF, polyurethane foam; SF, silicon foam; SS, stainless steel; PEF, polyester foam; NS, natural sponge; PVF, polyvinyl formal resin.



(a)



(b).

Figure 2 Micrographs of cross section of BSPs. (a) *Rh. chinensis* formed film densely near the surface of a BSP. (b) Electron micrograph of *Rh. chinensis* adhered to BSP matrix. (From Ref. 57.)

cultivation. Cell aggregation seems to be a common trigger for the enhancement of intracellular lipase production [70].

Some advantages of BSP technology over other methods of immobilization are

No chemical additions are required.
There is no need for preproduction of cells.
No aseptic handling of particles is required.
There is a large mass transfer rate of substrate and production within BSPs.
Particles are reusable.
Particles are durable against mechanical shear.
Bioreactor scale-up is easy.
Costs are low compared to some of the active techniques.

III. REACTOR CONFIGURATIONS FOR IMMOBILIZED CELLS

Immobilized cell reactors can be divided into several different categories, including stirred tank reactors, fixed bed reactors, and fluidized bed reactors, according to the flow pattern (Fig. 3). These reactor types can also be combined or modified, and a recycle reactor system can be used to improve the mass transfer characteristics and controllability of the reactor.

The choice of reactor design for an immobilized cell process would depend on several factors. The nature of the mass transfer requirements, i.e., oxygen supply and gas removal, plays an important part in this choice. When nonviable cells or viable but nongrowing cells are used, there is no requirement for oxygen or for carbon dioxide removal, so liquid-solid two-phase reactors can be used. Packed bed reactors are applicable primarily in this case.

In the case of living cells in an aerobic reaction, a sufficient supply of oxygen and removal of carbon dioxide generated by respiration will be needed, whereas in an anaerobic reaction the removal of gaseous end products is of great importance, necessitating the use of a three-phase reactor. Hence, a fluidized bed reactor or a trickle bed reactor, which is the three-phase corollary of the packed bed reactor, are suitable for this case, because they can provide excellent mass transfer capacity.

The immobilization method and particle characteristics must also be considered. In a stirred tank reactor, damage to particles through mechanical shearing is much greater than in a packed bed reactor. Thus, only relatively robust preparations of immobilized cells should be used in a stirred tank reactor. Whereas preparations of immobilized cells on very small particles can result in an unacceptably high pressure drop and clogging problems when used in a packed bed reactor, the use of a fluidized bed reactor can circumvent these problems.

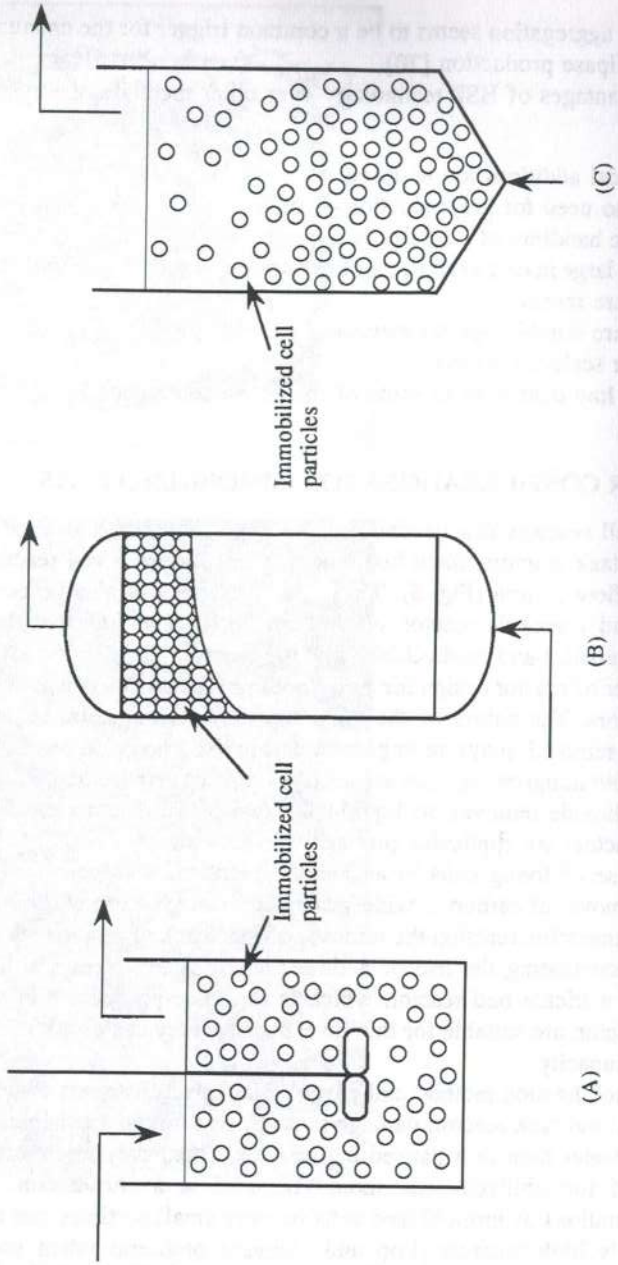


Figure 3 Most common types of reactors used for immobilized cells: (A) stirred tank reactor; (B) fixed bed reactor; (C) fluidized bed reactor.

Kinetic considerations are still another factor to be taken into account. In a continuous stirred tank reactor, the average reaction rate is lower than in a packed bed reactor due to the different operational concentration of the substrate. Conversely, a CSTR would be superior to any other kind of reactor in a substrate-inhibited reaction.

In addition to the foregoing requirements, the nature of the substrate, operational requirements, and hydraulic and economic considerations would also influence the choice of the reactor type [71].

A. Stirred Tank Reactors

The stirred tank reactor (STR) is the most commonly used reactor for freely suspended cell fermentation. It has the advantages of easy control of temperature and pH, since perfect mixing can easily be achieved. In a CSTR, all elements of the reactor have essentially the same concentration, and the substrate concentration inside the reactor is lower. Thus, the STR in continuous operation may be advantageous for a substrate inhibition reactor.

The problem associated with the use of immobilized cells in a STR is that the catalysts are exposed to a rather high degree of shear. This may severely damage the particles, especially in the case of gel particles. To alleviate the damage to particles by shear, a helical ribbon or screw-type impeller is usually preferable to a turbine or propeller-type impeller.

B. Fixed Bed Reactors

1. Packed Bed Reactors

This is by far the most frequently employed type of immobilized cell reactor. In a packed bed reactor (PBR) there is a steady movement of substrate across a bed of immobilized cells in a chosen spatial direction. Thus, a packed bed reactor can be used as a plug flow reactor (PFR), where the fluid velocity profile is perfectly flat over the cross section. A PFR may offer high rates of reaction due to high substrate concentration, but it has relatively poor mass and heat transfer coefficients due to low liquid velocities.

Some examples of reactor configurations of this type include collagen-bound cells of *Brevibacterium flavum* used in a spirally wound packed bed reactor for glutamic acid production [72], continuous ethanol production by *Saccharomyces cerevisiae* immobilized on calcium alginate beads [73], conversion of fumaric acid to aspartic acid by *Escherichia coli* entrapped in polyacrylamide gel [13], and *E. coli* immobilized by capture on the surface of insoluble pyridinium-type resin for L-aspartic acid production [74].

Packed bed reactors have the advantages of simplicity of operation and high reaction rates. The particulate catalysts which are packed in such reactors

have high specific interfacial areas of solid-liquid contact, and the velocity of liquid creeping over the static solid particles substantially alleviates the film resistance of mass transfer. However, in the case of immobilized living cells, efficient gas-liquid contact and carbon dioxide removal are often necessary. Under these conditions, a packed bed is often subject to accumulation of stagnant pockets of gas. This can cause gas flooding, which in turn produces poor liquid distribution and poor performance, and at worst the gels are abraded or ruptured. To avoid gas accumulation, a shallow horizontal packed bed reactor has been proposed by Shiotani and Yamane [75]. In the horizontal reactor, which has a free space above the gel bed, carbon dioxide gas can easily be released upward into the free space by its buoyancy. Ethanol productivity using the reactor with baker's yeast cells entrapped in calcium alginate gels was 1.5 times greater than that of a vertical packed bed reactor [75].

When high mass and heat transfer characteristics are required, or good mixing conditions are needed to improve the controllability of the reactor, a recyclable reactor system can be used. In this system, the reactor can be operated at high fluid velocities by recycling of the reaction mixture, which enhances bulk mass transfer coefficients between the substrate and the immobilized cell surface and also achieves perfect mixing of the reaction mixture. Kyotani et al. [76] developed a packed bed reactor with a recycling system, where acetone-dried *Rh. chinensis* were immobilized in polyurethane foam particles for an interesterification reaction between olive oil and methyl stearate, as shown in Fig. 4. The reaction mixture was circulated between the reactor and the agitation

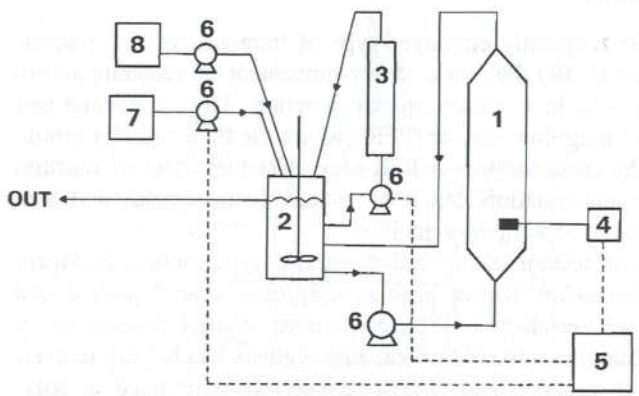


Figure 4 Schematic diagram of apparatus for continuous interesterification: (1) stainless steel reactor filled with immobilized fungus; (2) agitator as an adjustor for water content of reaction mixture; (3) silica gel column; (4) moisture analyzer; (5) microcomputer; (6) pump; (7) water storage tank; (8) reactant storage tank. (From Ref. 76.)

tank at an appropriate rate, by which means perfect mixing of the reaction mixture was achieved in the reaction system. This made it possible to control the water concentration of the reaction mixture at the optimal value of about 100 ppm; as a result, the half-life of the lipase catalyst in the cells could be prolonged up to 1200 h.

2. Other Fixed Bed Reactors

A trickle bed reactor (TBR), in which liquid flows down over a packed catalyst in the form of a thin film, can provide efficient oxygen supply and/or removal of gas from the reactor. Park et al. [54] studied acetone-butanol-ethanol fermentation using *Clostridium acetobutylicium* immobilized within natural sponge segments in a trickle bed reactor consisting of two serial columns. Because of the efficient removal by gas stripping of severely inhibitive products, the solvent productivity (acetone, butanol, and ethanol) was about 2.8 times greater than that of an immobilized CSTR. Briffaud and Engasser [77] also reported the use of a TBR for citric acid production by immobilized yeast cells within wood chips; the energy necessary for oxygen transfer was considerably lower in a TBR than in the stirred turbine reactor.

Oda et al. [39] have developed a parallel flow reactor, in which immobilized cell sheets are placed vertically in parallel, for ethanol production. This reactor, with yeast cells entrapped in a polyethylene glycolic photo-cross-linkable resin, had high productivity because of good gas release and sludge removability.

C. Fluidized Bed Reactors

A fluidized bed reactor (FBR) will provide a degree of mixing intermediate between the two extremes of the PFR and CSTR. The fluidization can be carried out by liquid, gas, or both. In the reactor, the substrate solution is passed upward at a velocity high enough to expand the bed of immobilized cells.

Fluidized bed reactors can offer good mixing, mass and heat transfer properties, and minimal pressure drops. In addition, advantages of using FBRs in bioreactors include (1) easy control and measurement of, for example, pH and dissolved oxygen; (2) easy particle sampling and replacement of the active fractions, even during operation; and (3) utilization of small particulates with a high specific surface area. These are important characteristics for the maintenance of operation. In a three-phase operation, gas-liquid contact and gas removal are facilitated compared with packed beds. Thus, this reactor, in particular, holds great potential for use with living immobilized cells where oxygen supply and/or carbon dioxide gas removal is necessary.

To obtain high conversion of the reaction or stability in the reactor at the minimum fluidizing velocity, as in the case of fixed bed reactors, a liquid re-

cycling system can be effectively employed. This procedure permits operation at relatively high flow rates, which minimizes mass transfer resistance for external diffusion in the reactor.

However, difficulties have been encountered with FBRs in operation. At relatively high bed expansion and low stability levels, fluidized beds have a narrow range of optimum operating conditions, making it rather difficult to maintain nonfluctuating operation. The problem is aggravated by the difficulty of modeling the behavior of fluidized bed reactors. In particular, it is not easy to predict accurately the effects of back-mixing on the overall reaction rate. This hampers the performance of scaled-up versions of the reactor. Another problem is related to the density difference between the immobilized cell particles and reaction mixture. Although this density difference should be as high as possible to achieve good mixing, most popular immobilized cell particles such as κ-carrageenan or alginate gels or foam biomass support particles (BSPs) show near-neutral buoyancy.

Application of the fluidized bed reactor with immobilized cells has been achieved in the initial stage of wastewater treatment [78–80], and the use of FBRs has been extended to aseptic systems.

Following the production of ethanol using immobilized *Zymomonas mobilis* in a columnar fluidized bed reactor by Davison and Scott [81], the same authors [82] proposed a bioreactor system consisting of a columnar fluidized bed of entrapped *Lactobacillus delbreukii* within κ-carrageenan gel for the simultaneous fermentation and separation of the desired product. Adsorbent particles of activated carbon added to the column absorb the product, lactic acid, and are removed from the base of the reactor. This biparticle fluidized bed reactor enhanced the fermentation and the control of pH. Kobayashi et al. [60], Fukuda and Morikawa [56], and Arcuri et al. [83] have also reported the use of the conventional FBRs for the production of penicillin, γ-linolenic acid, and thienamycin, where high production yields and successful dual-phase cultivation, i.e., a growth phase and a production phase, were achieved.

Busche and Allen [84] have developed a multiphase fluidized bed (MPFB) reactor for butanol extractive fermentation. In the MPFB reactor, a solvent is added to the fermentative environment to extract the product from the aqueous phase as fast as it forms, which improves fermentation in strong product inhibition. The MPFB reactor system enhanced the apparent product concentration and productivity, lead to a significant reduction in both fermentation and recovery costs [84].

The application of FBRs with a draft tube has been studied for penicillin production [85]. Using Celite as a support material, penicillin production under phosphate-limiting conditions could be maintained at a high level over a long period. Fan et al. [86] investigated this type of reactor for biological phenol degradation and achieved significantly higher treatment efficiency. Black et al. [12] have devised a circulating bed fermentor (CBF) that allows the use of low-density foam particles which are difficult to fluidize. In the CBF, air is introduced through the lower retaining plate/distributor, which is partly occluded, and good circulation of particles is effectively achieved at a comparatively low aeration rate. Thus, at a given aeration rate, the hydrodynamic stress around particles in the CBF might be lower than that in other types of reactor. The CBF was successfully operated continuously for extended periods for ethanol production by yeast cells.

Nakashima et al. [69] also studied a CBF–polyurethane foam particle system for cell-bonded lipase production of *Rhizopus chinensis* (Fig. 5). In this case, a high aeration rate significantly damaged the formation of intracellular lipase, and the results revealed that a comparatively low aeration rate capable of attaining good circulation of particles in the CBF was adequate for lipase production. Thus, the culture system shown in Fig. 5 could effectively produce cell-bonded enzymes with high lipase activity through control of the specific feed rate of the substrate within the optimal range.

To prevent blockage by a buildup of excess cells when filamentous fungi are employed, a spouted bed reactor (SBR) was examined by Webb et al. [55]. In the SBR, a recycled broth together with air is used to create a jet at the base of a bed of particles, causing the particles to spout and circulate. During the circulation, particles pass through a region of high shear near the jet inlet. This effectively prevents blockage, enabling steady-state conditions to be achieved during continuous operation.

Scott and Hancher [87] made a preliminary investigation of a tapered fluidized bed (TFB) using adhering *Pseudomonas* bacteria within and/or on coal particles for biodenitrification. In the TFB there is gradual expansion from a relatively small cross-sectional area of the entry to one that may be several times larger, and this makes the flow relatively stable throughout the reactor. Also, the TBF can either provide flow patterns that have minimal back-mixing, especially at the feed entry point, or operate in a similar way to a multistage unit in which the reactant and product concentrations vary throughout the reactor.

Examples of other variations of the conventional fluidized bed reactor that have been proposed include a three-stage conical fluidized bed reactor for ethanol production [88] and an airlift reactor with an external loop for cyclosporine production [89].

IV. MASS TRANSFER CONSIDERATIONS IN IMMOBILIZED CELL SYSTEMS

An essential requirement in immobilized cell systems is to enhance the activity of immobilized cell particles for application in industrial use. The activity is

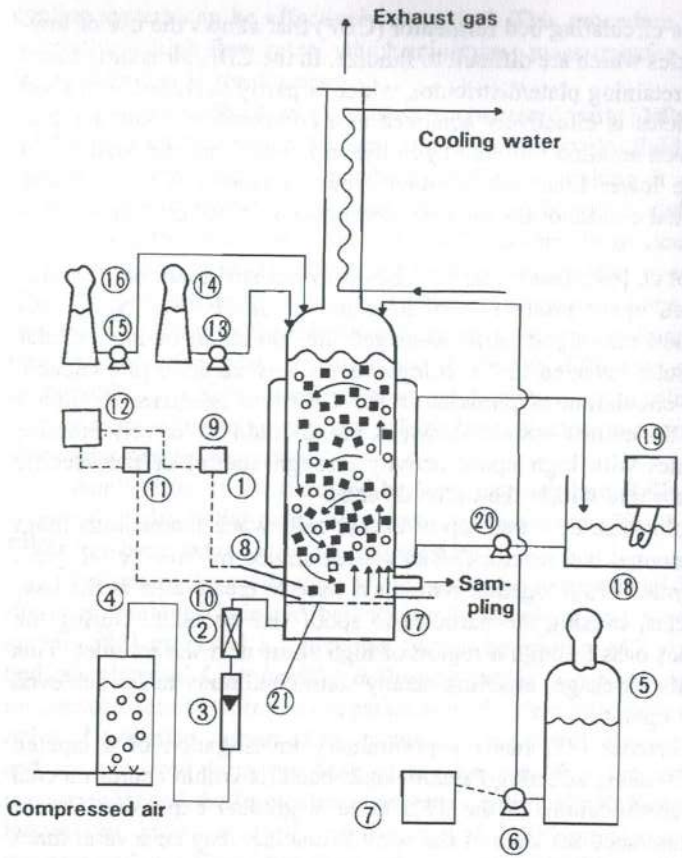


Figure 5 Schematic flow diagram of the circulating bed fermentor system: (1) circulating bed fermentor; (2) air filter; (3) rotameter; (4) water-saturated tower; (5) medium storage bottle; (6) medium feed pump; (7) microcomputer; (8) pH probe; (9) pH monitor/controller; (10) DO probe; (11) DO meter; (12) recorder; (13) NaOH feed pump; (14) NaOH storage bottle; (15) H_2SO_4 feed pump; (16) H_2SO_4 storage bottle; (17) thermometer; (18) water bath; (19) temperature controller; (20) circulation pump; (21) perforated plate. (From Ref. 69.)

significantly influenced by factors such as the mass transfer of either substrate or oxygen, the cell physiology, and the methods and materials used for immobilization; there are also mutual interactions among these factors [90].

As far as the mass transfer of substrate between the particles and the external bulk liquid is concerned, the diffusion resistance within the external

liquid film and the intraparticle diffusion resistance should be considered. In supplying oxygen to the cells immobilized within the particles through the gas-liquid interface, the liquid film resistance is usually limited compared with the gas film resistance, and thus the volumetric gas-liquid oxygen transfer coefficient based on the liquid phase becomes substantial.

A. External Mass Transfer

The phenomena involved are conveniently discussed with the aid of a simplified film model that assumes a stagnant layer of fluid adhering to the outer immobilized biocatalyst surface (Fig. 6). Substrates and products must penetrate this film by diffusion according to their concentration profile. Based on the assumption of $\delta \ll R$, the mass flux, N, through this film under steady-state conditions using Fick's law is described by

$$N|_{r=R} = \frac{D}{\delta}(S - S_s) = k_L(S - S_s) \tag{1}$$

where k_L and D are the mass transfer coefficient and the diffusion coefficient, respectively. The mass transfer coefficient depends on the hydrodynamics of the reactor system, and this can be estimated from a number of proposed cor-

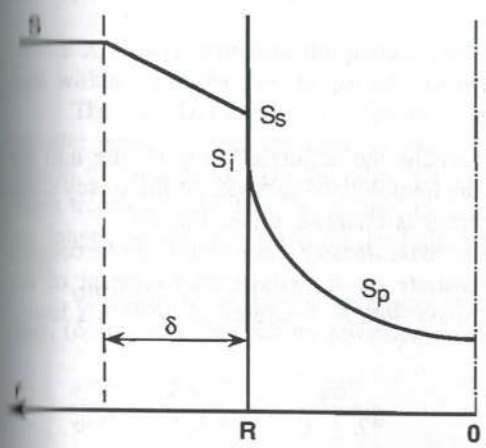


Figure 6 Simplified scheme for coupling of mass transfer and reaction; substrate concentrations in the bulk phase solution (S), at external surface of particle (S_s), just within the surface of particle (S_i), within particle (S_p), and external stagnant fluid film with thickness δ, and inside the particle with radius R.

relations. The correlations for mass transfer and experimental conditions are commonly expressed in terms of dimensionless groups such as the Sherwood number (Sh), Reynolds number (Re), and Schmidt number (Sc). One of the most broadly based correlations for fixed bed reactors was proposed by Wilson and Geankoplis [91]:

$$j = \frac{0.250}{\epsilon}\,\mathrm{Re}^{-0.31} \quad \text{for } 55 < \mathrm{Re} < 1500 \tag{2}$$

$$j = \frac{1.09}{\epsilon}\,\mathrm{Re}^{-2/3} \quad \text{for } 0.0016 < \mathrm{Re} < 55 \tag{3}$$

where j is the j-factor = $(k_L\rho/G)\mathrm{Sc}^{2/3} = \mathrm{Sh}/(\mathrm{Re}\mathrm{Sc}^{1/3})$, $\mathrm{Sh} = k_L d_p/D$, $\mathrm{Re} = d_p G/\mu$, $\mathrm{Sc} = \mu/\rho D$. G, d_p, ϵ, μ, and ρ are the mass velocity per unit superficial bed cross section, particle diameter, void fraction, liquid viscosity, and liquid density, respectively.

For fluidized bed reactors the following correlations may be introduced [92]:

$$j = 5.7\,\frac{\mathrm{Re}}{1-\epsilon} \quad \text{for} \quad \frac{\mathrm{Re}}{1-\epsilon} < 30 \tag{4}$$

However, the correlations available for the prediction of k_L in fluidized bed reactors have been inadequate, as it is rather difficult to evaluate dispersion characteristics accurately.

B. Internal Mass Transfer

Inside an immobilized biocatalyst particle, the substrate has to diffuse into the carrier in order to be converted by the immobilized catalyst. At the steady state, a profile of substrate through the carrier is established (see Fig. 6).

The simultaneous phenomena of mass transfer and reaction can be coupled through a mass balance for the substrate on an infinitesimal element of the particle. In a spherical particle, the mass flux, N, is expressed by Fick's law:

$$N = -D_e\,\frac{dS_p}{dr} \tag{5}$$

where D_e is the effective diffusion coefficient for the diffusing species in the particle.

From the mass balance for a spherical particle at steady-state using Eq. (5), the equation in terms of diffusion with reaction is described by:

$$D_e\left(\frac{d^2S_p}{dr^2} + \frac{2}{r}\,\frac{dS_p}{dr}\right) = (-r_s) \tag{6}$$

where $(-r_s)$ is the intrinsic reaction rate and generally can be expressed by the Michaelis-Menten equation (7):

$$(-r_s) = \frac{V_m S_p}{K_m + S_p} \tag{7}$$

where V_m and K_m represent the maximum reaction rate and Michaelis constant, respectively.

The boundary conditions used in the solution of the differential equation (6) are

$$\frac{dS_p}{dr} = 0 \quad \text{at } r = 0 \tag{8}$$

$$S_p = S \quad \text{at } r = R \tag{9a}$$

or

$$D_e\left(\frac{dS_p}{dr}\right) = k_L(S - S_s) = k_L\left(S - \frac{S_i}{K_p}\right) \quad \text{at } r = R \tag{9b}$$

where K_p and S_i represent the partition coefficient and substrate concentration just within the surface of the particle, respectively.

The boundary condition of Eq. (9a) assumes that there is no external mass transfer resistance and the concentration on the surface of the particle equals that in the bulk liquid. On the other hand, if there is considerable resistance to mass transfer across this liquid film, i.e., the concentrations at the surface will no longer be equal to those in the bulk liquid, Eq. (9b) should be employed [93].

When the mass transfer resistance is negligible, i.e., Eq. (9a) is available, Eqs. (6)–(9) and (9a) can be rewritten in dimensionless form:

$$\frac{d^2S^*}{dr^{*2}} + \frac{2}{r^*}\,\frac{dS^*}{dr^*} = \phi_1^2\,\frac{\beta S^*}{\beta + S^*} \tag{10}$$

$$\frac{dS^*}{dr^*} = 0 \quad \text{at } r^* \; 0 \tag{11}$$

$$S^* = 1 \quad \text{at } r^* = 1 \tag{12}$$

where the Thiele modulus, ϕ_1, and the dimensionless parameters, S^*, r^*, and β, are defined by the following equations:

$$\phi_1 = R\sqrt{\frac{V_m}{K_m D_e}} \tag{13}$$

$$S^* = \frac{S_p}{S}, \qquad r^* = \frac{r}{R}, \qquad \beta = \frac{K_m}{S} \tag{14}$$

The substrate concentration profile can be obtained by numerically integrating Eq. (10) with Eqs. (11) and (12), and then the overall reaction rate can be estimated.

It is convenient to use the effectiveness factor, η, which is the ratio of the reaction rate with and without diffusion effects, defined by

$$\eta = \frac{\text{observed rate of reaction with mass transfer resistance}}{\text{hypothetical rate of reaction without mass transfer resistance}} \tag{15}$$

Thus, based on the substrate concentration in the bulk medium, the observed overall reaction rate can easily be estimated when η is determined.

The effectiveness factor for a spherical particle using Eq. (15) would be

$$\eta = \frac{3}{\phi_1^2[\beta/(\beta + 1)]}\left.\frac{dS^*}{dr^*}\right|_{r^*=1} \tag{16}$$

When β tends to infinity, the reaction becomes a first-order reaction, and thus η is analytically obtained from Eq. (16), where $(dS^*/dr^*)|_{r^*=1}$ is calculated from the solution of Eq. (10) using Eqs. (11) and (12) as boundary conditions:

$$\eta = \frac{3}{\phi_1^2}(\phi_1 \coth \phi_1 - 1) \tag{17}$$

The effectiveness factor cannot easily be evaluated analytically when the reaction rate takes the nonlinear form indicated in Eq. (7). Thus, the numerical calculations of η versus ϕ_1 using Eqs. (10)–(12) and (16) are shown as a function of β in Fig. 7. When ϕ_1 is small, η is practically unity for all surface concentrations; i.e., there is no diffusional limitation, and the rate-controlling step is the reaction kinetics, whereas if $\eta < 1$ there is diffusional limitation and the rate-controlling step is mass transfer. The plots are identical with Eq. (17) when β is infinity.

An approximate expression of η for a sphere was proposed by Kobayashi et al. [94] as follows:

$$\eta = \frac{\eta_0 + 2.6\beta^{0.8}\eta_1}{1 + 2.6\beta^{0.8}} \tag{18}$$

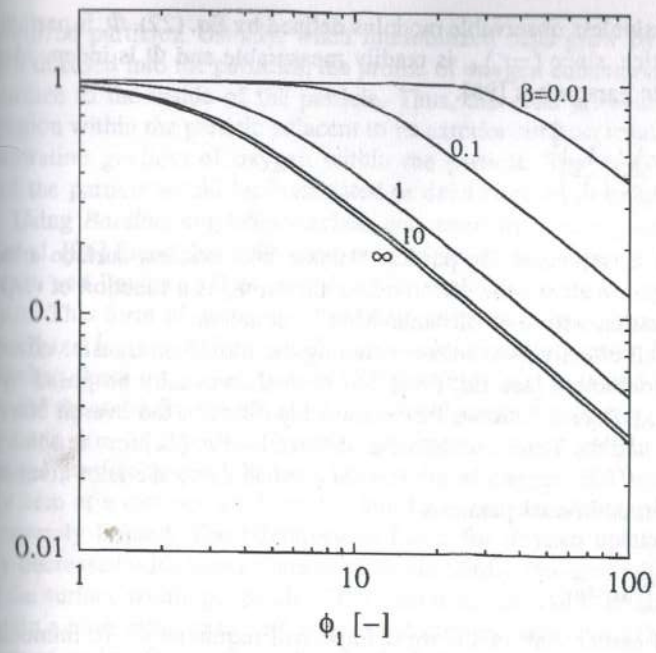


Figure 7 Effectiveness factors η versus ϕ_1.

where η_0 and η_1 are given by

$$\eta_0 \begin{cases} = 1 & \text{for } \Phi_a \le 3 \\ = 1 - \left[\dfrac{1}{2} + \cos\dfrac{1}{3}\left\{\cos^{-1}\left(\dfrac{6}{\Phi_a^2} - 1\right) + 4\pi\right\}\right]^3 & \text{for } \Phi_a \ge 3 \end{cases}$$

$$\eta_1 = \frac{3}{\Phi_a^2}(\Phi_a \coth \Phi_a - 1) \tag{19}$$

Φ_a is defined by

$$\Phi_a = \frac{\phi}{\sqrt{2(1 + \beta)\{1 - \beta \ln(1 + 1/\beta)\}}} \tag{20}$$

$$\phi = R\sqrt{\frac{V_m}{D_e(K_m + S)}} \tag{21}$$

To circumvent such difficulties in the calculating procedure, it is customary to use generalized plots of effectiveness factors.

A dimensionless observable modulus defined by Eq. (22), Φ, is particularly useful in practice, since $(-r_s)_{obs}$ is readily measurable and Φ is independent of intrinsic kinetic parameters [95].

$$\Phi = \frac{(-r_s)_{obs}}{D_e S}\left(\frac{V_p}{A_p}\right)^2 \tag{22}$$

where V_p and A_p represent the particle volume and external surface area, respectively. Figure 8 shows the effectiveness factor, Φ, as a function of $(1/\beta)$ for substrate utilization with the Michaelis-Menten equation.

An overall effectiveness factor including the film mass transfer effect and the partition coefficient [see Eq. (9b)] has been theoretically proposed by Yamane et al. [93]. Figure 9 shows the relationship between the overall effectiveness factor, η, and the Thiele modulus, ϕ, defined by Eq. (21) for the Michaelis-Menten equation with respect to a spherical particle using the Biot number (Bi $= k_L R/D_e$) as an additional parameter.

C. Oxygen Transfer

As mentioned earlier, one of the most important requirements in immobilized living or growing cell systems is the supply of oxygen to the cells within the

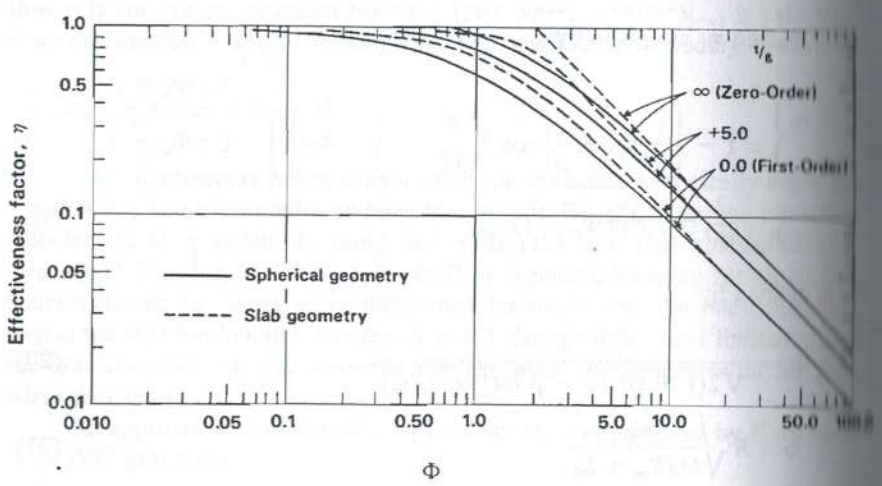


Figure 8 Effectiveness factors for substrate utilization with Michaelis-Menten kinetics. Φ is the observable modulus defined in Eq. (22). (From Ref. 95.)

immobilized particles. Usually, when immobilized cells grow by utilization of oxygen diffused into the particles, the profile of oxygen concentration falls from the surface to the inside of the particle. Thus, the cells grow abundantly in a thin region within the particle adjacent to its exterior surface, resulting in a steep concentration gradient of oxygen within the particle. The cells in the central part of the particle would be inactivated or dead because of oxygen starvation.

Using *Bacillus amyloliquefaciens* entrapped by κ-carrageenan gel, Shinmyo et al. [96] found that cells grew only within 50 μm of the exterior surface. Atkinson and Fowler [97] estimated this critical distance to be between 50 and 100 μm. This form of dense film has been observed even in immobilization by polyurethane foam particles, which have a significantly greater diffusion coefficient than those of gel entrapment [57] (see Fig. 2a). Toda and Sato [98] also estimated theoretically the effect of diffusional resistance to oxygen transfer on the growth rate of *Candida lipolytica* entrapped in agar gel. In the relatively larger gel particle (over 0.05 μm), the dissolved oxygen (DO) profile became nearly zero at a distance far from the interface, and simultaneously cell growth was severely limited. The effectiveness factor for oxygen uptake also significantly decreased with larger particle sizes but hardly changed with the distance from the surface within gel beads of 0.01 cm in radius. Another factor necessary to obtain a high value of the effectiveness factor for oxygen uptake is the maintenance of a high DO concentration in the bulk medium.

Thus, it is of great importance to increase the DO concentration at the particle-medium interface, and consequently the characteristics of the bioreactor in terms of oxygen transfer become one of the essential criteria for bioreactor design.

The presence of solid particles can significantly affect gas-liquid mass transfer characteristics. Hence, the effects of particle size, concentration, and density on the gas-liquid mass transfer have been studied by many investigators. Østergaard and Fosbol [99] investigated the effects of the particle size of glass ballotini, i.e., large and heavy particles, on the volumetric liquid-side mass transfer coefficient, $k_L a$, in a fluidized bed. The $k_L a$ values using 1-mm glass significantly decreased compared with those in bubble columns, whereas the $k_L a$ values using 6-mm glass were approximately twice those for bubble columns because of bubble disintegration. Similar observations were made by Dhanuka and Stepanek [100]. In the case of glass beads of 0.1 and 0.2 mm, the $k_L a$ values decreased considerably [101], whereas for 0.05-mm particle slurries they were significantly higher than in the two-phase system [102]. In the latter case, the authors suggested that the particles increased the liquid-side mass transfer coefficient, k_L, by penetrating the liquid-side diffusion film rather than affecting the specific interfacial area, a.

A modified empirical equation involving the liquid-phase mass transfer coefficient in a three-phase fluidized bed reactor was proposed by Fukushima [103]

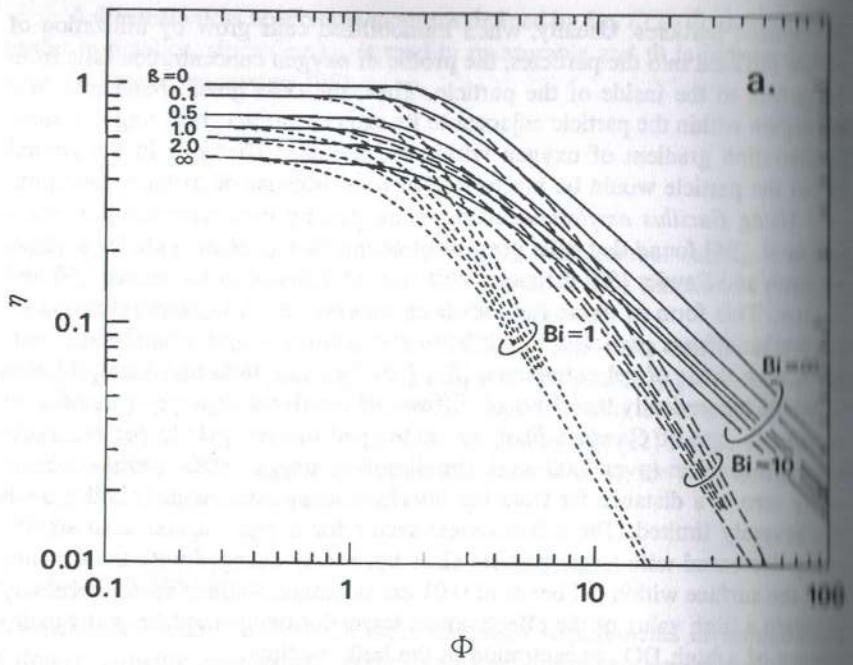


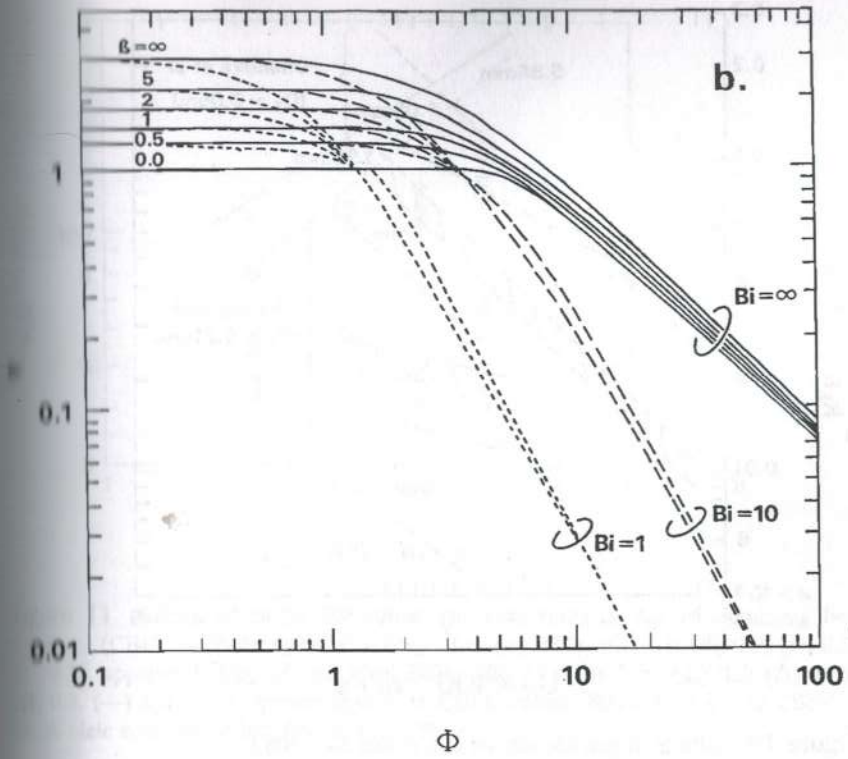
Figure 9 Overall effectiveness factor η versus ϕ for irreversible Michaelis-Menten kinetics when (a) $K_p = 0.5$ and (b) $K_p = 3.0$. (From Ref. 93.)

as follows:

$$\frac{k_L a d_p^2 \epsilon}{(D_l\, \epsilon_G)} = 1.4 \times 10^4 \mathrm{Re}_l^{0.38} \mathrm{Re}_g^{-0.2} \mathrm{Sc}_l^{0.5} \left(\frac{d_p}{d_T}\right)^{2.1} \tag{23}$$

where ϵ_G and d_T are gas holdup and column diameter, and the subscripts g and l represent the gas phase and liquid phase, respectively. The predictions for k_L agree fairly well with the literature data [99, 100, 104, 105] for the relatively larger particles. Usually, the particle size used for immobilized cells is beyond 1 mm in any of the immobilization techniques. The results of the relationships between $k_L a$ and superficial gas velocity, u_G, obtained by Østergaard [104] and Dhanuka and Stepanek [100] are shown in Fig. 10 [106].

Nakashima and Fukuda [107] have reported the effects of either empty or agar-filled 6-mm cubic polyurethane foam particles on $k_L a$ in a circulating bed fermentor (Fig. 11). In the presence of empty particles a slight decrease of $k_L a$ was observed, whereas there was a significant decrease of $k_L a$ values with the



agar-filled particles. These differences from Fig. 10 may be due to differences in particle density, shape, and reactor configuration.

V. CONCLUSIONS

Much of the recent research on immobilization has focused on more complex reactions using immobilized cell systems, which can offer a number of advantages over the use of soluble and immobilized enzymes, as well as chemical synthesis. Immobilized cell systems hold out the possibility of processing with relatively small investment costs, because of their high productivity, operational stability, the fact that recovery processes are unnecessary, etc.

However, severe biological and engineering constrains have frequently been encountered in the technical feasibility of employing these systems, and only a few industrial applications have actually been accomplished, as shown in Table 1. To apply immobilized cell systems in industrial processes, further studies to elucidate their properties—mainly involving cell physiology, immobilization methods, and mass transfer within bioreactors—will be required.

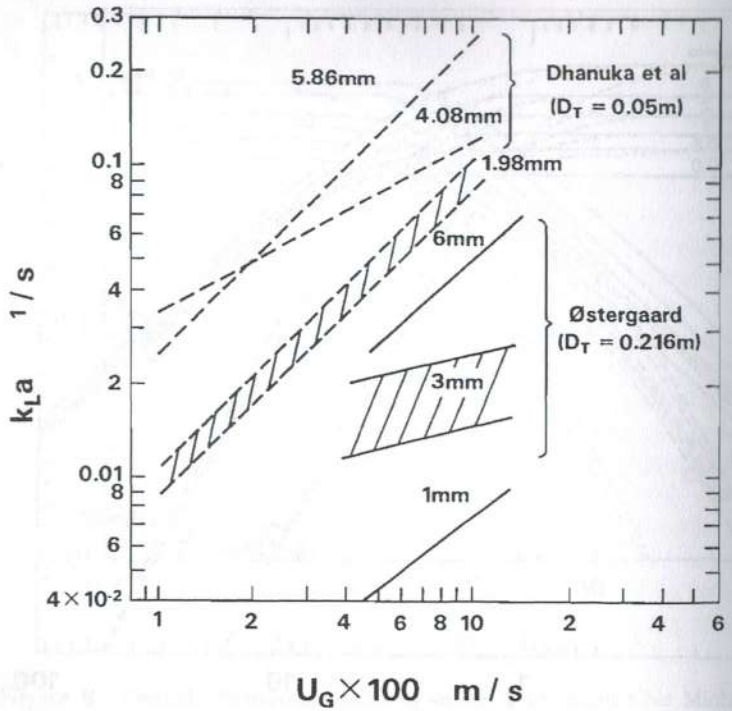


Figure 10 Effects of particle size on k_La. (From Ref. 106.)

Numerous unpredicted changes in growth and metabolism, some beneficial and some detrimental to the process, have been observed with immobilized cells. As a beneficial effect, a number of reports that cell immobilization significantly enhanced stability have been appeared. However, an interesting phenomenon can be observed in physiological changes with immobilized cells. As mentioned in an earlier section, when the fungus *Rh. chinensis* was immobilized within polyurethane foam particles, cells exhibited physiological and morphological changes, and the specific intracellular lipase activity was greatly enhanced by as much as severalfold compared with that of freely suspended cells [69, 70]. Also, some examples exhibiting unexpected phenomena have been observed, e.g., an increase in production yield [16] and a decrease in cell generation time [108] in cases absorbed or covalently immobilized.

Entrapment immobilization with synthetic polymers or polysaccharides involving active immobilization can be a satisfactory means of obtaining immobilized cells retaining full biochemical activity, and passive types of immobilization such as adsorption and colonization, which may be less expensive, provide even more natural methods of immobilization. In particular, to carry out a bioreactor operation stably and efficiently using growing cells, the cell concentration immobilized within the particles should be controlled at the optimal value during operation. The use of biomass support particles (BSPs) involving colonization makes it possible to perform such control readily. While cells are retained within the particles, any excess cells which form due to cell growth on the outside of the particles are effectively removed by the forces of attrition arising from interparticle and particle-wall contact. Advances in BSP technology should lead to a greater understanding of the factors involved in cell adhesion and to the development of novel support materials able to provide beneficial effects in immobilization.

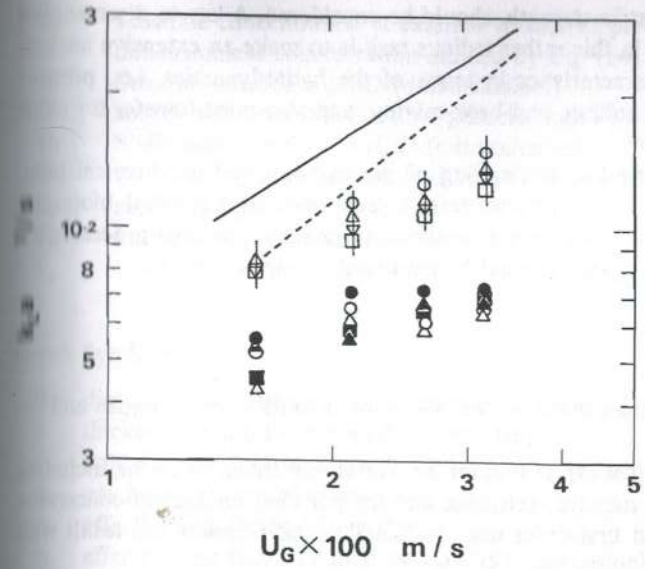


Figure 11 Effects of empty BSPs and agar-filled BSPs on k_La in circulating bed fermentor (CBF). Symbols: apparent holdup of empty BSPs, (Φ) 0.1; (Φ) 0.2; (Ψ) 0.3; (Ψ) 0.35. apparent holdup of agar-filled BSPs, (●) 0.1; (○) 0.15; (▲) 0.2; (△) 0.25; (■) 0.3. (—) k_La in an aqueous system in CBFs without BSPs; (---) k_La in CBFs in which oleic acid was added. (From Ref. 107.)

Cell aggregates are often subject to mass transfer limitations on the rate of reaction. In designing or selecting an appropriate bioreactor configuration, conflicting factors such as decreased particle size against problems of increased pressure drop in the bioreactor, or increased effective diffusion coefficients

against decreased matrix strength, should be considered. A key to disentangling the factors involved in this rather tedious task is to make an extensive analysis of the bioreactor characteristics in terms of the hydrodynamics, i.e., pressure drop, gas and liquid holdup, and back-mixing, and also mass transfer for either substrate or oxygen.

With an improved understanding of the physical and biochemical properties involved, innovative immobilization techniques and a novel bioreactor based on fundamental mass transfer analysis, promising industrial processes using immobilized cell systems could be exploited in various fields.

PROBLEMS

1. Discuss the characteristics of various immobilization technologies and reactor configurations.
2. Derive an analytical expression for the overall effectiveness factor, including the liquid-film mass transfer resistance and the partition coefficient concerning irreversible zero- and first-order reaction kinetics and compare the result with Fig. 9.

NOMENCLATURE

A_p	external surface area of immobilized biocatalyst particle (m^2)
a	gas-liquid specific interfacial area (1/m)
Bi	Biot number = $k_L R/D_e$ (dimensionless)
D	diffusion coefficient (m^2/s)
D_e	effective diffusion coefficient (m^2/s)
d_p	particle diameter (m)
d_T	column diameter (m)
G	mass velocity per unit superficial bed cross section (kg/m^2 s)
j	j-factor = $(k_L\rho/G)Sc^{2/3}$ (dimensionless)
K_m	Michaelis constant (kg/m^3)
K_p	partition coefficient (dimensionless)
k_La	volumetric mass transfer coefficient (1/s)
k_L	film mass transfer coefficient (m/s)
N	mass flux (kg/m^2 s)
R	radius (m)
Re	Reynolds number = d_pG/μ (dimensionless)
r	one-dimensional coordinate distance (m)
r^*	dimensionless coordinate of distance defined by Eq. (14) (dimensionless)
$(-r_s)$	intrinsic reaction rate (kg/m^3 s)
S	substrate concentration in bulk solution (kg/m^3)

S_s	substrate concentration at external surface of particle (kg/m^3)
S^*	dimensionless concentration defined by Eq. (14) (dimensionless)
Sc	Schmidt number = $\mu/\rho D$ (dimensionless)
S_p	substrate concentration within particle (kg/m^3)
Sh	Sherwood number = k_Ld_p/D (dimensionless)
S_i	substrate concentration just within the surface of particle (m)
u_G	superficial gas velocity (m/s)
V_m	maximum reaction rate (kg/m^3s)
V_p	volume of particle (m^3)

Greek Symbols

β	dimensionless Michaelis constant defined by Eq. (14) (dimensionless)
δ	thickness of diffusion boundary layer (m)
ε	void fraction (dimensionless)
ε_G	gas holdup (dimensionless)
η	effectiveness factor (dimensionless)
η_0	effectiveness factor defined by Eq. (19) (dimensionless)
η_1	effectiveness factor defined by Eq. (19) (dimensionless)
μ	liquid viscosity (kg/m s)
ρ	liquid density (kg/m^3)
Φ	observable Thiele modulus defined by Eq. (22) (dimensionless)
Φ_s	Thiele modulus defined by Eq. (20) (dimensionless)
ϕ	Thiele modulus defined by Eq. (21) (dimensionless)
ϕ_1	Thiele modulus defined by Eq. (13) (dimensionless)

Subscripts

g	gas phase
l	liquid phase
obs	observed

REFERENCES

1. I. Chibata, *Adv. Appl. Microbiol.* 22: 1 (1977).
2. T. R. Jack and J. E. Zajic, *Adv. Biochem. Eng.* 5: 125 (1977).
3. C. Bucke, *Process Engineering Aspects of Immobilized Cell Systems* (C. Webb, G. M. Black, and B. Atkinson, eds.), Institute of Chemical Engineers, Rugby, UK, 1986, p. 20.
4. G. Durand and J. M. Navarro, *Process Biochem.* 13: 14 (1978).
5. J. F. Kennedy and J. M. S. Cabral, *Applied Biochemistry and Bioengineering* (I. Chibata and L. B. Wingard, Jr., eds.), Academic Press, New York, 1983, p. 189.

6. F. B. Kolot, *Process Biochem. Aug./Sep.*: 2 (1981).
7. F. B. Kolot, *Process Biochem. Oct./Nov.*: 30 (1981).
8. J. Klein, *Bioreactor Immobilized Enzymes and Cells* (M. Moo-Young, ed.) Elsevier Applied Science, New York, 1988, p. 1.
9. S. Furusaki, *J. Chem. Eng. Jpn. 21*: 219 (1988).
10. A. Tanaka and H. Nakajima, *Adv. Biochem. Eng. Biotechnol. 42*: 97 (1990).
11. S. F. Karel, S. B. Libicki, and C. R. Robertson, *Chem. Eng. Sci. 40*: 1321 (1985).
12. G. M. Black, C. Webb, T. M. Matthews, and B. Atkinson, *Biotechnol Bioeng. 26*: 134 (1984).
13. I. Chibata, T. Tosa, and T. Sato, *Appl. Microbiol 27*: 878 (1974).
14. W. E. Workman and D. F. Day, *Biotechnol. Bioeng. 26*: 905 (1984).
15. R. Messing and R. A. Oppermann, *Biotechnol. Bioeng. 21*: 49 (1979).
16. J. M. Navarro and G. Durand, *Eur J Appl. Microbiol. Biotechnol. 4*: 243 (1977).
17. R. Messing, R. A. Oppermann, and F. Kolot, *ACS Symp. Ser. 106*: 13 (1979).
18. T. R. Jack and J. E. Zajic, *Biotechnol Bioeng. 19*: 631 (1977).
19. S. Shimizu, H. Morioka, Y. Tani, and K. Ogata, *J. Ferment. Technol. 53*: 71 (1975).
20. K. Yamamoto, T. Tosa, and I. Chibata, *Biotechnol. Bioeng. 19*: 1101 (1977).
21. S. Nishikawa, Y. Ashina, and I. Watanabe, *Kagaku Kogaku 51*: 423 (1987).
22. T. Omata, T. Iida, A. Tanaka, and S. Fukui, *Eur. J. Appl. Microbiol. Biotechnol 8*: 143 (1979).
23. K. Yokozeki, S. Yamanaka, T. Utagawa, K. Takinami, Y. Hirose, A. Tanaka, K. Sonomoto, and S. Fukui, *Eur J. Appl. Microbiol. Biotechnol. 14*: 225 (1982).
24. K. Sonomoto, M. M. Hoq, A. Tanaka, and S. Fukui, *J. Ferment. Technol. 59*: 463 (1981).
25. K. Sonomoto, M. M. Hoq, A. Tanaka, and S. Fukui, *Appl. Environ. Microbiol 45*: 436 (1983).
26. K. Yokozeki, S. Yamanaka, K. Takinami, Y. Hirose, A. Tanaka, K. Sonomoto, and S. Fukui, *Eur J. Appl. Microbiol. Biotechnol 14*: 1 (1982).
27. P. S. J. Cheetham, *Enzyme Microb. Technol. 1*: 183 (1979).
28. P. S. J. Cheetham, K. W. Blunt, and C. Bucke, *Biotechnol. Bioeng. 21*: 2155 (1979).
29. F. Paul and P. M. Vignais, *Enzyme Microb. Technol. 2*: 281 (1980).
30. J. Klein and F. Wagner, *DECHEMA-Monogr. 84*: 265 (1979).
31. S. Ohlson, P. O. Larsson, and K. Mosbach, *Eur J. Appl. Microbiol. Biotechnol 7*: 103 (1979).
32. S. Birnbaum, R. Pendleton, P. O. Larsson, and K. Mosbach, *Biotechnol. Lett. 3*: 393 (1981).
33. H. Tanaka, S. Irie, and H. Ochi, *J. Ferment. Bioeng. 68*: 216 (1989).
34. H. Tanaka, H. Kurosawa, and H. Murakami, *Biotechnol Bioeng. 28*: 1761 (1986).
35. R. Kaul, P. Adlercreutz, and B. Mattiason, *Biotechnol Bioeng. 28*: 1432 (1986).
36. J. Klein, J. Stock, and K.-D. Vorlop, *Eur J. Appl. Microbiol. Biotechnol. 18*: 86 (1983).
37. J. C. Ogbonna, M. Matsumura, T. Yamagata, H. Sakuma, and H. Kataoka, *J. Ferment. Bioeng. 68*: 40 (1989).

38. A. C. Hulst, J. Tramper, K. van't Riet, and M. M. Westerbeek, *Biotechnol. Bioeng. 27*: 870, (1985).
39. G. Oda, H. Samejima, and T. Yamada, Continuous alcohol fermentation technologies using immobilized yeast cells, *Proceedings of Biotech. 83*, Online Publ., Northwood, 1983, p. 597.
40. M. Wada, J. Kato, and I. Chibata, *Eur J. Appl. Microbiol. Biotechnol. 8*: 241 (1979).
41. I. Chibata, *Am. Chem. Soc. Symp. Ser. 106*: 187 (1979).
42. I. Takata, K. Kayaskima, T. Tosa, and I. Chibata, *J. Ferment. Technol. 60*: 431 (1982).
43. I. Chibata, T. Tosa, and S. Takamatsu, *Microbiol. Sci. 1*: 58 (1984).
44. T. Hattori and C. Furusaka, *J. Biochem. 48*: 831 (1960).
45. T. Hattori and C. Furusaka, *J. Biochem. 50*: 312 (1961).
46. A. J. Daugulis, N. M. Brown, W. R. Clutte, and D. B. Dunlop, *Biotechnol. Lett. 3*: 651 (1981).
47. T. A. Krug and A. J. Daugulis, *Biotechnol. Lett. 5*: 159 (1983).
48. E. Seyhan and D. J. Kirwan, *Biotechnol. Bioeng. 21*: 271 (1979).
49. D. E. Johnson and A. Ciegler, *Arch. Biochem. Biophys. 130*: 384 (1969).
50. B. Atkinson, G. M. Black, P. J. S. Lewis, and A. Pinches, *Biotechnol. Bioeng. 21*: 193 (1979).
51. P. F. Cooper, I. Walker, H. E. Crabtree, and R. P. Aldred, *Process Engineering Aspects of Immobilised Cell Systems* (C. Webb, G. M. Black, and B. Atkinson, eds.) Institute of Chemical Engineers, Rugby, UK, 1986, p. 205.
52. G. H. Fynn, and T. N. Whitemore, *Biotechnol. Lett. 4*: 577 (1982).
53. P. Oriel, *Biotecnol. Lett. 10*: 113 (1988).
54. C. H. Park, M. R. Okos, and P. C. Wankat, *Biotechnol. Bioeng. 34*: 18 (1989).
55. C. Webb, H. Fukuda, and B. Atkinson, *Biotechnol Bioeng. 28*: 41 (1986).
56. H. Fukuda, and H. Morikawa, *Appl. Microbiol. Biotechnol. 27*: 15 (1987).
57. T. Nakashima, H. Fukuda, S. Kyotani, and H. Morikawa, *J. Ferment. Technol. 66*: 441 (1988).
58. H. Kautola, and Y. Y. Linko, *Appl. Microbiol. Biotechnol. 31*: 448 (1989).
59. C. Capdevila, G. Corrieu, and M. Asther, *J. Ferment Bioeng. 68*: 60 (1989).
60. T. Kobayashi, K. Tachi, T. Nagamune, and J. Endo, *J. Chem. Eng. Jpn. 23*: 408 (1990).
61. C. Bailliez, C. Largeau, E. Casadevall, L. W. Yang, and C. Berkaloff, *Appl. Microbiol. Biotechnol. 29*: 141 (1988).
62. H. Armentia, and C. Webb, *Appl. Microbiol. Biotechnol. 36*: 697 (1992).
63. H. Yamaji, H. Fukuda, Y. Nojima, and C. Webb, *Appl. Microbiol. Biotechnol. 30*: 609 (1989).
64. K. Yanagi, H. Miyoshi, H. Fukuda, and N. Ohshima, *Appl. Microbiol. Biotechnol. 37*: 316 (1992).
65. T. Matsushita, M. Ketayama, K. Kamihata, and K. Funatsu, *Appl. Microbiol. Biotechnol. 33*: 287 (1990).
66. K. Lindsey, M. M. Yeoman, G. M. Black, and F. Mavituna, *FEBS Lett. 155*: 143 (1983).

67. M. J. C. Rhodes, and B. H. Kirsop, *Biologists 29*: 134 (1982).
68. G. R. Cysewski, and C. R. Wilke, *Biotechnol. Bioeng. 18*: 1297 (1976).
69. T. Nakashima, H. Fukuda, Y. Nojima, and S. Nagai, *J. Ferment Bioeng. 68*: 18 (1989).
70. T. Nakashima, S. Kyotani, E. Izumoto, and H. Fukuda, *J. Ferment. Bioeng. 70*: 85 (1990).
71. K. Venkatasubramanian, S. B. Karkare, and W. R. Vieth, *Appl. Biochem. Bioeng 4*: 311 (1983).
72. A. Constantinides, D. Bhatia, and W. R. Vieth, *Biotechnol. Bioeng. 23*: 899 (1981).
73. Y. Y. Linko and P. Linko, *Biotechnol. Lett. 3*: 21 (1981).
74. N. Kawabata and S. Nishimura, *Biotechnol Bioeng 35*: 1000 (1990).
75. T. Shiotani and T. Yamane, *Eur J. Appl. Microbiol. Biotechnol. 13*: 96 (1981).
76. S. Kyotani, T. Nakashima, E. Izumoto, and H. Fukuda, *J. Ferment. Bioeng. 71*: 286 (1991).
77. J. Briffaud and J. M. Engasser, *Biotechnol. Bioeng. 21*: 2093 (1979).
78. P. F. Cooper and D. H. V. Wheeldon, *Biological Fluidised Bed Treatment of Water and Wastewater* (P. F. Cooper and B. Atkinson, eds.), Ellis Horwood, Chichester, 1981, p. 121.
79. I. Walker and E. P. Austin, *Biological Fluidised Bed Treatment of Water and Wastewater* (P. F. Cooper and B. Atkinson, eds.), Ellis Horwood, Chichester, 1981, p. 213.
80. B. Atkinson, J. D. Cunningham, and A. Pinches, *Chem. Eng. Res. Des. 62*: 155 (1984).
81. B. H. Davison and C. D. Scott, *Appl. Biochem. Biotechnol. 18*: 19 (1988).
82. B. H. Davison and C. D. Scott, *Biotechnol. Bioeng. 39*: 365 (1992).
83. E. J. Arcuri, J. R. Nichols, T. S. Brix, and V. G. S. Santamarina, *Biotechnol Bioeng. 25*: 2399 (1983).
84. R. M. Busche and B. R. Allen, *Appl. Biochem. Biotechnol. 20/21*: 357 (1989).
85. J. H. Kim, D. K. Oh, S. K. Park, and Y. H. Park, *Biotechnol. Bioeng. 28*: 1838 (1986).
86. L. S. Fan, K. Fujie, T. R. Long, and W. T. Tang, *Biotechnol. Bioeng. 30*: 498 (1987).
87. C. D. Scott and C. W. Hancher, *Biotechnol. Bioeng. 18*: 1393 (1976).
88. H. Hatakeyama and H. Noda, *Kagaku Kogaku. 48*: 603 (1984).
89. B. C. Foster, R. T. Coutts, F. M. Pasutto, and J. B. Dossetor, *Biotechnol. Lett. 5*: 693 (1983).
90. F. Mavituna, *Process Engineering Aspects of Immobilised Cell Systems* (C. Webb, G. M. Black, B. Atkinson, eds.), Institute of Chemical Engineers, Rugby, UK, 1986, p. 134.
91. E. J. Wilson and C. J. Geankoplis, *Ind. Eng. Chem. Fundam. 5*: 9 (1966).
92. J. C. Chu, J. Kalil, and W. A. Wetteroth, *Chem. Eng. Prog. 49*: 141 (1953).
93. T. Yamane, S. Araki, and E. Sada, *J. Ferment. Technol. 59*: 367 (1981).
94. T. Kobayashi, K. Ohmiya, and S. Shimizu, *J. Ferment. Technol. 54*: 260 (1976).
95. J. E. Bailey and D. F. Ollis, *Biochemical Engineering Foundamentals*, McGraw Hill, New York, 1977, p. 395.
96. A. Shinmyo, H. Kimura, and H. Okada, *Eur. J. Appl. Microbiol. Biotechnol. 14*: 7 (1982).
97. B. Atkinson and H. W. Fowler, *Adv. Biochem Eng. 3*: 224 (1974).
98. K. Toda and K. Sato, *J. Ferment. Technol. 63*: 251 (1985).

99. K. Østergaard and P. Fosbol, *Chem. Eng. J. 3*: 105 (1972).
100. V. R. Dhanuka and J. B. Stepanek, *AIChE J. 26*: 1029 (1980).
101. K. D. P. Nigam and A. Schumpe, *AIChE J. 33*: 328 (1987).
102. K. Nguyen-Tien, A. N. Patwari, A. Schumpe, and W. D. Decker, *AIChE J. 31*: 194 (1985).
103. S. Fukushima, *J. Chem. Eng. Jpn. 12*: 489 (1979).
104. K. Østergaard, *AIChE Symp. Ser. 74*: 82 (1978).
105. J. C. Lee and H. Worthington, *Inst. Chem. Eng. Symp. Ser.* No. 38, *1*: B2 (1974).
106. H. Kojima and K. Asano, *Ryudousou Kogaku*, (K. Yoshida, and S. Furusaki, eds.), Society of Chemical Engineers, Japan, 1981, p. 79.
107. T. Nakashima and H. Fukuda, *J. Ferment Bioeng. 70*: 355 (1990).
108. K. K. Bandyopadhyay and T. K. Ghose, *Biotechnol. Bioeng. 24*: 805 (1982).

10
Immobilized Animal Cell Bioreactors

Matthew S. Croughan

Genentech, Inc.
South San Francisco, California

Tzyy-Wen Chiou

Academia Sinica
Taipei, Taiwan

Daniel I. C. Wang

Massachusetts Institute of Technology
Cambridge, Massachusetts

I. INTRODUCTION

A. Cell Attachment and Growth

To initiate a culture of animal cells, one removes the cells from the animal and often then immobilizes the cells on an appropriate surface [1,2]. If the subsequent culture environment is suitable, the cells will grow to cover the surface and can be used to inoculate a new, larger surface. The initial growth surface is generally the interior bottom wall of a simple vessel, such as a T-flask or petri plate [1,2]. For large-scale operations, which require billions of cells or more,

extensive surface area is often provided through highly engineered reactors. The design of such reactors is the topic of this chapter.

Many cells are anchorage dependent; they must attach and spread on a growth surface in order to synthesize DNA [3]. Such cells are often diploid and exhibit contact inhibition [2], wherein cell division is gradually inhibited by cell-cell contact as the culture reaches confluence (complete surface coverage). Anchorage dependence is generally documented under a limited set of operating conditions. Thus, it represents a practical operating requirement rather than a fundamental description. For any given cell line, one may find conditions which allow the cells to grow free-floating in suspension. To our knowledge, however, no diploid cells have been adapted to grow in suspension without first becoming aneuploid through the process of transformation, as described in Freshney [1].

Many aneuploid cell lines can be adapted to grow in suspension. The adaptation procedure simply involves periodic dilution of the cells in suspension into a new culture vessel with fresh medium. The cells in suspension eventually multiply, if possible. The cells which multiply in suspension may be genetically distinct from the original population. They often consist of a genetically heterogeneous population which is capable of adaptation to a wide variety of culture conditions. Such a population may contain cells both growing in suspension and attached to any available surface. There is often an equilibrium between the attached and suspended populations, wherein cells go back and forth between attachment and suspension, depending on circumstances such as the hydrodynamic environment [4]. The properties of the population depend on the culture protocols used. For instance, the tendency of the cells to attach to surfaces can be increased if only the attached cells are used to inoculate new cultures.

Immobilized cell reactors can be used for cells which are anchorage dependent or for those which can grow in suspension. Animal cells are generally immobilized through attachment to a surface, attachment to each other, entrapment in pores or a fiber matrix, and/or entrapment behind a membrane or screen.

B. Overview of Immobilized Animal Cell Bioreactors

Several potential advantages are gained upon cell immobilization:

1. As discussed, immobilization on a suitable surface allows for growth of anchorage-dependent cells.
2. For all cells, immobilization often allows simple cell-liquid separation. When cells are attached to a surface or held behind membranes, the culture fluid is more easily removed without carrying cells with it. Subsequent centrifugation and/or filtration steps may not be required to separate the fluid from the cells.

3. Cells may be immobilized in a region where they are not exposed to bubbles or high shear flows, which might otherwise damage them.
4. Immobilization may provide higher protein production per cell, improved vaccine production per cell, and/or other favorable changes in cell properties [5,6]

There are a few disadvantages of cell immobilization:

1. Immobilized cells must often be detached, removed, and/or disaggregated into a suspension of roughly single cells before they can be used to inoculate a new culture.
2. Immobilized cells cannot rotate or translate individually in response to fluid forces and thus may be more susceptible to damage upon any exposure to shear or collisions.
3. Entrapment membranes and/or multiple cell layers may hinder exchange of nutrients and waste products between the cells and medium.
4. The cost of the immobilization surface or matrix may be substantial.

The potential advantages and disadvantages of immobilization must be considered in the design and seletion of a cell culture reactor.

Immobilized bioreactors have been employed successfully in all areas of cell culture for many years [7]. As already discussed, petri plates and T-flasks have been extensively used to initiate primary cultures and, subsequently, to provide cells for experiments and/or further population expansion. The simple roller bottle, in which cells are grown on the inner surface of a rotating bottle [1], is used in the production of vaccines, such as rubella [8], and recombinant therapeutic proteins, such as erythropoietin, which have a low dose requirement. Microcarrier cultures, in which cells are attached to small beads suspended in a stirred tank [9,10], have been used for larger-scale production of vaccines and therapeutic proteins [6]. Packed bed bioreactors, in which cells are entrapped in a bed of spheres or fibers [5,11–17], show promise for even larger-scale production. Hollow-fiber reactors, in which cells are grown in or around fibers encased in a a reactor shell [18–23], are often used to manufacture antibodies for diagnostic use. A wide variety of other immobilized animal cell bioreactors have been developed, such as ceramic matrix reactors [24–27], fluidized beds or stirred tanks with porous microcarriers [6,28–33], stirred tanks with cells entrapped in microcapsules [34–40], electrode reactors [41], stirred tanks with cells grown as aggregates or periodically flocculated [42,43], and stacked plate reactors [1,44].

The design of each type of reactor is unique but follows the same principles. Nutrients must be provided to the cells, and waste products must be removed. If there are multiple layers of cells, diffusion through the layers must

be adequate. The medium must be replenished with any nutrients the cells deplete. As much as possible, the bioreactor should provide a homogeneous environment to all parts of the culture which is optimized with respect to environmental parameters such as temperature, pH, and nutrient levels. Nonetheless, convective flow for mixing should not be excessive, as animal cells are fragile and susceptible to death from hydrodynamic forces. The reactor should be as simple and inexpensive as possible. Because reactor costs often increase with reactor size, the surface area for cell attachment, or volume for cell entrapment, per total reactor volume should be as high as possible. The reactor should easily operate free of contamination with microorganisms.

In this chapter, the design of a stirred tank microcarrier bioreactor and a packed bed bioreactor with glass fibers is discussed in detail. These two examples are representative of two primary challenges a biochemical engineer may face in industry. In the first example, the operation of a traditional bioreactor is optimized. In the second example, the design and operation of an entirely new bioreactor are optimized. Both examples illustrate the application of both practical and fundamental principles to bioreactor design and optimization.

II. MICROCARRIER BIOREACTORS

A. Bioreactor Characteristics

In microcarrier bioreactors, animal cells are grown on the surface of small beads generally suspended in a stirred tank filled with aqueous culture medium [6,9,10]. The microcarrier beads are very small, with typical diameters of 100–200 μm, and almost neutrally buoyant, with typical densities of 1.03–1.05 g/ml. These density and size ranges allow both rapid suspension of the microcarriers under mild agitation and rapid gravity sedimentation of the microcarriers under static flow conditions. Thus, the microcarriers can easily be immersed in the culture fluid without subjecting the cells on their surfaces to unnecessary shear and yet easily separated from the culture fluid through sedimentation to allow medium replacement. The small microcarrier size further allows a high ratio of growth surface area to culture volume, as the microcarriers can easily be suspended at concentrations up to and over 500 million microcarriers per liter.

Precise optimization of microcarrier size is a complicated problem, yet to be thoroughly undertaken, with trade-offs between the cost of generating an inoculum of 1–6 cells per microcarrier [45–50], the ease of settling of microcarriers of different sizes, the cost and surface area per gram of microcarriers at different sizes, cell growth on microcarriers of different sizes (curvature effects), and hydrodynamic damage to cells on microcarriers of different sizes.

Many researchers and companies have employed the microcarrier technique and noted its advantages [1,6,9,10,45–64]. A thorough review of microcarrier culture systems is provided by Reuveny [6]. The use of a well-mixed stirred tank provides a homogeneous culture environment and good cell-liquid mass transfer, as will be discussed. Perfusion operation, with continuous cell-liquid separation and medium replacement, is easily obtained through the use of a microcarrier settling device [54,55,61,62] or filter [59].

The primary limitation to microcarrier cultures arises through the susceptibility of the cells to damage from fluid mechanical forces [60,63,64]. The susceptibility results from the lack of a protective cell wall, the relatively large size of animal cells, and the lack of individual cell mobility. Cells anchored to microcarriers cannot individually rotate or translate; they therefore cannot reduce the net forces and torques experienced upon exposure to fluid mechanical forces.

In microcarrier cultures, agitation is required for cell-liquid mass transport, gas-liquid mass transport (oxygenation), and liquid-phase mixing. For maximum cell growth, adequate mass transfer must be achieved with few or no detrimental effects from hydrodynamic forces. Although this condition can be readily attained in low-density laboratory cultures, it becomes more difficult to attain as cell densities or culture volumes are increased. Successful scale-up to high-density, large-volume cultures requires a thorough understanding of the hydrodynamic and mass transport phenomena.

B. Mass Transfer

1. Cell-Liquid Transport

For each chemical species which is consumed or produced by cells on microcarriers, the normalized gradient between the chemical concentration in the local bulk medium, X_b, and the concentration at the cell surface, X_c, can be calculated from mass transfer correlations for spheres in stirred tanks [65,66]. The result for cells on microcarriers can be expressed by the relation

$$\frac{X_b - X_c}{X_b} = \frac{YR_c d_p}{D_f X_b \, \mathrm{Sh}_c} \tag{1}$$

where Y is the surface coverage in cells/cm^2, R_c is the uptake rate per cell for a given chemical species, d_p is the microcarrier diameter, D_f is the diffusion coefficient for the given chemical species, and Sh_c is the Sherwood number for cell-liquid transport. This Sherwood number can be determined from published correlations [65,66] and has a minimum value of 2. For chemicals which are produced by the cells, R_c will be negative.

Substituting typical values for the parameters in Eq. (1), one finds that the normalized gradients are far less than unity, with values on the order of 0.001 to 0.1. The chemical concentrations at the cell surface are, therefore, essentially equal to the concentrations in the bulk medium. A similar Nusselt number analy-

sis shows that a parallel conclusion can be drawn with regard to temperature gradients.

Thus, mere suspension of microcarriers provides adequate mass and heat transport between the cells and medium. This conclusion is supported by the results in the literature [53,56,60,67,68]; uniform growth is observed over a wide range of nondetrimental levels of agitation that are sufficiently strong to suspend the microcarriers and provide adequate oxygen transfer to the bulk fluid.

2. Liquid-Phase Mixing

Given negligible gradients between the cells and bulk fluid, one should also consider the requirements for adequate mixing in the liquid phase. In general, mixing is required to provide a homogeneous environment in all parts of the culture which is optimized with respect to environmental parameters such as temperature, pH, and nutrient levels. To characterize the culture for research or regulatory purposes, a well-mixed culture is often useful, as samples can be withdrawn which are representative of the entire culture. If liquid base or acid is directly added for pH control, mixing is required to minimize the local pH excursion and any corresponding cell damage. Mixing is further required to minimize the lag between any acid or base addition and the corresponding response of the pH probe. Mixing is also required to ensure that appropriate nutrient and waste product levels are maintained in all regions of the reactor.

For any given circumstance, one must decide which liquid mixing criteria are critical. In this chapter, the criterion of adequate nutrient levels throughout a microcarrier bioreactor will be discussed.

Oxygen is often the nutrient most susceptible to depletion. Oxygen is often toxic or growth-inhibitory when present in excess of air saturation [69–72], which represents a very low molar concentration (~0.2 mM). Accordingly, the oxygen content of the medium must be limited in any region where oxygen is supplied. For sufficiently high cell concentrations and low mixing rates, the limited oxygen content may be completely consumed before the medium recirculates between any regions of oxygen supply and oxygen uptake.

For precise modeling of chemical gradients in the liquid phase, one can potentially employ detailed computer models of the fluid flow and chemical concentrations in a particular stirred tank reactor. Although this type of approach is becoming more common, it is expensive and not yet always reliable, because of the difficulties in modeling turbulent flow fields. A rougher but quicker picture can be obtained through analysis of mixing times. The mixing time is the time required for a chemical to reach a homogeneous concentration throughout the reactor after it has been added at a single position. To simplify measurements, mixing is often considered complete when there is less than 1% variation in concentration around the reactor [73]. For many reactors in the fully turbulent regime which are not being sparged with gas, the mixing time, M_t, for the fluid

phase can be estimated from [73]:

$$M_t = a_1\left(\frac{H}{D_a}\right)\left(\frac{D_o}{D_a}\right)^2 /N \tag{2}$$

where H is the liquid height, D_a is the impeller diameter, D_o is the tank diameter, N is the impeller rotation rate, and a_1 is a constant which is characteristic of the reactor and impeller geometry.

The mixing time generally represents the time required for about five complete circulations of the tank contents [73]. Thus, the time required for a fluid element to circulate once around a reactor can be estimated as one-fifth of the mixing time given by Eq. (2). If there is a distinct region where oxygen is supplied, such as at the culture surface, there will be a variation in dissolved oxygen level within any fluid element as it circulates around the bioreactor. This variation, ΔO_2, can be roughly estimated as

$$\Delta O_2 = \frac{R_o C M_t}{5} \tag{3}$$

where M_t is the mixing time given by Eq. (2), C is the viable cell concentration, and R_o is the oxygen uptake rate per cell. In the design of a microcarrier reactor, one might specify the maximum ΔO_2 based on the cell metabolism. The agitation should then be sufficient to provide a ΔO_2 less than the specified maximum.

3. Gas-Liquid Transfer

Given adequate cell-liquid transport and liquid-phase mixing, one should also consider the overall transfer of nutrients and waste products into and out of the bulk fluid. Oxygen is generally the most difficult nutrient to supply, as it is consumed at a considerable rate by the cells and yet is only sparingly soluble in cell culture medium. Nearly all other nutrients and waste products are highly soluble in aqueous solution; adequate levels can easily be maintained through fed-batch additions or medium exchange. Oxygen can potentially be supplied through continuous perfusion and recycle through an external oxygenator, although this technique requires very high recirculation rates. For high-density microcarrier cultures in a stirred tank, the perfusion technique is not a viable means of supplying oxygen.

Oxygen is generally supplied through continuous mass transfer between the culture fluid and a gas phase. The volumetric rate of oxygen transfer, N_o, is generally described by an equation of the form

$$N_o = K_L a(O_2^* - O_2) \tag{4}$$

where K_L is the overall oxygen transfer coefficient (cm/s), a is the interfacial gas-liquid surface area per unit culture volume (cm^{-1}), O_2^* is the dissolved oxygen concentration in equilibrium with the gas phase, and O_2 is the average

dissolved oxygen concentration in the bulk medium (mmol/ml). If oxygen is transferred from the gas headspace to the surface of the culture (surface aeration), the value of $K_L a$ is generally given by equations of the form [57,74,75]

$$K_L a = K_s a_s = \left(\frac{\mathrm{Sh}_s D_{f,o}}{D_o}\right)\left(\frac{1}{H}\right) \tag{5}$$

where

$$\mathrm{Sh}_s = a_2(\mathrm{Re})^{\partial 1}(\mathrm{Sc})^{\partial 2}(\mathrm{Fr})^{\partial 3} \tag{6}$$

$$\mathrm{Re} = \frac{ND_a^2}{\nu} \tag{7}$$

$$\mathrm{Sc} = \frac{\nu}{D_{f,o}} \tag{8}$$

$$\mathrm{Fr} = \frac{N^2 D_a}{g} \tag{9}$$

where a_2, $\partial 1$, $\partial 2$, and $\partial 3$ are empirical constants, Sh_s is the Sherwood number for surface aeration, K_s is the oxygen transfer coefficient for surface aeration, a_s is the gas-liquid surface area per unit culture volume for surface aeration as given by the reciprocal of the liquid height H, Re is the impeller Reynold's number, Sc is the Schmidt number, Fr is the Froude number, $D_{f,o}$ is the diffusion coefficient for oxygen, ν is the kinematic fluid viscosity in cm^2/s as given by the ratio of fluid viscosity to fluid density, and g is the acceleration due to gravity. If the tank is fully baffled, the effect of Froude number becomes negligible and $\partial 3$ is essentially zero. The kinematic fluid viscosity in the denominator in Eq. (7) is sometimes replaced by the kinematic suspension viscosity, as given by the ratio of suspension viscosity [76] to volume average density [77].

Oxygen can also be supplied to the culture through the use of gas-permeable tubing, such as silicone tubing [50,57,69,74,78]. In this technique, the sparge gas is generally passed through tubing suspended in the culture fluid. The tubing inlet and outlet are outside the culture fluid. Thus, the oxygen is not directly sparged as bubbles into the culture but instead passes through the walls or pores of the tubing into the liquid. This avoids the sparging damage discussed below.

Oxygen can potentially be supplied to microcarrier cultures through direct sparging with air or oxygen. However, direct sparging can cause damage to cells on microcarriers [79]. If foam is generated, transfer of the microcarriers into the foam phase may cause cell dehydration and death. For cultures with freely suspended animal cells, cell damage from sparging can often be largely eliminated through the use of protective agents, such as F-68 [69,80–85]. Use of such protective agents in microcarrier cultures may reduce damage from sparging,

although reports on this topic have not been found. However, because immobilization of cells on microcarriers makes them more susceptible to hydrodynamic damage, direct sparging in microcarrier cultures may involve a trade-off between cell damage and oxygen transfer. In this regard, sintered spargers might be investigated, as they deliver very small bubbles [86] without the need for high agitation from an impeller.

C. Design Principles and Optimization

The design of microcarrier bioreactors is a classic chemical engineering optimization problem. Upon scale-up, the maximum cell concentration is often determined by a trade-off between mass transfer and hydrodynamic cell death. To incorporate accurately such trade-offs in bioreactor design, one needs quantitative expressions for the rates of mass transfer and hydrodynamic death. In the previous section of this chapter, quantitative expressions for the rates of mass transfer were reviewed. Quantitative expressions for the rates of hydrodynamic death in microcarrier cultures have been derived for a particular cell line, human diploid FS-4 fibroblasts, grown on the surface of Cytodex 1 microcarriers [67,68,77,87]. In cultures which were well supplied with nutrients, including oxygen, controlled within a favorite pH range, and frequently depleted of any toxic metabolites, the cell population kinetics followed the expressions

$$\frac{dC}{dt} = \mu C - q_1 C - q_2 C_m C \tag{10}$$

$$q_1 = 6.2 \times 10^{-13}\left(\frac{\bar{\epsilon}}{\nu^3}\right)^{3/4} \tag{11}$$

$$q_2 = 7.6 \times 10^{-16}\left(\frac{\bar{\epsilon}}{\nu}\right) \tag{12}$$

where μ is the specific total growth rate in s^{-1} and is independent of the level of agitation, q_1 is the first-order specific death rate in s^{-1}, q_2 is the collision death rate constant in ml/microcarriers-s, and $\bar{\epsilon}$ is the average agitation power input per mass in cm^2/s^3. Agitation power inputs were estimated from the results of Nagata [88], which have since been shown to be accurate for the experimental system used [89].

Equation (11) describes cell death due to microcarrier-eddy interactions and is based on theoretical model [68]. Equation (12) describes cell death due to microcarrier-microcarrier collisions [67] and is a simple fit to experimental data [77]. For low levels of agitation, hydrodynamic death is essentially insignificant, and the values of q_1 and q_2 are generally negligible.

To illustrate the use of the kinetic expressions, one might consider the problem of maximizing the cell concentration in a large-scale microcarrier bioreactor. In such bioreactors, the maximum cell concentration is frequently limited by the rate of oxygen transfer [74]. The oxygen limitation occurs as the cells grow and the dissolved oxygen level drops below a critical or minimally acceptable value. Specification of this minimum value, $O_{2,min}$, and the gas phase composition will specify the maximum driving force for oxygen transfer. Specification of the reactor geometry, agitation power input, and fluid viscosity will specify the value of K_La. At the point of maximum oxygen transfer, an oxygen balance gives

$$R_oC_{max} = K_La(O_2^* - O_{2,min}) \tag{13}$$

where R_o is the oxygen uptake rate per cell and C_{max} is the maximum cell concentration which can be adequately supplied with oxygen.

For many cells, oxygen uptake is both growth and maintenance associated [57,58,70]. The specific oxygen uptake rate can generally be described by an equation of the form [90]

$$R_o = \frac{\mu}{Y_{n/o}} + M_o \tag{14}$$

where $Y_{n/o}$ is the growth yield of cells on oxygen in cells/mmol and M_o is the oxygen maintainence coefficient, or the oxygen uptake rate per cell under conditions of no cell growth.

For many cells, the specific growth rate is a function of both the cell and microcarrier concentration. For FS-4 cells, the mathematical dependence involves both the effects of contact inhibition and cell-derived growth inhibitors [67]. The effect of contact inhibition on growth can be incorporated with the following empirical equations, which fit the data presented in Hu et al. [45] for FS-4 cells:

$$\mu = \mu_{max}(100 - Z)/60 \qquad \text{for } Z > 40 \tag{15}$$

$$\mu = \mu_{max} \qquad \text{for } Z \leq 40 \tag{16}$$

where Z is the percent confluence. The growth rate free of contact inhibition, μ_{max}, and the maximum surface coverage depend weakly on the volumetric cell concentration [67]. Because FS-4 cells are inoculated at a roughly constant number of cells per microcarrier, the weak dependence on volumetric cell concentration can be roughly described by an empirical dependence on microcarrier concentration. Data for 5% serum [67], with microcarrier concentrations between 190 and 57,000 microcarriers/ml (0.05 to 15 g/L Cytodex 1), can be

described by the empirical relations

$$\mu_{max} = \frac{4.3 \times 10^{-5}}{C_m^{0.262}} \tag{17}$$

$$Z = \frac{100\ C}{500\ (C_m)^{0.80}} \tag{18}$$

where C_m is the microcarrier concentration in microcarriers/ml, C is the cell concentration in cells/ml, μ_{max} is the maximum growth rate in s^{-1}, and Z is the percent confluence.

If the oxygen transfer capabilities of a reactor are utilized to their fullest extent, the maximum cell concentration given by the limitations of oxygen transfer will equal the maximum cell concentration given by the limitations of hydrodynamic death and contact inhibition. This maximum cell concentration will be reached as the cells grow toward confluence and become contact inhibited. When the specific growth rate drops to a value equal to the total specific death rate, the rate of change in cell concentration will be zero and the cells will have reached the maximum cell concentration. At this point,

$$\mu = q_1 + q_2C_m \tag{19}$$

When the cells have reached their maximum concentration, the specific growth rate, as limited by contact inhibition and growth inhibitors, Eqs. (15)–(18), will be matched by the specific death rate, as determined by agitation and microcarrier concentration, Eqs. (11) and (12).

D. Illustration of Optimization

To fully illustrate how bioreactor design can be approached as an optimization problem involving quantitative rate expressions, optimizing calculations are described below for an FS-4 microcarrier culture. The optimum combination of agitation, microcarrier concentration, and fluid viscosity was determined for a given reactor. The objective was to maximize the maximum cell concentration.

The geometry of the reactor for this optimization is shown in Fig. 1 and in Table 1. The reactor has a liquid volume of 100 L and is equipped with baffles, a pitched-blade turbine (PBT), and a flat-blade turbine (FBT). The reactor is geometrically similar to the reactors which have been extensively studied by Aunins et al. [74]. For this optimization, it was assumed that oxygen transfer came solely through surface aeration. The oxygen transfer coefficient is then given by Eqs. (4)–(9), with $a_2 = 0.582$, $\partial 1 = 0.73$, $\partial 2 = 0.33$, and $\partial 3 = 0$. The constants were estimated from the results of Aunins et al. [74].

For operation in the completely turbulent regime, as required for microcarrier suspension, the average power input per unit mass, $\bar{\epsilon}$, was estimated

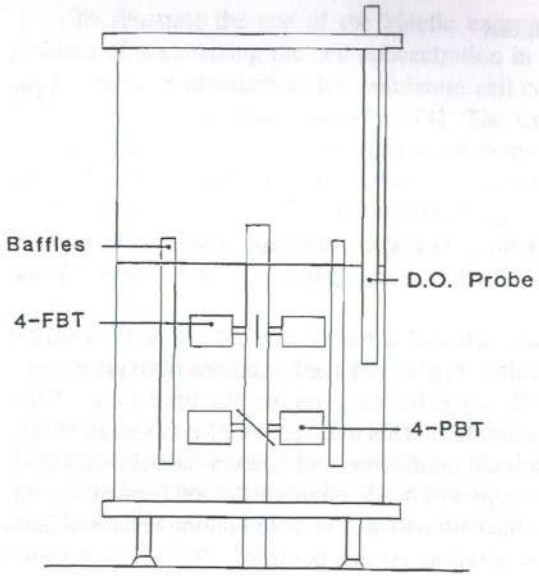


Figure 1 Traditional 100-L microcarrier bioreactor with four-bladed flat-blade turbine (4-FBT) and pitched-blade turbine (4-PBT).

by [73]

$$\bar{\epsilon} = \frac{0.8 N_p N^3 D_a^5}{V_c} \tag{20}$$

where N_p is the combined power number for the equal-sized turbines, V_c is the culture volume, and N is the impeller rotation rate, with all terms in metric units.

Table 1 Geometry of 100-L Microcarrier Bioreactor

Dimension	Size (cm)
Tank diameter	53.3
Liquid height	44.8
Impeller diameter	26.7
Baffle width	6.1
Radius to baffle inner edge	15.6
Impeller blade width	8.0
Height of center of pitched-blade turbine	15.0
Height of center of flat-blade turbine	30.0

The combined power number was estimated to be 5, the sum of 1 for the PBT and 4 for the FBT [91,92]. Because the spacing between the turbines is less than 60% of their diameter, the total power draw was reduced by a factor of 0.8, according to the results of Richards [93].

For the 10-L reactor investigated by Aunins et al. [74], the minimum speed for complete microcarrier suspension was approximately 30 rpm for medium with 10% serum, 10 g/L microcarriers, and no thickening agents. This corresponds to an estimated value of 16 cm^2/s^3 for the average power input per unit mass. Upon scale-up to the geometrically similar 100-L reactor, the agitation power per unit mass required for suspension will be reduced to 13 cm^3/s^3, according to the scale-up relations of Nienow [94]. From the results of Zweitering [95], one can incorporate the minor effects of fluid viscosity and volume fraction solids on the power required for microcarrier suspension.

This optimization was performed under the criteria of adequate liquid-phase mixing. The liquid-phase mixing was considered adequate if the estimated variation in dissolved oxygen content, as determined from Eq. (3), was less than 1% of saturation with air. As the culture surface is the region of oxygen supply, the variation in dissolved oxygen would occur as a fluid element left the culture surface and then recirculated back. The mixing time constant a_1 in Eq. (2) was assumed to be 4.3 [73].

This optimization was also performed under the criteria of adequate cell-liquid transport. This transport was considered adequate if the estimated normalized gradient in oxygen between the cells and local fluid, as determined from Eq. (1), was less than 0.10. The viscosity increase was limited so as not to introduce cell-liquid mass transport limitations at a Sherwood number of 2 or cell damage from time-average flow fields [68].

For this optimization, it was assumed that the culture medium was frequently replaced so as to eliminate nutrient and toxic metabolite limitations. To replace the medium in the small-scale cultures, the culture agitation was stopped, the microcarriers were allowed to settle, 60–80% of the culture medium was removed from the clear liquid above the settled microcarriers, fresh medium was added, and then the culture agitation was restarted [77]. In the 100-L reactor, the settling of the microcarriers might take too long, and one might employ a specific microcarrier settling device for cell-liquid separation [50,54,55,61,62]. For the culture kinetics to follow Eqs. (10)–(12) and (15)–(18), the interval length between medium exchanges should be determined according to the original protocols [67] as given by the relation

$$I_L = \frac{6.8 \times 10^5}{C_m} \tag{21}$$

where C_m is the microcarrier concentration in microcarriers per ml and I_L is the interval length in hours. This protocol maintains glucose levels between 1.0 and

4.5 g/L. From the amino acid results of Fleischaker [50] for the same culture system and same cell line, one can determine that this protocol maintains all amino acids at levels above 50% of their concentration in the fresh DME medium. Accordingly, cell death will arise primarily through hydrodynamic damage and not through nutrient depletion or toxic metabolite accumulation in the medium.

This optimization was further performed under the following additional assumptions:

1. The headspace gas contained a 90:10 air-CO_2 mixture.
2. The medium was supplemented with 5% (v/v) fetal calf serum.
3. The density and viscosity of this medium are 1.00 g/ml and 0.74 cp (0.0074 gm/cm-s), respectively [68].
4. The diffusion coefficient for oxygen in this medium at 37°C is 2.4×10^{-5} cm²/s, as given in Reid et al. [96] for oxygen diffusion in water and corrected for minor temperature effects from the correlation of Wilke and Chang [97].
5. For changes in viscosity, dextran of molecular weight 78,500 was assumed to be the thickening agent, which results in only minor changes in density [68].
6. The diffusion coefficient is inversely proportional to fluid viscosity [97].
7. The Henry's law coefficient for O_2 was estimated to be 0.86 mmol/L-atm [50], near the value of 0.95 mmol/L-atm since found by Wohlpart et al. [98]; thus, with 90% air containing 21% oxygen at 1 atm total pressure, $O_2^* = 0.86\ (0.90)(0.21)(10^{-3}) = 1.63 \times 10^{-4}$ mmol/ml.
8. The minimum acceptable level of average dissolved oxygen in the fluid was 10% of saturation with air. Below this value, oxygen can become growth-limiting [70,71]. Thus, $O_{2,min} = 1.81 \times 10^{-5}$ mmol/ml.
9. The growth yield of cells on oxygen, $Y_{n/o}$, was 3.0×10^8 cells/mmol and the maintenance coefficient on oxygen, M_o, was 3.61×10^{-11} mmol/cell-s [77].
10. The microcarriers (Cytodex 1) are solid spheres with an average diameter of 178 μm [67]. There are 3.8 million microcarriers per gram of dry Cytodex 1 [67].

After making these assumptions, one has reduced the number of independent variables to two: fluid viscosity and average power input per unit. The optimization involves calculation of the maximum cell concentration for each combination of fluid viscosity and average power input. The calculation involves an iterative procedure. For each value of fluid viscosity and agitation power, one calculates a value for K_La from Eq. (5) and then guesses an appropriate microcarrier concentration. The corresponding specific growth rate, based on the balance against hydrodynamic death, is then calculated from Eqs. (11), (12), and (19). The result is used to estimate the oxygen uptake rate per cell [Eq. (14)], the maximum cell concentration from the oxygen transfer limitations [Eq. (13)], and then the corresponding specific total growth rate as limited by contact inhibition and growth inhibitors at that particular cell density [Eqs. (15)–(18)]. If this rate [Eq. (15) or (16)] matches the rate based on the balance against hydrodynamic death [Eq. (19)], the calculation is complete. If not, a new microcarrier concentration should be guessed and the calculations repeated.

After having calculated the correct microcarrier concentration and maximum cell concentration, one can determine whether the criteria of adequate liquid mixing and cell-liquid transport were fulfilled. If they were not, the agitation power should be increased and/or the viscosity reduced until the criteria are fulfilled.

Figure 2 shows the results of the optimization calculations. Maximum cell concentration is plotted against average power input unit mass. Calculations are

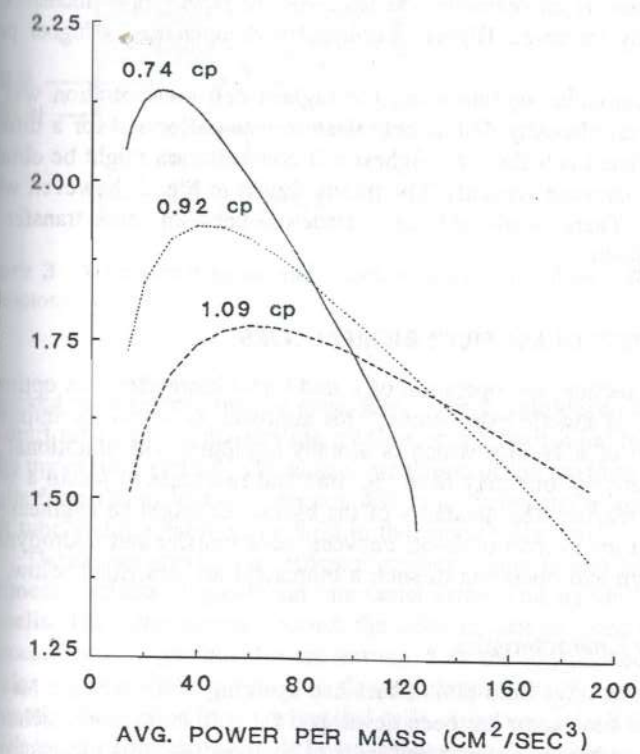


Figure 2 Estimated maximum cell concentration versus average power input per unit mass at different fluid viscosities for 100-L FS-4 microcarrier culture.

shown for three different viscosities: normal medium (0.74 cp) and medium slightly thickened (0.92 and 1.09 cp). For all three viscosities, the maximum cell concentration initially rises as the agitation is increased above the minimum level for microcarrier suspension. These increases are due to increased oxygen transfer capabilities. The highest cell concentration is obtained with the lowest viscosity, again due to oxygen transfer capabilities. If the agitation is increased above the optimum at the lowest viscosity, the maximum cell concentrations drop sharply. This is due to cell death resulting from excessive agitation. Eventually, at a power input of approximately 125 cm^2/s^3 for the viscosity of 0.74 cp, the specific death rate is greater than the maximum growth rate of the cells. The attached cell concentrations will then decrease from the time of inoculation.

If the viscosity is increased above 0.74 cp, the curves become flatter and less sensitive to agitation. In general, an increase in viscosity leads to a reduction in both oxygen transfer and hydrodynamic death. For any given power input in a given reactor, there is an optimum viscosity. As the power input increases, the optimum viscosity increases. Higher viscosities allow operation at higher power inputs.

For this particular optimization, the highest cell concentration was obtained with lowest viscosity. If this optimization was performed for a different reactor or a different cell line, the highest cell concentration might be obtained with a slightly increased viscosity. The trends shown in Fig. 2, however, would still be present. There would still be a trade-off between mass transfer and hydrodynamic death.

III. PACKED-BED GLASS FIBER BIOREACTORS

In the previous section, the operation of a traditional bioreactor was optimized through the use of kinetic expressions. This approach is useful for improving the performance of a facility which is already equipped with traditional bioreactors. Alternatively, one may have the time and resources to install a whole new type of bioreactor. The geometry of the bioreactor might be engineered so as to circumvent the normal trade-off between mass transfer and hydrodynamic death. The design and operation of such a bioreactor are described below.

A. Bioreactor Characteristics

Based on cell anchorage onto glass fibers and sparging of air through an inner draft tube, a new bioreactor has been developed for cultivating both anchorage-dependent and anchorage-independent cells [12]. Basically, this bioreactor has a modified configuration of a concentric cylinder airlift reactor as shown in Fig. 3. The cells are retained in the annulus section of the airlift bioreactor, which

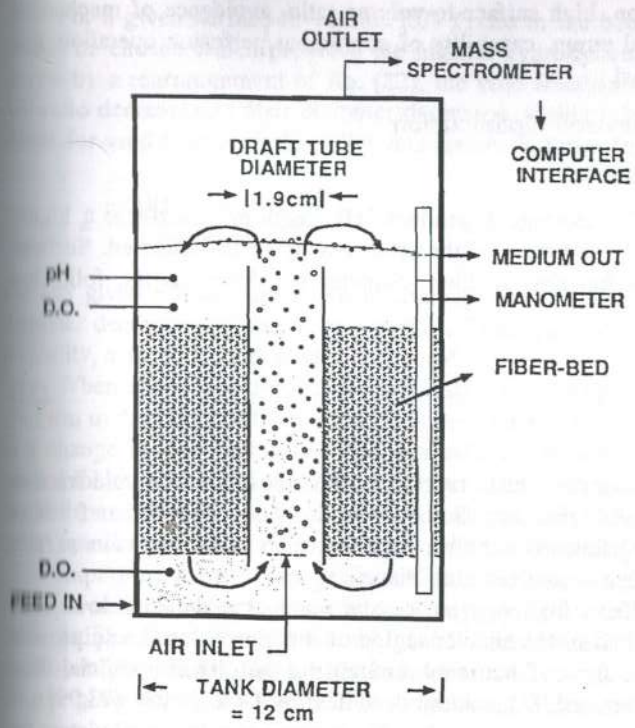


Figure 3 Schematic diagram and associated equipment of the 2.6-L airlift fiber bed bioreactor.

is packed with submicrometer glass fibers. Air is introduced from the base of the draft tube and oxygenates the medium in the draft tube; bubbles disengage at the upper fluid surface. The global circulation of the medium in the bioreactor results from the hydrostatic pressure difference between the aerated fluid in the draft tube and the bubble-free fluid in the annular fiber bed.

The design provides an efficient oxygen supply in situ and minimizes the detrimental effects of gas-liquid interfacial forces and hydrodynamic forces to the cells. The latter occurs because the cells are not exposed to gas-liquid interfaces, since no gas bubbles are entrained by the oxygenated medium in the annular region. When the fluid is flowing through the fiber bed, nutrients are convectively supplied to the entrapped cells. However, very little hydrodynamic cell damage can result because low turbulence and low shear stress occur due to the low liquid velocity in the annular fiber bed. Additional design objectives and unique characteristics of this bioreactor design include its simple construc-

tion, ease of operation, high surface-to-volume ratio, avoidance of mechanical agitation and external pump, capability of continuous/perfusion operation, and high scale-up potential.

B. Design Principles and Optimization

1. Glass Fiber Diameter

Since the growth of anchorage-dependent cells requires a surface, a higher surface-to-volume ratio allows a higher cell density to be achieved. Surface-to-volume ratio is a function of fiber diameter as shown in the following equation:

$$\frac{S}{V} = \frac{4(1 - E)}{d_f} \tag{23}$$

where S/V is the surface-to-volume ratio in the fiber bed, E is the void fraction of bed, and d_f is the fiber diameter. Since the surface-to-volume ratio of a fiber bed is inversely proportional to the fiber diameter, high surface-to-volume ratio can be achieved by decreasing the fiber diameter.

In order to achieve high oxygen transfer and yet maintain a low shear stress, an ideal fiber bed in the annular region of the bioreactor should provide for a high convective flow of nutrients through the bed. The superficial fluid velocity through a fiber bed, $U_{s/o}$, can be described by Darcy's law [73,99]

$$U_{s/o} = \frac{k\Delta P}{\eta L_o} \tag{23}$$

where k is the hydrodynamic permeability in cm^2, ΔP is the pressure drop across the bed in g/cm-s^2, L_o is the bed height in cm, and η is the fluid viscosity in g/cm-s. The theoretical value of permeability for various mat geometries without cells can be calculated via various models [99]. For laminar flow, the hydrodynamic permeability can be estimated by the Kozeny-Carman equation [73].

$$k = \frac{g_c \Omega_s^2 d_f^2 E^3}{150(1 - E)^2} \tag{24}$$

where Ω_s is the sphericity of the fibers (dimensionless) and g_c is Newton's law proportional factor, which has a value of 1.0 (dimensionless) when metric units are employed. The sphericity will depend on the orientation of the fibers relative to the direction of the fluid flow.

For a given surface-to-volume (S/V) ratio in the bed, the fiber diameter should be chosen which provides the highest hydrodynamic permeability. As shown by a rearrangement of Eq. (22), the void fraction required for a given S/V ratio decreases as fiber diameter decreases. If this relation is used to substitute for void fraction in Eq. (24), one arrives at following result:

$$k = \frac{g_c \Omega_s^2 [1 - (d_f S/V4)]^3}{150(S/V4)^2} \tag{25}$$

For any given S/V and sphericity, hydrodynamic permeability increases as fiber diameter decreases, as shown by Eq. (25). Consequently, to generate high permeability, a small fiber diameter is favored.

When anchorage-dependent cells attach to a support, they are likely to conform to the shape of the support. It is possible that this adaptation may result in a change in their metabolic rates. For cells on glass beads, Maroudas [100] has set the minimum threshold diameter for cell growth to be 50 μm. However, for cells on glass fibers, the minimum diameter may be smaller because the cells' cytoskeletal elements can align along the fiber axis [101,102].

Experiments have been performed to assess the possibility of using glass fibers of small diameters as an alternative support for cell growth. Perry and Wang [11] successfully grew recombinant Chinese hamster ovary cells (γ-CHO) on 80-μm glass fibers. We further found that γ-CHO cells growing on 24- and 40-μm glass fibers did not exhibit a significant difference in the growth rate and metabolic activity compared with cells growing in roller bottles. Singhvi et al. [103] are studying the effects of roughness on adhesion strength and cell function. More systematic research needs to be conducted in order to determine the optimum fiber diameter required for cell attachment and growth and the effect of curvature on cell metabolism. Decreasing fiber diameter is beneficial for obtaining high surface-to-volume ratio and high permeability, as mentioned earlier. However, to achieve the best reactor performance, it is important to consider the curvature effect on cell function when the fiber diameter is very small.

2. Airlift Design for Oxygen Transfer

In order to determine the optimal reactor configuration in terms of the ratio of inner draft tube diameter (D_i) to reactor diameter (D_o), a hydrodynamic model was developed to characterize the bioreactor with respect to the transport phenomena [13]. In this model, overall momentum can be balanced by equating the driving force resulting from the hydrostatic pressure difference against the hydraulic resistance of the draft tube and the annular packed bed. Using the model, one can predict liquid flow rates and volumetric mass transfer coefficients which

are in agreement with experimental results. Furthermore, the model can be used to optimize the D_i/D_o ratio to give the greatest oxygen transfer rate.

For the reactor shown in Fig. 3, Fig. 4 shows the calculated reactor oxygen transfer rate versus D_i/D_o ratio at various air flow rates. A distinctive maximum for the oxygen transfer rate was obtained for each air flow rate. Each maximum corresponds to an optimal D_i/D_o ratio. The optimal ratio shifts to higher values as the air flow rate is increased. The trend can clearly be seen by plotting the optimal D_i/D_o ratio versus air flow rate (Fig. 5). The quantitative relationship between optimal D_i/D_o and air flow rate (Q_g) was found to be

$$\left(\frac{D_i}{D_o}\right)_{opt} = 0.080(Q_g)^{0.22} \tag{26}$$

These optimal D_i/D_o ratios are much smaller than those found in traditional concentric airlift reactors without packing. The difference indicates that the in situ oxygenator occupies only a very small percent of the whole reactor. This is advantageous because most of the reactor space can be used to pack glass fibers and thus increase the surface area for cell growth.

Oxygen transfer in the system is a function not only of the D_i/D_o ratio but also of the hydrodynamic permeability of the fiber-bed. Figure 6 shows the effect of permeability of the fiber bed on the oxygen transfer rate at various

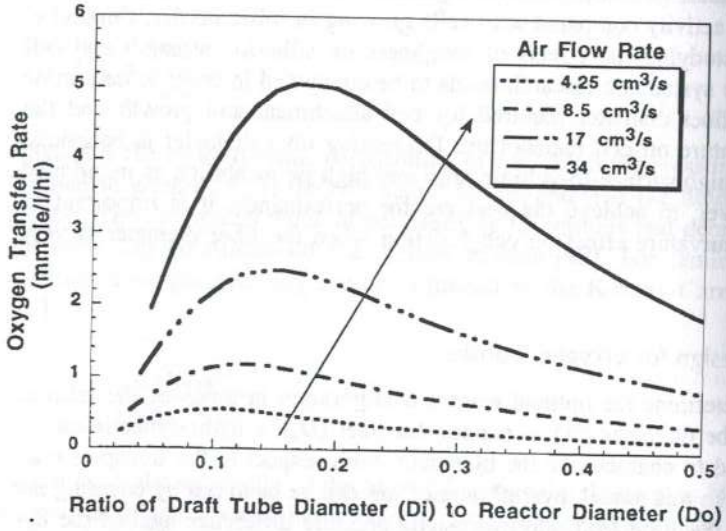


Figure 4 Effect of D_i/D_o on oxygen transfer rate at various air flow rates for fiber bed bioreactor.

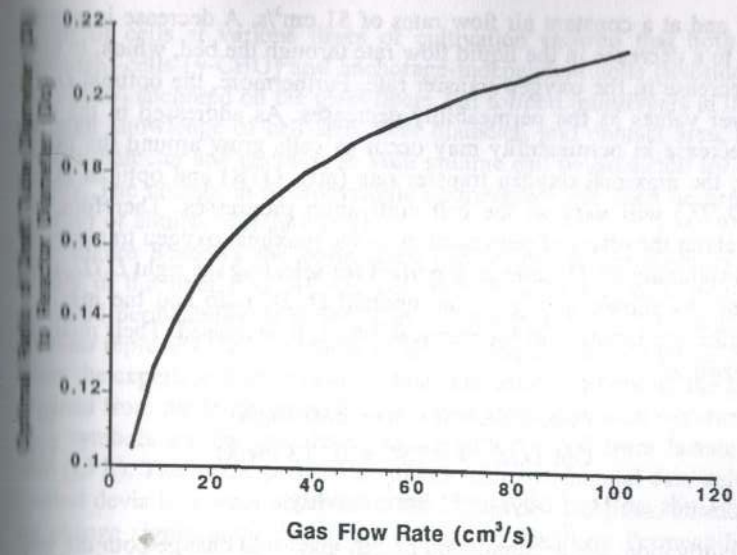


Figure 5 Optimal D_i/D_o at various air flow rates for fiber bed bioreactor.

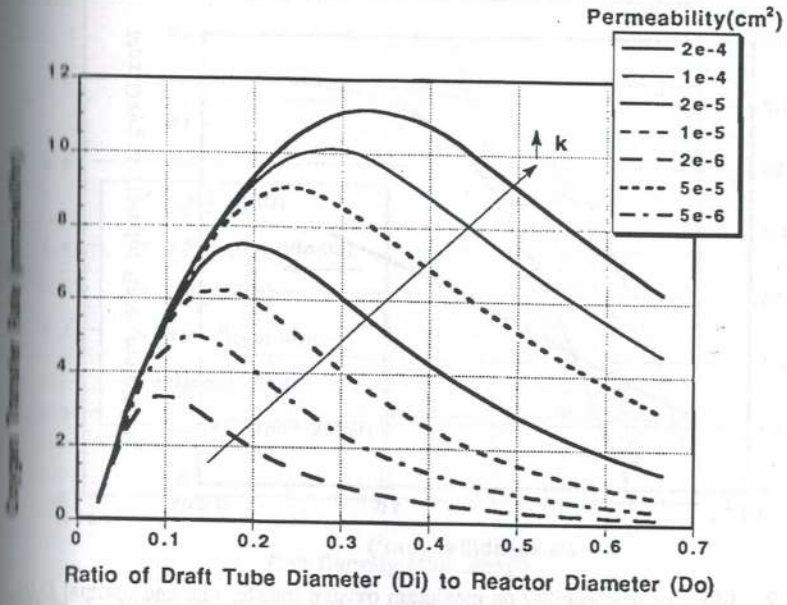


Figure 6 Effect of permeability on oxygen transfer rate for fiber bed bioreactor.

D_i/D_o ratios and at a constant air flow rates of 51 cm^3/s. A decrease in permeability leads to a decrease in the liquid flow rate through the bed, which, in turn, leads to a decrease in the oxygen transfer rate. Furthermore, the optimal D_i/D_o shifts to lower values as the permeability decreases. As addressed in the next section, a decrease in permeability may occur as cells grow around the fibers. Accordingly, the maximal oxygen transfer rate (max OTR) and optimal D_i/D_o ratio (Opt D_i/D_o) will vary as the cell cultivation progresses. Therefore, the information about the effect of permeability on the maximal oxygen transfer rate and the corresponding D_i/D_o ratio is important for selecting the right D_i/D_o ratio of the reactor. As shown in Fig. 7, the optimal D_i/D_o ratio and the maximal oxygen transfer rate increase as the permeability, k, is increased. Their relationships are shown as

$$\text{Max OTR} = 25.95 + 3.95 \log(k) \quad (27)$$

$$\text{Opt } D_i/D_o = 0.734 + 0.113 \log(k) \quad (28)$$

3. Effect of Cells on Hydrodynamic Permeability

During cell cultivation, the accumulation of cell mass will change both the void fraction and the "apparent" fiber diameter. Consequently, the permeability of the fiber bed is expected to change during the cultivation process. Photomicro-

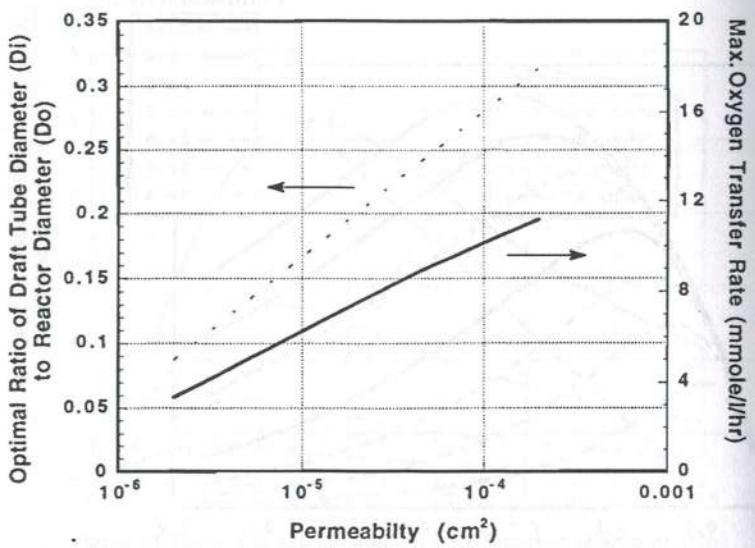


Figure 7 Effect of permeability on maximum oxygen transfer rate and optimal D_i/D_o ratio for fiber bed bioreactor.

graphs of cells at various times of cultivation showed that both anchorage-dependent cells (γ-CHO) and anchorage-independent cells (hybridoma ATCC-CRL-1606) anchored on the glass fibers and formed multilayers in the fiber bed. Through knowledge of cell size, fiber diameter, and contact area, the increase in fiber diameter and decrease in void volume can be predicted for a given cell density. The change in hydrodynamic permeability can then be predicted from Eq. (24) or similar correlations [104].

Figure 8 shows the normalized hydrodynamic permeability versus cell density in the fiber bed. All the permeability values were normalized by the measured permeability of a fiber bed without cells (2×10^{-5} cm^2). In Fig. 8, the lines represent the theoretical values and the square and circle symbols represent the experimentally measured data. The solid symbols are the cell densities obtained from nuclei counts (NC) after each cell cultivation experiment and the open symbols are the calculated cell density derived from lactate production rates (LPR). The model predictions match the experimental data quite well. The greatest deviations were observed at the higher cell densities. Permeability does not change significantly during the initial cell densities. Permeability does not change significantly during the initial cell growth but exhibits dramatic decreases

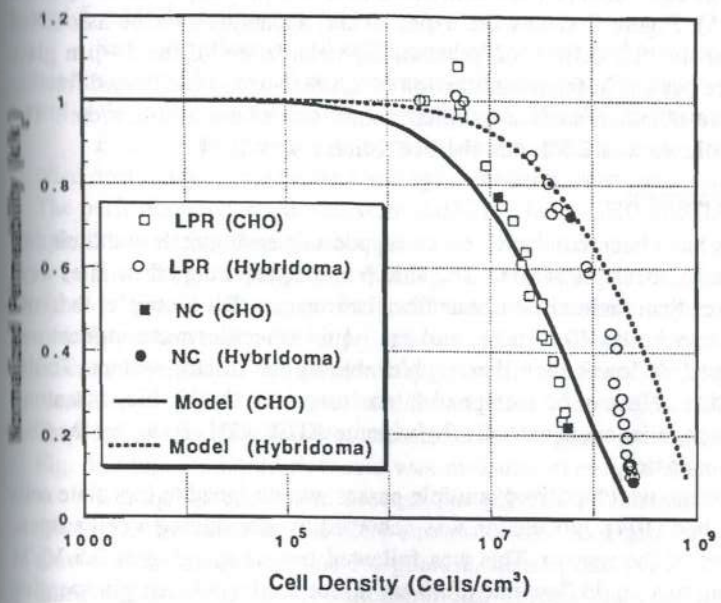


Figure 8 Effect of cell growth on permeability for fiber bed bioreactor. Experimental data and model predictions are shown for both γ-CHO and hybridoma cultures.

at high cell density. The critical cell density before the permeability is affected appears to be 1×10^7 cells/ml. Due to the difference in cell size and contact area, γ-CHO cells caused a more rapid change than the hybridoma cells. For example, it required about 1.5×10^7 γ-CHO cells/ml to reduce the permeability to 75% of its original value, whereas nearly 6×10^7 hybridoma cells/ml were required to generate the same effect.

Since the permeability is a function of cell density and permeability affects the maximal oxygen transfer rate and optimal D_i/D_o ratio, one can anticipate that the maximal oxygen transfer rate will change during the cell cultivation process. Increase in cell density is expected to cause reductions in maximal achievable oxygen transfer rate and optimal D_i/D_o value. A representative D_i/D_o of 0.15 was used for further cell cultivation experiments to demonstrate the feasibility of this bioreactor. Although this draft tube diameter is suboptimal during the initial phases of cell growth when the permeability is high, it provides higher oxygen transfer rate at the end of the cell cultivation when the permeability of fiber bed is low.

C. Operational and Technical Feasibilities

In order to examine the operational and technical feasibility of the bioreactor, cell cultivation experiments were conducted in a 4-L prototype reactor (with a D_i/D_o of 0.15). Figure 3 shows the expeirmental apparatus and the associated equipment for the cell cultivation process. Eighteen layers of the 24-μm glass fiber mat were packed in the annual region of the reactor to yield a void fraction of 91.5%. The obtained surface-to-volume ratio was about 150 cm^2/cm^3. The total liquid volume was 2.5 L and the bed volume was 1.2 L.

1. Cell Entrapment

Many studies have been conducted on entrapped suspension cells and their cultivation in packed beds [105–107]. The void fractions and liquid flow rates were all much lower than those used in our fiber bed reactor. For example, the void fractions were typically 40 to 60%, and the liquid velocities were at least one order of magnitude lower than those achievable in our reactor system. To determine whether cells can be entrapped in our fiber bed, the kinetics of entrapment of anchorage-independent cells (hybridoma ATCC-CRL-1606) by the fiber bed were investigated.

A procedure which utilized multiple passes was designed to inoculate cells into the fiber bed [104]. Inoculation was achieved by introducing a cell suspension at the top of the reactor. This was followed by gas sparging at 0.5 VVM (corresponding to a liquid flow rate of 40 cm^3/s) for 5 min and then gas sparging at 0.05 VVM (corresponding to a liquid flow rate of 4 cm^3/s) for the remainder of 1 h. During the first 20 min of the inoculation procedure, the concentration

of cells in suspension dropped by more than 99%, from 1.5×10^6 to 4×10^3 cells/ml. After the inoculation procedure was completed, there was a slight descending distribution of the cells from the top to the bottom of the fiber bed. The cell concentration at the bottom of the bed was about 70% of the cell concentration at the top.

Mechanistically, the fiber bed can immobilize cells through cell adhesion and at the same time can act as a depth filter to entrap the cells. Hence, our bioreactor can be used to immobilize both anchorage-dependent and suspension cells. It can also be effectively operated in a perfusion mode for both types of cells.

2. Operation and Control Strategies

Recombinant γ-CHO cells with encoded gene for γ-interferon and the murine hybridoma cell line, ATCC-CRL 1606, were used as model systems for anchorage-dependent and anchorage-independent cell lines, respectively [12,104]. Although the γ-CHO cells are aneuploid, they have not been adapted to suspension and are thus anchorage dependent [11]. For the γ-CHO cells, the medium was DMEM/F12 (1:1) supplemented with 1.2% dialyzed fetal bovine serum, 4 mM glutamine, and 0.125 μM methotrexate. For the hybridoma cells, the medium was Excell 300 supplemented with 0.125–0.3% fetal bovine serum and 5 mM glutamine.

Since the effective cell entrapment of the fiber bed had been demonstrated, continuous/perfusion cell cultivations were initiated. The methodology and control strategy were very similar for both types of cells, as described below.

Because the reactor was operated in a perfusion mode, the feed was supplied continuously and product and spent medium were continuously withdrawn. The perfusion rates employed were 0.074 h^{-1} for γ-CHO and 0.08–0.13 h^{-1} for hybridoma during the cell cultivation.

The oxygen uptake rate (OUR) in the reactor can be determined in situ either by liquid-side oxygen balance based on dissolved oxygen concentrations and liquid flow rate data or by gas-side oxygen balance based on mass spectrometer analysis of the gaseous oxygen concentration. The hydrodynamic permeability of the bed was calculated through Darcy's law [Eq. (23)]. The pressure drop across the fiber bed was measured with a glass manometer, as shown in Fig. 3, while the liquid flow rate was measured through a tracer method [13].

During the cultivation process, the pH was maintained within the range 6.7 to 7.3 by adjusting the CO_2 content in the inlet gas flow. Samples were drawn and analyzed off line for glucose, lactate, and product [γ-interferon or immunoglobulin G (IgG)] concentrations. Trypan blue cell counts and citric acid–crystal violet nucleus counts by hemocytometer were used to determine the cell numbers in the inoculum and the fiber bed, respectively.

The control strategy was to maintain the dissolved oxygen concentration at the bottom of the reactor greater than 15% air saturation through adjustments in gas flow rate. The gas flow rate was controlled by a mass flow controller (Brooks 5850E, Emerson Electric Co., Hatfield, PA). The gas flow rate required to meet the oxygen demand was low (0.05 VVM) initially and then increased up to 0.8 and 1 VVM for γ-CHO and hybridoma cultures, respectively, at the final stage of the cultivation processes. These high gas flow rates, which are similar to that used in microbial fermentation, have seldom been reported in animal cell culture. The high cell densities which require such high gas flow rates are not usually encountered in most cell culture systems. Furthermore, in many other types of culture vessels, such high gas flow rates may lead to unacceptable rates of cell death, as gas bubbles can be detrimental to cells [80–85].

At the highest gas flow rate (1 VVM), the liquid velocity was found to be about 0.13 cm/s, corresponding to a Reynolds number in the packed bed of less than 1. This indicates that the flow in the annular fiber bed section was in the laminar regime. The calculated shear stress was less than 1 dyne/cm^2, well below the range of 6.5 to 128 dyne/cm^2 which leads to cell damage [108–110].

Due to sparging, antifoam had to be added to suppress foaming throughout the course of cell cultivation. The concentration of antifoam (Dow Corning Medical Emulsion C) in the perfusion medium was approximately 850 ppm (for γ-CHO) and 1000 ppm (for hybridoma) at the end of the cultivation process. Based on our toxicity study, these concentrations are well within the biocompatible ranges for these cells.

3. Cell Growth and Product Formation

The cultivation processes lasted for 66 days (for γ-CHO) and 22 days (for hybridoma). The termination of each process was not due to contamination or the detrimental effects on the cells. Rather, it was due to our assurance that the reactor could, in fact, operate successfully over a prolonged period for each model cell type. The inoculated cell concentrations were 3.0×10^5 cells/ml for γ-CHO cells and 1.9×10^6 cells/ml for hybridoma. Based on the cell enumerations performed after dismantling the packed bed, final concentrations of 6.8×10^7 cells/ml for γ-CHO and 1.7×10^8 cells/ml for hybridoma were achieved. These are among the highest cell concentrations reported to date.

Because the cells in the fiber bed during the cell cultivation could not be enumerated directly, the growth kinetics were obtained through a time course analysis of oxygen uptake rates, lactate production rates, and fiber bed permeability. The volumetric uptake rate was divided by the uptake rate per cell, measured separately, to estimate the cell density at each time point. A second estimate was made through a parallel calculation based on lactate production. A third estimate was obtained from the correlation between bed permeability, which was measured on line, and cell density (Fig. 8).

For the γ-CHO culture, Fig. 9 shows the time course for cell density as estimated from lactate production (LPR), oxygen production (OUR), and bed permeability. The cell densities are normalized to the corresponding final cell densities obtained for each of the measurement techniques employed. All three techniques agreed well and showed rapid cell growth in the first 800 h followed by the attainment of a steady state. The initial net growth rate was 0.008 h^{-1}, comparable to the initial rate observed for batch roller bottle cultures. The net growth rate was gradually decreased to almost zero at 800 h. Since the dissolved oxygen was maintained above 20% air saturation and the glucose concentration in the spent medium was maintained above 0.6 g/L, it is unlikely that either oxygen or glucose was limiting.

In order to elucidate the cause of the decrease in net growth rate, the effect of specific perfusion rate was examined. The specific perfusion rate is the volumetric perfusion rate (L/h) divided by the total cell number at a given cultivation time. This parameter is an indication of the amount of medium (volume) supplied to each cell per unit time. A high specific perfusion rate means that a high rate of medium is fed and available to the cells for their metabolic needs.

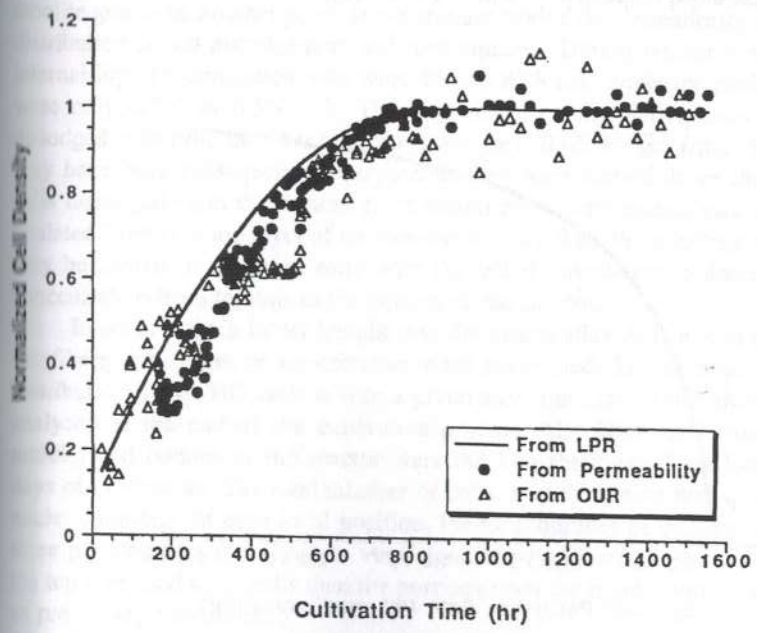


Figure 9 Normalized cell density versus cultivation time for γ-CHO culture in fiber bed bioreactor, as estimated by three methods.

As shown in Fig. 10, net growth rate was affected by the specific perfusion rate. At a high specific perfusion rate at which cell density was low, the net growth rate was maximum, and at a low specific perfusion rate at which cell density was high, the net growth rate was greatly reduced. Higher perfusion rates should lead to higher achievable cell densities. Although the limiting factor was not identified, Figure 10 provides useful information for process control. Specifically, for any given cell density, the required perfusion rate to support a desired net growth rate can be estimated.

Efficient oxygen transfer was achieved nondetrimentally throughout the reactor operation. During the cell cultivations, the oxygen transfer rate initially increased rapidly and then reached a steady state. High oxygen transfer rates (4 mmol/L-h for γ-CHO and 13 mmol/L-h for CRL-1606) were obtained at the steady states.

The desired products were synthesized continuously throughout the cultivation processes [12,104]. High productivities such as 2.1×10^6 IU γ interferon/L-h from γ-CHO and 28 mg IgG/L-h from hybridoma were achieved during the steady state. Even operating at the high perfusion rates, high product concentrations were obtained: 2.7×10^4 IU/ml for γ-interferon and 225 mg/L for IgG. Cumulatively, nearly 8×10^9 IU of γ-interferon and 23 g of IgG were produced in 66 and 22 days, respectively, with a 2.6-L reactor.

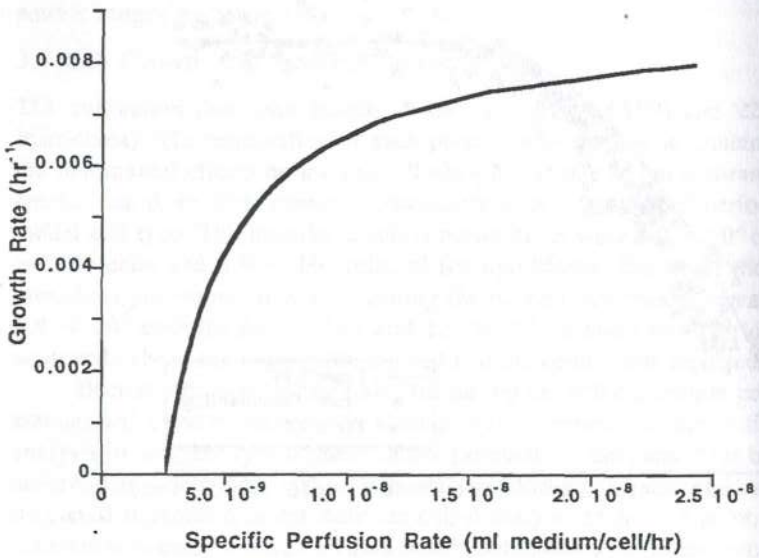


Figure 10 Effect of specific perfusion rate on specific net growth rate of γ-CHO cells in fiber bed bioreactor.

4. Hydrodynamic Consequences During Cell Cultivation

When high cell densities are achieved in this bioreactor, one of the potential problems is channeling, or the existence of stagnant regions in the fibrous bed. The inhomogeneity of the fluid flow may cause some uneven cell growth in the bed and this may affect the bioreactor performance. Cell distribution at the end of the cell cultivation experiments was examined axially and radially in order to assess the potential for channeling in the fiber bed at high cell densities [104].

Based on the nuclei counts performed on the fiber mats after the 66-day cell cultivations, γ-CHO cells showed a slight descending distribution along the axis from the top to the bottom of the reactor [12]. This may have resulted, at least in part, from the axial gradient in inoculum distribution, as discussed in Section III.C.1. It may also arise due to axial gradients in nutrient and waste product concentrations, which may become particularly important when cell density is high and/or specific perfusion rate is low. Interestingly, at the end of the cultivation, hybridoma cells also distributed in a descending manner from top to bottom, but the gradient in the case of hybridoma cells was steeper than in the γ-CHO case [104]. For hybridoma cells, the cell concentration in the bottom layer was only about 25% of that in the top layer.

Besides the uneven inoculum distribution and vertical nutrient or toxic metabolite gradients, another possible mechanism behind the dramatically descending distribution is cell dislodgement and reentrapment. During reactor operation, the internal liquid recirculation rates were 46.8 to 82.8 L/h, while the perfusion rates were only 0.216 to 0.350 L/h. The high internal recirculation rates may have dislodged cells from the fiber bed. However, the cells dislodged from the top layer may have been subsequently entrapped as they were carried down the bed. The cells dislodged from the bottom layer would have been recirculated and "reinoculated" onto the top layer of the bioreactor. Therefore, the resulting distribution may be similar to that seen early after the initial inoculation: a descending cell concentration from the top to the bottom of the reactor.

In order to gain better insight into the quantitative distribution of cells on the fibers, two types of experiments were performed. In one experiment, the distribution of γ-CHO cells within a given axial position in the bioreactor was analyzed at the end of the cultivation process. The fiber mats from the top, middle, and bottom of the reactor were cut into three equal portions after 66 days of cultivation. The total number of cells in each portion was quantified by nuclei counting. At each axial position, the total number of cells in each of the three portions was found not to vary significantly. However, the portions from the top layer had more cells than the portions from the middle and bottom layers, as previously mentioned.

For the second experiment, hybridoma cells were used, and the radial cell distribution was the focus of our study. The experiments were performed using

fiber mats from the middle of the reactor (8th layer) and bottom of the reactor (16th layer) after 22 days of cultivation. The fiber mats were sectioned radially and the cell concentration enumerated quantitatively through a DNA assay [104]. Both layers exhibited a "parabolic" cell distribution reminiscent of the velocity profile for laminar fluid flow: highest at the center and lowest at the walls. However, the difference between the highest and lowest cell concentrations was not significant. For example, at the bottom of the reactor, the highest and lowest cell concentrations were respectively 1.4×10^8 and 1.1×10^8 cells/ml. At the middle of the bioreactor, the highest and lowest cell concentrations were respectively 1.6×10^8 and 1.3×10^8 cells/ml, less than 25% different. In general, the distribution of the cells within each fiber mat layer was reasonably uniform.

The reasonable uniformity in cell distribution, for both the radial and axial directions, suggests that on a macroscale, no serious problem due to channeling was observed in the cell density ranges investigated. However, visual observation showed small regions of cell mass buildup, primarily in the form of big clumps around the intersections of fibers, which may have blocked small channels. The cells in the middle of these clumps may have been starved for nutrients. This potential problem might be addressed through the techniques used to analyze diffusion resistance through multiple cell layers in hollow-fiber bioreactors [18–22].

IV. CONCLUSION

The design of immobilized animal cell bioreactors is best approached through the application of both practical and fundamental engineering principles. The power of this approach has been illustrated through two examples in this chapter. In the first example, predictions were made on how to best operate a traditional microcarrier bioreactor. The predictions involved the use of kinetic expressions for mass transfer and hydrodynamic cell death. The combination of agitation and viscosity which would give the maximum cell concentration was calculated. In the second exmple, a new packed bed bioreactor was designed which circumvents the typical trade-off between mass transfer and hydrodynamic damage. Geometric and kinetic models were used to optimize the geometry and operation of the packed bed reactor. The performance of the packed bed reactor was shown to be impressive, particularly for extended perfusion culture at high cell density.

In the design of both the microcarrier and packed bed bioreactors, the processes which govern reactor performance were first identified. Mechanistic hypotheses were formulated and then described mathematically as a function of measurable variables. The mathematical descriptions were tested experimentally. If found valid, the descriptions were used to optimize the reactor geometry and/or operation. In the future, this classic engineering approach should be widely used to optimize the design and operation of immobilized animal cell bioreactors.

PROBLEMS

1. For reactors geometrically similar to the one depicted in Fig. 1 and Table 1, determine the tank diameter, liquid height, and impeller diameter for reactor volumes of 500 and 2500 L. What is the agitation speed required at the 100-, 500-, and 2500-L scales, respectively, to give an average power input per unit mass of 25 cm^2/s^3? What is the corresponding mixing time at each scale? What is the corresponding oxygen transfer rate, at the point of maximum cell concentration, through surface aeration at each scale? Use the same constants and assumptions as used in Section II.D for medium with 5% serum and no dextran (0.74 cp).
2. At an average power input per unit mass of 25 cm^2/s^3 and a fluid viscosity of 0.74 cp, the solution to the maximum cell concentration calculations described in Section II.D occurs at the microcarrier concentrations of 40,665, 20,806, and 10,365 microcarriers/ml, respectively, for the 100-, 500-, and 2500-L reactors described in Problem 1. For each scale, at the point of maximum cell concentration, determine the corresponding

   a. Percentage of hydrodynamic death due to microcarrier collisions
   b. Specific total growth rate, μ, based on the growth-death population balance
   c. Volumetric oxygen transfer rate
   d. Maximum cell concentration
   e. Percent confluence at the maximum cell concentration
   f. Specific total growth rate, μ, as limited by contact inhibition and cell-derived growth inhibitors
   g. Liquid mixing requirement
   h. Normalized gradient in oxygen between the cells and local bulk medium, with $Sh_c = 2$

Use the same constants and assumptions as in Section II.D. Are the liquid mixing and cell-liquid transport requirements fulfilled? How do the volumetric oxygen transfer rates compare to the values reported in Section III.C.3 for the 2.6-L packed-bed fiber bioreactor?

REFERENCES

1. R. I. Freshney, *Culture of Animal Cells*, Alan R. Liss, New York, 1983, pp. 9, 104–106, 231–238.
2. B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson, *Molecular Biology of the Cell*, Garland Publishing, New York, 1983, pp. 161–165, 769.
3. J. Folkman and A. Moscona, *Nature 273*: 345 (1978).
4. M. S. Croughan and D. I. C. Wang, *Biotechnol. Bioeng. 36*: 316 (1990).
5. R. E. Spier and J. P. Whiteside, *Biotechnol. Bioeng. 18*, 649 (1976).

6. S. Reuveny, *Large-Scale Mammalian Cell Technology* (A. S. Lubiniecki, ed.), Marcel Dekker, New York, 1990.
7. B. Griffiths, *Large-Scale Mammalian Cell Technology* (A. S. Lubiniecki, ed.), Marcel Dekker, New York, 1990.
8. A. Y. Elliot. *Large-Scale Mammalian Cell Technology* (A. S. Lubiniecki, ed.), Marcel Dekker, New York, 1990.
9. A. L. van Wezel, *Nature* 216: 64 (1967).
10. D. W. Levine, D. I. C. Wang, and W. G. Thilly, *Biotechnol. Bioeng. 21*: 821 (1979).
11. S. D. Perry and D. I. C. Wang, *Biotechnol. Bioeng. 34*: 1 (1989).
12. T.-W. Chiou, S. Murkami, W.-T. Wu, and D. I. C. Wang, *Biotechnol. Bioeng. 37*: 755 (1991).
13. S. Murakami, T.-W. Chiou, and D. I. C. Wang, *Biotechnol. Bioeng. 37*: 762 (1991).
14. S. Park and G. Stephanopoulos, *Biotechnol. Bioeng. 41*: 25 (1993).
15. D. Looby and J. B. Griffiths, *Cytotech.* 1, *339* (1988).
16. R. Bleim, R. Oakley, K. Matsuoka, and V. Taiariol, *App. Biochem. Biotechnol. 26*: 217 (1990).
17. B. Griffiths and D. Looby, *Animal Cell Bioreactors* (C. S. Ho and D. I. C. Wang, eds.), Butterworth-Heinemann, Boston, 1991.
18. L. G. Cima, H. W. Blanch, and C. R. Wilke, *Bioproc. Eng. 5*: 19 (1990).
19. J. Wei and M. B. Russ, *J. Theor. Biol. 66*: 775 (1977).
20. J. P. Tharakan and P. C. Chau, *Biotechnol. Bioeng. 28*: 329 (1986).
21. J. M. Piret and C. L. Cooney, *Biotechnol. Bioeng. 36*: 902 (1990).
22. R. A. Knazek, P. M. Gullino, P. O. Kohler, and R. L. Dedrick, *Science 178*: 65 (1972).
23. C. A. Heath and G. Belfort, *Animal Cell Bioreactors* (C. S. Ho and D. I. C. Wang, eds.), Butterworth-Heinemann, Boston, 1991.
24. B. K. Lydersen, G. G. Pugh, M. S. Paris, B. P. Sharma, and L. A. Noll, *Biotechnology 3*: 63 (1985).
25. M. A. Applegate, Doctoral dissertation. MIT, Cambridge, MA, 1991.
26. J. Robert, J. Cote, and J. Archambault, *Biotechnol. Bioeng. 39*: 697 (1992).
27. B. K. Lydersen, J. Putnam, E. Bognar, M. Patterson, G. G. Pull, and L. A. Noll, *Large-Scale Mammalian Cell Culture* (J. Feder and W. R. Tolbert, eds.), Academic Press, New York, 1985.
28. R. C. Dean, Jr., S. B. Karkare, N. G. Ray, P. W. Runstadler, Jr., and K. Venkatasubramanian, *Bioreactor Immobilized Enzymes and Cells* (M. Moo-Young, ed.), Elsevier Applied Science, New York, 1988.
29. K. Nilsson, F. Buzsaky, and K. Mosbach, *Bio/Tech. 4*: 989 (1986).
30. N. Shiragami, H. Honda, and H. Unno, *Bioproc. Eng. 8*: 295 (1993).
31. Product literature, Biomat Corporation, 57 Raleigh Road, Belmont, MA 02178, 1990.
32. M. Reiter, O. Hohenwarter, T. Gaida, N. Zach, C. Schmatz, G. Bluml, F. Weigang, K. Nilsson, and H. Katinger, *Cytotech. 3*: 2271 (1990).
33. J. N. Vournakis and P. W. Runstadler, Jr., *Animal Cell Bioreactors* (C. S. Ho and D. I. C. Wang, eds.), Butterworth-Heinemann, Boston, 1991.

34. F. L. Lim and R. D. Moss, *J. Pharm. Sci. 70*: 351 (1981).
35. U. Matulovic, D. Rasch, and F. Wagner, *Biotechnol. Lett. 8*: 485 (1986).
36. H. Uludag and M. V. Sefton, *Biotechnol. Bioeng. 39*: 672 (1992).
37. Ch. Cadic, B. Dupuy, I. Pianet, M. Merle, Ch. Margerin, and J. H. Bezian, *Biotechnol. Bioeng. 39*: 108 (1992).
38. A. H. Boag and M. V. Sefton, *Biotechnol. Bioeng. 30*: 954 (1987).
39. D. de la Broise, M. Noiseux, B. Massie, and R. Lemieux, *Biotechnol. Bioeng. 40*: 25 (1992).
40. R. G. Rupp, *Large-Scale Mammalian Cell Culture* (J. Feder and W. R. Tolbert, eds.), Academic Press, New York, 1985.
41. J. Kojima, H. Shinohara, Y. Ikariyama, M. Aizawa, K. Nagaike, and S. Morioka, *Biotechnol. Bioeng. 39*: 27 (1992).
42. J. G. Aunins and D. I. C. Wang, *Biotechnol. Bioeng. 34*: 629 (1989).
43. M. V. Peshwa, Y-S. Kyung, D. B. McClure, and W-S. Hu, *Biotechnol. Bioeng. 41*: 179 (1993).
44. Product literature, Costar Corporation, One Alewife Center, Cambridge, MA 02140, 1992.
45. W-S. Hu, J. Meier, and D. I. C. Wang, *Biotechnol. Bioeng. 27*: 585 (1985).
46. S. P. Forestell, N. Kalogerakis, L. A. Behie, and D. F. Gerson, *Biotechnol. Bioeng. 39*: 305 (1992).
47. J. M. Clark and M. D. Hirtenstein, *Ann. N.Y. Acad. Sci. 369*: 33 (1981).
48. M. Butler and W. G. Thilly, *In Vitro 18*: 213 (1982).
49. Product literature, Pharmacia Fine Chemicals, Uppsala, Sweden.
50. R. J. Fleischaker, Doctoral dissertation, MIT, Cambridge, MA, 1982.
51. D. J. Giard, D. H. Loeb, W. G. Thilly, D. I. C. Wang, and D. W. Levine, *Biotechnol. Bioeng. 21*: 433 (1979).
52. B. Mered, P. Albrecht, and H. E. Hopps, *In Vitro 16*: 859 (1980).
53. A. J. Sinskey, R. J. Fleischaker, M. A. Tyo, D. J. Giard, and D. I. C. Wang, *Ann. N. Y. Acad. Sci. 369*: 47 (1981).
54. J. Feder, and W. R. Tobert, *Sci. Am. 248*: 36 (1983).
55. A. T. Nahapetian, *Mammalian Cell Technology* (W. G. Thilly, ed.), Butterworth, Stoneham, MA, 1986, p. 151.
56. W. S. Hu, Doctoral dissertation, MIT, Cambridge, MA, 1983, p. 204.
57. J. M. Goldstein, Masters dissertation, MIT, Cambridge, MA, 1986.
58. M. Tyo and D. I. C. Wang, *Advances in Biotechnology.* Vol 1. *Scientific and Engineering Principles* (M. Moo-Young, ed.), Pergamon Press, New York, 1980, pp. 141–146.
59. R. Portner, M. Matsumura, T. Hatae, K. Murakami, and H. Kataoka, *Bioproc. Eng. 7*: 63 (1991).
60. M. S. Croughan, J.-F. Hamel, and D. I. C. Wang, *Biotechnol. Bioeng. 29*: 130 (1987).
61. M. Butler, T. Imamura, J. Thomas, and W. G. Thilly, *J. Cell Sci 61*: 351 (1983).
62. R. Fleischaker, *Large Scale Cell Culture Technology* (B. K. Lydersen, ed.), Hanser, New York, (1987).
63. R. S. Cherry and E. T. Papoutsakis, *Biotechnol. Bioeng. 32*: 1001 (1988).

64. S. Lakhotia, K. D. Bauer, and E. T. Papoutsakis, *Biotechnol. Bioeng. 40*: 978 (1992).
65. Y. Sano, N. Yagamuchi, and T. Adachi, *J. Chem. Eng. Jpn. 7*: 255 (1974).
66. R. V. Chaudhari, and P. A. Ramachandran, *AIChE J. 26*: 177 (1980).
67. M. S. Croughan, J-F. P. Hamel, and D. I. C. Wang, *Biotechnol. Bioeng. 32*: 973 (1988).
68. M. S. Croughan, E. S. Sayre, and D. I. C. Wang, *Biotechnol. Bioeng. 33*: 862 (1989).
69. D. G. Kilburn and F. C. Webb, *Biotechnol. Bioeng. 10*: 801 (1968).
70. A. K. Balin, D. B. Goodman, H. Rasmussen, and V. J. Christofalo, *J. Cell. Physiol. 89*: 235 (1976).
71. A. A. Lin and W. M. Miller, *Biotechnol. Bioeng. 40*: 505 (1992).
72. E. Meilhoc, K. D. Wittrup, and J. E. Bailey, *Bioproc. Eng. 5*: 263 (1990).
73. W. L. McCabe, J. C. Smith, and P. Harriot, *Unit Operations of Chemical Engineering*, McGraw-Hill, New York, 1985. pp. 138, 221–232.
74. J. G. Aunins, M. S. Croughan, D. I. C. Wang, and J. M. Goldstein, *Biotech. Bioeng. Symp. Ser. No 17.* 699 (1986).
75. J. Y. Oldshue, *Fluid Mixing Technology*, McGraw-Hill, New York, 1983, p. 213.
76. P. C. Hiemenz, *Principles of Colloid and Surface Chemistry*, Marcel Dekker, New York, 1977, pp. 62–80.
77. M. S. Croughan, Doctoral dissertation, MIT, Cambridge, MA, (1988).
78. E. K. Lee, L. Huang, and Y. H. Lee, *Biotechnol. Bioeng. 36*: 530 (1990).
79. M. S. Croughan and D. I. C. Wang, *Animal Cell Bioreactors* (C. Ho and D. I. C. Wang, eds.), Butterworth, Boston, 1991.
80. I. Jobses, D. Martens, and J. Tramper, *Biotechnol Bioeng. 37*: 484 (1991).
81. A. Mizrahi, *J. Clin. Microbiol. 2*: 11 (1975).
82. A. Handa, A. N. Emery, and R. E. Spier, *Proceedings of the 4th European Congress on Biotech.* 1987, Vol. 3, p. 601.
83. D. W. Murhammer and C. F. Goochee, *Biotechnol. Prog 6*: 391 (1990).
84. S. Goldblum, Y.-K. Bae, W. F. Hink, and J. Chalmers, *Biotechnol. Prog. 6*: 383 (1990).
85. D. R. Orton, Doctoral dissertation, MIT, Cambridge, MA, (1992).
86. S. Zhang, A. Handa-Corrigan, and R. E. Spier, *Biotechnol. Bioeng. 40*: 252 (1992).
87. M. S. Croughan and D. I. C. Wang, *Biotechnol. Bioeng. 33*: 731 (1989).
88. S. Nagata, *Mixing: Principles and Applications*, Wiley, New York, 1975, pp. 24–32.
89. J. G. Aunins, B. A. Woodson, Jr., T. K. Hale, and D. I. C. Wang, *Biotechnol. Bioeng. 34*: 1127 (1989).
90. S. J. Pirt, *Principles of Microbe and Cell Cultivation*, Blackwell Scientific Publications, London, 1975.
91. R. L. Bates, P. L. Fondy, and R. R. Corpstein, *Ind. Eng. Chem. Process Des. Dev. 2*: 310 (1963).
92. J. H. Rushton, E. W. Costick, and H. J. Everett, *Chem. Eng. Prog. 46*: 467 (1950).
93. J. W. Richards, *Br. Chem. Eng. 8*: 158 (1963).
94. A. W. Nienow, *Mixing of Liquids by Mechanical Agitation* (J. J. Ulbrecht, and G. K. Patterson, eds.), Gordon & Breach, New York, 1985, p. 273.

95. T. N. Zwietering, *Chem. Eng. Sci. 8*: 244 (1958).
96. R. C. Reid, J. M. Prausnitz, and T. K. Sherwood, *The Properties of Gases and Liquids*, McGraw-Hill, New York, 1977, p. 577.
97. C. R. Wilke and P. Chang, *AIChE. J. 1*: 264 (1955).
98. D. Wohlpart, D. Kirwan, and J. Gainer, *Biotechnol. Bioeng. 36*: 630 (1990).
99. G. W. Jackson and D. F. James, *Can. J. Chem. Eng. 64*: 364 (1986).
100. N. G. Maroudas, *Exp. Cell Res. 81*: 104 (1972).
101. G. A. Dunn and J. P. Heath, *Exp. Cell Res. 101*: 1 (1976).
102. P. E. Fisher and C. Tickle, *Exp. Cell Res. 131*: 407 (1981).
103. R. Singhvi, G. N. Stephanopoulos, and D. I. C. Wang, Paper no. 254d at AIChE Annual Meeting, Loss Angeles, 1991.
104. T-W. Chiou, Doctoral dissertation MIT, Cambridge, MA, 1992.
105. O. T. Ramirez, R. Mutharasan, and W. E. Magee, *Biotechnol. Tech. 1*: 245 (1987).
106. O. T. Ramirez and R. Mutharasan, *Biotechnol. Bioeng. 33*: 1072 (1989).
107. A. Marcipar, P. Henno, E. Lentwojt, A. Roseto, and G. Broun, *Ann. N.Y. Acad. Sci. 413*: 416 (1983).
108. R. F. Viggers, A. R. Wechezak, and L. R. Sauvage, *J. Biomech. Eng. 108*: 332 (1986).
109. N. A. Stathopoulos and J. D. Hellums, *Biotechnol. Bioeng. 27*: 1021 (1985).
110. C. F. Crouch, H. W. Fowler, and R. W. Spier, *J. Chem. Tech. Biotech. 35B*: 273 (1985).

11
Plant Cell Bioreactors

Peter D. G. Wilson and Martin G. Hilton

Institute of Food Research
Norwich Research Park
Colney, Norwich, England

I. INTRODUCTION

Plants produce a great diversity of products which over the years have been used for many purposes. These products include wood as a building material and fuel and primary metabolites such as sucrose, starch and vegetable oils as food commodities. Alongside these bulk products are the secondary metabolites, compounds not directly associated with the growth processes of the plant organism. These compounds are biochemically diverse, often of complex structure, and each one is generally specific to only a few species of plant. They have found their uses in human society as medicines and food flavoring ingredients. In some societies, the pharmacological properties of these metabolites are exploited by use of either parts of the intact plant or crude extracts. In Western medicine however, the tradition is to use plant-derived pharmaceuticals in the form of purified compounds. Such plant-derived compounds now constitute some 25% of prescribed drugs in the United States. The flavor compounds, apart from imparting the flavor characteristics of plants consumed directly as foods, are most often used as a whole plant materials such as herbs or as dried prep-

arations such as spices. As food production has become more industrialized, a market has also developed for purified flavor compounds, either as food ingredients themselves or as components of prepared flavor mixtures.

For economic reasons, the biotechnology of plant cell and organ culture is concerned with the production of the valuable secondary metabolites. A selection of important plant-derived secondary products amenable to biotechnological production is presented in Table 1. Biotechnology is a costly business, in terms of both the research effort required to develop processes and the capital cost of production, so when considering the use of plant biotechnology in this context, it is important to consider the alternatives, which broadly speaking are chemical synthesis, microbial synthesis, and extraction from whole plant material. Chemical synthesis is possible only for the simplest of plant secondary metabolites because of their complexity and stereochemistry. This is especially true of many pharmaceuticals and of many essential oils, which are in any case complex mixtures of compounds, whose organoleptic properties are markedly affected by small changes in the spectrum of compounds present. For these complex products, the alternatives are realistically either extraction from whole plants or in vitro production by plant or microbial biotechnology. The highly species-specific nature of phytochemicals precludes microbial production for the vast majority of compounds, although a few examples do exist [1]. Microbial production does, however, have potential advantages in terms of simpler bioreactor technology and faster growth rates compared with plant cells. Future developments in this approach will depend on the success of transferring partial or whole plant metabolic pathways into microbial hosts by the techniques of genetic engineering.

Table 1 Examples of Valuable Plant-Derived Secondary Products

Compound	Use	Plant
Cocaine	Anesthetic	*Etythroxylon coca*
Atropine	Anticholinergic	*Datura stramonium*
Vincristine	Antileukemic	*Catharanthus roseus*
Digoxin	Cardiac disorders	*Digitalis lanata*
Pyrethrin	Insecticide	*Pyrethrum*
Nepetalactone	Aphid pheromone precursor	*Nepeta* spp.
Quinine	Antimalarial and flavor	*Chinchona*
Codeine	Analgesic	*Papaver somniferum*
Reserpine	Circulatory disorders	*Rauwolfia serpentina*

Source: From Refs. 75–77.

The choice of plant biotechnology over conventional agriculture is concerned mainly with economics, stability of supply, and consistency of product quality. The economics of plant cell culuture have been reviewed by a number of authors [2,3], and as a rule a final product value of around $1000/kg is often seen as a good indicator for compounds amenable to biotechnological production for solely economic grounds. This figure excludes most of the food flavoring ingredients. However, many plants which produce high-value compounds can be grown only in tropical areas of the world, and this, in addition to problems of crop failure, pests and political factors, may lead to instability of supply. Plant biotechnology allows industry to produce their required compounds anywhere in the world. The technology also has the advantage of flexibility, in that the particular compound produced may be changed simply by using a different plant cell line, rather than having to establish new plantations. Furthermore, the quality of plant-derived biochemicals, especially those which are complex mixtures, is often dependent on seasonal and climatic factors, and in these cases the reproducibility of the biotechnology option may have advantages over agricultural practice.

Plant cells are utilized in three basic ways: as cells in suspension culture, as immobilized cells, or as cultures of plant organs. This chapter describes the properties of plant cells in culture, by comparison with microbial systems, and then examines each of the three types of culture system used with reference to examples from the primary literature. It is not intended to be a comprehensive review of published work in this field, and the reader is referred to other sources [4,5] for this information.

II. CHARACTERISTICS OF PLANT CELL CULTURES

The function of any bioreactor is to provide an appropriate environment for the growth and metabolite production of a particular organism, and thus it is clearly essential to understand the properties and requirements of the cells to be cultured in order to design the most productive system. For each class of organism (e.g., bacteria, filamentous fungi, mammalian and plant cells) there will be common properties, as well as requirements specific to the cell line in question. This chapter describes some of the common properties of plant cells in culture, as a guide to the important features which need to be considered when designing a plant cell bioreactor. As the vast bulk of large-scale fermentation is carried out for the exploitation of bacteria and filamentous fungi and the accumulated knowledge is often directed toward these systems, the special needs and properties of plant cells will be discussed in terms of the major differences from those of bacteria and fungi. Specific properties of each of the three types of plants cell cultures (suspension, immobilized, and organ) will be discussed.

Before starting to describe the particular properties of the different classes of plant cells, it will be instructive to remind ourselves of the general principles of scale-up. The science of scale-up emerged from the field of chemical engineering, where it was required to perform chemical conversions, conceived in the laboratory, in bulk. The basic principle is the separation of scale-independent features from scale-dependent features, i.e., those which are affected by the length over which they operate. The *scale-dependent* features are those concerned with transport processes—the transport of mass, of heat, and of momentum. These are thus the characteristics of a system which will change when we attempt to perform reaction on a different scale and whose effect we must allow for in the scale-up process. The *scale-independent* features are those which are intrinsic to the system—the thermodynamic and kinetic properties. The theory of scale-up requires us to perform experiments which separate these two kinds of processes to allow their independent quantification. The information resulting from these experiments may then, in principle, be recombined at any scale to predict the behavior of a scaled-up process.

When the process is chemical in nature, this philosophy is readily applied. In most cases, the identities of the reacting species are known, the reaction scheme can be elucidated, the thermodynamic parameters can be measured, and the intrinsic kinetics of the reaction can be determined. Thus, we can obtain good estimates for most, if not all, of the scale-independent variables. As far as the transport properties are concerned, we can estimate diffusion coefficients for diffusive mass transfer and can measure with some reliability the magnitude of interfacial mass transfer coefficients. The corresponding transport properties for heat transfer are similarly amenable to estimation or measurement. Momentum transport is somewhat more difficult to quantify, but much effort has been spent in the field of fluid dynamics with the result that laminar flow phenomena are reasonably well defined and some models exist for turbulent flow regimes. The fact that momentum transport is the least understood of the transport phenomena and that it has a profound effect on convective mass transfer is reflected in the large amount of chemical engineering research activity in this area.

Although the scale-up philosophy is readily applied to problems in chemical engineering, it is much more difficult in biochemical engineering, where the systems are much more complex. The "intrinsic kinetics," when referring to a biological system, should include (among many others) all the processes which lead to cell division, differentiation, and genetic regulation of primary and secondary metabolic pathways. Many of these processes are not understood, let alone quantified to any degree that would be useful in the design of biochemical reactors. This is not to say that the scale-up philosophy is useless in this context. Indeed, the concepts described above provide a useful guide not only for the design of bioreactor systems but also for interpretation of the results of experiments with bioreactor systems, and they should be at the core of our thinking when considering reactor design.

Unlike bacteria and fungi, the cells of higher plants do not naturally exist in isolation, but rather are part of the organized structure of the whole plant. Cell separation does not normally follow cell division, and cells remain in intimate contact with their neighbors. This contact allows direct chemical communication between cells through protoplasmic connections—the plasmodesmata—and also indirectly by the establishment of macrogradients of hormones and electrical potential which are intimately linked to the patterns of cell differentiation. When cells are put into suspension culture, these protoplasmic connections and macrogradients which allow cell-to-cell communication are largely destroyed. Consequently, the range of different morphological cell types produced does not mimic that of the parent plant. Indeed, it is often the case that repeated subculture of a cell line will tend to select faster-growing cells which are meristematic in nature and which in the whole plant are associated with the active growing region. These particular morphological forms are associated with particular patterns of gene expression, which are unlikely to be those related to the production of secondary metabolites. It is this feature of cell differentiation, with different cell types having different capacities for growth and product accumulation, which underlies the common practice of using different growth media for the phases of growth and product accumulation. This operating strategy is particularly common with cell suspension and immobilized cultures, where in the latter case a change of medium is also required to allow the cells to be maintained in a nongrowing but biosynthetically active state.

Individual plant cells are large (>100 μm) by comparison to bacterial cells (about 1–2 μm) and, in the absence of cell separation following division, often exist as aggregates with dimensions of several millimeters. This large size renders the cells and aggregates prone to mechanical damage from shear fields, turbulent eddies, and interactions with bioreactor equipment such as stirrers and pump impellers. Indeed, in the absence of shear fields, such as during callus culture, cultured plant cells grow as aggregates which may reach several centimeters across. The large size of plant cell aggregates results in a settling velocity much greater than that observed for microbial cells.

When plant cells are put into suspension culture they often release polysaccharide and glycoprotein materials into the growth medium. In intact plants and in plant organ cultures, these materials are normally deposited as part of the plant cell wall. As a result, suspension-cultured plant cells often have a thinner cell wall, making them more prone to mechanical damage. At high cell densities, the polymeric secretions increase the viscosity of the culture medium, which can hinder the mixing required to maintain homogeneity and to provide adequate mass transfer. In extreme conditions, the polymers may form a crust on the surface of the culture. For the case of immobilized plant cells the poly-

meric secretions play an important role in the adhesion of the cells to the inert support, as will be discussed.

As anybody entering the field of plant cell culture from a microbial background will quickly recognize, plant cells have a much slower growth rate than microbial cells. Whereas bacterial cells may have doubling times as short as 15 min, the fastest reported plant cell culture [6] has a doubling time of 15 h. Typical suspension cultures may have doubling times in the order of 1–2 days, and some of the slowest-growing cultures may exhibit doubling times as long as 5 days. This slow growth rate is clearly an important factor when considering the economics of a plant cell–based production system and poses stringent requirements on the mechanical design of bioreactors in order to maintain sterility for prolonged periods.

The slow growth of plant cell cultures is reflected in relatively low nutrient consumption and primary metabolite production rates, such as oxygen uptake and carbon dioxide production. These sink and source terms are among the scale-invariant properties of plant cells. Typical oxygen consumption rates for plant cells are of the order of 10^{-4} mol oxygen (g dry wt)$^{-1}$ h^{-1}. Stoichiometric considerations dictate a similar value for carbon dioxide production.

Whereas the low oxygen demand of plant cells lessens the mass transfer difficulties often experienced in microbial cultivations, some plant cell lines are very sensitive to their overall gaseous environment and, as illustrated later in this chapter, deleterious effects may occur when mass transfer coefficients are too high. A mathematical model describing the fate of volatile metabolites from plant cells was developed by Wilson and Rhodes [4]. The model demonstrates a direct relationship between the dissolved oxygen concentration in a growing culture and the concentration of dissolved volatiles and shows that the act of maintaining the dissolved oxygen concentration *above* a particular value automatically maintains the concentration of dissolved volatile *below* another value. The concentration of the dissolved volatile is given by the following equation:

$$v = v^* + \frac{r_v}{r}\left(\frac{D_O}{D_v}\right)^{0.5}(c^* - c) \tag{1}$$

Thus, as the mass transfer coefficient is increased to bring the oxygen concentration, c, closer to equilibrium with the gas stream, i.e., toward c^*, then the volatile concentration, v, will also move closer to equilibrium with the gas stream. i.e., toward v^*. For gaseous metabolites such as carbon dioxide and ethylene, v^* will be very low or perhaps even zero.

Thus, if there is an optimal concentration of the volatile for growth or product accumulation, then the concept of a maximum oxygen mass transfer coefficient may be as important as that of a minimum mass transfer coefficient. The model equations may be developed a little further to demonstrate the effect of oxygen mass transfer on the concentration of oxygen and produced volatiles.

It can readily be shown that

$$\frac{c}{c^*} = 1 - \frac{rX}{c^* k_l a} \tag{2}$$

This is shown parametrically in Fig. 1. It can readily be seen that values for $k_l ac^*/rX$ between unity (where the mass transfer capacity just meets the requirements of the cells) and about 10 have a profound effect on the dissolved oxygen concentration and thus also on the concentration of dissolved volatiles. Values higher than this have little effect on the oxygen concentration, but merely drive v a little closer to v^*. Application of this observation to effects reported in the literature is discussed further below.

III. BIOREACTORS FOR PLANT CELL SUSPENSION CULTURES

Plant cell suspension cultures are the most studied of all plant cell culture systems. As they are the closest relative to microbial systems, vessel designs and operating strategies can be readily borrowed from existing technologies. However, although reactor designs originate from microbial technologies, the mixing requirements of plant cell bioreactors are somewhat different. The primary fea-

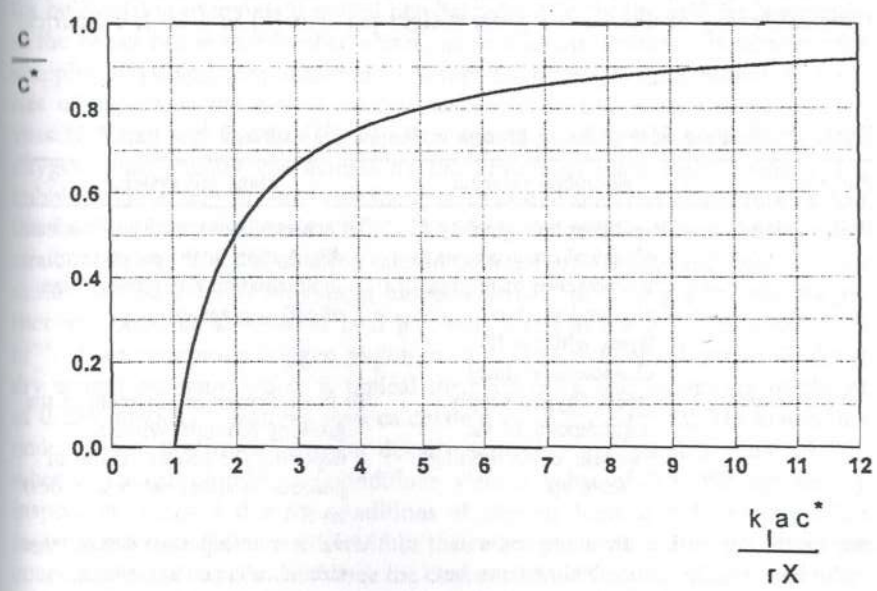


Figure 1 Effect of mass transfer coefficient and oxygen demand on dissolved oxygen concentration [see Eq. (2)].

tures of the mixing process are presented in Table 2 with the relative importance for microbial and plant cell systems.

Many of the bioreactor systems used for plant cell suspension culture are modifications of those used for microbial systems. Both mechanically agitated systems such as the stirred tank reactor (STR) and pneumatically agitated systems such as bubble column and airlift reactors have been used. In addition to these "traditional" designs, a number of novel reactor systems have been devised specifically for the growth of plant cells. These are described in the following.

The most common of the bioreactors is the stirred tank reactor, much used in the chemical industries and for which a wealth of design information exists. For this reason, as well as the economic benefits of being able to use existing fermentor designs and equipment, the stirred tank would be the reactor of choice, should it be able to meet the environmental requirements of the plant cells to be cultured. In order to meet the requirements for a low-shear environment, stirred tank reactors for plant cells are usually operated with lower stirrer speeds than would be used for microbial systems and often using a flat-bladed impeller in contrast to the Rushton turbine which is so prevalent in microbial reactors.

There are many examples of STR culture of plant cells in the literature, ranging in size from 1–2-L laboratory systems up to 20,000-L production systems. For example, cells of *Acer pseudoplatanus* were cultivated to a maximum biomass density of around 6.3 g dry weight per liter [7] in a 20-L cylindrical

Table 2 Features of the Mixing Process in Suspension Cultures

Function	Microbial systems	Plant cell systems
Bulk mixing	To achieve homogeneity of chemical environment in the presence of nutrient sink and product source terms with small characteristic times	To achieve homogeneity of biomass distribution in the presence of sedimentation due to large size of cell aggregates
Oxygen transfer	To meet oxygen supply requirements of the biomass; often limiting in scale-up	To meet oxygen requirement of the growing biomass, without depleting the concentrations of gaseous products below a critical level
Shear	Usually unimportant in microbial systems, but may be critical when handling fungal mycelia	Usually very important due to large size of cells and aggregates; some cell lines may adapt to high-shear conditions

glass vessel using a magnetically coupled agitator. Magnetically coupled stirring systems are often used in plant cell reactors because of their increased security against contamination compared with mechanical seals. On a larger scale, cells of *Nicotiana tabacum* have been cultivated in a 20,000-L stirred tank bioreactor for the production of tobacco biomass [6]. Biomass concentrations up to 16.4 g dry wt L^{-1} were obtained in this system, with a maximum productivity in continuous culture of around 6 g dry biomass L^{-1} d^{-1}.

However, stirred tank bioreactor systems are not always successful, failure often being ascribed to mechanical damage of the cells by the impeller system. This effect is not common to all plant cells but is dependent partly on the species of plant cell being cultivated. It has also been demonstrated that, at least for some species, cells may be adapted to grow in high-shear environments by use of shear as a selection pressure on a growing culture. As an example, Scragg et al. [8,9] describe the selection of a shear-resistant line of *Catharanthus roseus*.

Because of the need to maintain a low-shear environment, plant cells are often cultivated in air-agitated bioreactors operated either as bubble columns or airlift reactors. The difference between the two lies in the more defined flow patterns in airlift reactors due to the provision of a draft tube, either internal or external. A fuller description of these systems can be found elsewhere in this book. One drawback of air-agitated reactors is the link between the agitation capacity of the system and the oxygen mass transfer characteristics. The need for bulk mixing to maintain spatial homogeneity of cells and cellular aggregates in the vessel has been described above, as well as the problems associated with stripping physiologically important volatiles from the growing culture. In a series of papers on the growth of cell suspensions of *C. roseus* in air-agitated vessels, Smart and Fowler [10–12] demonstrate the deleterious effects of large oxygen mass transfer coefficients on the growth of plant cells. Using a 4-L bubble column, they showed that the growth rate of the cells was reduced when the k_la was increased above 25 h^{-1}. Similarly, in airlift reactors they demonstrated a strong effect of aeration rate on both the growth rate and final biomass density of the culture. Maximum biomass density of 14.3 g (dry weight) per liter was obtained at a k_la of 14.5 h^{-1}, which fell to 8.9 g L^{-1} at a k_la of 39 hr^{-1}. If we assume an oxygen requirement for the cells of 0.1 mmol O_2 per g dry weight per hour, which is typical for plant cells, and an oxygen solubility of 0.224 mmol L^{-1}, then we may calculate a value for k_lac^*/rX. The conditions under which maximum biomass density was observed yield a value of 2.3, whereas the less productive conditions yield a value of 9.8. We can see by inspection of Fig. 1 that the conditions of oxygen demand and supply put the mass transfer coefficients used within the range where they strongly affect the concentration of oxygen and hence the concentration of carbon dioxide and other dissolved volatiles. This may indicate the mechanisms behind these observed effects.

As a further example of the use of pneumatically agitated vessels and the importance of establishing a suitable gassing regime, cells of *Berberis wilsonae* were grown in a 20-L airlift fermentor by Breuling et al. [13]. In this system, the production of the required secondary products (the protoberberine alkaloids berberine and jatiorrhizine) increased with increasing aeration rate to a maximum and then fell as the aeration rate was further elevated. Breuling et al. also reported the use of 20- and 200-L airlift bioreactors for the production of protoberberine alkaloids from *B. wilsonae*, producing a maximum biomass density of 23 g dry wt L^{-1} in the 200-L bioreactor.

One way to separate the interdependence of mixing and mass transfer in pneumatically agitated systems is by partial recycling of the exit gases from the bioreactor while bleeding fresh air into the system. This permits a choice of gassing rate to ensure biomass homogeneity in the face of sedimentation, while allowing the concentration of dissolved oxygen and gaseous products to be controlled by manipulation of the gas recycle ratio. A bioreactor system employing this technique was described by Townsley and Webster [14], who used a 10-L airlift fermentor for the cultivation of cells of *Triptergium wildordii*. A gas recycle rate of 11 L min^{-1} was used for agitation purposes, and fresh air was bled into the system at a rate of 0.1 L^{-1} to supply the oxygen requirements of the cells. Using this system it was possible to fill the fermentor with cells in 80 days, effectively creating a packed bed.

A number of other systems for cultivating plant cells using mechanically agitated bioreactors have also been investigated. The rotating drum fermentor consists of a cylindrical vessel which rotates about its axis, which is mounted horizontally. The inside wall of the vessel is equipped with a series of baffles, which aid the mixing of the contents. The rotation of the drum also produces a thin film of liquid on the vessel wall, which enhances oxygen mass transfer. A air sparge line is also provided, which discharges below the surface of the cell suspension. The main advantage claimed for the rotating drum system is its ability to handle high-density suspension cultures, where the high viscosity of the suspension hinders mixing. Power requirements for such a system have been determined by Tanaka et al. [15] under high-viscosity conditions, using an agar-based pseudocell suspension. Tanaka [16] has also compared the rotating drum system with both conventional and modified stirred tank reactors and with the two classes of pneumatically agitated vessels. Using the solid-liquid mass transfer coefficients for the dissolution of solid particles of β-naphthol (an indicator of shear stress) as a scale-up criterion, a comparison was made between the different bioreactor systems at a number of scales up to 100 L. Using the production of shikonin by cells of *Lithospermum erythrorrhizon* as the test system, the rotating drum fermentor performed better at all scales.

A novel approach to mass transfer and mixing for plant cell culture was the devised by Janes et al. [17] and its use demonstrated for a suspension culture of *Beta vulgaris*. The vessel consists of two concentric cylinders, one of which rotates, setting up Taylor vortices in the annulus between the cylinders. These vortices provide the mixing for the cells. The inner cylinder of the reactor is composed of a gas-permeable membrane which provides bubble-free aeration of the growth medium.

A further system which provides oxygen via a membrane assembly was used by Piehl et al. [18] for the cultivation of cells of *Thalictrum rugosum*. The 20-L vessel was equipped with a stirrer, to which was attached a membrane assembly which provided bubble-free aeration. The authors report high biomass densities (up to 400 g fresh wt L^{-1} while maintaining the oxygen concentration at 30% of the saturation value.

IV. BIOREACTORS FOR IMMOBILIZED PLANT CELL CULTURES

The prime motivation for the culture of plant cells is the production of valuable secondary metabolites, rather than the plant biomass itself. The production of the biomass is slow and thus expensive, and common sense would indicate advantages in exploiting the biosynthetic capacity of biomass to the greatest extent to achieve the most economic benefit. This is one of the driving forces behind the use of immobilized cell systems, where the objective is to hold biomass in a productive but nongrowing state for extended periods of time.

Many forms of immobilization system have been used for plant cell culture, but all will involve at least some of the following processes.

1. Growth of free cells to provide material for immobilization
2. Bringing together of cells and immobilization support
3. Physicochemical interaction between support and cells
4. Physiological response of cells to support matrix
5. Further growth of plant cells
6. Supply of nutrients, including oxygen, to cells
7. Formation of desired product
8. Product release
9. Removal of product from growth medium

Four classes of immobilization systems may be identified: entrapment of cells within a gel bead, attachment of cells to the surface of beads, entrapment of cells within a solid reticulated matrix, and confinement of cells by the use of permeable membranes, including hollow fibers.

The earliest work on the immobilization of plant cells was by use of gel entrapment. Brodelius and his co-workers [19,20] used a number of different gels, including alginate, agar, κ-carageenan, and gelatin to investigate immobilization of plant cells. In addition to carbohydrate and protein gels, a wide range

of other materials have been used, including polyacrylamide [21], cross-linked polyphenylene oxide [22], and polyacrylamide hydrazine [23]. Of all these systems, the one in most common use is alginate. Alginate beads may be made by dispersing cells in a solution of sodium alginate, which is then introduced into a solution of $CaCl_2$, whereupon the replacement of monovalent sodium with divalent calcium ions leads to ionic cross-linking and subsequent gelation. Thus gel-entrapped plant cells may be formed without the possible ill effects of heating required for materials such as agar and gelatin.

Following immobilization of the cells, the gel beads may be used either in a packed bed configuration or as a fluidized bed. Using cells of *Catharanthus roseus* immobilized within calcium alginate beads, Brodelius et al. [20] used a packed bed reactor to investigate the production of alkaloids. In this system, the medium surrounding the bioparticles was passed through a layer of chloroform in order to collect the alkaloid. The biotransformation properties of plant cells have also been exploited by the use of packed beds of bioparticles. Again using an alginate immobilization system, Veliky and Jones [24] studied the 5' β-hydroxylation of gitoxigenin by cells of *Daucus carota*. Medium was recirculated through the 190-ml packed bed, and the cells remained viable and biochemically active for periods of up to 30 days.

The fluidized bed approach to exploiting gel-immobilized plant cells has been used for the culture of alginate-entrapped cells of *C. roseus* [25]. A conical reactor was used, the beads being fluidized by recirculating the growth medium through the bed. In this particular case, the reactor was operated for the purpose of producing fine cell suspensions, released from the surface of the bioparticles, as the immobilized cells grew within the gel, although the system would be equally applicable to secondary metabolite production or biotransformation. An important consideration in the design of such systems is the choice of an appropriate superficial velocity for the suspending fluid, in order to maintain the bed in a fluidized state without inducing disruption of the particles and subsequent release of the immobilized cells.

An alternative to immobilizing cells within a gel matrix is that of trapping cells within the interstices of a packed bed of gel beads. This approach was used by Kargi [26] for the production of alkaloids from the cells of *C. roseus*. The cells had been produced in a growth medium and were transferred to a production medium under a different hormonal regime before being inoculated into the packed bed. The bed consisted of 4-mm-diameter alginate beads in a 14 cm × 4 cm diameter column. The production medium was circulated through the bed, the cells attaching to the beads to fill the void volume. The bioreactor was operated for 12 days, and alkaloid production was higher than in a comparable free cell system.

A difficulty with using gel-entrapped cells is that growth of plant cells within the matrix can lead to disruption of the bioparticles and subsequent release of free cells. One way to overcome this problem is to use a more robust carrier, and several systems of this type have been investigated, including the use of spun nylon pads and reticulated polyurethane foam particles. In this type of system, cells are inoculated into the interstices of the support and allowed to grow to fill the void space.

In order to allow the cells to enter the spaces within the support, it has been found necessary to induce a flow of medium containing the free cells through and around the particles. In this way, the cells are filtered from the medium into the void spaces. This technique for inoculating supports has been achieved in two ways. First, the support particles may be held stationary and the cell suspension passed through the assembly of particles. Mavituna et al. [27] used this technique to inoculate cells of *Capsicum frutescens* into 1-cm polyurethane foam cubes. The cubes were held as a packed bed between stainless steel grids located in a 5-L air-agitated reactor. The bed was inoculated with cells by recirculating the cell suspension through the particles for a period of 25 days. During this time, the cells entered the voids of the particles and became firmly attached. After this initial phase, the steel grids were removed, and the system operated as circulating bed for a further 35 days. In this second phase, the growth medium was substituted for a production medium which contained precursors of the desired product, capsaicin, the pungent principle of chili pepper.

The second approach to inducing a flow through the uninoculated support matrix, that of passing the matrix through the cell suspension, was also demonstrated by Mavituna and her co-workers, again for the production of capsaicin from cells of *Capsicum* [28]. The reactor used consisted of polyurethane sheets mounted onto the impeller assembly of a stirred tank. The movement of the immobilization matrix through the free cell suspension caused the cells to enter the pores of the support matrix.

Once the cells have been brought into close proximity with the eventual support matrix, two phenomena operate in the immobilization process. The first is a weak physicochemical effect involving van der Waals forces and electrostatic effects. These effects have been described by Facchini et al. [29–32] in a study of the adhesion of plant cells to polymeric surfaces. The effectiveness of adhesion of plant cells to a given support depends on the relative interfacial tensions between the cells, the support, and the surrounding medium. The second phenomenon is the production of extracellular polymers which bind the cells to the surface of the support matrix. In a scanning electron microscopic study of plant cells immobilized in reticulated polyurethane foam, Rhodes et al. [33] and Robins et al. [34] observed that the cells were embedded in a layer of mucilaginous material.

Bioreactors in which cells are separated from growth medium by a permeable or semipermeable membrane have been in common use for the growth

of mammalian cells for a number of years, and their application to plant cells has been explored by a number of authors. Reactor types include simple single membranes, where cells are grown on the surface of a membrane floated on growth medium [35]; hollow-fiber systems [36–38] where cells may be grown around a bundle of semipermeable fibers located within an outer shell; and two-sided systems [39], where the cells are enclosed between two membranes with growth medium outside.

Shuler et al. [39] have investigated the scale-up of membrane reactors for plant cells using a form of tubular reactor, where the cells are entrapped within porous tubing, which is then suspended in growth medium within a bubble column. Using this system for *C. roseus,* with a dilute medium lacking inorganic phosphate, a fivefold stimulation of alkaloid production was observed, with 70% of the alkaloid being exported to the production medium.

As well as the benefit of allowing cells to grow in close proximity to each other and thus permitting cell-to-cell communications to be established, membrane systems offer the advantage of separating the metabolic products from the cells, resulting in easier product removal and avoidance of possible product inhibition effects. The application of membrane systems to plant cell culture has been reviewed by Shuler [40].

A common issue in all immobilized cell systems and indeed in all processes involving heterogeneous catalysis is that of simultaneous reaction and diffusion. This subject is widely covered in the chemical engineering literature [41–43], and the theories developed have been applied to a number of problems in biochemical engineering, most notably for the growth of fungal mycelia [44–46] and immobilized microbial systems [47,48]. Diffusion coefficients for nutrients [49], oxygen [50], and metabolites [51] have been determined for plant cell systems, and studies of oxygen diffusion limitation have been carried out [50,52,53]. As an example, Robins et al. [50], using an oxygen microelectrode, demonstrated that the oxygen tension within a bioparticle containing immobilized cells of *Cinchona pubescens* fell to below 10% of the saturation value at a depth of 1 mm.

The diffusion of compounds through immobilized cells may be affected by both the nature of the immobilization matrix and the cell loading within the system. This issue has been reviewed [54].

V. BIOREACTORS FOR PLANT ORGAN CULTURES

A recent development of plant biotechnology is the use of plant organ cultures. These cultures retain much of the high-level organization of plants, enabling more normal cell-to-cell communication to be established and, provided that this organization is maintained, this results in enhanced biochemical and genetic stability of the cultures. Two type of culture are recognized, transformed or so-

called hairy root cultures and shoot cultures. Root cultures may be formed by infection of plant material by the soil bacterium *Agrobacterium rhizogenes*. The formation and use of transformed root cultures has been reviewed by Toivonen [55]. Two major contraints exist with transformed cultures: the host range of *Agrobacterium,* which is restricted to monocotyledonous plants, and the fact that the products made by the cultures are likely to be restricted to those which are produced in the corresponding plant organ. It was this limitation and the desire to produce plant essential oils which led to the development of transformed shoot cultures, for example, of *Mentha* spp. [56], by transformation by *Agrobacterium tumefaciens.*

Apart from their enhanced stability, transformed plant organ cultures have the advantage that the transformation process may be used to introduce foreign genes into the genome of the transformed culture, thus offering the possibility of genetically manipulating the cultures for increased productivity. For example, the introduction and overexpression of a yeast gene coding for ornithine decarboxylase into *Nicotiana rustica* has led to increased nicotine accumulation in the resulting transformed root culture [57].

The organized nature of these cultures poses many novel problems for bioreactor development, particularly in the area of aseptic transfer of organized biomasss, and the bioreactor technology is at an early stage of development. The following sections review the current status of bioreactor systems for transformed root and shoot cultures.

A. Transformed Root Cultures

A bioreactor system for transformed root cultures was first described by Rhodes et al. [58] for the cultivation of *N. rustica* in a 1-L air-sparged vessel. The simple fermentor was constructed with an air sparger at the bottom of the vessel and an outlet weir near the top; it operated as a batch culture for the first 13 days and was then fed continuously for a further 16 days. The roots immobilized around the air sparger, and from an initial inoculum of 0.5 g, the biomass increased to fill the entire volume of the vessel to give a final weight of 144 g. Over the fermentor run, a total of 32.7 mg of nicotine was released into the effluent, representing 76% of the total available nicotine content of the roots.

The importance of immobilization for transformed root cultures and their inherent sensitivity to shear forces were demonstrated by Wilson et al. [59] with cultures of *N. rustica* grown in a conventional stirred tank reactor, 10 L in volume, and stirred at 200 rpm with a single Rushton turbine impeller mounted toward the base of the vessel. After inoculation, the roots rapidly lost their organized structure, and within 19 days the culture became morphologically a suspension culture of transformed cells of high density but of little biosynthetic capacity. This demonstrates the importance of cell–cell communication and

structure to the patterns of gene expression and consequent product formation described earlier.

By contrast, transformed roots of *Datura stramonium* and *N. rustica* retained both their organized structure and biosynthetic capacity when immobilized in a stainless steel cage immersed at the bottom of an air-sparged column fermentor. Approximately 2 g of transformed roots was placed aseptically into the cage (40 mm diameter, 50 mm high, and made from stainless steel mesh) and allowed to settle to the bottom of the glass column (60 cm high, 1.5 L in volume). The roots grew rapidly from the cage to fill the fermentor. Success of the system was demonstrated by the final biomass yield of 1.2 kg, after 60 days of growth with continuous feeding of fresh medium, representing a volume packing density of 75% v/v. Roots of *N. rustica* were also grown in the column fermentor, first in batch mode for 21 days and then with continuous nutrient feeding for a further 65 days. Analysis of the effluent for nicotine revealed that operation with a continuous flow of nutrient had led to a fourfold stimulation of alkaloid production (final nicotine yield of 132 mg) by comparison to batch-grown roots.

An immobilization device for transformed roots has also been developed by Jung and Tepfer [60] for cultures of *Atropa belladonna* and *Calystegia sepium* in 2- and 30-L stirred fermentors. The roots adhered to a stainless steel basket in a turbine-driven fermentor operated at 160 rpm. Biomass production rates of 0.8 g dry wt L^{-1} d^{-1} were achieved for *C. sepium* with the 2-L fermenter.

A different approach to producing an immobilized root culture was developed by Taya et al. [61], who used polyurethane foam as an immobilization matrix. The immobilized roots of *Armoracia rusticana* were grown in column fermentors (4.2 cm diameter by 35 cm high) operated in three modes: airlift, medium-trickling, and medium-filling siphon. The authors reported that the airlift system gave the best cultivation result (11.0 kg dry wt m^{-3}), which was comparable to that achieved in flask culture (7.7 kg dry weight m^{-3}). The medium-trickling and filling-siphon column supported less growth (6.8 and 4.7 kg dry wt m^{-3}, respectively).

Polyurethane foam was again employed as a means of root attachment in a rotating drum fermentor described by Kondo et al. [62] for culturing the roots of *D. carota*. A description of the rotating drum fermentor is given above. The foam prevented cell disruption from occurring through repeated dropping of the roots as the drum rotated. Without this modification, the growth rate of the roots was impaired. The authors also made modifications to a stirred tank reactor by placing a stainless steel mesh between the impeller and the growing space. This prevented damaging contact between the roots and the blades of the impeller. This configuration showed the most efficient growth (0.63 g dry w L^{-1} d^{-1}).

A modified stirred tank reactor was also developed by Hilton and Rhodes [63] for the growth of transformed roots of *Datura stramonium*. A stainless steel cage (135 mm diameter by 29 mm high) was attached to the baffles and positioned just above the impeller blades to ensure that the roots were not damaged. Under continuously fed conditions, final packing densities of 70% (v/v) were achieved after 37 days growth at 25°C. Hyoscyamine levels were equal to or higher than those found in roots grown in shake flasks, and production rates of up to 8.2 mg hyoscyamine L^{-1} d^{-1} were obtained. Based on the experience gained working with various bioreactors up to 20 L in volume, Wilson et al. [64,65] designed and commissioned a 500-L pilot plant bioreactor to study the problems of scale-up. They identified four areas requiring investigation:

1. The aseptic transfer of roots from seed vessel to pilot plant
2. Distribution of inoculum evenly throughout the growth vessel
3. The definition of a feeding regime to support rapid growth
4. Harvesting of the biomass produced

The 500-L vessel was designed to operate either as a droplet phase system or in submerged culture. Following the aseptic transfer of material from the seed vessel, the transformed roots are immobilized on an array of stainless steel barbs, linked together in the form of vertical chains arranged within a cylindrical framework at 10-cm spacing. The reactor is operated in submerged batch culture until the roots have established themselves on the matrix. The vessel is then drained and operated under droplet phase conditions in a fed-batch mode supplied with medium from a 50-L holding reservoir. The vessel is mounted on load cells, and any increase in the weight of the biomass can be logged by a computer control system and the data used to directly control the feeding regime by replacement of nutrients with stoichiometrically formulated medium additions pumped into the 50-L holding vessel from ancillary aspirators.

Fermentors using two liquid phases, one aqueous, the other solvent, have been developed to improve the release of secondary products from transformed root cultures. Builelaar et al. [66] studied the growth of transformed roots of *Tagetes patula* in a stirred tank bioreactor, a liquid-impelled loop reactor, and a bubble column with liquid-liquid extraction for recovery of the product thiophene. In the liquid-impelled reactor, the roots were immobilized in calcium alginate beads. The bubble column resulted in the greatest thiophene production, with up to 70% of the excreted product recovered in the hexadecane solvent phase.

Airlift bioreactors have been used successfully for transformed root cultures by Rodríguez-Mendiola et al. [67] for the growth of *Trigonella foenum-graecum* and *Nicotiana rustica*. However, distribution of the roots was uneven, due to the use of a glass draft tube. Immobilization occurred only at the centralizing arms of the draft tube inside the column. This problem was solved by the use of a wire mesh draft tube, which provided many more active growing points and resulted in even growth throughout the fermentor. The wire mesh

tube also facilitated the removal of biomass from the fermentor, as the roots tightly bound to the matrix are removed together. From an inoculum of 45 g fresh weight of *N. rustica*, 1350 g fresh weight of biomass was removed after 30 days of growth.

Growth of transformed roots of *N. rustica* was compared in novel bioreactors by Whitney [68]. Five types of bioreactor were compared: shaken bottle, aerated bottle, airlift, trickle film, and mist phase reactor. Biomass increases of 1000-fold were found in all the different configurations except the mist phase reactor, where the biomass increase was 3000-fold. The author also reported difficulty in removing the roots from the glass beads used in the trickle bed reactor, whereas roots were easily harvested from the other reactors.

Ultrasonics have been used to produce a fine mist (7–20 μm droplet diameter) for the distribution of nutrients in bioreactors. This method, developed by Weathers and Zobel [69], originally for the micropropagation of shoots or plantlets, has been used successfully to grow transformed roots of *Catharanthus* and *Beta* immobilized on a three-dimensional trellis of woven nylon string in a 1.29-L polycarbonate jar. The cultures were reported to have doubling times of 2.8 days, about the same as in flasks.

B. Transformed Shoot Cultures

A survey of the literature reveals a definite paucity of information on the growth of shoot cultures in bioreactors. This can be explained in part by the fact that shoot cultures are comparatively novel. In 1984, Hagimori et al. [70] reported on the growth of a shoot culture of *Digitalis purpurea* and the production of digitoxin in a 2.5-L jar reactor over a period of 15 days. A jar reactor was also used by Katagi et al. [71] for the growth of *Pelargonium graveolens*. In both cases, growth was supported in a medium containing phytohormones in vessels under illumination. It is a fact that such cultures require exogenous hormones for growth and for maintaining shoot structure and biosynthetic capacity. Transformed shoot cultures, on the other hand, do not require exogenous phytohormones to support growth and thus may be grown in a simple salts plus sugar medium. Saito et al. [72] described shooty teratomas in *Nicotiana tabacum* which were produced by strains of *Agrobacterium tumefaciens* mutated in the auxin genes (*aux* 1 and/or 2). These cultures were, however, unable to synthesize tropane alkaloids. Spencer et al. [56,73] developed transformed shoot cultures of *Mentha citrata* and *M. piperita* produced by *A. tumefaciens* T37. The shoot teratomas were investigated for their capacity to form the monoterpene components of mint oil. The findings presented so far have evolved from experiments in shake flasks and no work has yet been published on the growth of transformed shoot cultures in fermentors; however, in our laboratory, we have

been studying the growth of transformed shoot cultures of *M. citrata* and *M. piperita* in 14-L fermentors operated under different conditions and will present our own findings.

Two shoot cultures originally produced by Spencer et al. [56], derived from the transformation of *M. citrata* and *M. piperita* with *A. tumefaciens* T37, were maintained in 250-ml shake flasks containing 50 ml of MS0 plus 3% sucrose medium. Four such flasks containing 17-day-old cultures were used to inoculate a modified 14-L type of MF123 fermentor (New Brunswick Scientific, Watford, Herts, UK) containing 12 L of MS0 plus 3% sucrose medium. Fermentors prepared in this way were operated under different conditions: in a modified stirred tank, in hydroponic culture, or in a droplet system (Figs. 2–4). Growth was measured using a drain and weigh procedure [63].

The growth curves of *M. piperita* (Fig. 5) and *M. citrata* (Fig. 6) show that both the growth rate and the final yield were highest in fermentors operated with a droplet system. Nearly 3 kg fresh weight of *M. piperita* shoots were produced over a period of 53 days with a maximum growth rate of 0.10 d^{-1}. Slightly less growth (2.5 kg) and a slower maximum growth rate (0.08 d^{-1}) were attained by shoots of *M. citrata* over a period of 60 days. When the shoot cultures were grown under submerged conditions in a modified stirred tank, far less growth and a much slower growth rate were achieved. Growth may have been inhibited by the actual physical presence of the liquid medium continuously in contact with the leaves and stems, perhaps restricting gaseous exhange through the stomata. Problems were experienced after inoculation when the shoots would become immobilized at a few unplanned sites within the vessel. It was impossible to control the distribution of the inoculum, and this led to

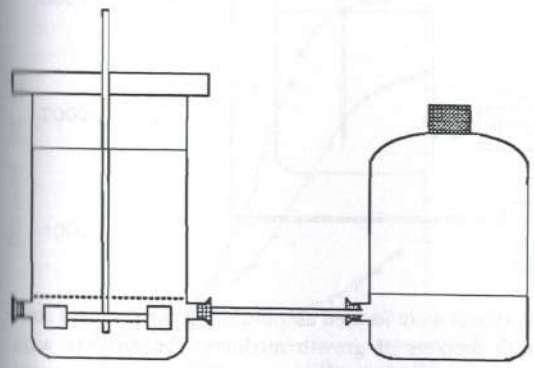


Figure 2 Submerged bioreactor. A stainless steel mesh was mounted just above the stirrer blades to protect the shoots from mechanical damage. The vessel was sparged continuously with air at a flow rate of 1 L min^{-1} while being stirred at 50 rpm.

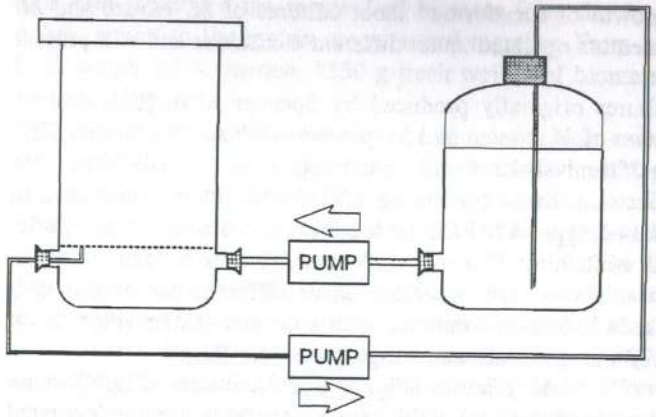


Figure 3 Hydroponic bioreactor. In this system, the vessel has two side ports, one allowing entry of fresh medium and the other controlling the liquid level. Peristaltic pumps were used to recycle the medium at a flow rate of 200 ml min^{-1}. During inoculation, the shoots were placed aseptically onto the stainless steel mesh located at the same level as the weir.

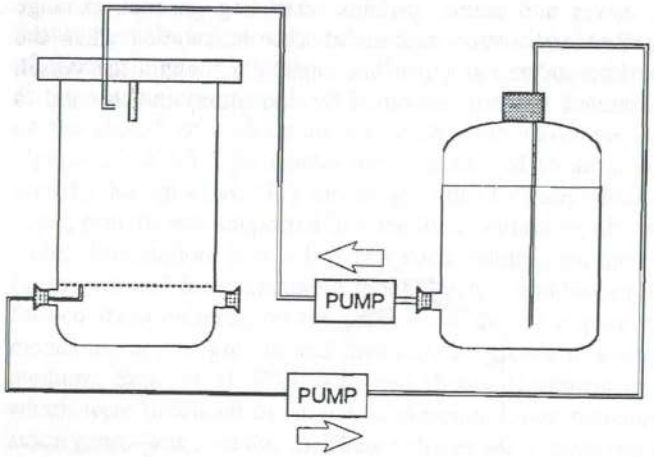


Figure 4 Droplet bioreactor. The shoots were located aseptically on the stainless steel mesh and continuously sprayed with droplets of growth medium. The droplets were formed and distributed by pumping a jet of growth medium onto a threaded stud mounted on the underside of the top plate. Medium was scavenged from the bottom of vessel and returned via a peristaltic pump to a holding reservoir. The vessel was aerated at a flow rate of 200 ml min^{-1}.

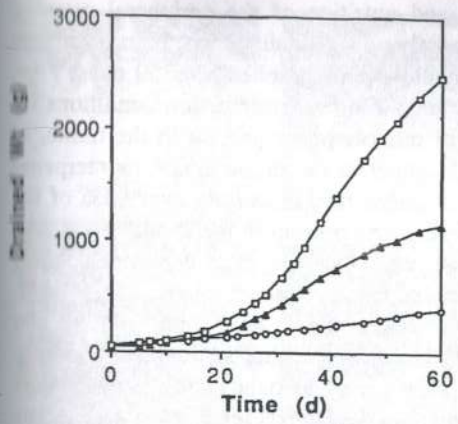


Figure 5 Growth of transformed shoots of *Mentha cirata* in bioreactors operated as (△) submerged, (○) hydroponic, and (□) droplet systems.

uneven growth in the fermentor. The poor growth of the shoots in fermentors operated under hydroponic conditions can probably be explained by limited nutritional absorption. At inoculation most of the shoots resting on the stainless steel mesh are individually in contact with the growth medium, but as they grow upward, the proportion of each shoot still in contact with the medium diminishes. Transport of nutrients from such single point contact with the growth

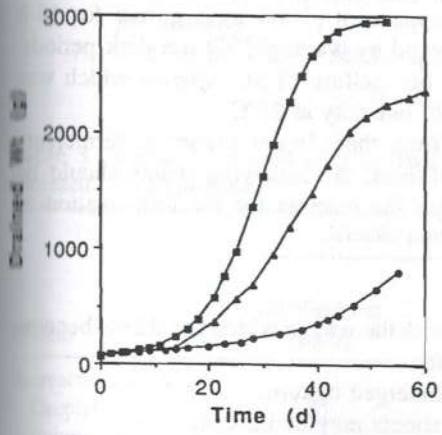


Figure 6 Growth of transformed shoots of *Mentha piperita* in bioreactors operated as (▲) submerged, (●) hydroponic, and (■) droplet systems.

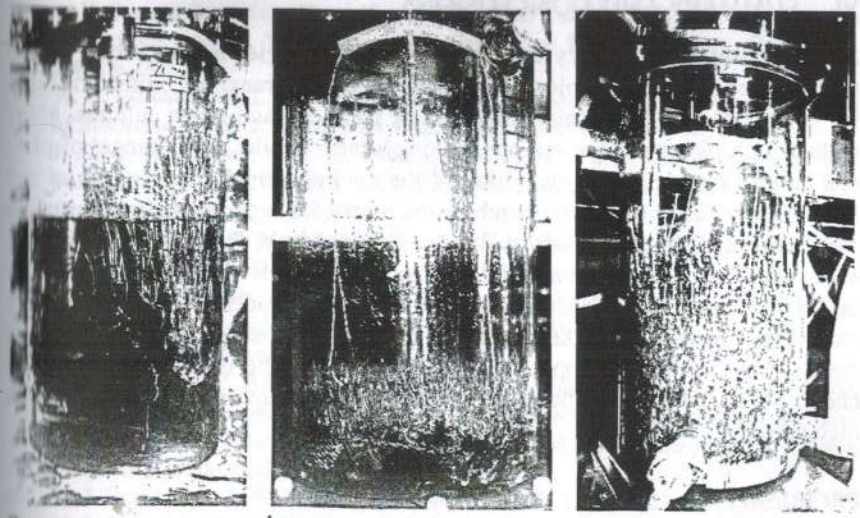
medium would appear to be limited and nutrition of the peripheral growing points of the shoot culture may operate only on a diffusion basis. Figure 7 shows the degree of growth in the three fermentors photographed after 50 days.

Analysis of the shoots grown under the three fermentation conditions revealed large differences in the levels of monoterpenes present in the tissue, as shown in Table 3. In shoots of *Mentha piperita* the predominant monterpenes were pulegone and menthofuran, and together they constitute over 90% of the total monoterpenes identified. The monterpenes present in the shoots of *Mentha citrata* were linalool and linayl acetate, with the acetylated component being dominant. Table 3 shows that the levels of the two monoterpenes found in *M. piperita* from a fermentation operated with a droplet system were vastly greater than those found in the same culture grown under either submerged or hydroponic conditions. Shoots of *M. citrata*, on the other hand, when grown under hydroponic conditions, were found to contain the highest levels of linalyl acetate (some 60% higher than in the droplet system). Spencer et al. [73] have shown that the levels of monterpenes in transformed shoots *M. citrata* grown in M50 medium in shake flasks can vary according to the age of the culture and that maximum concentrations are reached during the early growth phase.

The point at which levels of monoterpenes start to decline may be triggered by the depletion of one or more nutrients from the growth medium; thus shoots in hydroponic culture, if nutritionally restricted, could remain in early growth phase for a longer period of time, so maintaining these relatively high levels of monoterpenes. The nature of the monoterpenes present in the oils may be influenced by the environmental growth conditions, i.e., the duration of exposure to light, the intensity and quality of the light, and the temperature of the culture. Loomis [74] reported the accumulation of more oxidized products such as menthofuran and pulegone when peppermint plants were grown for 18-h photoperiods at low light intensity followed by warm (25°C) 6-h dark periods. Both of these products were found in our culture of *M. piperita* which was grown for 24-h photoperiods at low light intensity at 25°C.

From the results obtained so far from these initial studies of fermentors operated under the three different conditions, the following points should be borne in mind when considering designs for reactors for the fermentation of shoot cultures:

1. Morphology of culture influences the way in which the shoots become distributed within the fermentor.
2. Shoots may be stressed in submerged culture.
3. Nutrient transport within the shoots may be limited.
4. A droplet system of medium delivery overcomes the problems cited above.



a b c

Figure 7 Transformed shoots of *Mentha piperita* after 50 days in (a) submerged, (b) hydroponic, and (c) droplet systems.

5. The nutritional requirements of the culture in relation to growth and productivity need to be understood.
6. Environmental factors such as light and temperature periodic effects need to be characterized in order to maximize growth and productivity.
7. Can the process be scaled up for commercial exploitation?

Table 3 Essential Oil Production by Two Species of Mint Grown in Different Bioreactor Configurations

	Mentha piperita		*Mentha citrata*	
System	Menthofuran (μg/g)	Pulegone (μg/g)	Linalool (μg/g)	Linalyl acetate (μg/g)
Bioreactor system				
Droplet	87	255	32	209
Submerged	6	48	8	129
Hydroponic	10	27	34	343

VI. SUMMARY AND CONCLUSIONS

Although still in its infancy compared with technologies for microbial exploitation, plant cell biotechnology has spawned a wide range of bioreactor types for laboratory and pilot scale cultivation of plant cell and organ cultures. Many technical problems remain but one major obstacle for the development of plant cell culture systems is the definition of the environmental requirements of cell lines. This problem requires a combination of careful experimental studies under well-controlled conditions with a deep understanding of the physiological processes within the cells. Commercial success of plant cell biotechnology is likely to emerge from an understanding of the flux control mechanisms of secondary pathways, together with the ability to deregulate those pathways by genetic means. The rate of development of genetics, metabolic engineering, and biochemical engineering indicates that commercial success is not far from realization.

PROBLEMS

1. Describe the major differences between plant and microbial cells, and discuss the implications for bioreactor design.
2. Derive the equations, analogous to Eqs. (1) and (2), for the oxygen concentration where a fermentor is operated with gas recycling. The equations will include terms for both the gas recycle rate and the rate of gas bleed.

NOMENCLATURE

- c concentration of dissolved oxygen (mmol L^{-1})
- c^* concentration of dissolved oxygen in equilibrium with gas phase (mmol L^{-1})
- v concentration of dissolved volatile (mmol L^{-1})
- v^* concentration of dissolved volatile in equilibrium with gas phase (mmol L^{-1})
- D_o diffusion coefficient of oxygen in growth medium ($m^2\ s^{-1}$)
- D_v diffusion coefficient of volatile in growth medium ($m^2\ s^{-1}$)
- r oxygen consumption rate (mmol $kg^{-1}\ s^{-1}$)
- r_v volatile production rate (mmol $kg^{-1}\ s^{-1}$)
- k_la oxygen mass transfer coefficient (s^{-1})
- X biomass concentration (kg m^{-3})

REFERENCES

1. R. G. Berger, F. Drawert, and P. Tiefel, *Bioformation of Flavours* (R. L. S. Patterson, B. V. Charlwood, G. MacLeod, and A. A. Williams, eds.) Royal Society of Chemistry, Cambridge, 1992, p. 21.

2. A. H. Scragg, *Secondary Metabolism in Plant Cell Culture* (P. Morris, A. H. Scragg, A. Stafford, and M. W. Fowler, eds.), Cambridge University Press, Cambridge, 1986, p. 202.
3. W. E. Goldstein, L. L. Lasure, and M. B. Ingle, *Plant Tissue Culture as a Source of Biochemicals* (E. J. Staba, ed.), CRC Press, Baton Rouge, 1980, p. 191.
4. P. D. G. Wilson and M. J. C. Rhodes, *Advances in Plant Cell Biochemistry and Biotechnology* (I. M. Morison, ed.), JAI Press, London, 1992, p. 105.
5. A. H. Scragg and C. Arias-Castro, *Bioformation of Flavours* (R. L. S. Patterson, B. V. Charlwood, G. MacLeod, and A. A. Williams, eds.), Royal Society of Chemistry, Cambridge 1992, p. 131.
6. S. Azechi, T. Hashimoto, T. YuYama, S. Nagatsuka, M. Nakashizuka, T. Nishiyama, and A. Murata, *Hakkokaguku Kaishi 61*: 117 (1983).
7. R. Bligny, *Plant Physiol. 59*: 502 (1977).
8. A. H. Scragg, E. J. Allan, and F. Leckie, *Enzyme Microb. Technol. 10*: 362 (1988).
9. F. Leckie, A. H. Scragg, and K. C. Cliffe, *Progress in Plant Cellular and Molecular Biology* (H. J. J. Nijkamp, L. H. W. van der Plas, and J. van Aartrijk, eds.), Kluwer, Dordrecht, 1990, p. 689.
10. N. J. Smart and M. W. Fowler, *Biotechnol. Lett. 3*: 171 (1981).
11. N. J. Smart and M. W. Fowler, *Appl. Biochem. Biotechnol. 9*:209 (1984).
12. N. J. Smart and M. W. Fowler, *J. Exp. Bot. 35*: 531 (1984).
13. M. Breuling, A. W. Alferman, and E. Reinhard, *Plant Cell Rep. 4*: 220 (1985).
14. P. M. Townsley and F. Webster, *Biotechnol. Lett. 5*: 13 (1983).
15. H. Tanaka, F. Nishijima, M. Suwa, and T. Iwamota, *Biotechnol. Bioeng. 25*: 2359 (1983).
16. H. Tanaka, *Process Biochem. 22*: 106 (1987).
17. D. A. Janes, N. H. Thomas, and J. A. Callow, *Biotechnol. Techniques 1*: 257 (1987).
18. G. W. Piehl, J. Berlin, C. Mollenschott, and J. Lehmann, *Appl. Microbiol. Biotechnol. 29*: 456 (1988).
19. P. Brodelius, *Trends Biotechnol. 3*: 280 (1985).
20. P. Brodelius, B. Deus, K. Mosbach, and M. H. Zenk, *FEBS Lett. 103*: 93 (1979).
21. A. Rosevear and C. A. Lambe, *Adv. Biochem. Eng. Biotechnol. 31*: 37 (1985).
22. V. Jurku, T. Macek, K. T. Vane, V. Krumphansel, and V. Kubanck, *Biotechnol. Lett. 3*: 447 (1981).
23. A. Freeman, *Ann. N.Y. Acad. Sci. 434*: 418 (1984).
24. I. A. Veliky and A. Jones, *Biotechnol. Lett. 3*: 551 (1981).
25. P. Morris, N. J. Smart, and M. W. Fowler, *Plant Cell Tissue Organ Culture 2*: 207 (1983).
26. F. Kargi, *Biotechnol. Lett. 10*:181 (1988).
27. F. Mavituna, J. M. Park, P. D. Williams, and A. K. Wilkinson, *Plant and Animal Cells: Process Possibilities* (C. Webb and F. Mavituna, eds.), Ellis Horwood, Chichester 1987, p. 1.
28. F. Mavituna, A. K. Wilkinson, and P. D. Williams, *Bioreactors and Biotransformations* (G. W. Moody and P. B. Baker, eds.), Elsevier, London, 1987, p. 26.
29. F. DiCosmo, P. J. Facchini, and A. W. Neumann, *Trends Biotechnol. 6*: 137 (1988).
30. P. J. Facchini, A. W. Neumann, and F. DiCosmo, *Appl. Microbiol. Biotechnol. 29*: 346 (1988).

31. P. J. Facchini, A. W. Neumann, and F. DiCosmo, *Biotechnol. Appl. Biochem. 11*: 74 (1989).
32. P. J. Facchini, F. DiCosmo, L. G. Radvanyi, and Y. Giguere, *Biotechnol. Bioeng. 32*: 935 (1988).
33. M. J. C. Rhodes, R. J. Robins, R. J. Turner, and J. J. Smith, *Can. J. Bot. 63*: 2357 (1985).
34. R. J. Robins, D. O. Hall, E. J. Shi, R. J. Turner, and M. J. C. Rhodes, *FEMS Lett. 34*: 155 (1986).
35. R. M. Hamilton, Ph.D. Thesis, Rutgers University, 1987.
36. M. L. Shuler, *Ann. N.Y. Acad. Sci. 369*: 65 (1981). Abstract.
37. J. E. Prenosil and H. Pedersen, *Enzyme Microb. Technol 5*: 323 (1983). Abstract.
38. A. Jos, H. Pedersen, and C. K. Chin, *Ann. N.Y. Acad. Sci. 413*: 409 (1983) Abstract.
39. M. L. Shuler, G. A. Hallsby, J. W. Pyne, Jr., and T. Cho, *Ann. N. Y. Acad. Sci. 469*: 270 (1986). Abstract.
40. M. L. Shuler *Plant Cell Biotechnology* (M. S. S. Pais, F. Mavituna, and J. M. Novais) eds., Springer-Verlag, Berlin, 1988, p. 329.
41. J. M. Smith, *Chemical Engineering Kinetics,* McGraw-Hill, New York, 1981.
42. R. B. Bird, W. E. Stewart, and E. N. Lightfoot, *Transport Phenomena,* Wiley, Chichester, 1960, p. 529.
43. L. Lapidus and N. R. Amundson, *Chemical Reactor Theory—A Review,* Prentice-Hall, Englewood Cliffs, NJ, 1977.
44. T. Yano, T. Komada, and K. Yamada, *Agric. Biol. Chem. 25*: 580 (1961).
45. T. Kobayashi, G. van Dedem, and M. Moo-Young, *Biotechnol. Bioeng. 15*: 27 (1973).
46. S. M. Bhavaraju and H. W. Blanch, *J. Ferment. Technol. 53*: 413 (1975).
47. K. Toda and K. Sato, *J. Ferment. Technol. 63*: 251 (1985).
48. J. Radovich, *Enzyme Microb. Technol. 7*:2 (1985).
49. F. Mavituna, J. M. Park and D. Gardner, *Chem. Eng. J. 34*: B1 (1987).
50. R. J. Robins, A. J. Parr, S. R. Richards, and M. J. C. Rhodes, *Secondary Metabolism in Plant Cell Cultures* (P. Morris, A. H. Scragg, A. Stafford, and M. W. Fowler, eds.), Cambridge University Press, Cambridge, 1986, p. 162.
51. H. T. Pu and R. Y. K. Yang, *Biotechnol. Bioeng. 32*: 891 (1988).
52. A. C. Hulst, J. Tramper, P. Brodelius, L. J. C. Eijkenboom, and K. Ch. A. M. Luyben, *J. Chem. Tech. Biotechnol. 36B*: 198 (1985).
53. H. N. Chang and M. Moo-Young, *Appl. Biochem. Biotechnol. 29*:107, (1988).
54. B. A. Westrin and A. Axelsson, *Biotechnol. Bioeng. 38*: 439, (1991).
55. L. Toivonen, *Biotechnol. Prog. 9*: 12 (1993).
56. A. Spencer, J. D. Hamill, and M. J. C. Rhodes, *Plant Cell Rep. 8*: 601 (1990).
57. J. D. Hamill, R. J. Robins, A. J. Parr, D. M. Evans, J. M. Furze, and M. J. C. Rhodes, *Plant Mol. Biol. 15*:27, (1990).
58. M. J. C Rhodes, M. G. Hilton, A. J. Parr, J. D. Hamill, and R. J. Robins, *Biotechnol. Lett. 8*: 415 (1986).
59. P. D. G. Wilson, M. G. Hilton, R. J. Robins, and M. J. C. Rhodes, *Bioreactors and Biotransformations* (G. W. Moody and P. B. Baker, eds.), Elsevier, London, 1987, p. 38.

60. G. Jung and D. Tepfer, *Plant Sci. 50*:145 (1987)
61. M. Taya, A. Yoyama, O. Kondo, T. Kobayashi, and C. Matsui, *J. Chem. Eng. Jpn. 22*: 84 (1989).
62. O. Kondo, H. Honda, M. Taya, and T. Kobasyashi, *Appl. Microbiol. Biotechnol. 32*: 291 (1989).
63. M. G. Hilton and M. J. C. Rhodes, *Appl. Microbiol. Biotechnol. 33*: 132 (1990).
64. P. D. G. Wilson, M. G. Hilton, P. T. H. Meehan, C. R. Waspe, R. J. Robins, and M. J. C. Rhodes *Progress in Plant Cellular and Molecular Biology* (H. J. J. Nijkamp, L. H. W. van der Plas, and J. van Aartrijk, eds.), Kluwer, Dordrecht 1990, p. 700.
65. P. D. G. Wilson, M. G. Hilton, D. C. Steer, C. R. Waspe, R. J. Robins, and M. J. C. Rhodes, PCT Patent Application PCT/GB 89/00510, 1989.
66. R. M. Buitelaar, A. A. M. Langenhoff, R. Heidstra, and J. Tramper, *Enzyme Microb. Technol. 13*: 487 (1991).
67. M. A. Rodríguez-Mendiola, A. Stafford, R. Cresswell, and C. Arias-Castro, *Enzyme Microb. Technol. 13*: 697 (1991).
68. P. J. Whitney, *Enzyme Microb. Technol. 14*: 13 (1992).
69. P. J. Weathers and R. W. Zobel, *Biotech. Adv. 10*: 93 (1992).
70. M. Hagimori, T. Matsumoto, and Y. Mikami, *Agric. Biol. Chem. 48*: 965 (1984).
71. H. Katagi, E. Takahashi, K. Nakao, and M. Inui, *Nippon Nogeikagadu Kaishi 60*: 15 (1986).
72. K. Saito, I. Murakoshi, D. Inse, and M. Van Montagu, *Plant Cell Rep. 7*: 607 (1989).
73. A. Spencer, J. D. Hamill and M. J. C. Rhodes, *Phytochemistry 32*: 911 (1993).
74. W. D. Loomis, *Terpenes in Plants* (J. B. Pridham, ed.), Academic Press, New York, 1967, p. 59.
75. S. Thengane and A. F. Mascarenhas, *Indian Drugs 24(10)*: 460 (1987).
76. C. A. M. Campbell, G. W. Dawson, D. C. Griffiths, J. Petterson, J. A. Pickett, L. J. Wadhams, and C. M. Woodcock, *J. Chem. Ecol. 16(12)* 3455 (1990).
77. M. E. Curtin, *Biotechnology 1(8)*: 648 (1983).

12
Photobioreactors

Aleš Prokop

Vanderbilt University
Nashville, Tennessee

Larry E. Erickson

Kansas State University
Manhattan, Kansas

I. INTRODUCTION

Numerous applications of light in biological processes exist; these include the annual production of agricultural crops, where each field may be considered a bioreactor in which nutrients are applied and populations are managed through cultivation and application of chemicals. Forests may be viewed as managed bioreactors used to produce wood, paper, and other products. Seaweed and algae are produced in marine environments. Because most photobioreactors consume carbon dioxide and produce oxygen, they have global environmental importance. Photobioreactors have been designed for the following environmental applications: (1) wastewater treatment—wetland systems have been used; (2) nonpoint source water quality management—vegetation has been planted in waterways and along streams; (3) remediation of contaminated soil—the root zone of plants is rich in microbial communities which can degrade a wide variety of pollutants;

and (4) utilization of plants for the purification of indoor air. Although all of these applications are important, this work is directed toward photobioreactors for photosynthetic microbial populations. Many investigators have contributed to the advancement of the science and technology of photosynthetic microorganisms and the methods for their cultivation [1–102].

When light is needed for a chemical reaction to occur, the reactor must be designed with this in mind. Because light can't be stored in the reactor, it must be supplied continuously. Furthermore, light is readily absorbed; thus, the geometry of the reactor is important. These and other factors make photobioreaction engineering a challenging topic; however, the topic is too often neglected in educational programs. One of the reasons for this may be the instructional resources which are presently available. This contribution to the literature is directed toward both those who wish to teach photobioreaction engineering and those who need to design and build photobioreactors.

There are many contributions to the literature on photobioreactors, most of which are found in journals, conference proceedings, and reports. Edited works by Richmond [1], Borowitzka and Borowitzka [2], Stadler et al. [3], Cresswell et al. [4], Barclay and McIntosh [5], and Shelef and Soeder [6] include material on photobioreactors. There are also reviews on the design of photoreactors [7,8] and photobioreactors [9–11].

II. PHOTOTROPHS AND THEIR NUTRITIONAL REQUIREMENTS

A. Light and Its Utilization

1. Organisms

Several types of organisms depend on light. Those which use light as an energy source and carbon dioxide as the principal carbon source are called *photoautotrophs*. Among them, green and blue-green (bacteria) algae are mostly aerobic; other bacteria are anaerobic (e.g., *Chlorobium*). The latter use carbon dioxide as the nonlimiting carbon source and an inorganic compound (e.g., NO_2, H_2, H_2S, S, etc.; in the case of *Chlorobium* it is H_2S). In fact, such bacteria belong to *chemoautotrophs* by definition of their nutritional requirements. For our purpose, they can be denoted as photoautotrophs as well. The second major group, *photoheterotrophs*, use light as an energy source and an organic compound as the principal carbon source. Among algae, some strains are capable of photoassimilating organic substrates, such as acetate or glucose, and are denoted as *mixotrophs*. Others can even utilize organic compounds in the dark (*heterotrophs*). This review deals mostly with photoautrophic organisms, primarily dependent on light. Most of references and examples are drawn from the literature on algae. However, the majority of the conclusions will be transferrable to other

photosynthetic organisms. Reactor design for photosynthetic organisms is relevant no matter what the additional nutritional requirements may be.

The spectral quality of light utilized by photosynthetic organisms for growth and photosynthesis is defined by the absorption of major and accessory pigments (Fig. 1). Depending on the type of pigments, different light spectra can be utilized (absorbed); for practical reasons the most significant spectra are

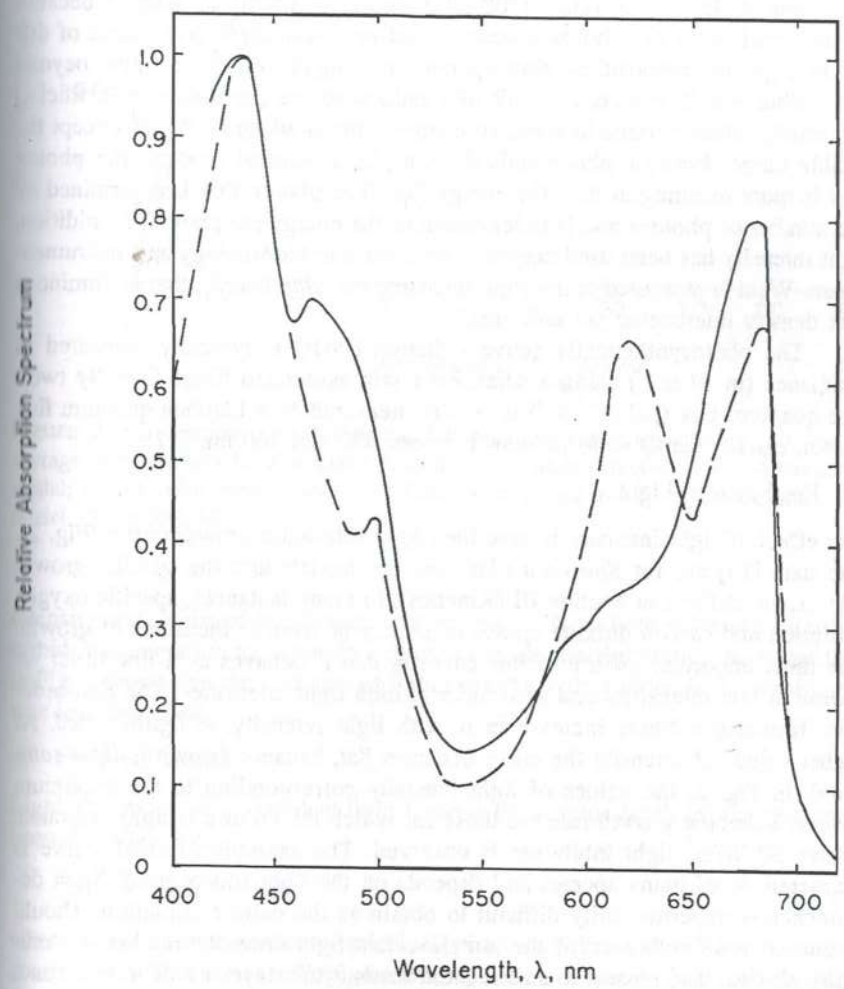


Figure 1 Examples of absorption spectrum characteristics for *Chlorella pyrenoidosa* (solid line) and *Oscillatoria redekei* (dashed line). (From Ref. 1.)

those of the visible region (400–700 nm), which make up about 40% of the total energy of solar radiation. Among artificial light sources the spectral quality and intensity vary considerably. Light beyond the visible region, on both sides of the spectrum, can have several biological effects (morphogenetic); some are detrimental. The photobioreactor design in terms of light spectrum adjustments is dealt with later.

2. Radiation and Measurement

Radiation is the visible range (400–700 nm) is of particular interest because those wavelengths are photosynthetically active. Some bacteria, because of different pigment composition, may operate in a slightly different range, beyond the visible one. The spectral bands of a radiant source (be it solar or artificial) are usually characterized in terms of energy. This is adequate for all except the visible range. Because photosynthesis is a photochemical process, the photon flux is more meaningful than the energy flux. The photon flux is determined by the number of photons and is independent of the energy per photon. In addition, light intensity has been used despite inaccuracies in terminology and instrumentation. What is measured is not light intensity, but *illuminance*, that is, luminous flux density intercepted per unit area.

The photosynthetically active radiation (PAR) is typically measured as irradiance (in W m^{-2}) using a solarimeter wih associated filters (usually two). The quantum flux (μE m^{-2} s^{-1}) is usually measured by a Lambda quantum flux sensor, equally sensitive to photons between 400 and 700 nm [12].

3. Response to Light

The effect of light intensity is described by a saturation growth curve (Fig. 2). The data [13] are for *Spirulina platensis*; the models and the specific growth rate, μ, are defined in Section III (Kinetics). In many instances, specific oxygen evolution and carbon dioxide uptake rates may be used as measures of growth. The most important feature of this curve is that it behaves as a first-order response at low intensities and zero order at high light intensities. The first-order part, featuring a linear increase in μ with light intensity, is *light-limited*. At higher values of intensity the curve becomes flat, because growth is *light-saturated*. In Fig. 2, the values of light intensity corresponding to the maximum observed specific growth rate are those for which the culture is light saturated. Above 50 W/m^2 light inhibition is observed. The saturation growth curve is characteristic of many species and depends on the light source used. Such dependence is experimentally difficult to obtain as the culture conditions should be such that all cells receive the same incident light intensity and are not mutually shaded. The closest to such conditions is a thin layer of algae (e.g., as a monocellular film attached to a platinum electrode), often called an optically thin culture [14]. For the experimental results in Fig. 2, a nearly uniform light

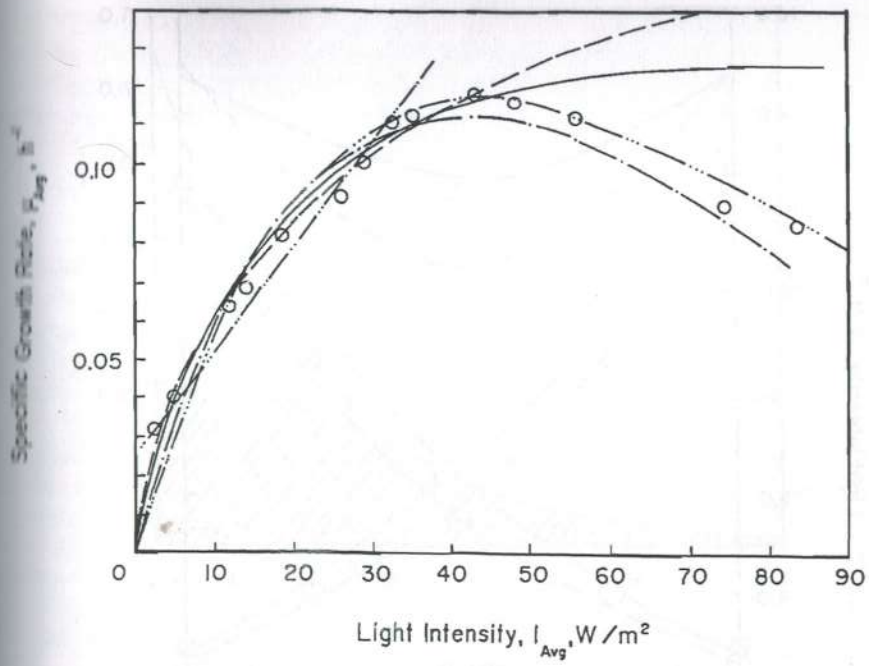


Figure 2 Comparison of experimental data relating average specific growth rate to average light intensity for X = 0.04 g/L with: (——) Monod model; (– – – –) Bannister model; (– · –) Aiba model; (— · · —) linear model; and (— · · · —) modified Aiba model. (From Ref. 13.)

intensity was achieved by illuminating the culture from both sides and by using turbidostat operation to maintain a dilute biomass concentration. The upper line in Fig. 3 shows that the variation of light intensity with position in the turbidostat was less than 20%.

4. Light Utilization

Light absorption of an incident light I_0 along the reactor depth (d) has generally been described by

$$I(d) = I_0 \exp(-\epsilon X d) \tag{1}$$

where X is the concentration of microbial biomass. In Eq. (1), ϵ is the extinction coefficient, which varies with pigment composition. Equation (1) is strictly valid for monochromatic light. For polychromatic light the wavelength dependence of ϵ must be considered. Often, an appropriate average value of ϵ for light of

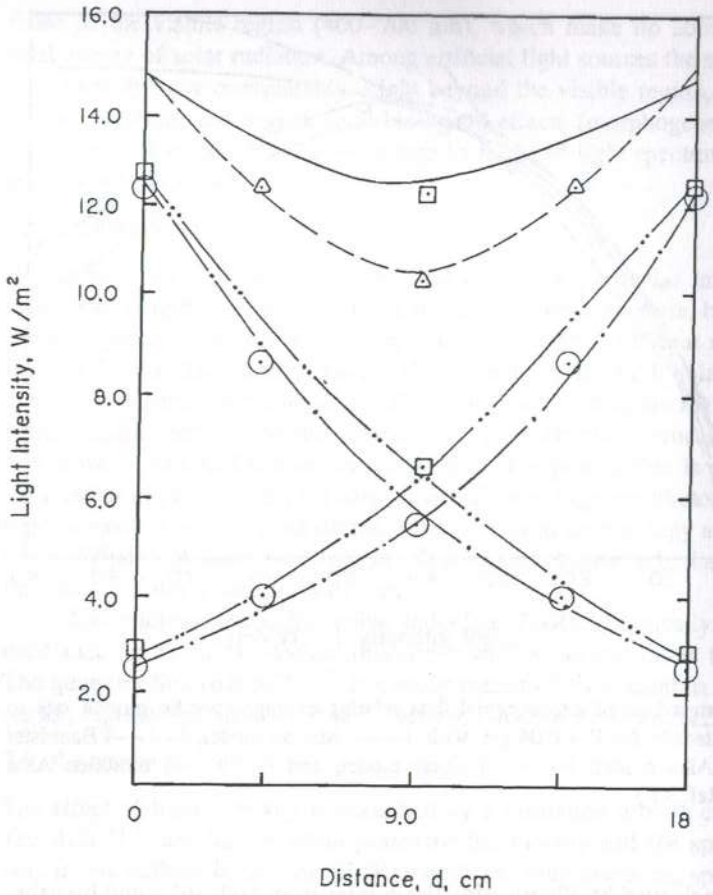


Figure 3 Variation of light intensity with position for I_{in} = 13.06 W/m² for X = 0.05 g/L. (○) Light shining from one side; (△) summation of light intensity from two sides based on measured values for light shining from one side at a time; (□) light intensity from one direction with light shining from both sides; and summation of light intensity measured in two directions with light shining from both sides. (From Ref. 13.)

a given quality is used. Figure 3 depicts the absorption characteristics for *S. platensis* [13].

When light passes through a dense culture, the irradiance decays along the depth (Fig. 4). The saturation light intensity I_s is the maximum that can be accommodated by the photosynthetic apparatus. When the intensities are higher, the excess light is not used and the photosynthetic activity proceeds only at a

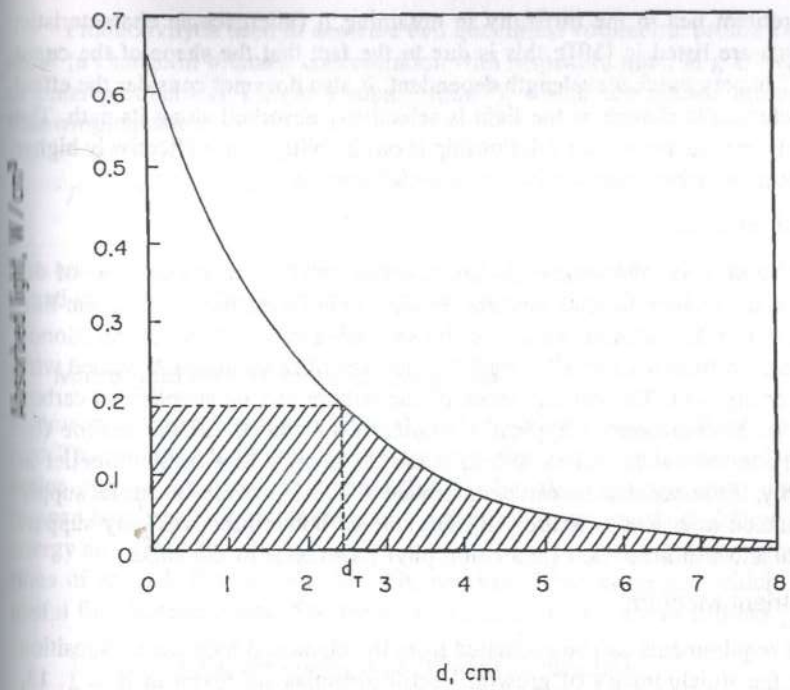


Figure 4 Variation of absorbed light with distance from the front of the vessel, d. The dashed horizontal line shows the energy requirement for growth of a culture with 0.4 g/L cell concentration at the maximum specific growth rate of 0.1 h^{-1} when η_{max} = 0.2711 and m_c = 0.0229 h^{-1}. The point d_T denotes the transition point where light-limited growth begins. (From Ref. 15.)

rate proportional to I_s. The shaded area in Fig. 4 represents the fraction of light being utilized effectively, E_s [9,15]. An example is available in which experimental data are analyzed and results are presented on light utilization efficiency [15]. Another expression for the fraction of light being utilized effectively, E_s, is [10,16]

$$E_s = \frac{I_s}{I_0}\left[\ln\left(\frac{I_0}{I_s}\right) + 1\right] \tag{2}$$

This formula is known as the Bush equation. Its derivation is given in Shelef et al. [16, p. 14] and it results from the ratio of shaded to total area underneath the curve. The total area is proportional to the light absorbed, the shaded area to the fraction utilized effectively. Such predictions are only qualitative. The

main problem lies in the difficulty in obtaining I_s (microscopic characteristic; some data are listed in [10]); this is due to the fact that the shape of the curve in Fig. 2 is very much wavelength dependent. It also does not consider the effect of polychromatic change as the light is selectively absorbed along its path. The practical consequence of this relationship is our inability to use effectively higher irradiances, whether from natural or artificial sources.

5. Carbon Source

As with light, photosynthetic organisms exhibit a similar saturation type of dependence for carbon dioxide. Lee and Erickson [17] have modeled carbon-limited growth of *S. platensis*; values of the saturation constant, K_s, in the Monod model ranged from 4.17 to 17.7 mg/L bicarbonate. The values of K_s varied with light intensity also. The carbon needs of the culture can be supplied as carbon dioxide or as bicarbonate. Typically, a mixture of 5% of carbon dioxide (by volume) and the balance of air (95%) is used. Contrary to a common belief in its toxicity, the concentration can be increased to 100% provided a careful supply is guaranteed to a dense culture [18]. Organic carbon compounds may support complete and continued cell (and chlorophyll) synthesis in the dark.

6. Nutrient Medium

Nutrient requirements can be estimated from the elemental biomass composition or from the stoichiometry of growth. Useful formulas are given in refs. 1, 13, and 19, featuring typical macro- and micronutrients. Some of the micronutrients may require a chelating agent to keep them in solution or to minimize toxicity caused by their excess.

III. KINETICS

A. Specific Growth Rate and Productivity

Specific growth rate is defined as a proportionality constant for the autocatalytic reaction of algal growth:

$$\frac{dX}{dt} = \mu X \tag{3}$$

where X is some measure of cell concentration and t is time. The dimension of μ is reciprocal time (e.g., 1/d, or d^{-1}). Specific growth rate can be measured in terms of any cell quantity, i.e., cell number, cell volume, cell dry weight, or optical density, the most meaningful being cell dry weight. Other measures are quite dependent on the cell physiological state. The values of specific growth rate reported in Fig. 2 are similar to those for many bacteria and yeasts.

Productivity is used to describe two quantities: volumetric productivity P_V, dX/dt (a change in biomass concentration with respect to time, in g L^{-1} $time^{-1}$), or areal productivity P_A (in g $area^{-1}$ $time^{-1}$), which are related through the following equation:

$$P_A = P_V\left(\frac{V}{A}\right) = \mu X d \tag{4}$$

The areal productivity is sometimes a more useful measure. The area A in the equation is that of illuminated surface; V is the culture volume.

B. Micro- and Macrokinetic Relationship

Microscopic characteristics refer to those for uniform light irradiance in a culture. The present microkinetic understanding of photosynthesis distinguishes two major events: light and dark reactions. During the light reactions, energy is trapped by a trapping center (pigments); the dark reactions which follow require energy and generate reducing equivalents and oxygen, each reaction lasting fractions of second. Several reaction schemes have been suggested which lead to useful final kinetic forms. The basic kinetic descriptions are as follows [10,13]:

1. First-order, zero-order type (Blackman kinetics)
2. Rectangular hyperbolic type (similar to Monod model and Michaelis-Menten in enzyme kinetics)
3. Bannister model
4. Aiba model
5. Modified Aiba model

Several of these are depicted in Fig. 2. The first model, unfortunately, is described mathematically by two separate segments as follows:

$$\mu = K_I I \qquad I < I_s \tag{5}$$

$$\mu = \mu_{max} \qquad I > I_s \tag{6}$$

Lee et al. [13] have shown that this model has a theoretical basis. For a constant energetic yield, they show that under light-limited growth conditions, the slope in Eq. (5) depends on the bioenergetic efficiency of light utilization by the culture. In Fig. 2, the linear model which fits the data does not pass through the origin; thus, it is not represented by Eq. (5). The Monod model, No. 2, is of the form

$$\mu = \frac{\mu_{max} I}{K_I + I} \tag{7}$$

The Bannister model, No. 3, is

$$\mu = \frac{\mu_{max} I}{(K_I^m + I^m)^{1/m}} \tag{8}$$

The foregoing models have been used successfully on different occasions and there is no agreement as to the best kinetic model. The nonlinear model No. 2 is the most frequently used, and the similarity to analogous microbial kinetic models should be noted. An extension of this model can easily be made for the case of excess light—an inhibition term is added to the denominator; this gives the Aiba model [13,20]:

$$\mu = \frac{\mu_{max} I}{K_I + I + K_i I^2} \tag{9}$$

where K_i is an inhibition constant. The modified Aiba model [20], No. 5, is obtained by removing the middle term from the denominator in Eq. (9).

Another useful extension of any of these models is obtained by incorporating a term for maintenance (endogenous metabolism, dark and photorespiration, cell death, etc.); this yields a net specific growth rate [10]:

$$\mu' = \mu - \mu_d \tag{10}$$

Note that μ' has a negative value at $I = 0$.

The foregoing microkinetic relationships have been observed to be valid for any monochromatic [14] or polychromatic (white) light source, and equally well for other measures of photosynthesis (e.g., oxygen uptake). A method of accounting for polychromatic light absorption has been proposed [21].

For optically dense cultures it is impossible to establish a uniform light condition within the culture. If the irradiance is high near a light source, it will be low far from the source because of absorption and scattering of light by the culture. The rate of photosynthesis will be different in different regions of the culture. According to the microscopic model, the rate of photosynthesis will depend on the local environment experienced by individual cells. The quantity governing this is a *time-averaged* irradiance [22]. In an optically dense culture, various cells in a culture are exposed to different irradiances, the rates of photosynthesis of individual cells differ, and the observed *macroscopic* rate represents an average of all the microscopic rates. The overall outcome depends very much on reactor design, light source characteristics, culture density, and particularly the mixing regime in the culture. From the analysis of several groups [10,22] it appears that the form of the macroscopic relationship will in the end be similar to one of the microscopic relationships for agitated cultures. In such cultures the time-averaged irradiance is the same for all cells and equal to the *space-averaged* irradiance. The practical outcome is the rectangular hyperbolic rate equation mentioned above, but with the space-average irradiance replaced

by the local light irradiance. Other types, formally identical to the rest of the suggested microscopic relationships, have also been suggested for macroscopic dependence. Because of its simplicity, the rectangular hyperbola is preferred. Obviously, any kinetic macroscopic model can be extended to include the effect of polychromatic light, considering the spatial distribution and the extinction coefficient variation.

C. Batch and Continuous Culture

The batch processing mode is a simple way to demonstrate a major kinetic difference between photosynthetic organisms and those dependent on an organic source (Fig. 5). For photosynthetic growth, the exponential growth phase is

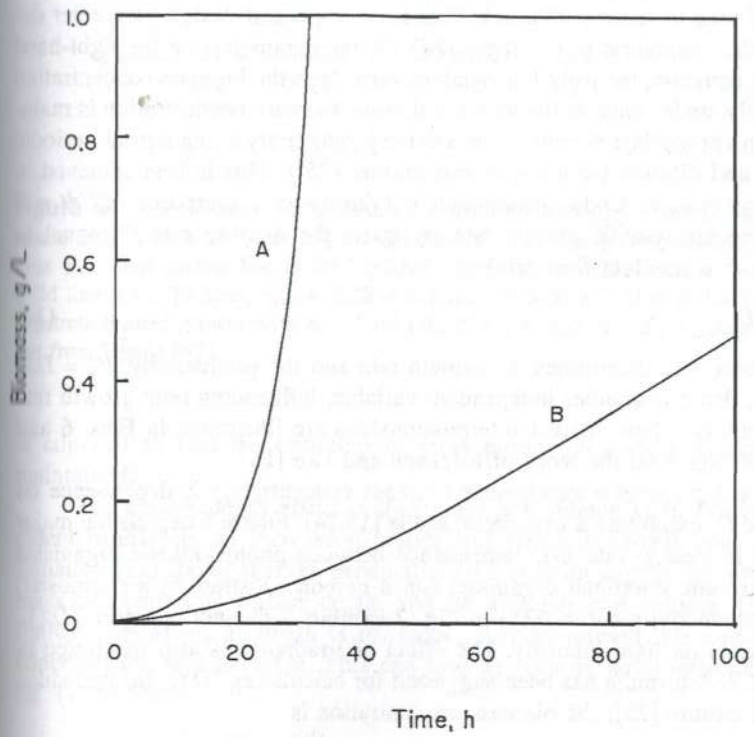


Figure 5 Comparison of simulated exponential growth (A) and light-limited growth (B) in batch culture for *Spirulina platensis* for the experimental conditions of Lee et al. [13]. The Monod model was used with $\mu_{max} = 0.1617\ h^{-1}$ and $K_I = 17.04\ W/m^2$; the Lambert-Beer law with $I_0 = 12.3\ W/m^2$, $\epsilon = 0.182\ m^2/g$, and $d = 0.18$ m was used, and the experimental chamber was lighted from both sides.

limited to optically dilute or thin suspensions. Typically, a long linear phase follows, a proof of light-limited conditions (see the data of Persoone et al. [23], for example). The slope evaluation (dX/dt) is a simple way to estimate the culture productivity (volumetric). The discussion of factors which control this productivity is best achieved on the basis of an equation derived from Eq. (4):

$$\frac{dX}{dt} = P_V = \mu X d_e \left(\frac{A}{V}\right) \tag{11}$$

where d is replaced by d_e, the equivalent thickness of the culture. This is a useful parameter for comparison of different algal cultivator geometries and represents a thickness as if the reactor were of a flat (rectangular) design. In Eq. (11) two terms (d_e and A/V) have the same dimension and cancel out. However, the equation is useful for demonstrating the effect of the ratio (A/V) (effective illuminated area to reactor volume). This is an important design parameter determining the volumetric productivity [24]. Of the parameters on the right-hand side of this equation, the truly independent variable is the biomass concentration X. Practically, under mass cultivation conditions, biomass concentration is maintained at an appropriate density to maximize productivity using partial periodic harvesting and dilution (semicontinuous culture [25]). This is best achieved in a continuous culture. Under steady-state conditions in a chemostat, $dX/dt = 0$ and macroscopic specific growth rate μ equals the dilution rate D (equal to F/V, where F is medium flow rate):

$$\mu = D \tag{12}$$

Thus, the flow rate determines the growth rate and the productivity, $P_V = DX$. The culture depth is another independent variable, influencing both growth rate and productivity. These mutual interrelationships are illustrated in Figs. 6 and 7, which are based on the work of Erickson and Lee [15].

Figure 6 presents a steady-state biomass concentration X dependence on dilution rate D, exhibiting a hyperbolic shape [15,24]. Figure 6 depicts the major differences in steady-state X-D dependence between photosynthetic organisms and heterotrophic microbial organisms (on a carbon substrate in a chemostat) [26]. The productivity curve (DX) in Fig. 7 exhibits a distinct optimum $(DX)_0$, which depends on light intensity. The effect of irradiance is also illustrated in Figs. 6 and 7. A formula has been suggested for calculating $(DX)_0$ for one-sided illuminated culture [25]; the biomass concentration is

$$(X)_0 = \left(\frac{1}{\epsilon d}\right) \ln\left(\frac{I_0}{I_c}\right) \tag{13}$$

where d is the depth of the vessel and I_c is the compensation light intensity, which is just sufficient to maintain the density of the culture. The dilution rate

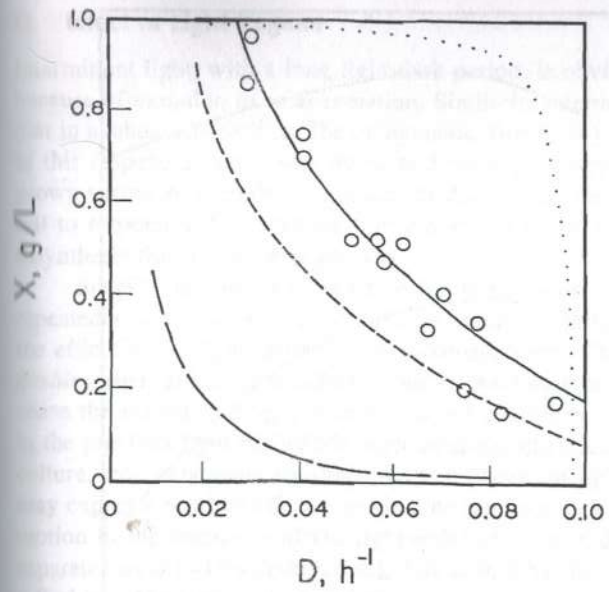


Figure 6 Model results for continuous cultivation with $\mu_{max} = 0.1\ h^{-1}$ based on the results of Erickson and Lee [15] for the data of Iehana [87]; long dashed line is for 2 klux and short dashed line is for 7.6 klux where $\eta_{max} = 0.2711$ and $m_c = 0.0229\ h^{-1}$; solid line is for 18 klux, $\eta_{max} = 0.2234$ and $m_c = 0.0400\ h^{-1}$. Dotted line is for carbon substrate limited growth with $K_s = 0.05$ g/L, $Y_s = 0.5$, and $S_0 = 2$ g/L. Data for 18 klux are from Iehana [87].

is adjusted so that the optimum biomass productivity at concentration X_0 is maintained.

The culture depth effect is illustrated in Goldman [10]. For constant incident irradiance, an increase in depth will result in growth rate depression. Equation (11) shows that the productivity depends on specific growth rate, biomass concentration, culture depth, and the area per unit volume. Under light-limited conditions, as depth is increased, specific growth rate and/or biomass concentration will decrease. This can best be seen by substituting Eq. (1) into Eq. (7) to obtain

$$\mu = \frac{\mu_{max} I_0 \exp(-\epsilon X d)}{K_i + I_0 \exp(-\epsilon X d)} \tag{14}$$

which shows that μ decreases if the value of d is increased.

The overall suggestion from the preceding discussion is as follows: to reach an optimal productivity, one should adopt an appropriate flow rate-depth

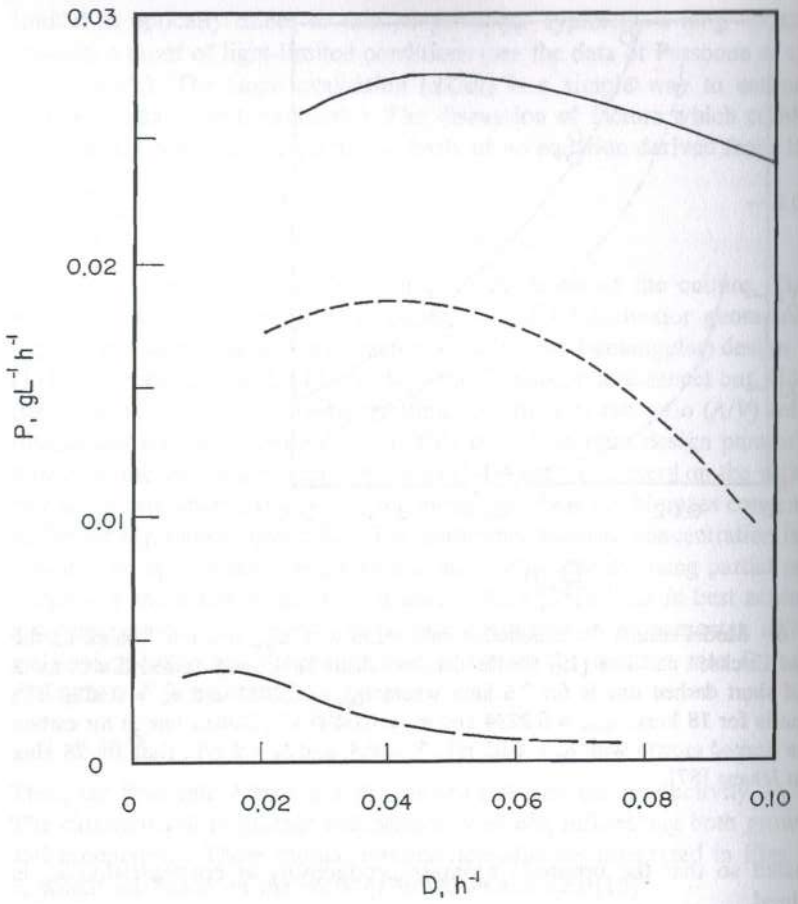


Figure 7 Productivity (DX) as a function of dilution rate for 2 klux, 7.6 klux, and 18 klux model results shown in Fig. 6. The maximum productivity is found at larger dilution rates for higher light intensities.

combination that will result in an optimal biomass concentration for productivity [10]. As the depth is usually a fixed design parameter, adjustment of density via flow rate in a continuous culture is the best strategy. This is quite possible for artificially illuminated cultures. For outdoor mass culture, however, as the incident irradiance varies over the daytime and season, some flow rate adjustments should be attempted. The most common is a semicontinuous culture, with partial daily harvest and medium replacement, adjusted to seasonal variations.

D. Effect of Light Regime

Intermittent light, with a long light-dark period, is obvious for outdoor cultures because of variation in solar radiation. Similarly, intermittent light became popular in a laboratory setting. The ontogenetic (life-cycle) factors are of importance in this respect. Later, it was noted that some photosynthetic organisms can be grown successfully under continuous artificial irradiation. Many algae, however, fail to respond and exhibit inhibitory phenomena in terms of growth and photosynthesis (bleaching of pigments).

Special attention has been directed to short-term light effects. It has been repeatedly demonstrated that turbulence in an optically dense culture increases the efficiency of light utilization by photosynthesis. This effect is denoted as a *flashing light* effect. This effect is due to the fact that photosynthesis does not cease the instant that light is removed but continues until the energy absorbed in the previous light period has been used and chemically stored. In a turbulent culture, individual cells are subjected to a pattern of light and dark periods. One may expect a positive effect provided the time scale characterizing the turbulent motion in the reactor is of the right order of magnitude (10–70 ms light flash separated by 0.1–10 s dark period). Although it has been demonstrated that such a flashing effect takes place, it has never been conclusively shown that algae in turbulent flow can take advantage of this effect [27]. However, it has been demonstrated that light modulation at a frequency which is attained in outdoor mass cultures with a mechanism for ordered rather than turbulent mixing with longer time constants is adequate to stimulate productivity significantly [11].

Another aspect of light utilization is that of nonuniform illumination. Because of absorption of light by a culture and reactor design considerations (e.g., a storage, nonilluminated tank for culture recirculation), the suspension volume can be divided into two parts: in one the irradiance is lower than or equal to the compensation point for individual cells (light-limited, nonilluminated, or dark zone), and in the other the irradiance is higher than the compensation one (illuminated zone). Because of biological inertia (a dark reaction following a light one as discussed above), photosynthesis proceeds for a certain period even after the transition of the cells from the illuminated to the nonilluminated zone. The productivity then depends on the relative size of the two parts of suspension volume or on the corresponding residence time in them. Based on time constants of the light and dark reactions, as well as residence times in the illuminated and nonilluminated zones, Lívanský [28] suggested a formula accounting for these effects. A similar analysis, using a maintenance concept, has been applied to experimental data [29]. From these investigations it can be inferred that bioreactors for photosynthesis should be designed to minimize the fraction of the culture in the dark.

IV. STOICHIOMETRY AND BIOENERGETICS

A. Stoichiometry and Balances

The mass and energy balances are presented for processes in which an organic substrate is present and used with light energy; Lee et al. [30,31] have analyzed photoautotrophic, mixotrophic, and photoheterotrophic growth. The mass balance equation for photoheterotrophic and mixotrophic growth is

$$CH_mO_\ell + aNH_3 + bO_2 = y_cCH_pO_nN_g + cH_2O + dCO_2 \tag{15}$$

where CH_mO_ℓ denotes the elemental composition of the organic substrate and $CH_pO_nN_g$ is the elemental composition of these elements in the biomass. The radiant energy which is supplied is included in the energy balance

$$Q_s\gamma_s + Q_R = Q_by_c\gamma_b + Q_H \tag{16}$$

where Q_R is the radiant energy supplied per g atom carbon of substrate consumed and Q_H is the heat evolved per g atom carbon of substrate consumed.

The regularities used in prior work [31–34] may be used to obtain numerical values of the weight fraction carbon σ_b, reductance degree γ_b, and energy content per equivalent of available electrons, Q_O, for the microbial biomass. These regularities have been shown to be similar for many photoautotrophic, mixotrophic, and heterotrophic cultures [32,33]. Where cellular elemental composition is provided, it should be used to calculate values of σ_b and γ_b. The mean values of $\sigma_b = 0.4766$ and $\gamma_b = 4.402$ for ammonia as the nitrogen source [32] can be used when elemental composition is not given.

For Eq. (1), the carbon balance is

$$y_c + d = 1 \tag{17}$$

where y_c is the stoichiometric coefficient for organic substrate carbon converted to biomass and d is the coefficient for organic substrate carbon converted to CO_2. The value of d may be either negative, positive, or zero. In many mixotrophic applications, d is positive. When d is negative and CO_2 is used as a carbon source for growth, the carbon incorporated into biomass should equal the sum of the organic substrate carbon and the carbon from CO_2; that is,

$$y_c = 1 - d \tag{18}$$

For photoautotrophic growth $y_c = -d$.

The coefficient b in Eq. (15) may be negative, positive, or zero for algae which can produce oxygen; however, because purple and green bacteria do not produce oxygen, b is positive or zero for these bacteria.

The available electron balance for Eq. (15) is

$$\frac{4b}{\gamma_s} + \frac{y_c\gamma_b}{\gamma_s} = 1 \tag{19}$$

or

$$\epsilon + \eta = 1$$

where $\epsilon = 4b/\gamma_s$ is the fraction of available electrons transferred to oxygen and $\eta = y_c\gamma_b/\gamma_s$ is the fraction of available electrons incorporated into microbial biomass. For photoautotrophic growth, $-4b = y_c\gamma_b$, where the value of b is negative because oxygen is produced.

The energy for growth is supplied by light and the organic substrate. Equation (16) may be written in the form

$$\epsilon_E + \eta_E = 1 \tag{20}$$

where

$$\epsilon_E = \frac{Q_H}{Q_s\gamma_s + Q_R} \tag{21}$$

is the fraction of energy evolved as heat, and

$$\eta_E = \frac{Q_by_c\gamma_b}{Q_s\gamma_s + Q_R} \tag{22}$$

is the fraction of energy incorporated into microbial biomass. For photoautotrophic growth, $Q_s\gamma_s = 0$ in Eqs. (21) and (22). In Eqs. (16), (20), and (21) Q_b is the energy regularity of Thornton [35], Kharasch [36], and Minkevich and Eroshin [37], which is approximately 113 kJ/g equivalent of available electrons for microbial biomass [33]. Values for Q_s can usually be found in the literature, but 113 kJ/g equivalent may be used for Q_s as well.

Lee et al. [30,31] have developed equations in which the energy requirements associated with photosynthetic growth and maintenance are each clearly identified. The model is

$$\frac{\mu}{\eta_E} = \frac{\mu}{\eta_E^{max}} + m_E \tag{23}$$

where μ/η_E is the specific rate with which radiant energy and organic chemical energy are utilized, and μ/η_E^{max} and m_E are the specific rates of energy utilization for growth and maintenance, respectively. The parameters μ/η_E^{max} and m_E are the true growth yield and maintenance coefficient, respectively. Several other alternative forms of Eq. (23) may be written. If Eq. (20) is included in the numerator of the left-hand side of Eq. (23),

$$\frac{\mu(\epsilon_E + \eta_E)}{\eta_E} = \frac{\mu}{\eta_E^{max}} + m_E \tag{24}$$

Equation (24) uses the measured values of the terms on the right-hand side of Eq. (16); Eq. (24) is independent of the measured values on the left-hand side of Eq. (16).

Values of the regularities for photosynthetic microorganisms have been analyzed and the results are reported in Lee and Erickson [32]. For freshwater algae, average values of $\sigma_b = 0.4766$ and $\gamma_b = 4.402$ have been reported. For marine algae, the salt content of the algae is often significant; thus, the average value of the weight fraction carbon, $\sigma_b = 0.3354$, is lower. The value of the reductance degree, $\gamma_b = 4.062$, is also lower.

Lee et al. [30,31] have analyzed literature data and reported values for bioenergetic yields and maintenance coefficients for photoautotrophic, mixotrophic, and photoheterotrophic growth. Some of the bioenergetic yield values for photoautotrophic growth are presented in Table 1; these values are selected from a large number of values reported by Lee et al. [31]. Some of the values in Table 1 and others in Lee et al. [31] exceed the estimated maximum bioenergetic yield of 0.29. Because there are many opportunities for errors, it is desirable to conduct the experiments in such a way that balances can be used to check the consistency of the data that are collected.

In Tables 2 and 3, results from Lee et al. [30] are presented for data of Ogawa and Aiba [38]. Note in Table 2 that the available electron yield ranges

Table 1 Comparison of the True Biomass Energetic Yield and Maintenance Coefficient for Photosynthetic Organisms

Species	η_{max}	m_e (h^{-1})	References
Chlorella[a]	0.27	−0.015	[88]
Chlorella,[a] mixed culture with bacteria	0.40	0.0029	
Chlorella pyrenoidosa	0.64	0.030	[24]
	0.35	0.016	
Spirulina platensis	0.115	0.0255	[89]
Oscillatoria agardhii	0.23	0.0255	[90]
O. agardhii	0.19	0.0211	
O. agardhii	0.15	0.00667	
Scenedesmus protuberans[a]	0.18	0.0444	[90,91]
S. protuberans[a]	0.13	0.0692	
S. obliquus	0.04	0.225	[90,92]
Chlorella[b]	0.08	0.187	[93]

[a]The nitrogen source was urea.
[b]The light source was approximately full sunlight.
Source: Ref. 31.

Table 2 Carbon, Available Electron, and Bioenergetic Yields in Mixotrophic Growth of *Chlorella vulgaris* Grown on Glucose Media Under Aerobic Light Conditions[a] by Ogawa and Aiba [38]

Dilution rate D (h^{-1})	X (g/L)	S_0 (mM)	S (mM)	Carbon yield[b] y_c	Available electron yield η	Energetic yield η_E
0.085	0.276	2.78	0	0.657	0.723	0.359
0.116	0.274	2.78	0	0.652	0.718	0.356
0.140	0.269	2.78	0	0.641	0.705	0.436
0.170	0.282	2.78	0	0.672	0.739	0.490
0.190	0.253	2.78	0.106	0.626	0.689	0.468

[a]Light intensity; $I_oA = 0.104$ (kcal/h).
[b]Reductance degree and weight carbon fraction of biomass are $\gamma_b = 4.402$ and $\sigma_b = 0.4766$, respectively, for ammonia as the source of nitrogen.
Source: Analysis and results from Ref. 30.

Table 3 Estimation of Growth Yield Parameters for *Chlorella vulgaris* Grown on Glucose Media Under Aerobic Light Conditions in Table 2

		η_E^{max}		m_E (h^{-1})	
Data used	Form	Point estimate	95% confidence interval	Point estimate	95% confidence interval
η_{kcal}	I	0.682	0.356, 0.853	0.123	−0.044, 0.290
	II	0.735	0.383, 0.963	0.134	−0.047, 0.320

Source: Ref. 30.

from 0.689 to 0.723 while the energetic yield is considerably smaller. In Table 3, the estimated values of the growth yield and maintenance coefficient are both quite large. The large 95% confidence interval is commonly found. The theoretical yields, which are presented in Lee and Erickson [39], are of similar magnitude.

B. Photosynthetic Efficiency

Estimation of the total photosynthetic efficiency E_T is a complicated matter, primarily because the absorptive characteristics of light vary with wavelength. The second factor considered is the light utilization efficiency E_S, defined in Section II.A.4.

Quantum yield (efficiency; reciprocal is denoted as quantum requirement, Q_Λ) has been the subject of intense debate. The energy equivalent of light quantum is related to the wavelength and true quantum yield is species related. Figure 8A depicts experimental data on the relative quantum requirement for *Chlorella pyrenoidosa* as a function of visible light wavelength. The *thermodynamic efficiency* $E_q(\Lambda)'$ has been shown to be directly related to wavelength [10]. This linear relationship is depicted in Fig. 8B. The thermodynamic efficiency, however, must be corrected for the effect of wavelength, as photosynthesis does not occur with equal efficiency throughout the visible light; that is, by means of quantum requirement. Thus the *weighted thermodynamic efficiency* $E_q(\Lambda)$ results from (Fig. 8C) [10,40]

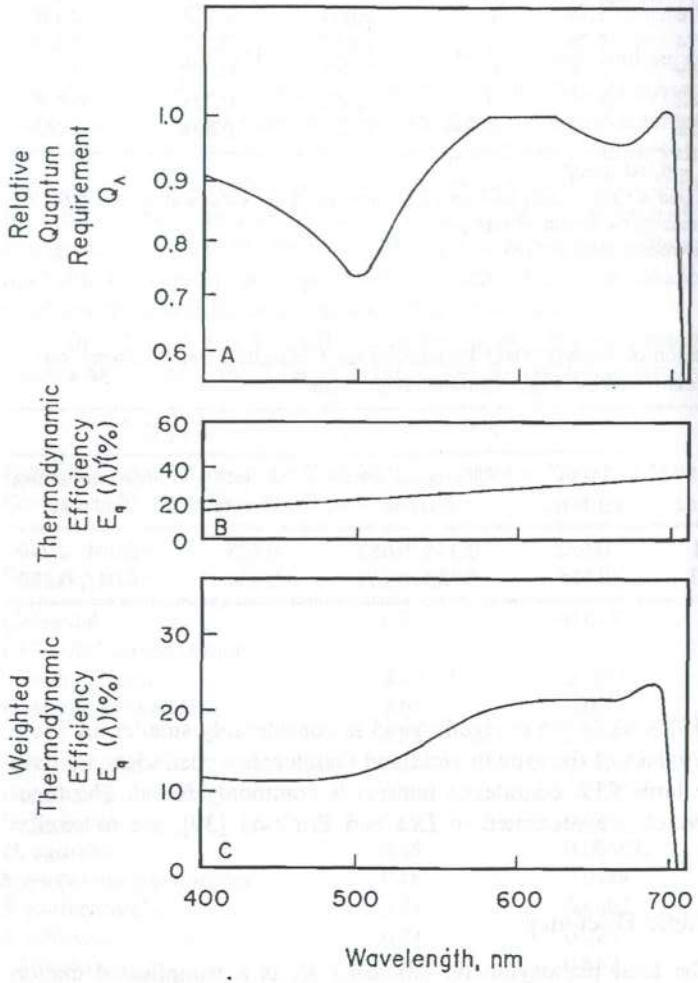


Figure 8 Effect of relative quantum requirement (A) on weighted thermodynamic efficiency (C) for an assumed thermodynamic efficiency (B) of 1 mole of CO_2 assimilated per 10 quanta of light. Part (A) is for *Chlorella pyrenoidosa* [10].

$$E_q(\Lambda) = Q_\Lambda E_q(\Lambda)' \tag{25}$$

In terms of oxygen evolution (OE)

$$\left(\frac{\text{OE}}{\text{energy}}\right) = \left(\frac{\text{OE}}{\text{quanta}}\right)\left(\frac{\text{quanta}}{\text{energy}}\right) \tag{26}$$

Looking at Fig. 8C, we see the highest weighted efficiencies in the region 600–700 nm. Similar results would be expected for carbon dioxide fixation or growth (in the latter case the curve is called the *growth action spectrum*). The two terms on the right-hand side of Eq. (25) are Q_Λ, originating from physical laws, and $E_q(\Lambda)'$. As the latter is extremely difficult to measure, the lumped term $E_q(\Lambda)$ in terms of growth response is feasible to obtain experimentally. In this case, separate experiments are to be conducted at a set of monochromatic light conditions varying from 400 to 700 nm [41,42].

For visible light, the average value for $E_q(\Lambda)' = 0.23$ would be thus lower by Q_Λ down to $Q_q(\Lambda) = 0.20$. This efficiency should be reduced by an additional multiplicative term due to light efficiency E_s, accounting for irradiances in excess of I_s. The total photosynthetic efficiency is thus

$$E_T = E_q(\Lambda)E_s \tag{27}$$

As values of E_s could be in the range 1 to 0.2, it can push E_T down from 0.2 to 0.04, a rather low number. The conversion of radiant energy into biomass as represented by an energy balance [10] is then as follows:

$$P_A = (E_T J)(I_0 - I_d) - P_D \tag{28}$$

in which J is an average caloric content of biomass (kcal g^{-1}), I_0 and I_d are, respectively, the visible radiant energy incident to the surface and at depth d in a culture [the difference $(I_0 - I_d)$ is the amount of energy absorbed] and P_D is the overall decay (maintenance) term in units of areal productivity. P_D is a sum of photorespiration for light and dark (in shaded areas and in outdoor culture) respiration reactions. Photorespiration may be a rather small (less than 10%) part of the maximal specific growth rate [29]. This means that, especially at lower growth rates, substantial energy is diverted from growth to maintenance [30,31,43]. The dark respiration could be substantial and is dependent on the relative residence time between light and dark zones. It has been evaluated as 10–30% of daylight productivity for outdoor culture of *Spirulina* and is a com-

plex function of temperature and light conditions under which the culture is grown, the carbohydrate content being the main determinant [44]. The respiration rate and cellular carbohydrate content are closely related. Only part of the carbohydrate stored during the light period is utilized for protein synthesis at night, the majority being respired. The growth temperature affects the maintenance term in an indirect manner as shown for *Scenedesmus* [43]. Extrapolation of data of Prokop and Řičica [24] to zero dilution rate ($1/X$ versus D) yields values up to 0.7 d^{-1} for *Chlorella pyrenoidosa* 7-11-05 grown under continuous illumination at 38.5°C. Gons and Mur [43] report values for *Scenedesmus* at 20°C which are less than 0.2 d^{-1}.

V. PHOTOBIOREACTOR DESIGN

A. Introduction and Classification

An excellent summary of photobioreactor design configurations has been presented [11]. The culture system classification used in this chapter is adopted from the same article. It is further extended to account for more recent developments as well as for other laboratory types of cultivation in conventional or adapted reactors, gaining popularity. A historical synopsis of evolution of algal technology has also been presented [11].

The reactor classification used in this chapter is as follows:

- Culture systems utilizing ponds or rectangular tanks with limited mixing
- Deep channeled culture systems with a closed circulating loop and better mixing
- Shallow circulating systems mostly with an ordered mixing pattern
- Reactor (chemical) type design with many modifications:
  - Columnar or rectangular, with airlift mixing
  - Tubular, with enclosed hydraulic system and pump
  - Cylindrical, with mechanical mixing
  - Special types

B. Pond Culture [11]

Numerous pond designs have been tested, some being of circular or oval shape (from top view); others are tanks of different provenience. Some systems are provided with a rotating paddle or pump mixing, including an old Japanese system with a rotating arm. The latter is based on a hollow arm driven by suspension discharge, using the Archimedes wheel principle. As they feature depths from 10 to 100 cm, the mixing intensity is rather limited. They have been largely abandoned except for rural technology applications. Productivities are rather low. Some design parameters are given in Ref. 11.

C. Deep Channeled Systems [11]

In these systems suspension is circulated through channels in most cases in the form of a closed, recirculating loop. The intensity of turbulence is typically selected just to maintain the culture in suspension. The serpentine channels are driven by a paddle wheel or by a recirculating pump (this version is often called a *raceway* system). These systems have been very popular in wastewater treatment (high-rate oxidation ponds) as well as for "clean" culture systems and are presently being utilized. The latter culture system is used in Israel for large-scale production of glycerol and β-carotene and several production facilities exist in the United States. They offer satisfactory productivities at moderate cost. A special closed alternative of a deep type cultivator was suggested; a hemispherical dome made of plastic covers the circular culture pond, and the algae suspension is agitated by a paddle [45].

D. Shallow Circulating Systems

These systems have been extensively discussed [11]. The common feature is that they are intensely mixed (linear velocities 6–60 cm s^{-1}) and most of them take advantage of ordered mixing with regular pattern of light and dark, rather than a flashing light effect. This is achieved by means of a pump or airlift system and by insertion of baffles or special foils. Typically, units are sloped and their depth is in the range of 2–15 cm. Only two examples are presented here at length. The first one is of Czechoslovak origin. The more advanced version utilizes a series of baffles positioned across the flow in a 5-cm-deep inclined channel. The algal suspension is forced to flow over and under the baffles, generating flow eddies. The baffles are spaced about 15 cm apart along long channels. The suspension is collected in an auxiliary tank downstream and recirculated back by means of a pump. Provisions were made for culture storage in an auxiliary tank overnight or in foul weather. Several units of this design [46,47] up to 900 m^2 in size have been built in Europe and tested and similar ones have been used in several different countries, including Kuwait [48].

Another nonrandom mixing design was described by Laws et al. [49] using foils similar in design to airplane wings, immersed in a dense, shallow algal culture. The foils were positioned every 1.2 m along the culture channel. Cells were exposed to a patterned (as opposite to random) light–dark cycle time of 1–2 s. The productivity in this system was increased by a factor of 2.2 over the productivity in the same system without the foils.

Compared with previous systems, the shallow circulation system has a higher capital cost, mainly due to special requirements for mixing devices. Overall, this system is the best available for the mass culture of photosynthetic organisms, offering effective mixing energy utilization.

E. Reactor Type Design

1. Columnar or Rectangular Reactors

Airlift driven columns or rectangular tanks have frequently been used in laboratory investigations, alternatively with a recirculation loop (e.g., [50]). Illumination is provided by an external light source or a bank of lights. A battery of airlift units has been patented [51].

2. Tubular Bioreactor

Such reactors are made of transparent plastic or glass and tube diameter is typically 5–15 cm. The tube arrangement can be in spiral or meandering (flat) mode. In the latter case, the closed loop is either lying on the ground or submerged in a pool of water, suitable for temperature control. Illumination for spiral tubular reactors can be optimized [49] based on similar studies with chemical photoreactors. For the flat design, the ratio A/V is close to 10 m^{-1}. Large-scale versions up to 100 m^2 in size have been designed for mass outdoor culture of algae [52,53].

3. Cylindrical Reactor Type

Many different types have been suggested. Mechanically mixed fermentors represent one type; they may be illuminated externally [24]. Alternatively, an external jacket (annulus) may be provided for heat removal with light via a hollow shaft [54] or a finger. Several commercial bioreactors with internal illumination are available. The second type is an annular design with internal (e.g., [55]; an additional annulus between the internal light source and the algal annulus can be provided for cooling) and/or external light sources. An internal source has been used with a Couette device providing a thin layer of algal culture between a stationary outer cylinder and a rotating inner cylinder (e.g., [56]), generating Taylor vortices. An enhancement in photosynthesis has been noted for this type of ordered mixing compared with turbulent mixing. Hollow light-transmitting baffles provided with fluorescent tubes have been suggested for insertion into a rectangular tank [57]. Usually, one-sided or two-sided illumination is provided for rectangular tanks. Such geometry is, in fact, the easiest to treat quantitatively. A special type of annular reactor is that of Algatron [10]. In this reactor, a thin algal film is held against the internal wall of a rotating cylinder by centrifugal force. The light source can be located both internally and externally of the culture annulus. In addition, a falling film photobioreactor has been suggested. Algae are allowed to fall in a thin film down through a corrugated panel and are eventually recycled [58].

4. Special Reactor Types

A hexagonal column filled with vertically arranged tubular fiber-optic light radiators provides a very high A/V ratio, close to 600 m^{-1} [59,60]. A special sunlight-harnessing device has been developed to focus visible light into the fiber optic, resulting in very high algal productivity. Another optical fiber light source-reactor has also been described [61,62]. In the former case, an optical fiber bundle, with introduced sunlight radiation, is set inside the rotating basket-annulus filled with an algal culture. In the latter case, the internal surface of the fiber is not smooth, allowing better lateral light diffusion. A similar concept has been described for hydrogen gas production by *Rhodospirillum rubrum*. In addition, a microporous membrane on the outer side of an immobilized algal layer provides for medium exchange and product harvesting [63].

All of these approaches are in line with attempts to maximize productivity in photobioreactors. Another patent uses several high-intensity light sources attached to transparent tubes inside a cylindrical vessel, provided with reflectors at the other end. Tubes are made of internally reflective material [64]. All these approaches are distinguished by a high A/V ratio and high productivities. Cell immobilization, microfiltration, or dialysis can be accommodated as an extra option.

5. Light Modulation

In addition to the use of fiber-optic light sources, allowing more concentrated light inputs (and high A/V), several light modulation techniques are available to adjust for light quality. They are all based on the use of convenient dyes which absorb a certain part of the light spectrum.

The detrimental effect of near-ultraviolet light on algal growth can be prevented by means of polymer sheets or glass incorporating an appropriate dye over the surface of algae under outdoor conditions [65,66]. Similar films have been used in greenhouses for higher plants [67,68]. Among the different organic dyes, the following were used: derivatives of benzophenone, phthalocyanine, and triazole. Substantial increases in algal (plant) productivities have been claimed.

Infrared light, on the other side of the spectrum, which primarily causes overheating of the culture, can be removed [66]. Traditionally, a copper sulfate solution or films have been used.

An interesting effect is achieved with laser dyes. Some of them are characterized by high quantum yields and high stability (exposure to light). Laser dyes are organic dyes with different absorption and emission spectra, the latter always being shifted to longer wavelength, typically by 30–50 nm. Thus a convenient laser dye (e.g., thiophene derivative) can be used to shift UV light to the visible part of spectrum [69]. The overall effect is an increase in the photosynthetically active component of the transmitted light. Other wavelength shifters have been tested as enhancers of algal growth [42]. As most of the laser dyes tested showed toxicity to algae, an indirect method of application has been suggested. A double-layer tubular reactor with a dye outside and algae inside is

a possibility. Such light modification by dye shifting may be also used to match the growth action spectra of particular photosynthetic organisms. An interesting matching of light quality to photosynthesis was suggested [70]. Because of their different action spectra, the maximum utilization of sunlight was achieved by combining green algae (upper layer) with photosynthetic bacteria (lower layer) in a double-layer tubular photobioreactor.

Dyes can be incorporated into transparent capsules and suspended in the algal culture. Methodically similar is an attempt cited by Tamiya [71] to use suspended phosphorescent particles in a dense algal suspension. This would serve as an internal source of radiation, as the particles which absorbed light near the culture surface would irradiate algae in shaded areas. A patent was obtained on the use of luminescent coating applied to an internal (and back-reflective) surface of a tubular reactor. An electroluminophore emits light when a voltage is applied to the conductors [72].

VI. OTHER ENGINEERING CONSIDERATIONS

A. Mass Transfer

The major concern related to mass transfer is the carbon dioxide demand and supply. As it is not clear which form of carbon dioxide (free or bicarbonate) is utilized by algae, this adds to uncertainty in the kinetic treatment of supply and utilization. The CO_2 demand has been related to the biomass concentration [73]. Brune and Novak [74] concluded that the Monod equation was appropriate for modeling the algal response to free carbon dioxide. It also appears that the rates of various carbonate reactions associated with dissociation are many times higher than the maximal carbon dioxide fixation rate observed; thus CO_2-bicarbonate equilibrium can be assumed. Curless and co-workers [76,76] have modeled CO_2 mass transfer and the kinetic transformation to other inorganic carbon forms.

The carbon dioxide supply (absorption) in a tubular reactor has been quantified for CO_2 surplus as well as for CO_2-limiting conditions, assuming both free CO_2 and bicarbonate are utilized [77]. Energy demands for mixing to satisfy homogeneity and carbon dioxide supply in tubular reactors have been evaluated [78]. For many production systems a special absorption tower (nonilluminated) is typically designed and used to achieve good mass transfer for supplying CO_2 to the culture.

The oxygen demand and supply for aeration at night have also been predicted [78].

B. Heat Transfer

A heat balance for a closed tubular reactor has been proposed, enabling a determination of the total photosynthetic efficiency [78]. For open outdoor units

an evaporation term should be added. Considerable evaporation under arid conditions helps to maintain optimal culture conditions [48,49].

C. Reactor Operation

Batch and continuous operations have been discussed in the kinetics section. Another important operational aspect is whether the culture is freely suspended or immobilized. The use of immobilized photosynthetic organisms for biotransformation or energy production (hydrogen) has been noted [79–81]. Immobilized processing offers some advantages, among them higher stability of the photosynthetic system.

D. Bioreactor Control

Several algorithms have been proposed and tested to control algal growth, some in closed tubular bioreactors, another in an open pond. All are based on an indirect method of biomass estimation by means of mass balancing. Nitrogen balance [82], oxygen balance or oxygen production rate [83], and CO_2 balance [84] have been used successfully. The latter method allowed control of the carbon-nitrogen ratio. The balancing via CO_2 uptake is achieved through pH control. One possible source of error in this case is production of OH^- by algae growing in nitrate-containing media [74]. A frequency response method has been tested for evaluation of dynamic characteristics of algae to different light regimes (step, pulse, or sine functions). The advantage of such dynamic testing is the extraction of time constants necessary for rational design of a control strategy [85].

E. Scale-Up

The scale-up of many algae systems (pools and deep systems) has been largely done empirically. Some rational concepts have been utilized to scale up shallow systems with ordered mixing. Because of the importance of depth, scale-up often results in an increase in photobioreactor area while keeping reactor depth constant. In the special reactor category, only tubular reactors have a good potential for scale-up. At the same time, temperature can easily be controlled and cultivation season considerably extended. Some overheating is observed in summer; this requires spraying with water. One problem in tubular reactors is removal of oxygen, requiring an appropriate design (degassing column). Carbon dioxide can be supplied in the same column.

F. Comparison of Reactor Designs

The comparison is difficult because no single unifying aspect is available: many variables and factors are involved. The ratio A/V characterizes production *potential*; the reciprocal of this ratio represents an equivalent culture thickness and

is helpful for characterizing global light conditions within the reactors. As demonstrated on a collection of data from the literature [24], as A/V increases, both P_V and P_A increase, provided light conditions are somewhat comparable. In Table 4 a range of values for A/V is presented for different reactor categories. Real numbers of productivities are not provided. P_V can reach a few g L^{-1} h^{-1} and P_A up to 75 g m^{-2} d^{-1} in a laboratory setting.

VII. APPLICATIONS

A summary of applications is presented in Table 5. Many significant opportunities to produce specialty chemicals remain to be exploited. Photoreactors developed for algae and other organisms can be used for plant tissue culture. However, they require special low-shear mixing because the cells are sensitive to shear stress.

VIII. CONCLUSIONS

This chapter presents the important concepts and relationships for photobioreaction engineering; however, no attempt has been made to provide a complete review of the available literature, which is very extensive. Because of the limited space available, the interested reader will have to go to the primary literature for additional information. We have tried to include most of the major engineering contributions on photobioreactors in the reference list.

Efficient distribution and conversion of light energy to useful products through photosynthesis is the major challenge of photobioreactor design and synthesis. The area of the photobioreactor, the depth of the light path into the culture, and the concentration of photosynthetic biomass are important design variables. Since the cost of product recovery is often proportional to the quantity of broth processed, a high concentration in the reactor reduces separation cost;

Table 4 Comparison of Photobioreactors

Reactor type	A/V (m^{-1})	P_V	P_A
Ponds	1–100		
Deep channeled systems	2–10		
Shallow circulating systems	10–30		
Column reactors	20–40	increases	increases
Rectangular reactors	30–50	↓	↓
Tubular reactors	10–100		
Film reactors	200–300		
Fiber-optic reactors	600–700		

Table 5 Applications of Photobioreactors

Application area	Product	References
Biomass		
Protein, etc.	Traditional and health foods	
Methane	Energy	
Space	Atmosphere regeneration in closed systems	
Products	Secondary metabolites	
	Fatty acids (γ-linolenic acid)	[94]
	Vitamins and coloring agents (β-carotene, chlorophylls)	
	Polyols (glycerol)	
	Polysaccharides	[72]
	Hydrocarbons	[95]
Energy	Hydrogen generation	[81]
Waste treatment	High-rate oxidation ponds (e.g., nitrogen/	[96]
	phosphorus removal, organochlorine)	[97]
	Heavy metal adsorption (lead)	[98]
Plant tissue culture	Secondary metabolites	[99]
		[100]
		[101]
	Flavoring agents, etc.	[102]

however, this requires a photobioreactor with large area and shallow culture depth.

The intensity of the light varies within the photobioreactor and light energy is not easily stored within the reactor. Thus, the variation of light intensity as a function of position must be considered in design. Complete characterization of light conditions within a reactor would include the light intensity as a function of wavelength and position. The kinetics and bioenergetics of light limited growth are coupled. Although there is some evidence that cells can store light energy for brief periods of time, a design based on a model of the dynamics of light absorption, transformation, and chemical concentrations of high-energy compounds such as ATP is not presently available. Where sunlight is used at full intensity, it is clear that systematic mixing is beneficial. Mixing sufficient to maintain a uniform biomass concentration within the reactor appears to be desirable in most applications.

PROBLEMS

1. *Effect of temperature on kinetics.* Several values of activation energy are reported by Lee and Erickson [17]. Review the prior work cited by Lee and

Erickson. What experimental design considerations are required to obtain data that can be used to estimate the activation energy when growth is light limited?

2. *Estimation of true growth yield and maintenance coefficient.* Find an appropriate set of literature data and estimate the values of the true growth yield and the maintenance parameter. For additional information on methods, please see Lee et al. [13,30,31] and Erickson and Fung [86].

3. *Consistency of literature data.* Using mass balances, energy balances, and the laws of thermodynamics, identify data sets in the literature in which experimental errors of more than 10% are present. Make a list of methods that can be used to check the consistency of experimental data if the appropriate measurements are made. Also list the required measurements.

4. *Kinetic and bioenergetic parameters.* Lee et al. [13,15] have considered the kinetics and bioenergetics of light-limited growth. Show how the values of kinetic parameters will vary with changes in the bioenergetic yield.

5. *Light saturation conditions.* In Section II, the light intensity, I_s, was introduced to identify the maximum intensity at which light can be effectively absorbed. Examine several sets of kinetic data to determine the value of I_s for each data set. Is I_s related to any of the parameters in any of the kinetic models? How is I_s related to K_I in the Monod model? See Eq. (7).

6. *Photobioreactor design.* Using kinetic, bioenergetic, and light absorption models, design an optimal continuous photobioreactor system for the case in which solar energy is distributed uniformly with mirrors and reflectors to the surfaces of cylindrical tubes. Find optimum values for tube diameter, tube length, dilution rate, biomass density, and incident light intensity. Assume there are costs which are proportional to tube area, recirculation flow rate, and product separation flow rate. Minimize cost per kg of biomass produced.

7. *Mixing in photoreactors.* Using the structured model of Frohlich et al. [26] and the bioenergetic analysis of Lee et al. [13,15] as a starting point, develop a dynamic structured cell model that can be used to simulate the effects of mixing on bioenergetic yield for high light intensities.

NOMENCLATURE

A	area
b	stoichiometric coefficient for oxygen; see Eq. (15)
D	dilution rate
d	depth into bioreactor; stoichiometric coefficient for carbon dioxide in Eq. (15)
d_c	equivalent depth in Eq. (11)
E_q	weighted thermodynamic efficiency
E'_q	thermodynamic efficiency

E_s	fraction of light utilized effectively
E_T	total photosynthetic efficiency
I	light intensity
I_c	compensation light intensity
I_d	light intensity at depth d
I_0	incident light intensity
I_s	saturation light intensity
J	average caloric content of biomass
K_I	saturation coefficient in Monod model for light intensity
K_i	inhibition coefficient in Aiba model for light intensity
K_s	saturation coefficient in Monod model for organic substrate
k_1	kinetic constant in Blackman model
m	exponent in Bannister model
m_e	maintenance coefficient
P_A	areal productivity
P_D	maintenance or decay term in units of areal productivity
P_V	volumetric productivity
Q_b	enthalpy per equivalent of available electrons for biomass
Q_H	heat evolved per g mole of carbon substrate consumed
Q_R	radiant energy supplied per g mole of carbon substrate consumed
Q_S	enthalpy per equivalent of available electrons for substrate
Q_Λ	relative quantum requirement
S_0	inlet substrate concentration
t	time
V	volume
X	concentration of microbial biomass
Y_s	biomass yield on organic substrate in mass units
y_c	biomass yield or organic substrate based on mass of carbon consumed and incorporated

Greek Symbols

γ_b	reductance degree of biomass per g mole of carbon
γ_s	reductance degree of substrate per g mole of carbon
ϵ	extinction coefficient in Eq. (1); fraction of available electrons transferred to oxygen in Eq. (19) = $4b/\gamma_s$
ϵ_E	fraction of energy evolved as heat
η	fraction of available electrons incorporated into microbial biomass = $y_c\gamma_b/\gamma_s$
η_E	fraction of energy incorporated into microbial biomass
μ	specific growth rate

μ_d specific endogenous cell death coefficient
μ_{max} maximum specific growth rate
μ' net specific growth rate
σ_b weight fraction carbon in microbial biomass

REFERENCES

1. A. Richmond (ed.), *Handbook of Microalgal Mass Culture*, CRC Press, Boca Raton, FL, 1986.
2. M. A. Borowitzka and L. J. Borowitzka (eds.), *Microalgal Biotechnology*, Cambridge University Press, New York, 1988.
3. T. Stadler, J. Mollion, M. C. Verdus, Y. Karamanos, H. Morvan, and D. Christiaen (eds.), *Algal Biotechnology*, Elsevier Applied Science, New York, 1988.
4. R. C. Cresswell, T. A. V. Rees, and N. Shah (eds.), *Algal and Cyanobacterial Biotechnology*, Wiley, New York, 1989.
5. W. P. Barclay and R. P. McIntosh (eds.), *Algal Biomass Technologies, An Interdisciplinary Perspective*, Beihefte zur Nova Hedwigia, Heft 83, J. Cramer, Berlin, 1986.
6. G. Shelef and C. J. Soeder (eds.), *Algae Biomass Production and Use*, Elsevier, New York, 1980.
7. E. R. De Bernardez, M. A. Claria, and A. E. Cassano, *Chemical Reaction and Reaction Engineering* (J. J. Carberry and A. Varma, eds.), Marcel Dekker, New York, 1987, pp. 839–921.
8. J. S. Dranoff, *Diamond Jubilee Historical/Review Volume* (L. Resen, ed.), *AIChE Symp. Ser. 79* (235):65 (1983).
9. J. Myers, Algal photosynthesis, *Kirk-Othmer Encyclopedia of Chemical Technology*, 2nd ed., Vol. 1, Interscience, New York, 1963, pp. 649–668.
10. J. C. Goldman, Outdoor algal mass cultures. II. Photosynthetic yield limitations, *Water Res. 13*: 119–136 (1979).
11. K. L. Terry, L. P. Raymond, System design for the autotropic production of microalgae, *Enzyme Microbiol. Technol. 7*: 474–487 (1985).
12. M. M. Ludlow, Measurement of solar radiation, temperature and humidity, *Techniques in Bioproductivity and Photosynthesis* (J. Coombs and D. O. Hall, eds.), Pergamon Press, Oxford, 1982, pp. 5–16.
13. H. Y. Lee, L. E. Erickson, and S. S. Yang, *Biotechnol. Bioeng. 29*: 832 (1987).
14. J. M. Pickett and J. Myers, Monochromatic light saturation curves for photosynthesis in *Chlorella*, *Plant Physiol. 41*: 90–98 (1966).
15. L. E. Erickson and H. Y. Lee, *Beihefte zur Nova Hedwigia 83*: 197 (1986).
16. G. Shelef, W. J. Oswald, and C. G. Golueke, Kinetics of algal systems in waste treatment. Light intensity and nitrogen concentration as growth-limiting factors. NITS Report PB-260 088 (SERL Rept. 68-4, Sanitary Eng. Res. Lab, University of California, Berkeley). National Technical Information Service, Washington, DC, 1968.

17. H. Y. Lee and L. E. Erickson, Kinetic and bioenergetic studies of *Spirulina platensis* in chemostat and turbidostat cultures, Eighth Symposium on Biotechnology for Fuels and Chemicals, *Biotech. Bioeng. Symp. 17*: 463–479 (1986).
18. S. J. Pirt, Microbial photosynthesis in the harnessing of solar energy, *J. Chem. Technol. Biotechnol. 32*: 198–202 (1982).
19. S. Aaronson, *Experimental Microbiology, Ecology*, Academic Press, New York, 1970.
20. S. Aiba, *Adv. Biochem. Eng. 23*: 85–156 (1982).
21. J. Simmer, Absorption of radiant energy in algal suspensions, *Arch. Hydrobiol. Suppl. 51* (Algological Studies vol. 20): 337–348 (1978).
22. A. G. Fredrickson, A. H. Brown, R. L. Miller, and H. M. Tsuchiya, Optimum conditions for photosynthesis in optically dense cultures of algae, *Am. Rocket Soc. J. 31*: 1429–1435 (1961).
23. G. Persoone, J. Morales, H. Verlet, N. DePauw, *Algal Biomass Production and Use* (G. Shelef and C. J. Soeder, eds.), Elsevier, New York, 1980, pp. 505–522.
24. A. Prokop and J. Řičica: *Chlorella pyrenoidosa* 7-11-05 in batch and in homogeneous continuous culture under autotrophic conditions. I. Growth characteristics of the culture, *Folia Microbiol. 13*: 353–361 (1968).
25. K. Lívanský, Semicontinuous cultivation of autotrophic algae, *Folia Microbiol. 24*: 346–351 (1979).
26. B. T. Frohlich, I. A. Webster, M. M. Ataai, and M. L. Shuler, Photobioreactors: Models for interaction of light intensity, reactor design, and algal physiology, Fifth Symposium on Biotechnology for Fuels and Chemicals, *Biotechnol. Bioeng. Symp. 13*: 331–350 (1983).
27. C. K. Powell, J. B. Chaddock, and J. R. Dixon, *Biotechnol. Bioeng. 7*: 295–308 (1965).
28. K. Lívanský, Effect of the nonilluminated part of suspension on biomass production in an algal reactor, *Folia Microbiol. 24*: 339–345 (1979).
29. Y.-K. Lee and S. J. Pirt, Energetics of photosynthetic algal growth: Influence of intermittent illumination in short (40 s) cycles, *J. Gen. Microbiol. 124*: 43–52 (1981).
30. H. Y. Lee, L. E. Erickson, and S. S. Yang, *Biotechnol. Bioeng. 27*: 1640 (1985).
31. H. Y. Lee, L. E. Erickson, and S. S. Yang, *Biotechnol. Bioeng. 26*: 926 (1984).
32. H. Y. Lee and L. E. Erickson, *Biotechnol. Bioeng. 26*: 758 (1984).
33. L. E. Erickson, *Biotechnol. Bioeng. 22*: 451 (1980).
34. L. E. Erickson, I. E. Minkevich, and V. K. Eroshin, *Biotechnol. Bioeng. 20*: 1595 (1978).
35. W. M. Thornton, *Philos. Mag. 33*: 196 (1917).
36. M. S. Kharasch, *J. Res. Natl. Bur. Stand. 2*: 359 (1929).
37. I. E. Minkevich and V. K. Eroshin, *Folia Microbiol. 18*: 376 (1973).
38. T. Ogawa and S. Aiba, *Biotechnol. Bioeng. 23*: 1121 (1981).
39. H. Y. Lee and L. E. Erickson, *Biotechnol. Bioeng. 29*: 476 (1987).
40. J. H. Ryther, Potential productivity of the sea, *Science 130*: 602–608 (1959).
41. Š. Kubín, E. Borns, J. Doucha, and U. Seiss, Light absorption and production rate of *Chlorella vulgaris* in light of different spectral composition, *Biochem. Biophys. Pflanz. 178*: 193–205 (1983).

42. A. Prokop, M. F. Quinn, M. Fekri, M. Murad, and S. A. Ahmed, Spectral shifting by dyes to enhance algae growth, *Biotechnol. Bioeng. 26*: 1313–1322 (1984).
43. H. J. Gons and L. R. Mur, Energy requirements for growth and maintenance of *Scenedesmus protuberans* Fritsch in light-limited continuous cultures, *Arch. Microbiol. 125*: 9–17 (1980).
44. G. Torzillo, A. Sacchi, R. Materassi, and A. Richmond, Effect of temperature on yield and night biomass loss in *Spirulina platensis* grown outdoors in tubular photobioreactors, *J. Appl. Phycol. 3*: 103–109 (1991).
45. R. D. Walmsley and S. N. Shillinglaw, Mass algal culture in outdoor plastic-covered minipond systems, *Ann. Appl. Biol. 104*: 185–197 (1984).
46. I. Šetlík and Š. Kubín, Industrialization of agriculture. A challenge to photosynthetic research, *Acta Univ. Carolinae Biol. Suppl. (1/2)*: 77–88 (1966).
47. I. Šetlík, V. Šust, and I. Málek, Dual-purpose open circulation units for large scale culture of algae in temperature zones. I. Basic design considerations and scheme of pilot plant, *Algol. Studies (Třeboň) 1*: 111–164 (1970).
48. A. Prokop and M. Fekri, Potential of mass algae production in Kuwait, *Biotechnol. Bioeng. 26*: 1282–1287 (1984).
49. E. A. Laws, K. L. Terry, J. Wickman, and M. S. Chalup, *Biotechnol. Bioeng. 25*: 2319–2335 (1983).
50. H. J. Silva, T. Cortiñas, and R. J. Ertola, Effect of hydrodynamic stress on *Dunaliella* growth, *J. Chem. Technol. Biotechnol. 40*: 41–49 (1987).
51. Algatech, Inc., Apparatus for Microalga Cultivation in Fluid-based Medium, Swedish Patent 003,801, 1991.
52. G. Torzillo, B. Pushparaj, F. Bocci, W. Balloni, R. Materassi, and G. Florenzano, Production of *Spirullina* in closed photobioreactors, *Biomass 11*: 61–74 (1986).
53. D. Chaumont, C. Thepenier, C. Gudin, and C. Junjas, Scalling up a tubular photobioreactor for continuous culture of *Porphyridium cruentum* from laboratory to pilot plant (1981–1987), *Algal Biotechnology* (T. Sadler, J. Mollion, M.-C. Verdus, Y. Karamanos, H. Morvan, and D. Christinen, eds.), Elsevier Applied Science, London, 1988, pp. 199–208.
54. PCC Technology, Inc., Stirring-type Cultivation Device, Japan Patent 01-190,619, 1991.
55. Institute of Soil Science and Photosynthesis, Fermentor for Phototrophic Microorganisms, USSR Patent 240,565, 1989.
56. R. L. Miller, A. G. Fredrickson, A. H. Brown, and H. M. Tsuchiya, *Ind. Eng. Chem. Process Des. Dev. 3*: 134–143 (1964).
57. Martek, Inc., New Fermentor for Photosynthetic Microorganisms, e.g., alga, WO Patent 9,107,080, 1989.
58. R. Roubicek, J. T. Patton, K. H. McCorkle, and A. L. Rakow, *Algal Biomass Technologies* (W. R. Barclay and R. P. McIntosh, eds.) Beihefte zur Nova Hedwigia, Heft 83, J. Cramer, Berlin, 1986, p. 218. (Also, R. V. Roubicek and V. Feres, Equipment for Enhanced Mass Transfer in Chemical and Biochemical Processes, U.S. Patent 4,657,677, 1987.)
59. K. Mori, Photoautotrophic bioreactor using visible solar rays condensed by fresnel lenses and transmitted through optical fibers, *Biotechnol. Bioeng. Symp. 15*: 331–345 (1985).

60. K. Mori, Apparatus for Photosynthesis, U.S. Patent 4,900,678, 1990.
61. H. Kikuchi, A Culturing Apparatus for Algae, Japan Patent 03-112481, 1991.
62. T. Matsunaga, H. Takiyama, H. Sudo, N. Oyama, S. Ariura, H. Takano, H. Hirano, J. G. Burgess, K. Sode, and N. Nakamura, Glutamate production from CO_2 by marine cyanobacterium *Synechococcus* sp. using a novel biosolar reactor employing light-diffusing optical fibers, *Appl. Biochem. Biotechnol. 28/29*: 157–167 (1991).
63. L. Mignot, G. A. Junter, and M. Labbé, A new type of immobilized photobioreactor with internal illumination by optical fibres, *Biotechnol. Technol. 3*: 299–304 (1989).
64. R. J. Radmer, Photobioreactor, U.S. Patent 4,952,511, 1990.
65. I. Harasawa, Y. Hariki, K. Maeda, and K. Nakamura, Method for Cultivating Algae and a Covering Material Used Therefor, U.S. Patent 4,235,043, 1980.
66. L. P. Raymond, Mass Algal Culture System, U.S. Patent 4,320,594, 1982.
67. F. Glatti, Materials with a Specific Photo-sensitivity Based on Synthetic Thermoplastic Polymers and Their Use in the Agricultural Field, U.S. Patent 3,911,620, 1975.
68. L. E. Amborski, Organic Polymeric Structures Having an Ultraviolet Light Absorbent Compound Incorporated Therein, U.S. Patent 3,043,709, 1962.
69. M. F. Treadaway, Polymer-optical Brightener Combinations in Transparent Film Form Useful as Glazing Materials Capable of Modifying Plant Growth Rate, U.S. Patent 4,189,866, 1980.
70. C. Gudin, D. Chaumont, and E. Berra, New concepts in solar biotechnology, *Energy from Biomass* (W. Palz, P. Chartier, and D. O. Hall, eds.), Applied Science Publishers, Barking, England, 1981, pp. 659–664.
71. H. Tamiya, Mass culture of algae, *Annu. Rev. Plant Physiol. 8*: 309–334 (1957).
72. Stavropol Agricultural Institute, Photobioreactor for Microalga Culture, USSR Patent 397,957, 1990.
73. S. J. Pirt, Y. K. Lee, M. R. Walach, M. W. Pirt, H. H. M. Balyuzi, and M. J. Bazin, A tubular bioreactor for photosynthetic production of biomass from carbon dioxide: Design and performance, *J. Chem. Technol. Biotechnol. 33B*: 35–58 (1983).
74. D. E. Brune and J. T. Novak, The use of carbonate equilibrium chemistry in quantifying algal carbon uptake kinetics, *Eur. J. Appl. Microbiol. Biotechnol. 13*: 71–76 (1981).
75. L. E. Erickson, C. E. Curless, and H. Y. Lee, *Ann. N.Y. Acad. Sci. 506*: 308 (1987).
76. C. E. Curless, Mathematical Modeling and Simulation of Photosynthetic Growth in Continuous Culture under Bicarbonate and Light Limited Conditions, M.S. thesis, Kansas State University, Manhattan, 1986.
77. Y.-K. Lee and S. J. Pirt, CO_2 absorption rate in an algal culture: Effect of pH, *J. Chem. Technol. Biotechnol. 34B*: 28–32 (1984).
78. J. C. Weisman, R. P. Goebel, and J. R. Benemann, Photobioreactor design: Mixing, carbon utilization, and oxygen accumulation, *Biotechnol. Bioeng. 31*: 336–344 (1988).
79. M. D. Trevan and A. L. Mak, Immobilized algae and their potential for use as biocatalysts, *Trends Biotechnol. 6*: 68–73 (1988).

80. G. C. Papageorgiou, K. Kalosaka, G. Sotiropoulou, J. N. Barbotin, B. Thomasset, and T. Thomas, Entrapment of active ion-permeable cyanobacteria (*Anacystis nidulans*) in calcium alginate, *Appl. Microbiol. Biotechnol. 29*: 565–571 (1988).
81. I. H. Park, K. K. Rao, and D. O. Hall, Photoproduction of hydrogen, hydrogen peroxide and ammonia using immobilized cyanobacteria, *Hydrogen Energy 16*: 313–318 (1991).
82. M. R. Walach, H. H. M. Balyuzi, M. J. Bazin, Y. K. Lee, and S. J. Pirt, Computer control of an algal bioreactor with simulated diurnal illumination, *J. Chem. Technol. Biotechnol. 33B*: 59–75 (1983).
83. S. Ben-Yakov, H. Guterman, A. Vonshak, and A. Richmond, An automatic method for on-line estimation of the photosynthetic rate in open algal ponds, *Biotechnol. Bioeng. 27*: 1136–1145 (1985).
84. M. R. Walach, M. J. Bazia, S. J. Pirt, and H. H. M. Balyuzi, Computer control of carbon-nitrogen ratio in *Spirulina platensis*, *Biotechnol. Bioeng. 29*: 520–528 (1987).
85. H. L. Y. Lam and H. R. Bungay, Frequency reponse analysis of oxygen evolution by algae, *J. Biotechnol. 4*: 125–142 (1986).
86. L. E. Erickson and D. Y. C. Fung (eds.), *Handbook of Anaerobic Fermentations*, Marcel Dekker, New York, 1988.
87. M. Iehana, *J. Ferment. Technol. 61*: 457 (1983).
88. S. J. Pirt, Y. K. Lee, A. Richmond, and M. W. Pirt, *J. Chem. Technol. Biotechnol. 30*: 25 (1980).
89. S. Aiba and T. Ogawa, *J. Gen. Microbiol. 102*: 179 (1977).
90. L. Van Liere and L. R. Mur, *J. Gen. Microbiol. 115*: 153 (1979).
91. H. J. Gons and L. R. Mur, *Vet. Int. Ver. Theor. Angew. Limnol. 21*: 125 (1978).
92. W. J. Oswald, *Proceedings of the International Biological Program/Primary Productivity*, Pergamon, London, 1970, p. 473.
93. J. Myers, *Primary Productivity in the Sea* (P. G. Falkowski, ed.) Plenum, New York, 1980, pp. 1 and 447.
94. M. Hirano, H. Mori, Y. Miura, N. Matsunaga, N. Nakamura, and T. Matsunaga, γ-Linolenic acid production by microalgae, *Appl. Biochem. Biotechnol. 24/25*: 183–191 (1990).
95. C. Bailliez, C. Largeau, and E. Casadevall, Growth and hydrocarbon production of *Botryococcus branuii* immobilized in calcium alginate gel, *Appl. Microbiol. Biotechnol. 23*: 99–105 (1985).
96. J. B. Carberry and F. F. Henshaw, Biokinetic parameters of a photosynthetic waste stabilization process, *Water Sci. Technol. 21*: 647–658 (1989).
97. X. F. Wu and N. Kosaric, Removal of organochlorine compounds in an upflow flocculated algae photobioreactor, *Water Sci. Technol. 24*: 221–232 (1991).
98. Z. Aksu and T. Kutsal, A Bioseparation process for removing lead (II) ions from waste water by using *C. vulgaris*, *J. Chem. Technol. Biotechnol. 52*: 109–118 (1991).
99. W. Husemann, K. Fischer, I. Mittelbach, B. Hubner, G. Richter, and W. Barz, Photoautotrophic plant cell cultures for studies on primary and secondary metabolism, *Primary and Secondary Metabolism of Plant Cell Cultures II* (W. G. W. Kurz, ed.), Springer-Verlag, New York, 1989, pp. 35–46.

100. W. J. Treat, J. Castillon, and E. J. Soltes, Photobioreactor culture of photosynthetic soybean cells. Growth and biomass characteristics, *Appl. Biochem. Biotechnol. 24/25*: 497–510 (1990).
101. W. J. Treat, C. R. Engler, and E. J. Soltes, Culture of photomixotrophic soybean and pine in a modified fermentor using a novel impeller, *Biotechnol. Bioeng. 34*: 1191–1202 (1989).
102. J. E. Lancaster, E. M. Dommisse, and M. L. Shaw, Production of flavor precursors (*S*-alke(en)yl-L-cysteine sulphoxides) in photomixotrophic callus of garlic, *Phytochemistry 27*: 2123–2124 (1988).

13
Bioreactor Operation Modes

Tsuneo Yamane

Nagoya University
Nagoya, Japan

I. INTRODUCTION

As for purely chemical processes, there are several operation modes for industrial biotechnological processes in which (micro)organisms are involved. In this chapter, industrial operation modes suitable for organisms (microorganisms, animal cells, or plant cells) suspended uniformly in culture fluid are discussed. Growing organisms immobilized within particles suspended uniformly in the culture fluid are also included in the discussion.

A. Classification of Operation Modes

Table 1 shows a classification of major operation modes often applied to the growing organisms suspended in the culture liquid [1]. Basic modes are batch and continuous operations, but semibatch, repeated batch, and repeated semibatch operations have developed as modifications of batch ones. The differences among the operation modes classified in Table 1 can be well understood when changes in the volume of the culture broth with elapsed time are illustrated as in Fig. 1.

In the batch biotechnological process (mode 1), all nutrients required during one run of cultivation, except for molecular oxygen in an aerobic process

Table 1 Classification of Bioreactor Operation Modes

1. Batch
2. Fed-batch (or semibatch)
3. Repeated fed-batch (or cyclic fed-batch)
4. Repeated batch (or cyclic batch or semicontinuous)
5. Continuous

Source: Ref. 1.

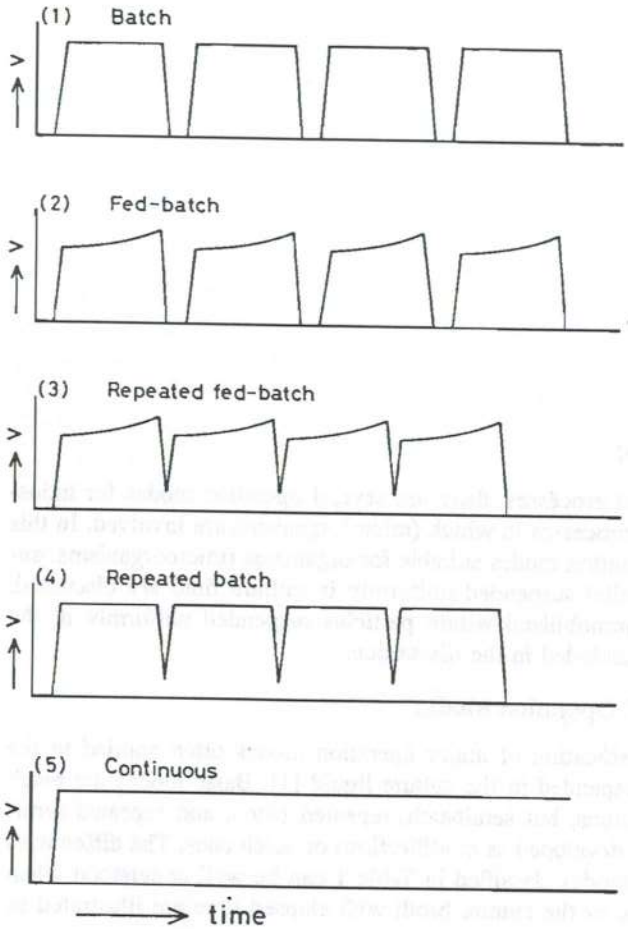


Figure 1 Changes in volume of culture broth with elapsed time for various operation modes of (micro)organisms.

and ammonia or other chemicals for pH adjustment, are added to the medium before cultivation is started, and the broth containing products is withdrawn only at the end of each batch run. In a continuous biotechnological process (mode 5), all nutrients are added continuously to the bioreactor and fractions of the culture broth are removed at the same flow rate as that of supplied nutrients to maintain a constant culture volume. Between these "ideal" modes there are several modifications concerning the mode of addition of raw material(s) and withdrawal of products. The fed-batch mode (mode 2), in the broadest sense, is defined as a biotechnological operation mode in which one or more or in some cases all the required nutrients are being supplied to the bioreactor during cultivation but the culture broth containing the metabolite(s) is taken out once only at the end of each run. Because of the feeding of the nutrient(s) solution, the volume of the broth increases more or less. In repeated batch operation (mode 4), not all the culture broth is taken out when the cell mass or product concentration reaches a desired level, and some of the culture broth remains and is used as an inoculum for the next batch run supplemented with freshly prepared medium. This mode is particularly useful for the immobilized growing cells. It is also possible to retain a portion of a fed-batch culture at the end of one run and to use the residual part of the broth as an inoculum for the next fed-batch culture, the whole mode of operation being called repeated fed-batch culture (mode 3). These modified modes of operation have been devised to overcome problems the biotechnological processes have specifically envisaged. A continuous operation has been generally regarded as the most efficient one, but maintenance of high activity of (micro)organisms in the continuous culture is difficult owing to their genetic variations and long-term continuation of pure culture is also hard because of microbial or phage contaminations; thus in reality we do not have many continuous biotechnological processes on an industrial scale. Also, we have often admitted that a steady state is not always the best environmental condition for the (micro)organisms to exhibit their full biosynthetic potential. On the other hand, batch operation is the most primitive mode and the least efficient; the most serious drawback is that the environment (nutrient concentrations) surrounding the organisms is not controlled at all in a batch culture even though their functions and activities vary greatly depending on changes in the environment, and it is laborious and risky that each main batch culture requires several "seed" cultures whose volumes increase consecutively. A fed-batch operation has been developed to deal with the former disadvantage, a repeated batch operation alleviates the latter burden, and a repeated fed-batch operation improves both. The number of repetitions of the operation modes 3 and 4 is not, however, limitless because of microbial contamination and/or reduced biosynthetic activity of cells, and modes 3 and 4 require another tank of almost the same size as the main tank for the medium preparation.

Among these five modes, progress curves for cell, substrate, and product concentrations in batch, fed-batch, and continuous cultures are depicted conceptually in Fig. 2, which is also helpful for recognizing the differences in these three operation modes. At present, most industrial biotechnological processes are being operated in batch or fed-batch mode.

B. Basic Equation of Operation for Stirred Tank Bioreactor

Referring to the single stirred tank bioreactor sketched in Fig. 3, the balance equations of viable cells, dead cells, the ith nutrient (substrate), and the jth metabolite (product) are written as follows.

Growth of viable cells:

$$\frac{d(vx_{out}^{(v)})}{dt} = vr_{xv} - vr_{xd} + f_{in}x_{in}^{(v)} - f_{out}x_{out}^{(v)} \tag{1}$$

Accumulation of dead cells:

$$\frac{d(vx_{out}^{(d)})}{dt} = vr_{xd} + f_{in}x_{in}^{(d)} - f_{out}x_{out}^{(d)} \tag{2}$$

Consumption of ith substrate:

$$\frac{d(vs_{out}^{(i)})}{dt} = -vr_s^{(i)} + f_{in}s_{in}^{(i)} - f_{out}s_{out}^{(i)} \qquad i = 1, 2, \ldots, m \tag{3}$$

Formation of jth metabolite:

$$\frac{d(vp_{out}^{(j)})}{dt} = vr_p^{(j)} + f_{in}p_{in}^{(j)} - f_{out}p_{out}^{(j)} \qquad j = 1, 2, \ldots, n \tag{4}$$

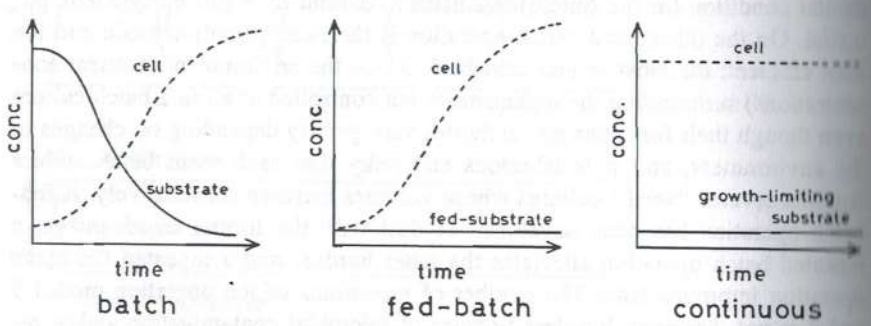


Figure 2 Conceptional progress curves of cell mass and substrate and product concentrations in batch, fed-batch, and continuous cultures.

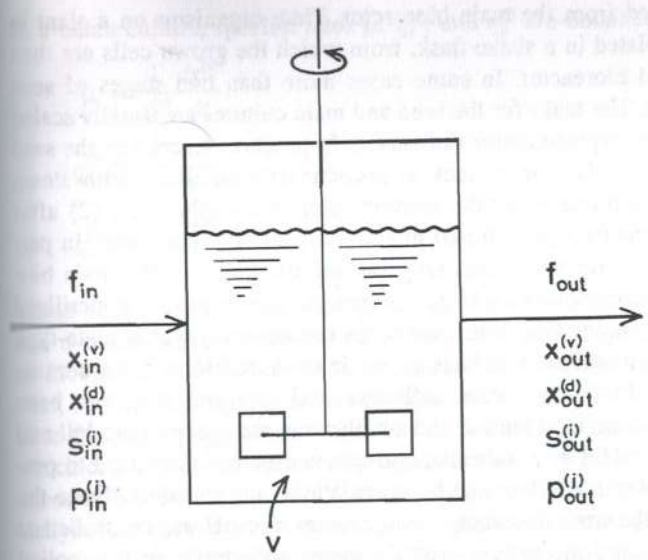


Figure 3 General biotechnological process for a single stirred tank bioreactor.

Culture volume in the bioreactor:

$$\frac{dv}{dt} = f_{in} - f_{out} \tag{5}$$

For the meanings of symbols in Eqs. (1)–(5), see the Nomenclature at the end of the chapter. Equations (1)–(5), together with initial conditions for the variables and the constraints on the variables, constitute a general mathematical model for a single stirred tank bioreactor when suitable kinetic expressions for r_x, r_d, $r_s^{(i)}$, and $r_p^{(j)}$ are identified.

II. BATCH OPERATION

A. Procedure for Batch Culture

Procedures for any batch cultures are basically the same, although some small differences are observed depending on the kind of (micro)organism and the kind of metabolite desired. Major equipment consists of the main bioreactor and seed bioreactor, both being submerged bioreactors (stirred tanks). They have ancillary instruments for medium preparation, steam sterilization, cooling, air compression, air filtering, stirring, antifoam, and so on. The seed bioreactor is used to cultivate cell mass initially necessary for the main bioreactor, and the desired

metabolite is obtained from the main bioreactor. First, organisms on a slant in a test tube are inoculated in a shake flask, from which the grown cells are then transferred to a seed bioreactor. In some cases more than two stages of seed culture are necessary. The tanks for the seed and main cultures are usually scaled up by one order of the representative dimension. In practice, a series of the seed cultures work first; (1) the vacant tank is preliminarily sterilized with steam followed by steam sterilization of the medium prepared in the tank, (2) after cooling, the microorganisms precultured in a shake flask are inoculated. In parallel with cultivation in the seed tank, preparations are made for the main bioreactor. When the bioreactor has a large volume, the medium is not sterilized inside but is normally made free of contaminants continuously with a plate-type or tubular sterilizer. The microorganisms grown in the seed bioreactor are transferred to the main bioreactor after sufficient cell concentration has been achieved. Sufficient steam sterilization and air filtration are necessary to kill and remove included microbial contaminants, and various methods are used to prevent invasion of contaminants during the inoculations and transfers. Once the cultivation starts in the main bioreactor, temperature and pH are controlled at their optimum values and the culture broth is stirred and sterile air is supplied (aeration) during the whole cultivation. The cultivation is finished when the desired level of cell growth and metabolite accumulation is attained, and the culture broth is subjected to next downstream treatment for product recovery. One run of batch operation ranges from several tens of hours to more than several weeks. The sequential operation of a batch culture is governed by the main bioreactor procedure, and the total process time for one run of the batch culture is the sum of the times required for input of the sterilized medium in the main bioreactor, transfer of inoculum, lag of cell growth, major microbial reaction, harvesting (broth out), inside cleaning, and vacant sterilization.

B. Basic Equation of Batch Operation

In the batch operation there is neither input nor output, so the volume is unvaried and we can put $f_{in} = f_{out} = 0$ and v = constant in Eqs. (1)–(4), which become

$$\frac{dx^{(v)}}{dt} = \nu_{xv} - r_{xd} = (\mu - k_{xd})x^{(v)} \tag{6}$$

$$\frac{dx^{(d)}}{dt} = r_{xd} = k_{xd}\, x^{(v)} \tag{7}$$

$$\frac{ds^{(i)}}{dt} = -r_s^{(i)} = -q_s^{(i)}x^{(v)}, \qquad i = 1, 2, \ldots, m \tag{8}$$

$$\frac{dp^{(j)}}{dt} = r_p^{(j)} = q_p^{(j)}\, x^{(v)}, \qquad j = 1, 2, \ldots, n \tag{9}$$

In a batch culture, specific rates μ, $q_s^{(i)}$, and $q_p^{(j)}$ are obtained from

$$\mu \equiv \frac{1}{x^{(v)}}\frac{dx^{(v)}}{dt} \tag{10a}$$

$$q_s^{(i)} \equiv \frac{1}{x^{(v)}}\frac{ds^{(i)}}{dt} \tag{10b}$$

$$q_p^{(j)} \equiv \frac{1}{x^{(v)}}\frac{dp^{(j)}}{dt} \tag{10c}$$

These equations, together with the kinetic rate equations of r_{xv}, r_{xd}, $r_s^{(i)}$, and $r_p^{(j)}$ and a set of initial conditions (concentrations of x_v, x_d, $s^{(i)}$, and $p^{(j)}$ at the time of inoculation), constitute an unstructured model of the batch operation. However, a batch culture is an unsteady-state process and changes in the environment alter the physiology of the cells. No useful, simple kinetic model is available which can express complicated progress curves of a batch culture including lag, exponential, stationary, and declining phases. Rather it is more important to analyze the batch culture from the viewpoint of relative pattern of the specific growth rate μ and the specific metabolite formation rate q_p. In this connection, metabolite formation in a batch culture is classified as growth-associated, non-growth-associated, and complex growth-associated.

Medium composition of a batch culture is usually optimized by statistical optimum design of experiments, and pH or temperature can be optimized by applying modern optimization theory. The objective function of a batch operation is either the final metabolite concentration, p_f, or productivity of the metabilite or net profit ($= k_1p_f - k_2t_f - \Sigma_{i=1}^{m} k_{3i}s_0^{(i)}$, where k_1, k_2, and k_{3i} are constants). In a batch operation volumetric productivity (or simply productivity, typically having units of g metabolite L^{-1} h^{-1}) must be calculated over an entire cycle time, t_c, which includes the actual microbial reaction period, t_f, and the downtime necessary for cleaning, refilling, sterilizing, and emptying, t_d, and hence is often called overall productivity. The maximum productivity may be found graphically as shown in Fig. 4. This level of overall productivity often occurs at a cell or metabolite concentration less than the maximum. Optimum productivity must therefore be decided by making allowance for economics, in particular nutrient costs and running costs (mostly costs of agitation and aeration).

III. FED-BATCH OPERATION

Fed-batch operation was defined in the Introduction. It is shown schematically in Fig. 5. Industrial microbial processes are often operated by fed-batch mode. The basic advantage of fed-batch processes is that the concentrations of the fed nutrients in the culture liquid in the bioreactor can be controlled voluntarily by changing the feed rate. Therefore the fed-batch mode of operation is usually

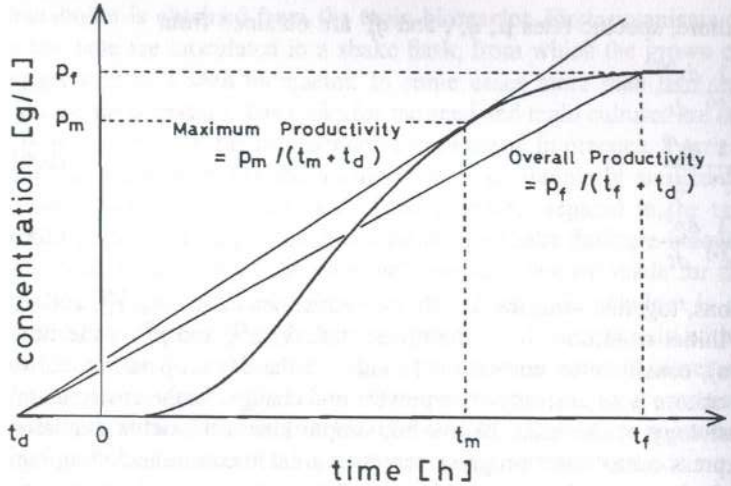


Figure 4 Productivity in batch operation.

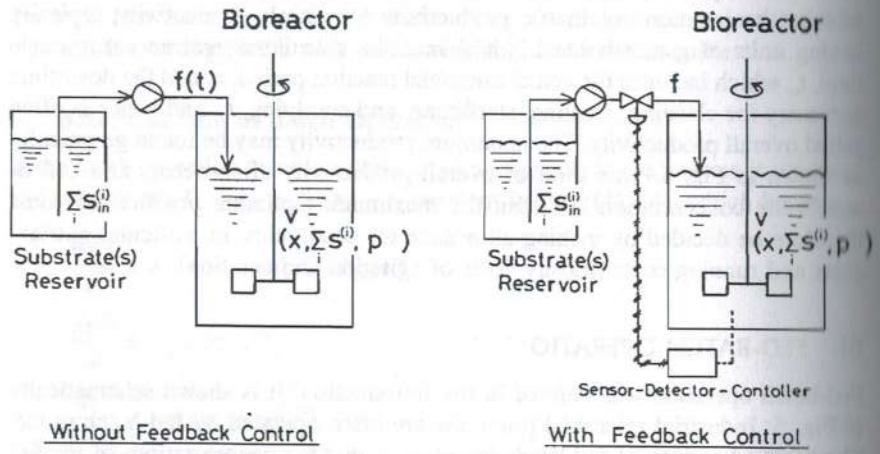


Figure 5 Schematic diagram of fed-batch culture. When $i = 1$, a single substrate is fed; when $i > 1$, multiple substrates are fed (multisubstrate fed-batch culture).

superior to conventional batch operation when changing concentrations of a nutrient (or nutrients) affect the yield or productivity of the desired metabolite. Our previous review article [2] emphasized this point and covered many important aspects of the fed-batch technique.

A. Utility of Fed-Batch Culture

There are many types of processes for which fed-batch operation is effective, as follows:

(1) Nutrients such as methanol, ethanol, acetic acid, and aromatic compounds inhibit the growth of microorganisms even at relatively low concentration. By feeding such substrates slowly so that their concentration becomes never high, the inhibition can be markedly reduced.

(2) High cell density cultivation. If very high cell concentrations are required, the high substrate concentration that would be required in normal batch cultivation may well prove inhibitory, even if there was no such effect at more normal concentrations.

(3) Glucose effect (Crabtree effect). In the production of baker's yeast from malt wort or molasses, ethanol is produced even in the presence of sufficient dissolved oxygen (DO) if an excess of sugar is present in the culture liquid. Ethanol is the main cause of low cell yield. Aerobic ethanol formation in the presence of a high glucose concentration is known as the Crabtree effect (or glucose effect). To reduce this effect, a fed-batch process is generally employed for baker's yeast production. In aerobic cultivation of bacteria such as *Escherichia coli*, a high glucose concentration results in the formation of organic acids such as acetic, lactic, and formic acids, which cause the physiological activity of the cells to deteriorate. This phenomenon is called bacterial Crabtree effect. To reduce this effect, fed-batch culture is being tried for recombinant *E. coli* too.

(4) Catabolite repression. When a microorganism is provided with a rapidly metabolizable carbon-energy source such as glucose, there may be a repression of enzyme biosynthesis, causing slower metabolism of energy source. If the glucose concentration is kept low by its feeding, the enzyme biosynthesis is derepressed.

(5) Auxotrophic mutants. In a microbial process employing an auxotrophic mutant (nutrition-requiring mutant), excess supply of the required nutrient results in abundant cell growth with less accumulation of the desired metabolite due to feedback inhibition and/or end-product repression. In such a process, the accumulation of the desired metabolite can be maximized by growing the mutant on a limited amount of the required nutrient, which is fed at a controlled rate.

(6) Non-growth-associated products. In a non-growth-associated microbial process, such as antibiotic production, microorganisms initially rapidly util-

ize the carbon-energy source for growth and then synthesize the desired secondary metabolite in the subsequent declining phase and early stationary phase. In a conventional batch process, this production phase is short. To prolong and maintain high synthesis activity of the semistarved microorganism, the carbon-energy source and/or precursors are fed into the bioreactor at a controlled rate.

(7) On-off control of gene expression in recombinant microorganisms. When the promoter of a foreign gene encoded on a plasmid is regulatable, its expression is controlled by addition of a compound (inducible gene) or by depletion of a nutrient (responsible gene). Early expression of the regulatable gene puts an excess burden on the host cells, resulting in suppressed growth and often in instability of the plasmid, so that careful feeding at a later phase of culture is important for achieving high accumulation of the foreign protein [3].

(8) Others. In some microbial reaction processes, replacing water lost by evaporation through exhaust gas and decreasing viscosity of the broth are achieved by water supply.

B. Basic Equation of Fed-Batch Operation

Referring to the definition of the fed-batch culture ($f_{out} = 0$) and taking the volume increase into consideration, and because usually $x_{in}^{(v)} = x_{in}^{(d)} = p_{in}^{(j)} = 0$, Eqs. (1)–(5) reduce to

$$\frac{d(vx^{(v)})}{dt} = vr_{kv} - vr_{xd} = (\mu - k_d)vx^{(v)} \tag{11}$$

$$\frac{d(vx)^{(d)})}{dt} = vr_{xd} = vk_d\, x^{(v)} \tag{12}$$

$$\frac{d(vs^{(i)})}{dt} = -vr_{s}^{(i)} + f_{in}s_{in}^{(i)}, \quad i = 1, 2, \ldots, m \tag{13}$$

$$\frac{d(vp^{(j)})}{dt} = vr_{p}^{(j)}, \quad j = 1, 2, \ldots, n \tag{14}$$

$$\frac{dv}{dt} = \alpha f_{in} \tag{15}$$

In Eq. (13), only a single substrate is supplied if $m = 1$ and a mixture of more than one substrate is supplied if $m > 2$ (multisubstrate fed-batch culture). In Eq. (15), α is the coefficient of increment in the volume of the culture broth due to the nutrient solution feeding. To minimize the increase in v, as highly concentrated a nutrient solution as possible is preferably supplied so that $\alpha < 1$. The value of α depends not only on f_{in} but also on amounts of acid and alkaline solutions added for pH adjustment, on the amount of antifoam solution added, and on the amount of water lost through exhaust gas.

In a fed-batch culture, specific rates, μ, q_s, and q_p are obtained from

$$\mu \equiv \frac{1}{vx^{(v)}}\frac{d(vx^{(v)})}{dt} - k_{xd}$$

$$q_s^{(i)} \equiv \frac{1}{vx^{(d)}}\left[f_{in}s^{(i)} - \frac{d(vs^{(i)})}{dt}\right] \tag{16}$$

$$q_p^{(j)} \equiv \frac{1}{vx^{(d)}}\frac{d(vp^{(j)})}{dt}$$

Equations (11)–(16)—together with kinetic expression of μ, $q_s^{(i)}$, and $q_p^{(j)}$ and a set of the initial conditions—constitute the operation equations of fed-batch culture. Because Eqs. (11)–(15) are based on mass balance principles, they are applicable for any kind of fed-batch culture, whether with or without feedback control (Fig. 5).

C. Classification of Fed-Batch Culture

The major purpose of fed-batch operation is to control the substrate (nutrient) concentration in the culture liquid. In establishing a fed-batch operation, two decisions must be made, (1) the type of nutrient(s) to be fed and (2) the mode of feeding. To identify the most effective nutrient(s), knowledge of microbial physiology, biochemistry, and genetics is essential. Sometimes it is inevitable to work by trial and error to find an effective substrate. On the other hand, biochemical engineering may play an important role in deciding the optimum feeding mode. It is possible to classify fed-batch microbial processes according to the feeding mode. Table 2 shows the classification by the author [2,4]. This classification divides fed-batch operations broadly into processes without feedback control and those with feedback control. The former are further subdivided with reference to feed rates into constant, exponential, and optimized, intermittent, and others.

Table 2 Classification of Fed-Batch Cultures

(1) Without feedback control	(2) With feedback control
1.1 Intermittent addition	2.1 Indirect feedback control
1.2 Constantly fed-batch	2.2 Direct feedback control
1.3 Exponentially fed-batch	2.2a Constant-value control
1.4 Optimized fed-batch	2.2b Optimum control
1.5 Others	

Source: Ref. 2.

D. Fed-Batch Culture Without Feedback Control

In a fed-batch operation without feedback control, the feed rate is changed during the operation according to a predetermined pattern. In the most primitive fed-batch culture without feedback control, the substrate is added intermittently, most likely because of easy handling. It is obvious that frequent intermittent addition approaches continuous feeding, and we can find no particular advantages in intermittent feeding.

1. Constant Fed-Batch Culture

A fed-batch culture with a constant feed rate of a growth-limiting substrate is called a constantly fed-batch culture and is regarded as the simplest fed-batch culture system. Mathematical analyses of this type of operation were carried out by the Yamane and Hirano [5], Pirt [6], and Dunn [7], and experimental studies were made by Yamane and Hirano [8].

The most prominent characteristic of the constantly fed-batch culture when a single substrate is supplied is the occurrence of (arithmetically) linear growth:

$$\frac{d(vx)}{dt} = k_1 \text{ (const.)} \tag{17}$$

The linear growth rate constant, k_1, is related to f_{in} and s_{in} by

$$k_1 = Y_{s/x} f_{in} s_{in} \tag{18}$$

The linear growth appears, however, only when the following inequality holds:

$$f_{in}s_{in} < \mu_{max}vx/Y_{x/s} \tag{19}$$

When Monod's equation is applied as a kinetic expression of r_x and the maintenance coefficient m is negligible, x and s are expressed by

$$x = \frac{Y_{x/s}[f_{in}s_{in}t + v_0(s_0 + x_0/Y_{x/s})]}{\alpha f_{in}t + v_0} \tag{20}$$

$$s = \frac{K_s f_{in}s_{in}}{(\mu_{max}t - 1)f_{in}s_{in} + \mu_{max}v_0(s_0 + x_0/Y_{x/s})} \tag{21}$$

where x_0, s_0, and v_0 are the values of x, s, and v at the start of feeding. The value of s in the linear growth phase is generally very small as can be seen by a calculation from Eq. (21) and was verified by experiments [8].

2. Exponentially Fed-Batch Culture

Under ideal conditions microorganisms grow exponentially. In order to keep the exponential growth in a fed-batch culture, the required flow rate (volumetric or mass) must be increased exponentially with time, and therefore this mode of

fed-batch culture is called exponentially fed-batch culture [9,10]. The necessary and sufficient conditions for the exponential growth are

$$s_0 = g^{-1}(\mu)$$

$$f_{in} = \frac{\mu/Y_{x/s} + m}{s_{in} - s_0} v_0x_0 \exp(\mu t) \tag{22}$$

Note that $s_{in} >> s_0$ when a concentrated nutrient solution is supplied, so that

$$f_{in}s_{in} = (\mu/Y_{x/s} + m)v_0x_0 \exp(\mu t) \tag{23}$$

On the other hand, when a dilute nutrient solution is supplied, $dv/dt = f_{in}$, so that

$$v = \frac{v_0x_0}{s_{in}Y_{x/s}} \exp(\mu t) + v_0\left(1 - \frac{x_0}{s_{in}Y_{x/s}}\right) \tag{24}$$

provided that $s_{in} >> s_0$. When s_{in} is preset so as to satisfy $s_{in} = x_0/Y_{x/s}$, then from Eq. (24) one obtains

$$v = \frac{v_0x_0}{s_{in}Y_{x/s}} \exp(\mu t) \tag{25}$$

Since the microorganisms grow exponentially,

$$vx = v_0x_0 \exp(\mu t) \tag{26}$$

and one obtains from Eqs. (25) and (26)

$$x = s_{in}Y_{x/s} = \text{const.} \tag{27}$$

Thus, the cell concentration is unvaried, resembling a continuous culture. The main features of exponentially fed-batch culture are

1. The constant substrate concentration; $s = s_0 = \text{const.}$
2. The voluntary control of the specific growth rate of microorganisms by external variation of the exponent μ of the feed rate, provided that $\mu \leq \mu_{max}$

Exponentially fed-batch culture is, therefore, somewhat similar to a chemostat with constant dilution, although it is, in fact, a modification of batch culture.

3. Optimized Fed-Batch Culture

Whenever a microbial process is operated semibatchwise under real economic circumstances, the feed rate of the substrate should be changed so that a judiciously chosen objective function may be maximized or minimized. A number of applications of modern mathematical optimizations for microbial processes have been reported involving optimization of the feeding mode. In 1978, Ya-

mane et al. [11] proposed a simple optimization technique by regarding the specific growth rate, μ, as an operating variable and the total cell mass, vx, as a state variable instead of solving the original optimization problem directly. Advances in the application of process systems engineering to fed-batch culture have been reviewed [12].

In Japan several researchers are studying the optimization of fed-batch culture. Shioya [13] has shown the usefulness of and difficulties involved in the μ-optimization technique for the optimization of f_{in} by conducting a number of fed-batch cultures to produce several metabolites. Kishimoto et al. developed a statistical approach to determine an optimum feed rate [14] and studied on the application of fuzzy theory to fed-batch culture [15].

E. Fed-Batch Operation with Automatic Feedback Control

It is desirable to incorporate a feedback control system into the feeding system if possible. Fed-batch operation with automatic feedback control is divided into two categories: those with indirect feedback control and those with direct feedback control (Table 2).

Fed-batch operation with indirect feedback control utilizes an observable parameter that is closely connected with features of the microbial reaction. Several such feedback parameters have been reported, including dissolved oxygen (DO) concentration, respiratory quotient (RQ), pH, metabolite concentration, partial pressure of CO_2 in exhaust gas (PCO_2), and turbidity of culture broth. In the case of pH, two limits are utilizable, a low limit when pH tends to drop during cultivation and a high limit when pH tends to rise during cultivation. pH is a very useful control index, especially when a complex medium containing corn steep liquor, defatted soybean, yeast extract, or peptone is used. The author and colleagues [16,17] reported effective productions of thiostrepton and a foreign green product (enzyme) by pH-stat modal fed-batch culture using a high limit. Konstantinov and Yoshida [18] have reported a DO-stat modal fed-batch culture combined with knowledge-based software from artificial intelligence.

Turbidity of the culture broth is also an useful control index; it is applicable to a complex medium but limited to the culture of suspended unicellular microorganisms. The culture turbidity is converted to the dry cell concentration, so it is actually a utilization of the dry cell concentration as a feedback control parameter. Yamane et al. [19] have reported that the data from an on-line laser turbidimeter combined with the data on the instantaneous volume of the culture broth can be used to control automatically a specific consumption rate of the substrate in fed-batch culture.

In semibatch operation with direct feedback control, which is usually automatic and negative, the concentration of the fed substrate in the culture liquid measured continuously or intermittently is utilized directly as a feedback parameter. It is not always necessary to fix a value of its concentration; this may be changed in the optimized pattern during operation. To execute fed-batch culture with direct feedback control, it is a prerequisite to be able to measure the concentration of the fed substrate either continuously in situ or intermittently with a proper sensor-detector system. If the fed substrate is volatile, such as methanol or ethanol, a tubing sensor is excellent [20,21]. Yamane et al. [22] succeeded in mass production of poly-3-hydroxybutyrate by fed-batch culture on methanol automated by a porous Teflon tubing sensor. Various biosensors that have been advanced in the past decade are also applicable.

In industrial fed-batch cultures various kinds of fed substrate may be used, and Table 3 summarizes the possible applicability of various control indices for these fed substrates.

IV. CONTINUOUS OPERATION

Continuous culture has a long history since the early twentieth century but its kinetic theory was established in the 1950s. As mentioned previously, there are not many industrial continuous culture at present, even though academic studies have been carried out all over the world.

A. Classification of Continuous Operation

Continuous operation of a stirred tank bioreactor is classified in Table 4 according to the mode of attaining its steady state. Among them, the chemostat is the simplest and the most widely applied, so that it is mainly described in this section.

Table 3 Possible Applicability of Various Control Indices for Fed-Batch Cultures with Feedback Control

	C source	Single (nonvolatile)	Complex (nonvolatile)	Single (volatile)	Complex (volatile)
Index	N source	NH_4^+	Organic N (+ NH_4^+)	NH_4^+	Organic N (+ NH_4^+)
DO		A	B	A	B
Tubing method		D	D	A	A
pH (low limit)		C	D	C	D
pH (high limit)		B	A	B	A
Turbidity		A	A	A	A

A, excellent; B, good; C, not bad; D, inapplicable.
Source: Ref. 1.

Table 4 Classification of Continuous Culture

(1) Continuous culture without any feedback chemostat control (Monod, 1950; Novick and Szilard, 1950)
(2) Continuous culture with feedback control
  2.1 Turbidostat (Bryson, 1952)
  2.2 Nutristat (Edwards et al., 1972)
  2.3 Auxostat
    pH auxostat (Martin and Tempfling, 1976)
    DO stat
    Others

Source: Ref. 1.

B. Basic Equation of Chemostat

The technical term chemostat is an alternative name for a single continuously stirred tank bioreactor without feedback control of the feed rate of the nutrient solution. Quantitative analysis of the chemostat is based on the expectation that in the chemostat the growth of cells and product formation are coupled in some manner to nutrient utilization and that product formation will be coupled to growth and/or cell mass concentration, depending on the metabolic regulatory controls. The only assumption made to derive the basic equation of the chemostat is that the growth rate is determined by the rate of supply of a single essential nutrient (called the growth-limiting substrate), which could be the carbon source, nitrogen, phosphorus, trace element, or vitamin, depending on the relative concentration in the feed medium. All other nutrients must be present in sufficient but not inhibitory concentrations.

Referring to the chemostat in a single bioreactor shown in Fig. 6, we have $f_{in} = f_{out} = f$, $x_{in}^{(v)} = x_{in}^{(d)} = p_{in}^{(j)} = 0$, v = constant, and $s_{out}^{(j)} = s$ (growth-limiting substrate concentration) in Eqs. (1)–(5), so that the general forms of balance of the chemostat become

$$v\frac{dx^{(v)}}{dt} = vr_{xv} - vr_{xd} - fx^{(v)} \tag{28}$$

$$v\frac{dx^{(d)}}{dt} = vr_{xd} - fx^{(d)} \tag{29}$$

$$v\frac{ds}{dt} = -vr_s + f(s_{in} - s) \tag{30}$$

$$v\frac{dp^{(j)}}{dt} = vr_p^{(j)} - fp^{(j)}, \qquad j = 1, 2, \ldots, n \tag{31}$$

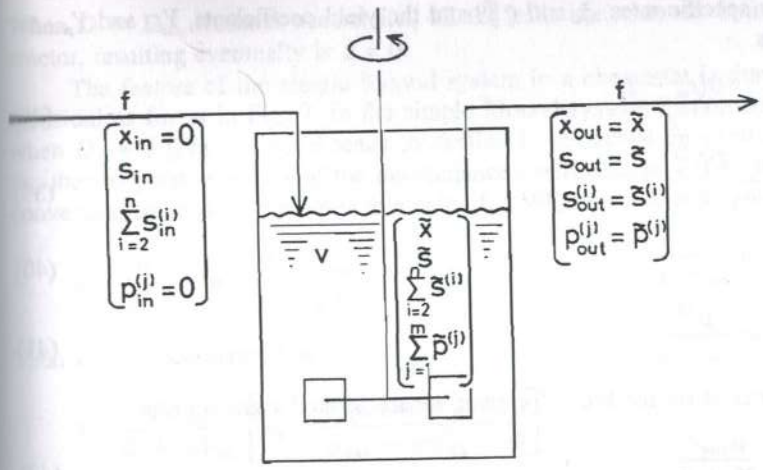


Figure 6 Chemostat at a steady state in a single stirred tank bioreactor.

At the steady state, where any transient changes due to fluctuations in the flow rates, medium compositions, or environmental conditions have died out, all the terms on the left-hand side of the balance Eqs. (28)–(31) can be set equal to zero to give

$$\tilde{r}_{xv} - \tilde{r}_{xd} = D\tilde{x}^{(v)} \tag{32}$$

$$\tilde{r}_{xd} = D\tilde{x}^{(d)} \tag{33}$$

$$\tilde{r}_s = D(s_{in} - \tilde{s}) \tag{34}$$

$$\tilde{r}_p^{(j)} = D\tilde{p}^{(j)}, \qquad j = 1, 2, \ldots, n \tag{35}$$

where a tilde (~) means the value at steady state, and D is called the dilution rate:

$$D \equiv f/v \tag{36}$$

D is the reciprocal of the mean residence time of all materials flowing through the bioreactor.

When $\tilde{x}^{(d)}$ is negligibly small, $\tilde{r}_{xd} = 0$ and $\tilde{r}_{xv} = \tilde{r}_x = \tilde{\mu}\tilde{x}$, so that from Eq. (32) we have

$$\tilde{\mu} = D \tag{37}$$

The other specific rates, $\bar{q}_s$ and $\bar{q}_p^{(j)}$, and the yield coefficients, $Y_{x/s}$ and $Y_{p/s}$, are defined as

$$\bar{q}_s \equiv \frac{D(s_{in} - \bar{s})}{\bar{x}} \tag{38}$$

$$\bar{q}_p^{(j)} \equiv \frac{D\bar{p}^{(j)}}{\bar{x}} \tag{39}$$

$$Y_{x/s} \equiv \frac{\bar{x}}{s_{in} - \bar{s}} \tag{40}$$

$$Y_{p/s} \equiv \frac{\bar{p}^{(j)}}{s_{in} - \bar{s}} \tag{41}$$

In the so-called simple Monod system, where Monod's growth model

$$\bar{\mu} = \frac{\mu_{max}\bar{s}}{K_s + \bar{s}} \tag{42}$$

and $Y_{x/s}$ = constant, $\bar{x}$ and $\bar{s}$ can be expressed as functions of operating variables, s_{in} and D, and kinematic parameters, μ_{max}, K_s, and $Y_{x/s}$:

$$\bar{x} = Y_{x/s}\left(s_{in} - \frac{K_s D}{\mu_{max} - D}\right) \tag{43}$$

$$\bar{s} = \frac{K_s D}{\mu_{max} - D} \tag{44}$$

The productivities of cell mass and metabolite are defined as $D\bar{x}$ and $D\bar{p}^{(j)}$, having units of g L^{-1} h^{-1}:

$$D\bar{x} = Y_{x/s}D\left(s_{in} - \frac{K_s D}{\mu_{max} - D}\right) \tag{45}$$

$$D\bar{p}^{(j)} = Y_{p/s}D\left(s_{in} - \frac{K_s D}{\mu_{max} - D}\right) \tag{46a}$$

When the objective metabolite is intracellular,

$$D\bar{p}^{(j)} = D\gamma\bar{x} = \gamma Y_{x/s}D\left(s_{in} - \frac{K_s D}{\mu_{max} - D}\right) \tag{46b}$$

where γ is the metabolite content.

The constraint imposed on $\bar{x}$, $\bar{x} > 0$, leads to the limitation of D, the maximum admissible dilution rate called the critical dilution rate, D_{cri}, which is slightly less than μ_{max}.

$$D < D_{cri} = \frac{\mu_{max}s_{in}}{K_s + s_{in}} \approx \mu_{max} \tag{47}$$

When $D \geq D_{cri}$, washout occurs; i.e., all the cells are removed from the bioreactor, resulting eventually in $\bar{x} = 0$.

The feature of the simple Monod system in a chemostat is drawn in dimensionless forms in Fig. 7. In the simple Monod system, $\bar{x}$ approaches $Y_{x/s}s_{in}$ when $D \to 0$ (Fig. 7), but $\bar{x}$ tends to decline in the actual chemostat. This is ascribed to positive values of the maintenance coefficient, m, and/or the rate of conversion to dead cells from viable cells, k_{xd}. When $m > 0$, q_s is given by

$$q_s = \frac{\mu}{Y^*_{x/s}} + m = \frac{1}{Y^*_{x/s}}\frac{\mu_{max}s}{K_s + s} \tag{48}$$

Then $\bar{x}$ and $\bar{s}$ are derived as

$$\bar{x} = \frac{DY^*_{x/s}}{D + mY^*_{x/s}}\left[s_{in} - \frac{(D + mY^*_{x/s})K_s}{\mu_{max} - mY^*_{x/s} - D}\right] \tag{49}$$

$$\bar{s} = \frac{(D + mY^*_{x/s})K_s}{\mu_{max} - mY^*_{x/s} - D} \tag{50}$$

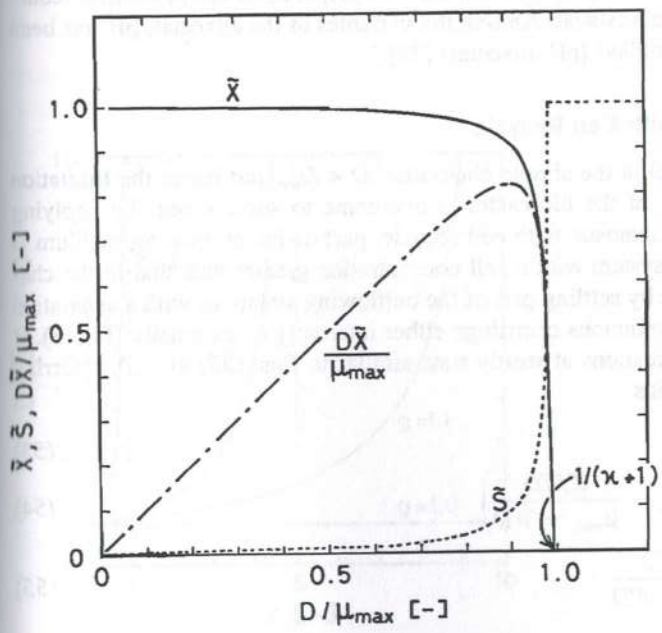


Figure 7 Profiles of $\bar{x}$, $\bar{s}$, and $D\bar{x}$ of the simple Monod system in a chemostat ($k = 0.01$). $k \equiv K_s/s_{in}$, $\tilde{X} \equiv \bar{x}/(Y_{x/s}s_{in})$, $\tilde{S} \equiv \bar{s}/s_{in}$.

And, when $k_{xd} > 0$, $\bar{x}$ and $\bar{s}$ become

$$\bar{x}^{(v)} = \frac{D}{(D + k_{xd})/Y^*_{x/s}} \left[s_{in} - \frac{K_s(D + k_{xd})}{\mu_{max} - (D + k_{xd})} \right] \tag{51}$$

$$\bar{s} = \frac{K_s(D + k_{xd})}{\mu_{max} - (D + k_{xd})} \tag{52}$$

In Eqs. (43) and (44), $Y_{x/s}$ is assumed constant and in Eqs. (48)–(52), $Y^*_{x/s}$ is assumed constant, but $Y_{x/s}$ again often decreases as D increases to D_{cri}, for several reasons.

C. Continuous Operation with Feedback Control

In a chemostat without any feedback control, the system is unstable when D is near D_{cri} and $\bar{s}$ is quite small or in the range of the semistarved condition, which is not always preferable for producing metabolite. These demerits can be overcome by introducing a sort of feedback control. There are several control indices as listed in Table 4. They are turbidity of the culture broth in a turbidostat, concentration of the growth-limiting substrate in a nutristat, and a variable related indirectly to the culture system, such as pH, DO, or Eh (oxidation-reduction potential) in an auxostat. Among the variables in the auxostat, pH has been most extensively studied (pH auxostat) [23].

D. Chemostat with Cell Recycle

The limitation on D in the simple chemostat, $D < D_{cri}$, and hence the limitation of the productivity of the bioreactor is overcome to some extent by applying cell recycle. In a chemostat with cell recycle, part of the outflowing medium is removed from the system with a cell concentration greater than that in the chemostat; this is done by settling part of the outflowing stream or with a separation device such as a continuous centrifuge either internally or externally (Fig. 8). If one sets balance equations at steady state similar to Eqs. (32) to (35), referring to Fig. 9, one obtains

$$\bar{\mu} = WD \tag{53}$$

$$\bar{x} = \frac{Y_{x/s}}{W} \left(s_{in} - \frac{K_s WD}{\mu_{max} - WD} \right) \tag{54}$$

$$\bar{s} = \frac{K_s WD}{\mu_{max} - WD} \tag{55}$$

$$D_{cri} = \frac{1}{W} \frac{\mu_{max} s_{in}}{K_s + s_{in}} \tag{56}$$

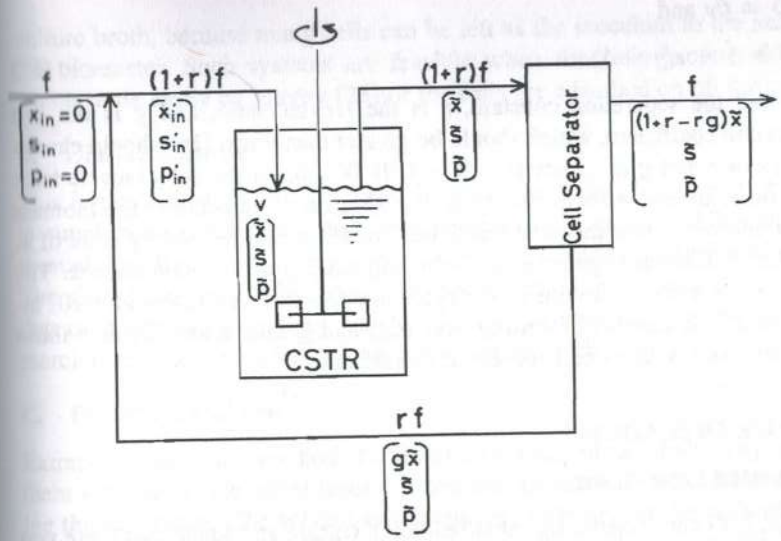


Figure 8 Chemostat with cell recycle.

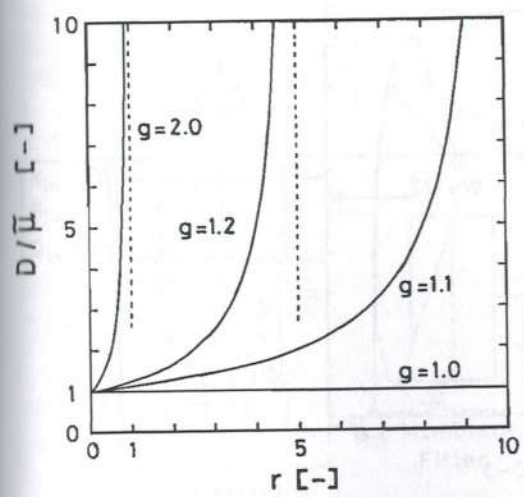


Figure 9 Increased D by setting proper values of r and g in a chemostat with cell recycle.

where $D \equiv f/v$ and

$$W \equiv 1 - r(g - 1) \tag{57}$$

W is called the separation constant, r is the recycle ratio, and g is the cell concentration coefficient, which should be greater than unity. One should choose the values of r and g to satisfy $0 < W < 1$. If $W = 0$, all the cells recycle back to the bioreactor and without cell lysis or endogeneous respiration the biomass in the bioreactor would increase limitlessly, which means no steady state of s. This point is discussed again in the following section as filtration culture. The features of the chemostat with cell recycle are depicted in Figs. 9 and 10, indicating that D is capable of getting over μ_{max} and greater $\bar{x}$ and $D\bar{x}$ than those in the chemostat without cell recycle are attainable.

V. OTHER OPERATIONS

A. Repeated Operations

Mori et al. [24] conducted a repeated fed-batch culture and found that there was an optimum volume of the culture broth that gave the highest volumetric productivity. Repeated operations are especially effective when microorganisms are naturally separated from culture broth at the time of partial removal of the

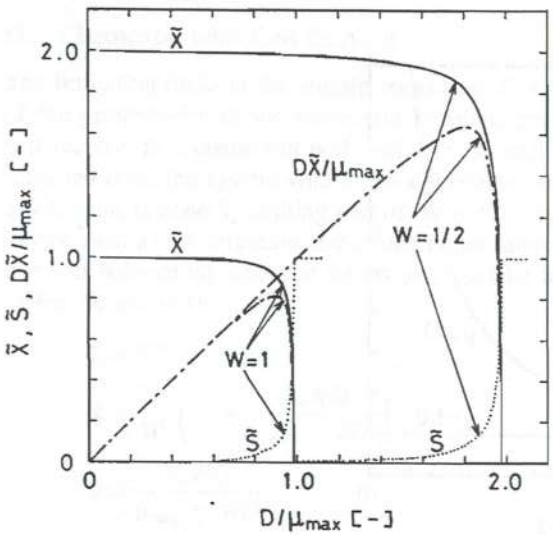


Figure 10 Profiles of x, s, and Dx of the simple Monod system in chemostat with cell recycle ($k = 0.01$). $k \equiv K_s/s_{in}$, $\bar{X} \equiv \bar{x}/(Y_{x/s}s_{in})$, $\bar{S} \equiv \bar{s}/s_{in}$, $W \equiv 1 - r(g - 1)$.

culture broth, because many cells can be left as the inoculum to the next run in the bioreactor. Such systems are feasible when the cells become flocculants which settle easily by gravity [25] or the cells are adsorbed on oil droplets [26].

B. Dialysis Culture

This is a combination of culture and dialysis. The culture broth in the bioreactor is supplied to a dialyzer, where metabolites (often growth-inhibitory) diffuse through the dialysis membrane from the culture liquid to another medium tank. The size of the medium tank must be approximately the same as or greater than that of the bioreactor, so an industrial dialysis culture has not yet been commercialized although it has a long history of research.

C. Extractive Culture

Extractive culture is a method of alleviating toxicity of metabolites by extracting them selectively into water-insoluble organic solvent added in a bioreactor during the cultivation. The solvent should not be inhibitory for the cells and should preferably be cheap. Its application to acetone-butanol fermentation has been studied [27].

D. Filtration Culture

In filtration culture, the culture broth is filtered through a microporous membrane which cannot let the cells pass (Fig. 11). All the outflowing medium is removed

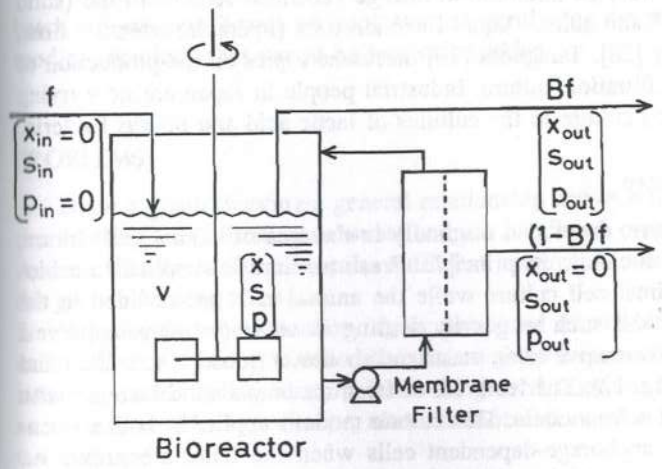


Figure 11 Filtration culture. B is bleed rate. When $B = 0$, system is a purely filtration culture and when $B > 0$, system is a chemostat with cell recycle.

free of cells. Fresh medium must be fed together with filtering in order to keep the culture broth volume constant; therefore one can regard the filtration culture as a kind of fed-batch culture. An industrial sterilizable membrane suitable for this purpose is composed of ceramics or synthetic polymer and is now commercially available in Japan.

The culture broth flows on the surface of the membrane while filtrate flows out from the membrane rectangularly, so that it is called crossflow. Once the cell concentration in the bioreactor rises up to a high cell density, the filtration culture can be extended to a chemostat with cell recycle by "bleeding" a definite amount of the culture broth. In this particular case, referring to Fig. 11, the balances of x, s, and p are

$$\frac{dx}{dt} = (\mu - BD)x \tag{58}$$

$$\frac{ds}{dt} = D(s_{in} - s) - \frac{\mu x}{Y_{x/s}} \tag{59}$$

$$\frac{dp}{dt} = -Dp + q_p x \tag{60}$$

The condition for no washout at the steady state is

$$D < \frac{\mu}{B} \tag{61}$$

Unlike the chemostat with cell recycle mentioned in the Section IV.C, the continuous filtration culture permits one to change cell mass retention time (solid retention time, SRT) and culture liquid retention time (hydraulic retention time, HRT) independently [28]. Taniguchi [29] discussed topics on the production of useful materials by filtration culture. Industrial people in Japan are now trying to apply the filtration culture to the cultures of lactic acid and *Bifidus* bacteria.

E. Perfusion Culture

Ideal perfusion culture, developed originally in the area of animal cell culture, is similar to filtration culture in principle. Fresh medium is supplied to a bioreactor used for animal cell culture while the animal cells are retained in the bioreactor by a method such as gravity settling in order not only to harvest product(s) but also to remove toxic waste metabolite(s) (mostly lactic acid and ammonium ions) (Fig. 12). The feed rate of fresh medium should increase with increasing in animal cell numbers. This culture mode is applied to both suspension-type cells and anchorage-dependent cells when the latter are grown on microcarriers. Various effective microcarriers have been commercialized in Japan.

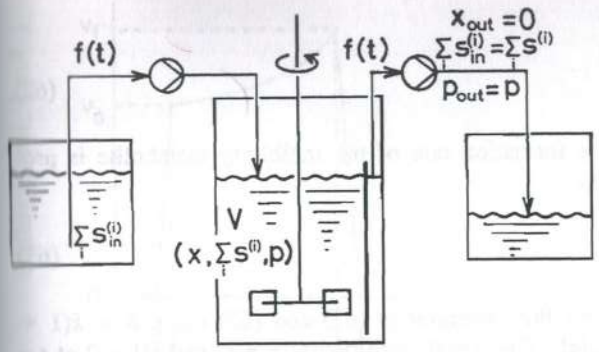


Figure 12 Perfusion culture. $\Sigma_i s^{(i)}$ represents complex medium. Bioreactor configuration is not always stirred tank.

VI. CONCLUDING REMARK

Traditionally, biotechnological processes have been operated batchwise. This is because the products of biotechnological reactions have been categorized as fine chemicals or speciality chemicals whose annual productions are not large. Progress in the technique of fed-batch culture has made industrial people understand its usefulness, and the number of fed-batch operations has been gradually increasing. Little specific detail is available in the public domain regarding fed-batch operations as commercial secrecy affords a competitive advantage.

Continuous culture gives much greater productivity than batch and fed-batch culture, and it will be implemented mostly for the productions of commodity chemicals that would be less value-added.

PROBLEMS

1. Draw a graph showing a general relationship between the initial concentration of the growth-limiting substrate and the maximum crop in a batch culture. Assume that the medium contains sufficient amounts of all nutrients other than the growth-limiting substrate.
2. Suppose that the inoculum, x_0, is composed of viable cells, $x^{(v)}$ and dead cells, $x^{(d)}$, and that the viable cells start to grow exponentially with a growth rate, μ_{max}, after the lag time, t_1. Draw the growth curve of the batch culture, x, as a function of culture time, t, after the inoculation when $x^{(d)}/x_0 = 0$, 0.2, 0.4, 0.6, 0.8, and 1.0.
3. In a batch culture, it is assumed that the growth rate, dx/dt, is proportional to x and is reduced by an inhibitory metabolite as a first-order function of its

concentration:

$$\frac{dx}{dt} = kx(1 - a[\mathrm{I}]) \tag{62}$$

It is also assumed that the formation rate of the inhibitory metabolite is proportional to the growth rate:

$$\frac{d[\mathrm{I}]}{dt} = b\frac{dx}{dt} \tag{63}$$

Derive the growth curve on the assumptions (62) and (63) using $K = k(1 + abx_0)$ and $x_{st} = (1 + abx_0)/ab$. The initial condition is $x = x_0$ and $[\mathrm{I}] = 0$ at $t = 0$. Draw the growth curves for $x_0/x_{st} = 0.01$, 0.05, 0.1, 0.2, and 0.4.

4. As mentioned in Section III.D.1, exponential growth followed by linear growth is observed in a constantly fed-batch culture. Answer the following questions.

(a) x and s in the linear growth phase are given by Eqs. (20) and (21), respectively. When the initial culture condition is set to satisfy the equation $s_{in} = s_0 + x_0Y_{x/s}$, verify that $x = Y_{x/s}s_{in}$ = constant throughout the linear growth phase. Also show that $s = v_0s_0/(f_{in}t + v_0)$.

(b) Verify that the transition time, t_T, from the exponential growth phase to the linear growth phase is obtained by solving the following equation:

$$v_0x_0 \exp(\mu_{max}t_T) = Y_{x/s} f_{in}s_{in}t_T + v_0(x_0 + Y_{x/s}s_0)$$

5. Consider a fed-batch culture in which the substrate feed rate is automated by the culture turbidity. The balance of the substrate is given by

$$\frac{d(vs)}{dt} = f_{in}s_{in} - q_{sc}(vx) \tag{64}$$

where q_{sc} is the specific substrate consumption rate. If the volumetric substrate feed rate, f_{in}, is controlled to satisfy $f_{in} = q_{sf}(vx)/s_{in}$ (q_{sf} is the specific substrate feed rate), verify that $ds/dt = (q_{sf} - q_{sc})x$ and that s is constant when $q_{sf} = q_{sc}$. It is assumed that $s_{in} >> \alpha s$.

6. Profiles of x and v versus t depicted in Fig. 13 were obtained in a fed-batch culture. From the profiles you want to estimate a repeated fed-batch culture. Suppose that in the repeated fed-batch culture the culture volume, v_{out}, is taken out from v_f and the fresh culture medium, amounting to v_{in}, is put into the bioreactor so that the volume of the culture broth becomes v_R at the beginning of each culture. Also suppose that the culture time is t_k and t_s is the time required for taking out the culture broth and filling in the fresh medium.

(a) Derive the cell productivity (time-average value), P_{RFB}, in repeated fed-batch

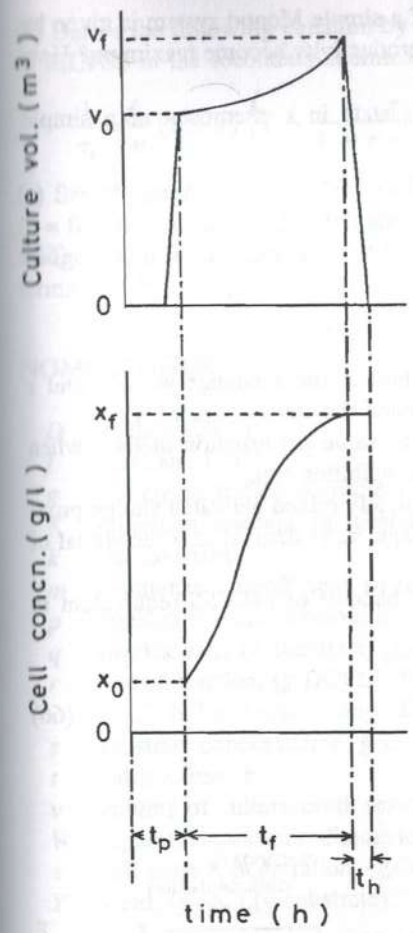


Figure 13 Profiles of x and v versus t in a fed-batch culture.

culture, which is defined as (total cell mass drawn out and harvested after cultivation) (final culture volume in bioreactor)$^{-1}$ (total time for culture operation)$^{-1}$, as a function of t_p, t_f, t_k, t_h, n (number of repetitions) and v_{total}/v_f.

(b) Sketch the estimated cell productivity, P_{RFB}, as a function of v_{out}/v_f for the cases of n = 1, 2, 4, 8, 12 and ∞ when x_f, v_f, t_f, t_p, and t_s are fixed at 119 gDC/L, 1.10 m^3, 12 h, 5 h, and 1.0 h, respectively.

7. The operating variables of a chemostat are f, s_{in}, and v. When one of them is varied keeping the remaining ones constant, how do $\bar{x}$, $\bar{s}$, and $\bar{\mu}$ change? Sketch profiles of them roughly with graphs.

8. The cell productivity in a chemostat of a simple Monod system is given by Eq. (45). At what value of D does the cell productivity become maximum? How much is the maximum value?

9. The system equations of the unsteady state in a chemostat of a simple Monod system are given by

$$\frac{dx}{dt} = x\left(\frac{\mu_{max}s}{K_s + s} - D\right)$$
$$\frac{ds}{dt} = D(s_{in} - s) - \frac{x}{Y_{x/s}}\frac{\mu_{max}s}{K_s + s} \tag{65}$$

(a) Show by a dynamical system analysis how x and s change when x and s fluctuate as $x = \bar{x} + \Delta x$ and $s = \bar{s} + \Delta s$ around the steady state $(\bar{x}, \bar{s})$.

(b) Analyze the stability of chemostat by the same perturbation method when the growth rate kinetics are of the substrate inhibition type.

10. Figure 14 is a flow diagram of a completely mixed activated sludge process. In the activated sludge process, sludge age, τ_s, is defined as a reciprocal of the specific sludge formation rate.

(a) Derive the following equation from the balance of MLVSS (equivalent to biomass) in the aeration tank.

$$\frac{1}{\tau_s} = \frac{f_{in}}{v}\left(1 + r - \frac{rx_r}{\bar{x}}\right) \tag{66}$$

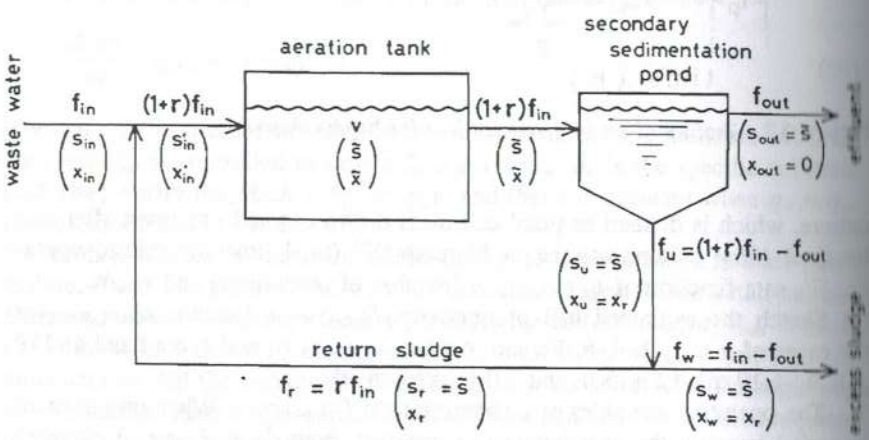


Figure 14 Flow diagram of a completely mixed activated sludge process.

(b) Derive the following equation by substituting $x_r/\bar{x}$ obtained from the balance of MLVSS in the secondary sedimentation pond.

$$\frac{1}{\tau_s} = \frac{f_{in}}{v}(1 + r)\left(1 - \frac{r}{1 + r - f_{out}/f_{in}}\right) \tag{67}$$

(c) Sketch graphs of $(f_{in}/v)/(1/\tau_s)$ as functions of r for the following cases: $f_{out}/f_{in} = 0, 0.6, 0.8, 0.9, 0.95, 0.98$, and 1.0. From the graphs, discuss the effect of sludge return on the efficiency of biological oxygen demand (BOD) removal in terms of f_{in}/v.

NOMENCLATURE

D	dilution rate, h^{-1}
f	feed rate, L h^{-1}
g	cell concentration coefficient, dimensionless
K	saturation constant, (g substrate) L^{-1}
k	rate constant
m	maintenance coefficient, (g-substrate) (g-DC)$^{-1}$ h^{-1}
p	metabolite concentration, g L^{-1}
q	specific rate, (g-substrate) (g-DC)$^{-1}$ h^{-1} or (g-metabolite) (g-DC)$^{-1}$ h^{-1}
r	rate of reaction, (g-DC) L^{-1} h^{-1} or (g-substrate) L^{-1} h^{-1} or (g-metabolite) L^{-1} h^{-1} or recycle ratio, dimensionless
s	substrate concentration, g L^{-1}
t	culture time, h
v	volume of culture broth (excluding gas bubbles), L
W	separation constant, dimensionless
x	cell mass concentration, (g-DC) L^{-1}
Y	yield, (g-DC) (g-substrate)$^{-1}$ or (g-metabolite) (g-substrate)$^{-1}$

Greek Symbols

α	coefficient of increment in the volume of the culture broth, h^{-1}
μ	specific growth rate, h^{-1}

Subscripts

0	initial
b	bleed
cri	critical
f	final
in	inflow

l	linear growth
max	maximum
out	outflow
p	metabolite (product)
s	substrate
xd	dead cell
xv	viable cell
x/s	cell mass from substrate
p/s	metabolite from substrate

Superscripts

(d)	dead
(*i*)	*i*th substrate
(*j*)	*j*th metabolite
(v)	viable
~	at steady state
*	true

REFERENCES

1. T. Yamane, *Bioreaction Engineering*, 2nd ed., Sangyo Tosho, Tokyo, 1991, p. 179.
2. T. Yamane and S. Shimizu, *Adv. Biochem. Eng. Biotechnol. 30*: 147 (1984).
3. S. Iijima, *Hakkokogaku Kaishi 69*: 507 (1991).
4. T. Yamane, *Hakko Kogaku 56*: 310 (1978).
5. T. Yamane and S. Hirano, *J. Ferment. Technol. 55*: 156 (1977).
6. S. J. Pirt, *Ann. N.Y. Acad. Sci. 326*: 119 (1979).
7. I. J. Dunn, *Ann. N.Y. Acad. Sci. 326*: 127 (1979).
8. T. Yamane and S. Hirano, *J. Ferment. Technol. 55*: 380 (1977).
9. T. Yamane, M. Kishimoto, and F. Yoshida, *J. Ferment. Technol. 54*: 229 (1976).
10. H. C. Lim, *Biotechnol. Bioeng. 18*: 1635 (1976).
11. T. Yamane, T. Kume, E. Sada, and T. Takamatsu, *J. Ferment. Technol. 55*: 587 (1977).
12. A. Johnson, *Automatica 23*: 691 (1987).
13. S. Shioya, *Ferment. Industry 45*: 90 (1987).
14. M. Kishimoto, T. Sawada, T. Yoshida, and H. Taguchi, *Biotechnol. Bioeng. 26*: 871 (1984).
15. M. Kishimoto, *Biosci. Industry 49*: 383 (1991); *49*: 724 (1991).
16. T. Suzuki, T. Yamane, and S. Shimizu, *Appl. Microbiol. Biotechnol. 25*: 526 (1987).
17. E. E. Tulin, S. Ueda, H. Yamagata, S. Udaka, and T. Yamane, *Biotechnol. Bioeng. 40*: 844 (1992).
18. K. Konstantinov and T. Yoshida, *J. Ferment. Biotechnol. 70*: 420 (1990).

19. T. Yamane, W. Hibino, K. Ishihara, Y. Kadotani, and M. Kominami, *Biotechnol. Bioeng. 39*: 550 (1992).
20. K. Dairaku and T. Yamane, *Biotechnol. Bioeng. 21*: 1671 (1979).
21. T. Yamane, M. Matsuda, and E. Sada, *Biotechnol. Bioeng. 23*: 2493, 2509 (1981).
22. T. Yamane, T. Suzuki, and S. Shimizu, *Bioproducts and Bioprocesses* (A. Fiechter, H. Okada, and R. D. Tanner, eds.), Springer-Verlag, Berlin, 1986, p. 321.
23. S. P. Fraleigh, H. R. Bungay, and L. S. Clesceri, *Trends Biotechnol. 7*: 159 (1989).
24. H. Mori, T. Yamane, T. Kobayashi, and S. Shimizu, *J. Ferment. Technol. 61*: 391 (1983).
25. S. Limtong, M. Nakata, H. Funahashi, T. Yoshida, T. Seki, J. Kumnuanta, and H. Taguchi, *J. Ferment. Technol. 62*: 55 (1984).
26. T. Furukawa and T. Ogino, *J. Ferment. Technol. 60*: 377 (1982).
27. M. Taya, *Hakko Kogaku 65*: 249 (1987).
28. H. N. Chang and S. Furusaki, *Adv. Biochem. Eng. Biotechnol. 44*: 27 (1991).
29. M. Taniguchi, *Kagaku Kogaku 55*: 119 (1991).

14
Scale-Up

Carles Solà and Francesc Gòdia

Universitat Autònoma de Barcelona
Bellaterra, Catalonia, Spain

I. INTRODUCTION

Transferring to an industrial scale processes successfully developed at the laboratory (bench) scale is not a simple procedure. According to Bisio [1], scale-up is defined as

> The successful startup and operation of a commercial size unit whose design and operating procedures are in part based upon experimentation and demonstration at a smaller scale of operation.

It is well known in the chemical and pharmaceutical industries that a commercial process must be based on experimental data obtained at laboratory and pilot scales. When dealing with biochemical reactions these data are still more necessary, as in general we are facing a much more complex entity. A satisfactory scale-up can require an empirical procedure in which the reactor size is progressively increased, having as a target the industrial one. Such a procedure would be very expensive in both time and cost.

In the chemical industry the old rule of thumb used in the 1930s, that each individual increase of scale should not surpass a factor of 10, is now considered obsolete. In fact, the American Oil Company built an ultracracking

unit from the data obtained in a small pilot plant, using a scale factor of 80,000 [2]. It is a matter of fact that in fermentation technology 10 is still considered the safe scale factor, as a consequence of the lack of control on all the elements having an effect in the development of a fermentation process.

Scale-up is intrinsically complicated due to the combination of different causes: the representative equations of the process cannot be solved (frequently even written!); physical, chemical, and biological parameters are interconnected; solutions obtained in the laboratory or pilot plant are not valid for a commercial one; and the actual performance of the full-scale equipment is unknown (if it is still not built or assayed).

Process complexity and the level of understanding of it determine the recommended rate of scale-up. In the field of chemical reactions there are scale-up examples using scale factors of 50,000 or 80,000 in the case of fixed-bed reactors (reactors with a diameter between 2.5 to 9 m have been designed using data obtained in reactors 13 to 44 mm in diameter), whereas when scaling up triphasic fluidized beds the scale factor lies between 10 and 100. In the latter case the main difficulty is in the complex and still poorly known hydrodynamic characteristics of the reactor [3].

In fermentation technology the biggest reactor volumes are found in antibiotic production, ethanol fermentation, and wastewater treatment plants. New fermentation plants for antibiotic production have been built as batteries of 12 fermentors of 250,000 L each, to be compared with those of 75 m^3 used before. In the case of citric acid production there are stirred fermentors up to 500 m^3 and even 1000 m^3 of the tower type. Sewage is treated by Bayer AG in a 20,000-m^3 reactor which has a height of 30 m and a diameter of 26 m, using 7 MW of power for mixing and aeration. In most cases there are mechanically stirred bioreactors. In general, it is not possible to design these systems from the data obtained in a small laboratory installation. Moreover, the consideration of the enormous power requirements has boosted the development of new types of fermentors for use at industrial scale, such as the airlift reactor.

One exception to this general trend occurs in the field of wastewater biological treatment plants, in particular those using anaerobic digestion. For instance, Amoco Co. [4] built a plant to treat the wastewater produced by the manufacture of terephthalic acid (containing more than a dozen organic species, especially aromatic compounds), scaling up directly from a 10-L pilot unit to a 10,000-m^3 operating unit, with no adjustments in the original design parameters.

However, sometimes there is no need for scale-up or a need for only a very limited extent. Let us consider, for instance, the production of a therapeutic protein by means of a genetically modified strain of *Escherichia coli*. If this protein represented 20% of the overall bacterial protein, a wet biomass yield of 50 g/L and an overall downstream process efficiency of 50%, we could obtain

0.5 g per liter fermentor. Thinking of a normal dose as 1 mg, a fermentor of "only" 10 m^3 would supply 5 million doses per batch.

The purpose of scale-up is the selection of design conditions and operational procedures to ensure that the effect of the different variables on the process is the same in units of different size. The objective is then to obtain similar yields with the same product distribution (quality). This process starts at the laboratory scale and frequently must continue at the smallest possible scale, for practical and economic reasons. When knowledge of the process is incomplete or when relatively important amounts of product are needed (for market evaluation, regulatory issues, etc.) it is necessary to employ pilot plants of bigger size, currently referred to as demonstration units.

Stirred tank bioreactors have received predominant attention regarding scale-up, and therefore most of this chapter describes methods of scale-up developed for this type of bioreactor. Other bioreactor types, such as columnar fermentors, that present more difficulties in finding general scale-up criteria, due to their higher complexity, are discussed in Section IV.B.

II. CONSEQUENCES OF CHANGING THE SCALE OF OPERATION

Theoretically, a chemical or biochemical reaction proceeds at a rate that is independent of the vessel size or geometric structure. However, the reaction rate is affected by the physical processes of mass, heat, and momentum transport, normally controlled by the reactor size and structure. Thus the reaction becomes indirectly influenced by the reactor type and dimensions, in a manner that frequently is not quantitatively foreseeable.

In the case of cell growth there is, however, an effect of a direct nature, as a consequence of the change of the scale of operation. In fact, the number of generations required to achieve a final biomass concentration starting from a given inoculum is a function of the final fermentor volume. Assuming for simplicity that exponential growth occurs throughout, then

$$N_g = 1.44(\ln V + \ln x - \ln X_0) \tag{1}$$

where X_0 is the number of cells in the original inoculum, x the final biomass concentration to achieve, V the fermentor volume, and N_g the number of generations. This equation shows that in a fermentation employing a microorganism characterized by a certain rate of appearance of undesired variants, its final proportion in the population increases with fermentation scale, as a consequence of the appearance of new variants at each generation and the multiplication of those that previously appeared.

Denoting by x_n and x_m the concentrations of parent and variant species, λ the rate of appearance of variants, expressed as number of variants produced

per genome per generation, and α the ratio of the specific growth rate of the variant to that of the parent organism, the fraction of parent organisms after N_g generations will be

$$\frac{x_n}{x_n + x_m} = \frac{\alpha + \lambda - 1}{(\alpha - 1) + \lambda \cdot 2^{N_g(\alpha+\lambda-1)}} \tag{2}$$

Figures 1 and 2 show the effect of different values for α and λ. In spite of the approximate nature of the equation, the dramatic consequences are easily observed.

There are other indirect consequences of increasing the operational scale, such as the influence of the batch sterilization cycle: large-scale fermenters need longer sterilization time, and as a consequence the quality of the medium is affected by the loss of thermolabile compounds, such as vitamins.

Although the most important effect is in general on agitation and aeration, difficulties are also found in scaling up the associated heat transfer equipment, because the heat produced in the fermentation increases in direct proportion to vessel volume, whereas the available area is proportional to its external surface.

The objective of using a plant of reduced dimensions can also be to study the behavior of an industrial one, the small one being a class of mock-up. The main target in this case would be to show the result of changes in the operational procedures or conditions, in a safer and less risky way than acting directly on the industrial installation.

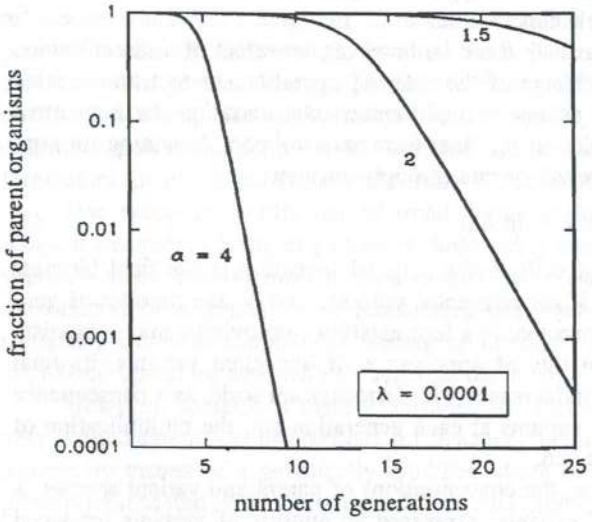


Figure 1 Effect of different growth of parent and variant strains.

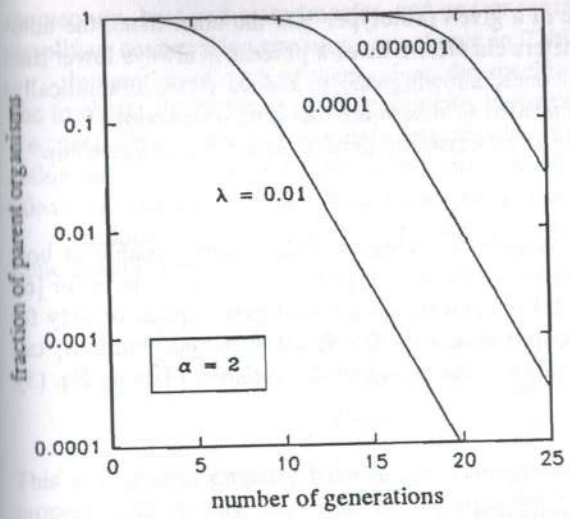


Figure 2 Effect of rate of appearance of variants.

We will concentrate mainly on the scale-up of mechanically stirred bioreactors, which are in most use currently. However, we must not forget that a biotechnological plant consists of more elements that a bioreactor and that all the components must be correctly scaled up. In the next section we present a method of general (and limited) application that constitutes the scientific base for developing, organizing, or understanding different shortcut methods.

III. SIMILARITY THEORY

The quantitative description of biological processes stumbles on the difficulty (even the impossibility) of writing down and solving the set of mathematical equations describing them, especially at a large scale. Using the techniques of dimensional analysis, one is able to obtain a certain degree of knowledge and also some guidance for design and scale-up [5].

The application of purely dimensional reasoning to a problem does not give a complete mathematical description; however, basic relationships between dependent and independent variables can be written down and, later on, they can be tested and completed through experimental measurements. These relationships are frequently very useful in deducing criteria for scale-up, e.g., what magnitudes have to be held constant in order that a physical model, or mock-

up, would be representative of a given prototype.* On the other hand, the number of dimensionless parameters characteristic of a process is always lower than the number of dimensional ones, allowing one to reduce (often dramatically) the number of experiments needed to determine the system behavior.

The basis of similarity is expressed in general as a lineal relationship:

$$m' = km \tag{3}$$

between the values of any magnitude, being an independent variable in both systems, in the mock-up (m) and in the prototype (m'); k is the scale factor [6]. Depending on whether Eq. (3) is valid for all the variables implied or only for some of them, similarity between systems will be total or partial. Similarity can be divided into classes according to the nature of the variables obeying Eq. (3):

Geometric similarity
Dynamic similarity
Thermal similarity
Mass (concentration) similarity
(Bio)chemical similarity

Each one is a prerequisite for the others, precisely in the order shown. So, geometric similarity is needed before one could study whether two systems are dynamically similar (similarity in the distribution of fluid velocities), etc.

In fact, the underlying idea in the concept of similarity is as follows: if two systems are described by the same differential equation, having the same contour constraints, then both systems will behave identically. In the case that the set of equations is not amenable to being solved mathematically, one can experimentally "see the solution," that is, how it behaves, in a mock-up. This approach starts from the fact that the equations describing a phenomenon consist of a series of terms having dimensional homogeneity. Each one of them represents a magnitude of interest. Then, if the equation is divided by one of the terms, the new equation will be dimensionless. This can also be done by defining a new set of variables, made dimensionless by giving their value relative to a fixed one or dividing all the equations by a term corresponding to a contour condition. Thus a dimensionless equation is obtained in which a series of moduli without dimensions appear. Their value will determine the system behavior and can be considered as the "natural" dimensionless numbers for that system. A series of frequently used moduli have evolved over the years that do not always coincide with those obtained by the procedure already described, although they are closely related. For a very general system in which there are simultaneously

Model is used with the meaning of mathematical model, where *mock-up* refers to an installation smaller than the one we want to design or analyze, which is also designated as *prototype*.

momentum, heat and mass transfer, and one or more biochemical reactions, the simplified conservation equations are shown in Table 1 [5].

Integration of each of them, given the specific contour constraints, allows one to obtain the different profiles: velocity, temperature, and concentrations for a system. From them one can calculate physical transport phenomena and reaction rates. This approach is scarcely applicable. From these dimensional equations it is possible to deduce their corresponding dimensionless versions.

An analysis of the equations in Table 1 reveals that all of them have the same general form:

$$\underbrace{\frac{\partial\psi}{\partial t}}_{\text{accumulation}} + \underbrace{(\mathbf{U}\cdot\nabla)\psi}_{\text{convective transport}} = \underbrace{\psi_G}_{\text{generation}} + \underbrace{\delta(\nabla^2\psi)}_{\text{gradient transport}} \tag{4}$$

This is a general property balance for incompressible conditions and constant properties. Obviously, each term in this equation has the same dimensions, regardless of which concentration or property is substituted for ψ. Therefore, the ratio between the various terms must be dimensionless and form some sort of dimensionless number or group.

A frequent starting point is to take the ratio of the convection term $(\mathbf{U}\cdot\nabla)\psi$ to the molecular transport term $\delta(\nabla^2\psi)$. The operator ∇ has units of reciprocal length. Let us replace the operator ∇ with $1/L$ (a reciprocal characteristic distance) and the velocity vector $\mathbf{U}$ with U, a characteristic velocity. Then the ratio of the second and fourth terms in Eq. (4) becomes

$$\frac{(\mathbf{U}\cdot\nabla)\psi}{\delta(\nabla^2\psi)} = \frac{U\psi/L}{\delta\psi/L^2} = \frac{UL}{\delta} \tag{5}$$

The term UL/δ, where U and L are generalized in concept to signify a velocity and a length appropriate to the experiment, represents the ratio of the convection transport to the molecular transfer. This very important dimensionless number in the case of momentum transfer becomes the Reynolds number, taking into account that now the diffusivity δ is the kinematic viscosity $\nu = \mu/\rho$:

$$\mathrm{Re} = \frac{UL}{\nu} = \frac{LU\rho}{\mu} \tag{6}$$

The Reynolds number is then the ratio of the convection to molecular momentum transport; it is also often referred to as the ratio of the inertial to viscous forces. A low Reynolds number means viscous forces (molecular transport) are large compared with inertial forces (convective transfer). What is more important is that its value will characterize this behavior in geometrically similar systems regardless of differences in size. Similar analyses can be done between

Table 1 Microscopic Balances with Respect to Fixed Coordinates

Vector notation	Cartesian tensor notation
Mass balance for ath species	
$\underbrace{\frac{\partial}{\partial t}\rho_a}_{\text{Accumulation}} = \underbrace{-(\nabla \cdot \mathbf{n}_a)}_{\text{Transport through surface}} + \underbrace{r_a}_{\text{Generation}}$	$\underbrace{\frac{\partial \rho_a}{\partial t}}_{\text{Accumulation}} = \underbrace{-\frac{\partial}{\partial x_i}(\rho_a v_i + j_i^a)}_{\text{Transport through surface}} + \underbrace{r_a}_{\text{Generation}}$
$(\mathbf{n}_a = \rho\omega_a v + j_a)$	
Mass balance (total)	
$\underbrace{\frac{\partial \rho}{\partial t}}_{\text{Accumulation}} + \underbrace{(\nabla \cdot \rho \mathbf{v})}_{\text{Transport through surface}} = 0$	$\underbrace{\frac{\partial \rho}{\partial t}}_{\text{Accumulation}} + \underbrace{\frac{\partial}{\partial x_i}(\rho v_i)}_{\text{Transport through surface}} = 0$
Momentum balance	
$\underbrace{\frac{\partial}{\partial t}(\rho v)}_{\text{Accumulation}} = \underbrace{-[\nabla \cdot \phi]}_{\text{Transport through surface}} + \underbrace{\sum_{a=1}^{a} \rho_a g_a}_{\text{Generation}}$	$\underbrace{\frac{\partial(\rho v_i)}{\partial t}}_{\text{Accumulation}} = \underbrace{-\frac{\partial}{\partial x_i}(\rho v_i v_j + \tau_{ij} + p\delta_{ij})}_{\text{Transport through surface}} + \underbrace{\sum_{a=1}^{a} \rho_a g_i^a}_{\text{Generation}}$
$(\phi = \rho v v + \tau + p\delta)$	
Energy balance	
$\underbrace{\frac{\partial}{\partial t}\rho\left(\hat{U} + \frac{v^2}{2}\right)}_{\text{Accumulation}} = \underbrace{-(\nabla \cdot \mathbf{e})}_{\text{Transport through surface}} + \underbrace{\sum_{a=1}^{a}(n_a \cdot g_a)}_{\text{Generation}}$	$\underbrace{\frac{\partial}{\partial t}\rho\left(\hat{U} + \frac{v^2}{2}\right)}_{\text{Accumulation}} = \underbrace{-\frac{\partial}{\partial x_i}\left(-k\frac{\partial T}{\partial x_i} + \sum_{a=1}^{a}\frac{n_a}{M_a}h_a + \tau_{ij}v_j\right)}_{\text{Transport through surface}} + \underbrace{\sum_{a=1}^{a} n_i^a g_i^a}_{\text{Generation}}$
$\left(e \simeq -k\nabla T + \sum_{a=1}^{a}\frac{n_a}{M_a}h_a\right)$	
Mechanical energy balance	
$\underbrace{\frac{\partial}{\partial t}\left(\frac{\rho v^2}{2}\right)}_{\text{Accumulation}} = \underbrace{-(\nabla \cdot \Gamma)}_{\text{Transport through surface}} + \underbrace{p(\nabla \cdot \mathbf{v}) + \tau{:}\nabla\mathbf{v} + \rho(\mathbf{v} \cdot \mathbf{g})}_{\text{Generation (consumption)}}$	$\underbrace{\frac{\partial}{\partial t}\left(\frac{\rho v^2}{2}\right)}_{\text{Accumulation}} = \underbrace{-\frac{\partial}{\partial x_i}(\tfrac{1}{2}\rho v^2 v_i + p v_i + \tau_{ij}v_j)}_{\text{Transport through surface}} + \underbrace{p\frac{\partial v_i}{\partial x_i} + \tau_{ij}\frac{\partial v_i}{\partial x_j} + \rho v_i g_i}_{\text{Generation (consumption)}}$
$\left(\Gamma = \frac{\rho v^2 v}{2} + pv + [\tau \cdot \mathbf{v}]\right)$	

Source: Ref. 5.

the different terms in all the equations, thus defining an important number of dimensionless moduli.

To see how this approach works and at the same time reveal its limitations, let us refer to a classical example, taken from Bird et al. [7]. It is desired to predict the vortex depth in a big stirred tank, unbaffled, in function of the stirrer speed. The sought approach is to build a mock-up in which the phenomenon will be observed.

After considering mass and momentum balances, as well as the boundary conditions, all of them in dimensionless form, the conclusion is reached that both systems will behave equally if the quantities

$$\underbrace{\frac{D_i^2 N \rho}{\mu}}_{\text{(Reynolds number)}} \quad \text{and} \quad \underbrace{\frac{D_i N^2}{g}}_{\text{(Froude number)}} \tag{7}$$

have the same value in two tanks that are geometrically similar, that is, have the same ratio of height to diameter and of height to impeller diameter.

Then imposing that $Re_1 = Re_2$ and $Fr_1 = Fr_2$ results in

$$\frac{N_2}{N_1} = \sqrt{\frac{D_{i_1}}{D_{i_2}}} \tag{8}$$

and

$$\frac{\mu_2}{\rho_2} = \frac{\mu_1}{\rho_1}\left(\frac{D_{i_2}}{D_{i_1}}\right)^{3/2} \tag{9}$$

The result is then that dynamic similarity, needed in order to have similar vortexes, is not possible using the same fluid in both tanks. If, for instance, a mock-up with lineal dimensions half of those of the big tank is desired, a liquid whose kinematic viscosity was $1/\sqrt{8}$ times the kinematic viscosity of the liquid to be stirred in the big tank would be needed. If we decide to use the same liquid, it is not possible to have equal Re and Fr in both tanks. The selection of equal Re implies that Fr will be higher in the small tank, giving a deeper vortex.

It can be concluded that strict similarity between two systems of biotechnical interest is, in fact, impossible. For instance, dynamic similarity, giving only velocity similarity, needs Re, Fr, and We to be the same in both systems. This would require simultaneously that the velocity variation is proportional to the scale factor to -1, $\frac{1}{2}$, and $-\frac{1}{2}$.

Another clear example is related to temperature control: heat is produced in all the fermentor volume and transferred through its surface. When its capacity is increased, the volume increases in proportion to the diameter cubed but the available area is augmented only to the square.

The consequence is that if a mock-up has to be built to study a biochemical process, thinking of a later prototype, it is limited to partial similarity, maintaining constant perhaps one or two dimensionless groups, and even that is not always possible. This reasoning is on the basis of the concept of rate-limiting mechanism or regime and its detection approach, regime analysis. The idea is that as processes normally take place through several steps, if one of them is sufficiently slower than the others it will control (limit) the overall process.

IV. SCALE-UP METHODS USED IN BIOREACTORS

As previously seen, it is not possible to apply similarity theory in a complete form to the scale-up of bioreactors. However, this theory has guided the formulation of a series of rules of thumb. In fact, many of them are actually the result of regime analysis. Based on the work of Sweere et al. [8] scale-up methods can be classified as

1. Fundamental method
2. Dimensional analysis
3. Rules of thumb
4. Scale-down approach/regime analysis
5. Trial and error

This classification is, of course, questionable and has some degree of arbitrariness, but it allows a certain organization of a complex reality. Next, some of the methods most often employed for scale-up are studied; most of them could be classified as rules of thumb evolved by means of dimensional and regime analysis.

A. Methods Frequently Used

The scale-up of geometrically similar bioreactors is carried out in a large proportion of the cases demanding equality in both systems of

Agitation power per volume
Volumetric oxygen mass transfer coefficient
Maximum shear stress
Mixing time

In fact, all these parameters are related to each other and also to the system variables, but in different forms. Thus very different results for the same problem can be obtained depending on the criteria employed. It is worth mentioning a pioneering paper by Oldshue [9] on the scale-up from an 80-L fermentor to another of 10,000. In this case the increase is 5 times in the tank diameter and 125 times in volume. In Table 2 the 1.0 in the second column denotes the

Table 2 Relationship Between Properties for Scale-Up

Property	Small scale, 80 L	Large scale, 10,000 L			
P	1.0	125	3125	25	0.2
P/V	1.0	1.0	25	0.2	0.0016
N_i	1.0	0.34	1.0	0.2	0.04
D_i	1.0	5.0	5.0	5.0	5.0
F_1	1.0	42.5	125	25	5.0
F_1/V	1.0	0.34	1.0	0.2	0.04
N_iD_i	1.0	1.7	5.0	1.0	0.2
Re_i	1.0	8.5	25	5.0	1.0

Source: Ref. 9.

magnitude that is kept constant in both tanks. Values for each property have been normalized to the values for the 80-L tank, so the "small-scale" column shows all 1.0. For example, scale-up based on constant P/V will increase the maximum shear rate by 70% ($ND_i = 1.7$) and increase the circulation time about threefold. On the other hand, scale-up based on constant circulation time (that is, F_1/V) requires 3125 times more power in the large tank.

The different dependences of important transport properties on agitator design make scale-up of agitated vessels a bit of an art. One must always try to select as a scale-up basis the transport property most critical to the performance of the bioprocess. This is not easy given the potentially sensitive and diverse responses of cells to each of the transport phenomena influenced by the mixer size and rotation speed.

When the scale-up is carried out on the basis of only one magnitude, it is necessary to inspect the situation in which other important characteristics are left. In this context the mixing time, maximum shear stress, and oxygen transfer are essential. Figure 3 is a qualitative representation of factors limiting the zone where satisfactory fermentor operation can be found when performing reactor scale-up [10].

The practical application of some of the most popular criteria is described in the following.

1. Constant Power per Unit of Volume

The power supplied to the system by agitation and aeration has a direct effect on the system hydrodynamic behavior and on mass transfer characteristics. A simplified explanation is that P/V determines the Reynolds number value, and this influences the degree of turbulence and hence mass transfer coefficients, especially oxygen transfer from gas bubbles. On the other hand, the lineal stirrer

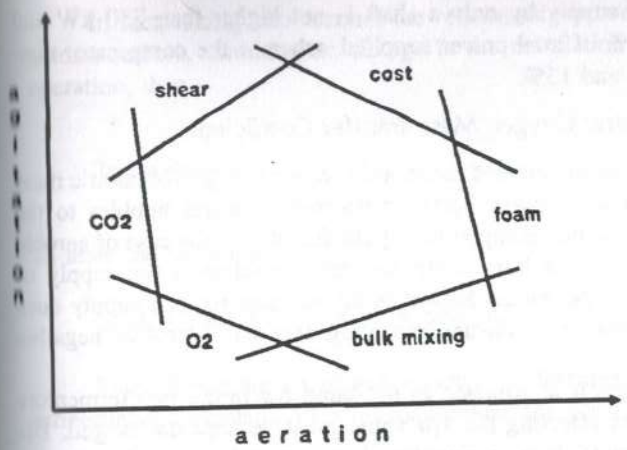


Figure 3 Operating boundaries for fermentation scale-up. (From Ref. 14.)

speed, πND_i, determines the maximum shear stress in the tank and also influence the stable size of flocs and gas bubbles, beside possible cell damage.

When the fluid in the tank is fully turbulent, then the power number* is constant, so

$$P \propto N^3 D_i^5 \tag{10}$$

as the volume will vary proportionally to D_i^3, then

$$\frac{P}{V} \propto N^3 D_i^2 \tag{11}$$

Imposing the equality of P/V in both tanks then implies that

$$N_2^3 D_{i_2}^2 = N_1^3 D_{i_1}^2 \quad \rightarrow \quad N_1 = N_2 \left(\frac{D_{i_2}}{D_{i_1}}\right)^{2/3} \tag{12}$$

This method was employed in the scale-up of the first penicillin fermentation plants. In general, fermentors had a power of 1 hp/100 gal (2 kW/m^3) with the rotation speed calculated according to the former equation. Vessels had also geometric similarity.

In fact, P/V has limited practical value that in large fermentors is about 5 kW/m^3 and is a magnitude that can have very different value depending on the

*The power number N_p is a dimensionless number defined as $N_p = P/(\rho N^3 D_i^5)$. It depends on the type of impeller and Re, becoming constant for Re > 10^4 approximately. P is the power consumed by the agitation in absence of gas bubbling.

working scale. Power supply by only a shaft is not higher than 750 kW and accounts for 85 to 95% of total power supplied, whereas the compressor contribution is between 5 and 15%.

2. Constant Volumetric Oxygen Mass Transfer Coefficient

Most often the magnitude maintained constant in scale-up is the volumetric mass transfer coefficient for the oxygen being transferred from gas bubbles to the microorganisms. This practice is supported by the fact that in the case of aerobic fermentations developed on a large scale, the main problem is the supply of enough oxygen from the gas phase. However, an adequate oxygen supply does not guarantee good mixing, a misunderstanding that has potential negative consequences.

The basic approach is as follows: as the same k_La in the two fermentors is desired, the variables affecting the k_La value must be kept unchanged. Dimensional analysis helps to develop equations in terms of dimensionless groups (through experiments or/and analysis of the equations of change). The equality of these moduli will assure the constancy of the k_La value.

In fact, there are many equations relating k_La with different variables, depending on the experimental data used to obtain the correlation (fermenter, impeller, medium composition, microorganism, etc.). For instance,

$$k_Lap = a_1\left(\frac{P_g}{V}\right)^{0.95}(v_s)^{0.67} \tag{13}$$

where a_1 is a function of v_s (for instance, $a_1 = 100$ when $v_s = 1$ m/min) or

$$k_Lap = 0.6\left(\frac{P_g}{V}\right)^{0.4}(v_s)N^{0.5} \tag{14}$$

or

$$k_Lap = 0.18(2.0 + 2.8N)\left(\frac{P_g}{V}\right)^{0.77}(v_s)^{0.67} \tag{15}$$

where P_g is the power consumption of liquid agitation in gassed systems, usually a fraction, 0.4–0.6, of the nongassed power. It can be determined from empirical correlations between P_g/P and the aeration number ($N_a = F/ND_i^3$) [11].

The use of one or another of these equations will give different results, even quite separate. For instance, the power required for a fermenter of 100 m^3, 4.0 m diameter, equipped with two turbine impellers of 1.6 m diameter, rotating at 120 rpm, an air flow rate of 20 Nm^3/min, and assuming a broth viscosity of 1 cp, is calculated by means of Michel-Miller and Oyama-Endoch correlations [11] giving 895 and 548 hp, respectively; that is a 60% deviation, which is not unusual [12].

In fact, taking into account the relationship between the power and Reynolds numbers, assuming no change in the correlation form with the scale of operation, then

$$\mathrm{Re}_2 = \mathrm{Re}_1\left(\frac{v_{s_1}}{v_{s_2}}\right)^{a_1/a_2} \tag{16}$$

To have the same k_La,

$$(k_La)_1 = \alpha\mathrm{Re}_1^{a_1}v_s^{a_2} \qquad (k_La)_2 = \alpha\mathrm{Re}_2^{a_1}v_s^{a_2} \tag{17}$$

Thus, fixing the lineal gas velocity v_{s_2} determines Re_2. If fluid physical properties are constant, all other magnitudes are also fixed; for instance,

$$h_2 = f(\mathrm{Re}_2, v_{s_2}) \qquad (P/V)_2 = f(N_2, \mathrm{Re}_2, Q_2) \tag{18}$$

$$N_2 = \frac{\mathrm{Re}_2\mu}{D_{i_2}^2\rho} \qquad \dot{\gamma}_2 = \dot{\gamma}_1\left(\frac{N_2}{N_1}\right) \tag{19}$$

Some of the problems coupled to the procedures just seen are the following:

- In the case of non-Newtonian fluids, the hydrodynamic parameters change along the fermentation, producing a variation in the applicable scale-up relationships.
- Many available correlations depend on the work scale.
- Physical properties (σ, μ) change during the fermentation in a form that, in general, is scale dependent.
- Gas lineal velocity, v_s, at the large scale can be more than an order of magnitude that at the small scale (even if the value of VVM is lower).
- It is almost impossible to maintain the effect of fermentor internals scale independent.
- The effect of surface tension is strongly dependent on the scale.
- There is a lack of reliable correlations.

Obviously, there is no strict necessity to maintain geometric similarity and often it is not useful to do so. The gain in flexibility is at the price of losing more reliability in using the correlations.

Taking into account the latter points, it is not surprising that the usual methods have an important component of art and empiricism. One of the more used practices is based in maintaining constant k_La and the maximum shear rate. In order to apply this technique successfully the following are needed [13]:

- A reliable method for calculating the power required in order that k_La and the maximum shear rate have the desired value
- A reliable method of measuring k_La
- Ability to predict the effect of some factors such as the increase of pressure and superficial aeration
- Ability to predict how the spatial distribution of important variables will change when changing the scale of operation

Lilly [14] emphasizes checking three specific issues once the scale-up has been performed:

(1) Bulk mixing: the danger of thinking that good oxygen transfer warrants good mixing has already been mentioned. This is not necessarily true. In general, for larger scales the mixing time is larger. Some typical values from Lilly [14] are:

Volume (m^3)	Mixing time (s)
0.01	1 to 5
0.05	2.2
1.0	20
1.8	29
60	67
100	100
120	140

It has been observed that in fermentors with volumes between 16 and 100 L, stagnant zones are formed when the mixing time is greater than 15 min and cells suffer damage when it is less than 1.5 min. In this case a mixing time between 2 and 8 min is recommended. Poor mixing in the fermentor can cause severe control problems: formation of concentration and temperature gradients. If acid or base is being added for pH control and the inlet is over the free surface or far away from the impeller, microorganisms can be subjected to damaging pH oscillations.

(2) Shear stress: In the absence of a good knowledge of the interaction between shear stress and morphology, especially in the case of micelle-forming microorganisms, the impeller maximum lineal velocity (tip velocity) is used as a rule of thumb in scale-up. Surprisingly, in most of the industrial fermentors tip velocity is always between 5 and 6 m/s, regardless of the implied process.

(3) Oxygen transfer: a series of additional effects must be taken into account, for instance, that the height/diameter ratio is larger on the industrial scale than in the laboratory; the gas flows upward, transferring oxygen and being enriched in CO_2. There is also a noticeable hydrostatic head, typically of 1 atmosphere. If mass transfer is more rapid than vertical mixing, a vertical gradient of oxygen concentration will be developed. Also CO_2 profiles can cause problems, especially in the upper part and when the fermentor is working under overpressure. Radial gradients can also be formed, in particular with high-viscosity broths, in which the zone with good mass transfer rate can be limited at the impeller proximity. In that event, microorganisms must be recycled to this zone before the oxygen concentration in the fluid near them falls below a critical value. It is important to bear in mind that the oxygen solubility is very low (< 10 ppm) and that its supply to cells can become critical in a short time (< 30 s) [15].

3. An Algorithm to Scale Up Stirred Fermenters

Hubbard [16], after an analysis of scale-up problems, in particular those associated with non-Newtonian fluids, proposes a scale-up methodology consisting of a combination of the different published procedures that are critically revised in his paper.

Once a fermentation has been accomplished successfully on the laboratory scale and the criterion for success has been established, scale-up calculations can be made. The procedure recommended is shown in Fig. 4.

The scale-up methods described involve measuring only the rheological properties of the broth, the system dimensions, and the directly controlled variables Q and N. k_La is not a directly controlled variable even though it is crucial for the successful operation of the fermentor. Applying the methods is not completely straightforward. k_La can be calculated directly for the laboratory-scale fermentor. Calculating Q and N for the plant-scale fermentor usually involves some iteration, because Q and N appear in more than one factor in the mass transfer coefficient correlations. If the impeller speed is to be maintained constant as in method 2, N for the plant scale can be calculated directly, and the effective viscosity can be estimated from the rheological equation (power law, for instance). Then Q can be calculated from the appropriate correlation for k_La. Since Q appears implicitly, iterative calculations are required. If either VVM or v_s is to be maintained constant as in method 1, Q for the plant scale can be calculated directly. Then N and effective viscosity for the plant must be determined simultaneously from the rheological equation and the appropriate k_La correlation. If (Q/ND_i^3) is to be maintained constant (method 1), a trial value for Q for the plant scale must be chosen. Then the calculations can proceed as in method 2. The value of Q calculated is compared to the trial value, and the trial value is modified until convergence is attained.

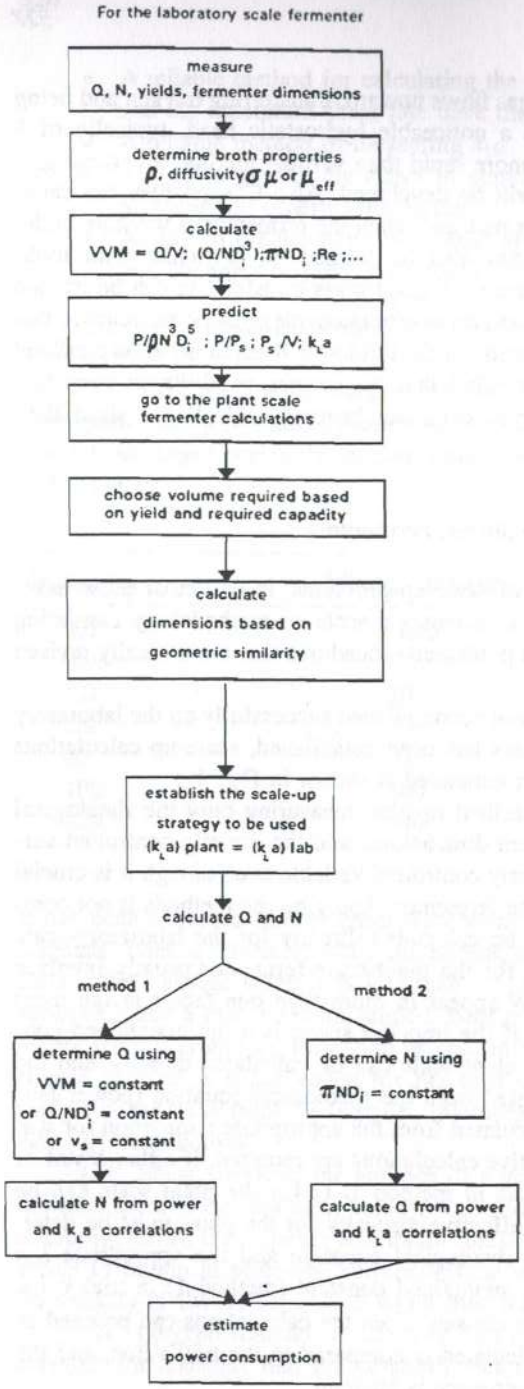


Figure 4 Hubbard's methodology for scale-up. (From Ref. 16.)

B. Regime Analysis and Scale-Down

1. Methodology

This method is based on performing a regime analysis of a production scale bioreactor in order to build a laboratory scale bioreactor in which the mechanisms controlling the process are essentially the same. Then, the process can be optimized at the laboratory scale and finally transferred to the production scale. This method is reviewed in Sweere et al. [17] and its conception is shown schematically in Fig. 5.

Regime is used in this context to indicate the rate-controlling mechanism. Thus, a kinetic regime means that the bioreactor performance is dominated by kinetic processes (cell growth and product formation) rather than physical transport phenomena (heat exchange, oxygen transfer), which are more rapid in this case. If the rate is controlled by more than one process, then there exists a mixed rate-controlling mechanism.

The approach consists essentially of answering the following three questions: Is there a rate-controlling mechanism? Which is it? Will it be a change in regime when scaling up? The calculation of the characteristic times for the different processes is a particularly revealing way to answer these questions when mass transfer and conversion processes are involved. The characteristic time is inversely related to the rate of a mechanism. The comparison of time constants for conversion, mixing, flow, diffusion, mass transfer, and residence time can give a good idea of which is the rate-controlling mechanism. Some of

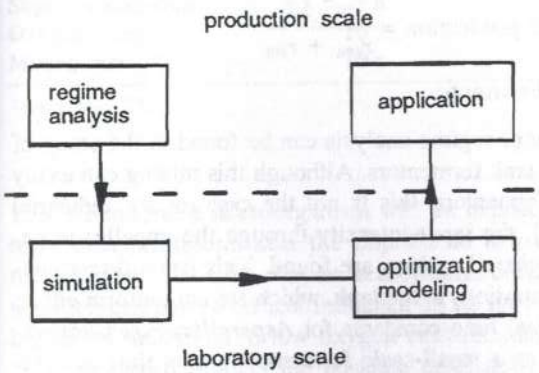


Figure 5 Regime analysis and scale-down method for the scale-up of bioreactors. (From Ref. 17.)

the main time constants are defined as follows, according to Oosterhuis [18]:

$$\text{Mixing time: } t_m = \frac{V}{2.6ND^3}$$

(Re > 5000, Rushton impeller)

$$\text{Conversion time: } t_c = \frac{C}{r}$$

$$\text{Diffusion time: } t_D = \frac{L^2}{D_{eff}}$$

$$\text{Liquid residence time: } \tau = \frac{V}{F}$$

$$\text{Gas residence time: } \tau_G = \epsilon \frac{V}{F}$$

$$\text{Mass transfer time: } t_{mt} = \frac{1}{k_L a}$$

$$\text{Oxygen consumption } (C_{O_2} > K_{O_2}): t_{oc} = \frac{C_{O_2}}{r_{O_2}^{max}}$$

$$\text{Sugar consumption } (C_s > K_s): t_{sc} = \frac{C_{s_0}}{r_s^{max}}$$

$$\text{Cell growth: } t_g = \frac{1}{\mu_{max}}$$

$$\text{Heat transfer: } t_{ht} = \frac{V\rho C_p}{h_b A}$$

$$\text{Heat production} = \frac{\rho C_p \Delta T}{r_{HM} + r_{HS}}$$

2. Application to a Stirred Fermentor

One example of the application of regime analysis can be found in the study of the degree of mixing in stirred tank fermentors. Although this mixing can easily be ideal for laboratory scale fermenters, this is not the case for the industrial scale. As shown in Fig. 6 [19], the input intensity through the impeller is unequally distributed and poor agitated regions are found. This has a direct consequence for the oxygen concentrations in the tank, which are not uniform either. Taking as an example the typical time constants for *Aspergillus niger* fermentation [20], shown in Table 3, in a small-scale fermentor mixing time is comparable to oxygen transfer time (about 10 s). However, moving to large-scale fermentors increases mixing time to 100 s and oxygen transfer time only to

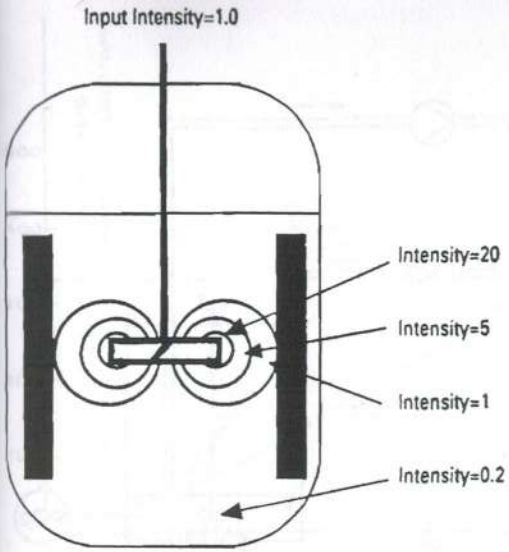


Figure 6 Scheme of local relative turbulence intensities in a stirred fermentor. (From Ref. 19.)

Table 3 Typical Time Constants for *Aspergillus niger* Fermentation

Time constant	Small scale	Large scale
Cell growth	5 h	5 h
Sugar consumption	5 h	5 h
Oxygen transfer	10 s	18 s
Mixing time	10 s	100 s

Source: Ref. 20.

18 s. Therefore, a microorganism will be exposed to cycles ranging from high oxygen concentration near the impeller to low oxygen concentration near the reactor wall. The time that an aerobic microorganism is exposed to the latter situation can have a critical influence on its growth and product yields, depending on its sensitivity to low oxygen concentrations.

One way to study the possible deleterious effects of scale-up on the behavior of a sensitive aerobic microorganism is to scale down the industrial scale fermentor to two connected laboratory scale CSTR fermentors (Fig. 7), one of

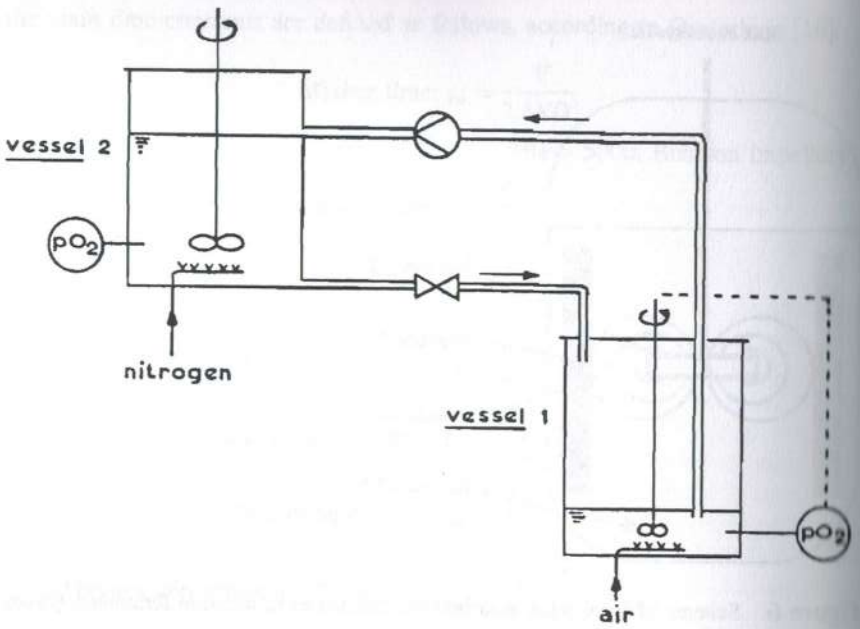


Figure 7 Setup for two interconnected CSTR systems. (From Ref. 18.)

them aerated and the other sparged with nitrogen, thus simulating the two different microenvironments that the microorganisms will experience in the industrial scale fermentor. Such an approach has been applied successfully to the analysis of scaling up gluconic acid production by *Gluconobacter oxidans* [18]. Figure 8 shows some of the experimental results obtained in this case. The evolution of gluconic acid concentration in the medium with time is given for three situations: experiment 1, a single vessel with 30% air saturation level; experiment 2, two connected vessels, both at 30% air saturation; experiment 3, two connected vessels, one of them at 30% saturation and the other at 0%. From the marked decrease in gluconic acid production rate for experiment 3, it is evident that the two interconnected vessels constitute an adequate system for studying the restrictions imposed by poor aerated zones in production scale fermentors.

Another possibility is to scale down the industrial stirred reactor to a tubular loop reactor, with a highly aerated zone (riser) and a poorly aerated one (downcomer), as depicted in Fig. 9. The application of such a system to simulate the circulation loops in CSTR reactors has been studied in the production of pullulan by *Aureobasidium pullulans* [20]. Figure 10 shows the effect of differ-

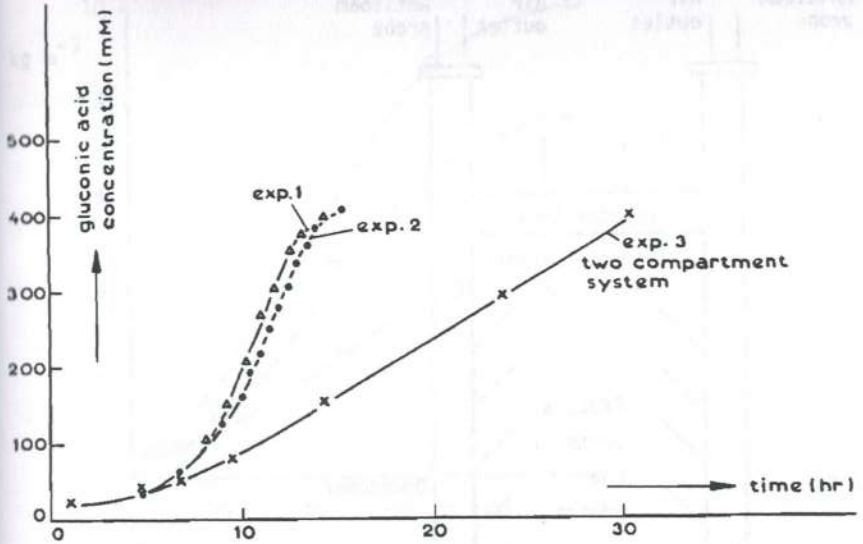


Figure 8 Gluconic acid production for different experiments as referred to in the text. (From Ref. 18.)

ent circulation times on the pullulan production in a batchwise fermentation. Clearly, as the circulation time increases the activity of the cells decreases, as a consequence of being exposed for longer periods to low oxygen concentrations. Therefore, this is a promising system for scaling down the effect of circulation loops in industrial scale CSTR and studying and optimizing the different parameters influencing the fermentation before its final scale-up.

3. Application to Columnar Fermentors

A different type of reactor to which the regime analysis and scale-down methodology has been applied is the columnar reactor, in particular bubble columns, airlift, and fluidized-bed fermentors. Although airlift reactors, for example, have been extensively used at the industrial scale and many papers in the literature describe their operation, the important geometric differences between reactors and in the physical properties of the fermentation media make it very difficult to generalize its behavior [21]. Therefore, in this case there are no accurate correlations to describe the effect of scale-up on the hydrodynamic characteristics of the fermentor (gas holdup, mixing) or on the oxygen transfer rate. Moreover, one general trend of this type of fermentors is that, opposite to stirred tanks, there exist close interrelationships between the different performance

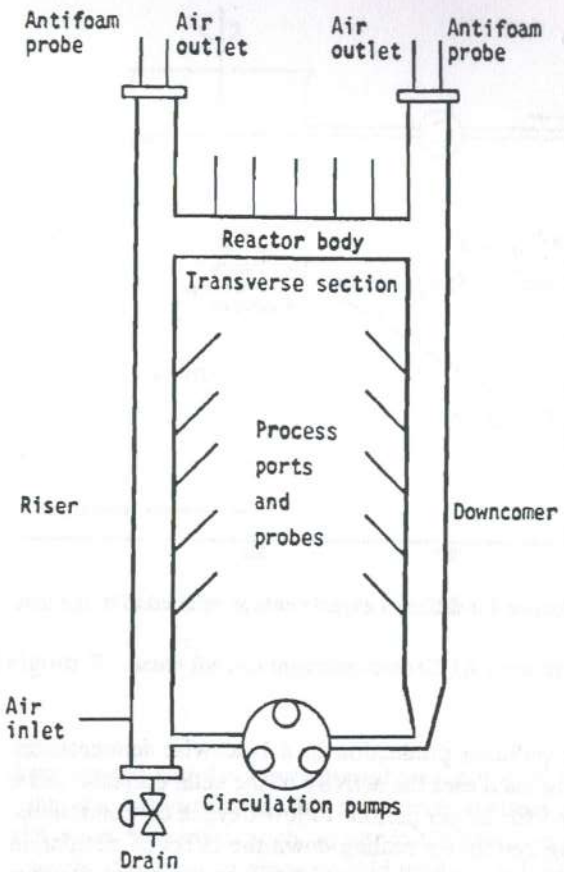


Figure 9 Scheme of a tubular loop reactor. (From Ref. 20.)

characteristics [22]. As an example, Fig. 11 shows the influence of liquid velocity on different aspects of the behavior of an airlift reactor.

This complex situation makes it more difficult to derive clear and straightforward criteria for scale-up. As an example, bubble columns can be scaled up on the basis of constant superficial gas velocity (v), as this quantity governs the gas-liquid mass transfer in these reactors. However, for mixing of the liquid content in the column, v is not the only relevant parameter. In fact, it has been demonstrated [23] that when a bubble column is called up by a factor of 10, the superficial gas velocity has to be increased by a factor of 32 in order to have the same liquid mixing time in both reactors. Another important point to

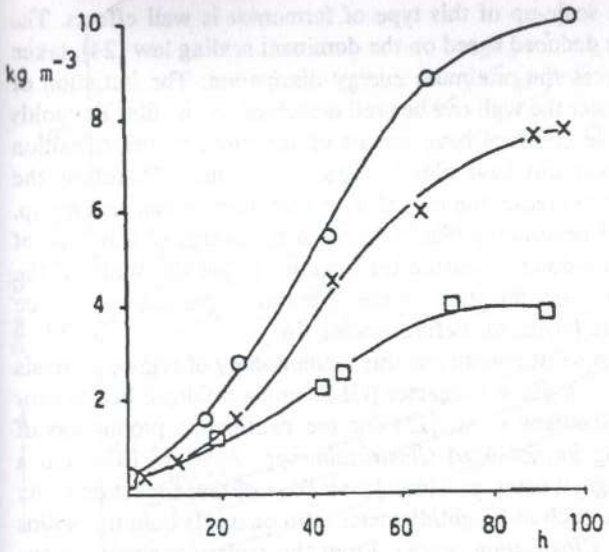


Figure 10 Production of pullulan in a loop reactor at circulation times of 15 s (○), 30 s (×), and 62 s (□). (From Ref. 20.)

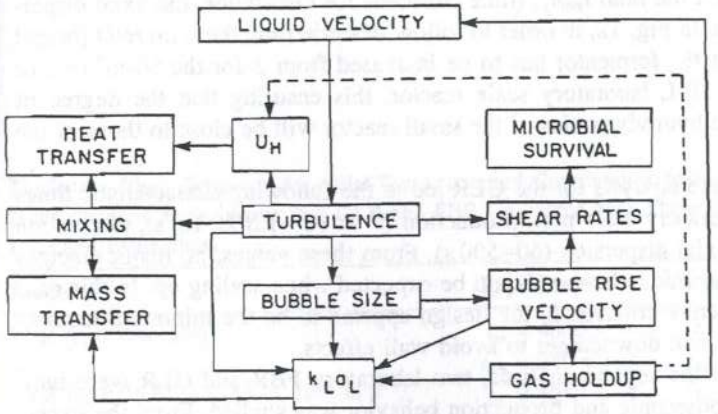


Figure 11 Interrelationships between the liquid circulation velocity and other performance characteristics in an airlift fermentor. (From Ref. 22.)

take into account in the scale-up of this type of fermentor is wall effects. The effect of scale-up can be deduced based on the dominant scaling law [24], taken as the length that produces the minimum energy dissipation. The initiation of turbulence in the liquid near the wall can be well described by the film Reynolds number (Re_f). Small-scale columns have values of Re_f close to the transition one (1160), and larger columns have clearly higher Re_f values. Therefore, the value of Re_f can be used to predict the initiation of turbulence when scaling up.

The high degree of uncertainty that is found in the design of this type of fermentor makes the scale-down approach interesting, in that the study of the different variables and the optimization of the operational parameters can be done in a laboratory scale fermentor before scaling up.

An exhaustive example of how to use this methodology of regime analysis and scale-down to scale up a gas loop reactor (GLR) and a fluidized-bed reactor (FBR) is presented in Shoutens et al. [25] for the continuous production of isopropanol-butanol using immobilized *Clostridium* spp. A 50-m^3 FBR and a 65-m^3 GLR are first designed using previous knowledge of these reactors to fix their main characteristics, such as height/diameter ratio or solids hold-up (solids meaning the Ca-alginate *Clostridium* beads). From the regime analysis for the FBR, characteristic times for production (7.2×10^3–1.2×10^4 s), liquid circulation (600–1200 s), and axial dispersion (6.9–69×10^5 s) are determined. Also, time constants for diffusion and reaction inside the Ca-alginate beads are not taken into consideration, as previous studies of a CSTR fermentor showed that effectiveness factors in this case are close to unity; thus diffusion is faster than reaction. From the values of the characteristic times it can be deduced that mixing in the FBR is caused mainly by circulation. In order to have the same regime in full-scale and laboratory scale reactors, the scale-up criterion used is to keep constant the ratio t_{cir}/t_{ax} (time constants for circulation and axial dispersion). As shown in Fig. 12, in order to follow this criterion, the ratio H/D (height to diameter) of the fermentor has to be increased from 3 for the 50-m^3 reactor to 25 for the 10-L laboratory scale reactor, this ensuring that the degree of mixing and the hydrodynamics of the small reactor will be close to those of the large reactor.

The regime analysis for the GLR led to the following characteristic times for a 10-L laboratory scale unit: production (9×10^3–1.8×10^4 s), circulation (5–20s), and axial dispersion (60–500 s). From these values, no major changes in the hydrodynamic behavior should be expected when scaling up. In this case the most restrictive criterion in the design appears to be the minimum requirements for riser and downcomer to avoid wall effects.

Based on the regime analysis, two laboratory FBR and GLR were built and their hydrodynamic and production behavior was studied. From the experiments, the real time constants could be compared to those estimated in the previous regime analysis, as shown in Table 4.

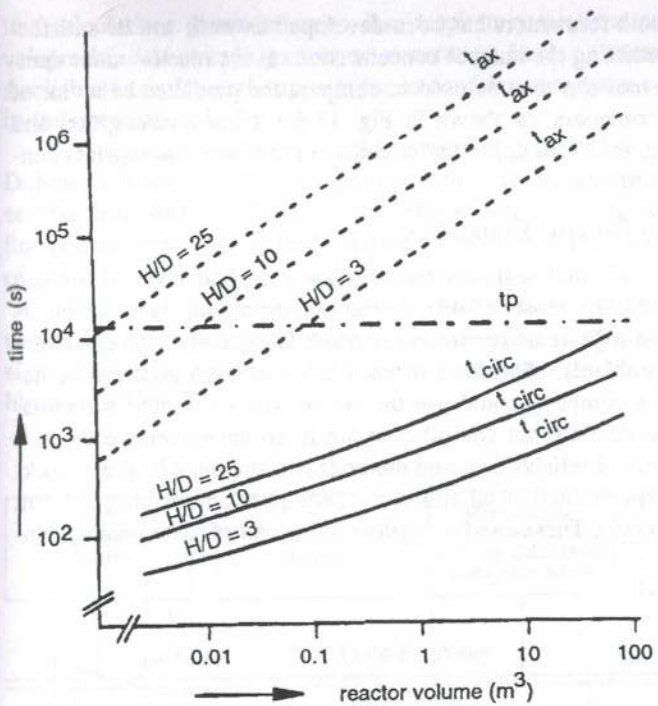


Figure 12 Estimated values of the characteristic times in an FBR. t_{ax} = axial dispersion time; t_{circ} = liquid circulation time; t_P = product formation time. (From Ref. 25.)

Table 4 Time Constants for Axial Dispersion and Circulation Obtained from Hydrodynamic Experiments in a GLR and FBR Compared with Those from Literature Data and Estimations

Reactor	Experiments	Literature estimated
FBR (ϵ_s = 44–45%)	t_{ax} = 4000 s	t_{ax} = 20000 s
	t_{circ} = 250 s	t_{circ} = 200 s
GLR (ϵ_s = 35%; $v_s = 2.5 \times 10^{-2}$ m s^{-1})	t_{ax} = 1250 s	t_{ax} = 700–1000 s
	t_{circ} = 10 s	t_{circ} = 20–40 s

Source: Ref. 25.

A model for both fermentors has been developed as well, and its satisfactory behavior for predicting the butanol concentrations at the reactor outlet demonstrated [24]. This tool can then be used to compare the predicted behavior of the two full-scale fermentors, as shown in Fig. 13 for sugar consumption and productivity, helping, for example, in the selection of the most appropriate configuration for that particular case.

4. Scale-Up Using On-Line Computers

It has already been said that scale-up methods have a good dose of art and empiricism. Obviously, the most reliable source of information is experiments performed on existing large-scale fermentors, but this is expensive and can cause serious production problems. Moreover, it tends to discourage innnovative designs. Alternatively, a company could use the stored data on similar fermentations, but often these data are not compiled or are in an unrecoverable form.

The development of reliable scale-up methods can and must be done working on appropriate experimental installations, well equipped and making efficient use of modern computers. These can be employed for control, data logging, on-

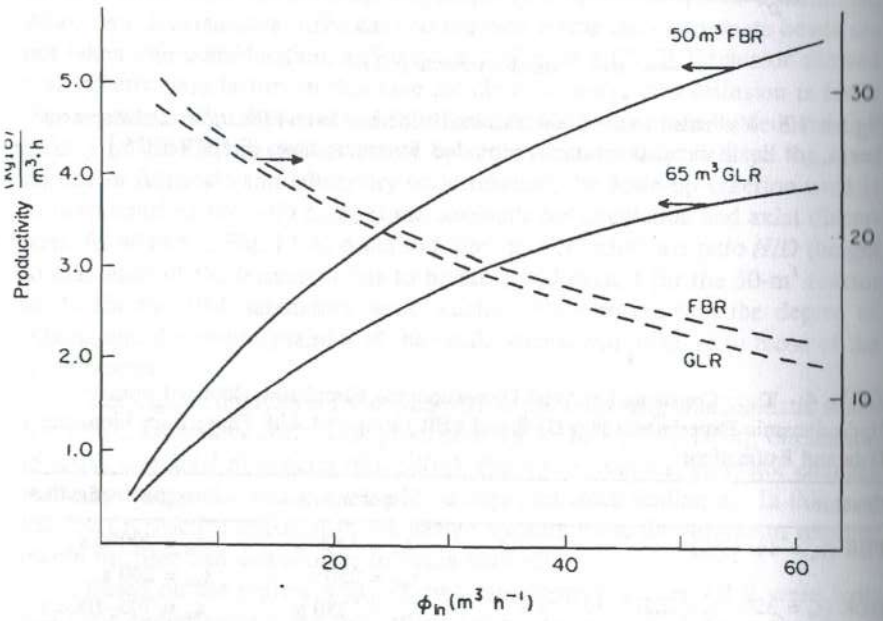


Figure 13 Reactor productivity and sugar consumption as a function of feed flow rate for the full-scale FBR and GLR. (From Ref. 25.)

line calculations (mass balances, k_La, respiratory quotient, etc.), correlation development, data storage, and report generation. Such a system can indeed supply a reliable basis for scale-up, similar in confidence to those routinely performed in the chemical industry.

As an example, let us consider the pilot plant built by Merck, Sharp and Dohme in Rahway, N.J. [26] equipped with a control computer system in which several processes have been successfully scaled up taking advantage of the on-line determination of key fermentation parameters. Figure 14 shows the structure of the data acquisition and control system.

This plant has been used to perform the scale-up of efrotomycin production [27]. The experiments were conducted using fermentors of three different sizes, of similar geometry, baffled and equipped with six-bladed Rushton turbines (see Table 5).

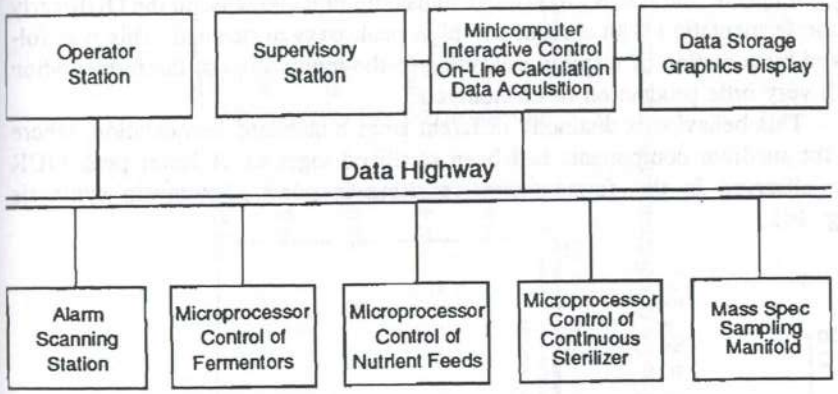


Figure 14 Distributed digital control system. (From Ref. 26.)

Table 5 Geometry of Fermenters

Property	Tank size (m³) 0.8	1.9	19
Number of impellers	2	3	4
Tank diameter (m)	0.81	1.07	1.98
Impeller diameter (m)	0.30	0.42	0.69
Ratio *T/D*	0.38	0.39	0.35
Range of rpm	33–338	22–230	15–155

Source: Ref. 27.

One of the main concerns in scale-up of fermentations with heat-sensitive medium compositions using batch sterilization is the effect of the excessive heat stress on the medium due to the longer heat-up and cool-down time needed for the larger tanks. These sterilization conditions can be simulated at the smaller-scale fermentors in the pilot plant and with the use of on-line computer-generated calculations, an empirical model of the metabolic behavior of the fermentation on scale-up can be prepared. For the efrotomycin fermentation these changes in sterilization conditions make a dramatic difference in process performance (see also Section V.A).

An overall batch sterilization cycle time of heat-up and cool-down at the 1.9-m^3 scale is 70 min, compared with 240 min at the 19-m^3 scale fermentor (Fig. 15).

Pilot plant trials at the 0.8- and 1.9-m^3 fermentors using a medium with separately sterilized glucose to simulate anticipated sterilization conditions on the production scale showed dramatic results. On-line computer calculations for oxygen uptake rate (OUR) showed an unusually rapid increase in the OUR early in the fermentation with a relatively high peak oxygen demand. This was followed by cessation of respiration leading to the termination of the fermentation with very little production of efrotomycin.

This behavior is distinctly different from a standard fermentation, where all the medium components had been sterilized together. A lower peak OUR was observed in this fermentation, resulting in good efrotomycin synthesis (Fig. 16).

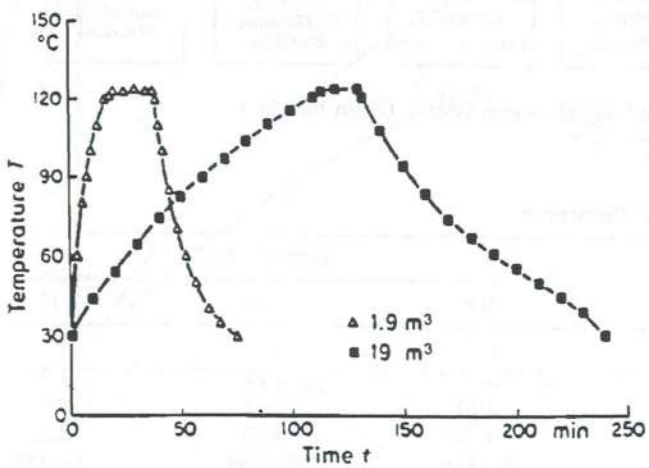


Figure 15 Temperature-time profile for a batch sterilization. (From Ref. 27.)

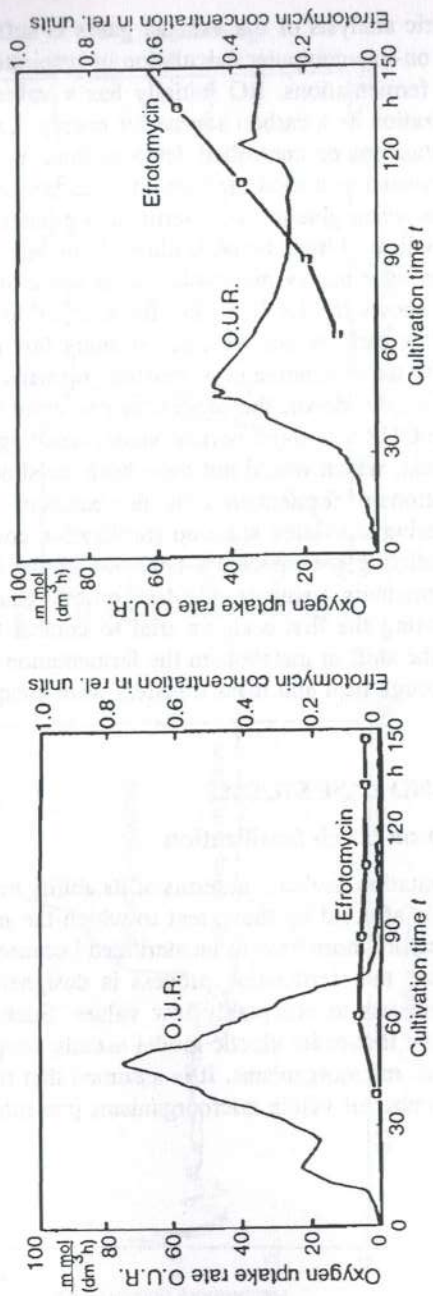


Figure 16 Oxygen uptake rate versus time for two different fermentation media. Left: separate glucose sterilization. Right: glucose sterilized with the rest of the medium. (From Ref. 27.)

Mass spectrometric analysis of the exhaust gases is sufficiently accurate to provide meaningful on-line computer calculation of respiratory quotient during the course of the fermentations. RQ initially has a value of around 1.0, typical of glucose utilization as a carbon source for energy. Later it drops to a value of 0.7 for the standard or controlled fermentation, a value typical for utilization of oil (it was used as a seed medium) as a carbon source for energy (Fig. 17, left). However, when glucose was sterilized separately the RQ profile showed that the metabolism shifted to utilization of oil but was not able to sustain it for long, showing runaway metabolism after some time.

Figure 17, right, shows the OUR profile for a 19-m^3 fermentation. The high OUR observed at its early stages indicates strongly that the fermentation is rapidly moving toward the phenomenon of runaway fermentation. In order to force the metabolism to slow down, the process temperature was lowered by 2.5°C. This lowered the OUR to a more normal value, resulting in a reasonable production of efrotomycin, which would not have been possible otherwise.

Computer calculations of fermentation kinetics based on data from small pilot plant fermentors using simulated scale-up sterilization conditions provide a useful model for predicting the metabolic behavior of the fermentation on scale-up to the larger fermenters. Using on-line data, process changes were made based on this model during the first scale-up trial to control the metabolism. Without knowledge of the shift in metabolism the fermentation would not have been successful even though heat and mass transfers were adequate.

V. APPLICATIONS AND CASE STUDIES

A. Effect of Scale-Up on Batch Sterilization

The quality of the fermentation medium, in terms of its ability to support growth and product formation, is affected by the extent to which the medium is overheated. Large batches require more time to be sterilized because there are more contaminants. In practice, the sterilization process is designed to reduce the probability of lack of success to acceptably low values. Such a probabilistic approach follows from the first-order kinetic model usually adopted to describe the thermal destruction of microorganisms. It is assumed that the rate of death is proportional to the number of viable microorganisms present, i.e.,

$$\frac{dX}{dt} = -k_r X \tag{20}$$

Integrating,

$$\ln X = \ln X_0 - \int_0^t k_r \, dt \tag{21}$$

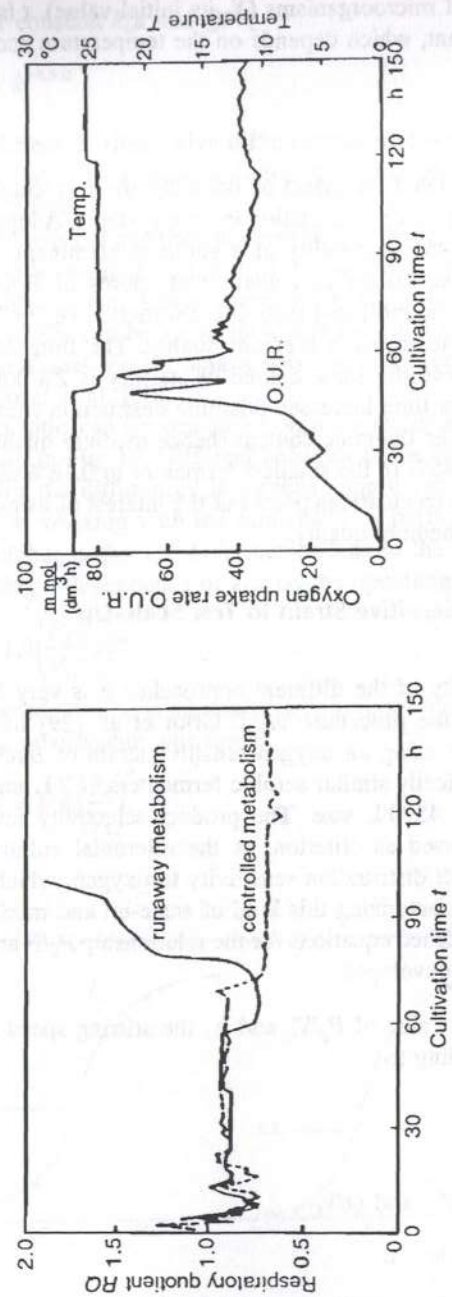


Figure 17 Respiration quotient and oxygen uptake rate versus time: control of metabolism using a step-down change in temperature. (From Ref. 27.)

where X is the number of microorganisms (X_0 its initial value), t is time, and k is the thermal rate constant, which depends on the temperature according to

$$k = A \exp\left(-\frac{E_a}{RT}\right) \tag{22}$$

Humphrey [28] has calculated the effect of doubling the fermentor volume on medium quality, assuming batch sterilization in both systems. Adopting a typical design value of 10^{-3} for the probability of a single contaminant remaining in the tank after sterilization, taking as contaminant spores of *Bacillus stearothermophilus* ($E_a \sim 100$ kcal/mol) and thiamine destruction ($E_a \approx 22$ kcal/mol) as the model compound to measure medium quality. The time needed in the largest fermentor to achieve the same degree of sterility is 2% longer than in the smallest, but this extra time increases thiamine destruction from 99.45% to 99.82%; in other words, the thiamine content (hence medium quality) has been reduced by 60%, from 0.45% in the smallest fermentor to 0.18% in that having double the volume. These calculations point out the interest of using continuous sterilization to maintain medium quality.

B. Use of an Oxygen-Sensitive Strain to Test Scale-Up Procedures

In order to test the validity of the different approaches it is very important to have criteria sensible to the procedure used. Griot et al. [29] have analyzed several scale-up strategies using an oxygen-sensitive strain of *Bacillus subtilis* in various nearly geometrically similar aerobic fermenters of 7 L and 150 L size and of 45 L, 450 L, and 4500 L size. The product selectivity ratio (acetoin/butanediol, Ac/Bu) was used as criterion, as the microbial culture employed showed an unusual product distribution sensitivity to oxygen, which makes it a suitable parameter for characterizing this kind of scale-up and mixing studies.

Using different published equations for the relationship P_g/P and k_La, three criteria for scale-up were developed:

1. Using the same value of P_g/V_L and v_s, the stirring speed depends on the scale according to

$$\frac{N_{\text{large}}}{N_{\text{small}}} = k^{-0.719} \tag{23}$$

2. For constant P_g/V_L and Q/V_L

$$\frac{N_{\text{large}}}{N_{\text{small}}} = k^{-0.632} \tag{24}$$

3. For constant k_La and Q/V_L

$$\frac{N_{\text{large}}}{N_{\text{small}}} = k^{-0.932} \tag{25}$$

These three different criteria give different ratios of stirrer speeds, 0.576, 0.616, and 0.489, taking into account a factor of 10 in volume, hence a linear scale factor of $k = 2.154$.

In a first series of experiments, working with the 7- and 150-L total volume fermentors (with working volumes of 5 and 100 L, respectively), the product ratio was plotted versus stirrer speed using a constant value of $Q/V_L = 0.42$ VVM. In Fig. 18 it can be appreciated that the curves have the same shape but shifted on the N axis. Power uptake was not measured but calculated using the equation of Joshi et al. [30].

Product ratio can be compared on a power uptake basis with Q/V_L constant, as shown in Fig. 19. Different behavior in the two tanks can be observed. The reason for the differences in oxygen supply can perhaps be found in the gassing rate, as working with the same Q/V_L, superficial gas velocity is almost three times higher in the 150-L fermentor than in the 7-L one.

Assuming a dependence of k_La on the operating parameters such as

$$k_La = 1.2\left(\frac{P_g}{V_L}\right)^a v_S^{0.6} \tag{26}$$

and that the product ratio is approximately proportional to OTR, Ac/Bu = $k'k_La$, the following relationship was tested:

$$\frac{\text{Ac}}{\text{Bu}} v_S^{0.6} = k'K\left(\frac{P_g}{V_L}\right)^a \tag{27}$$

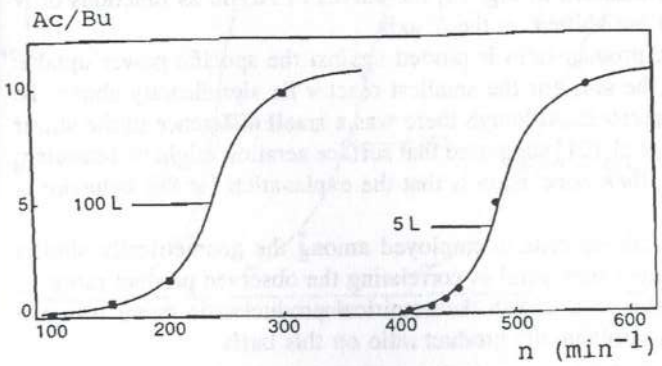


Figure 18 Dependence of product ratio on stirrer speed. (From Ref. 29.)

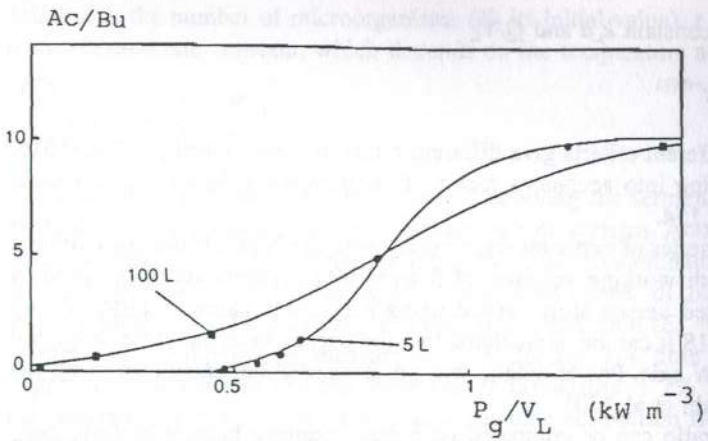


Figure 19 Power uptake per unit volume as scale-up criterion using Q/V_L = 0.42 VVM. (From Ref. 29.)

Plotting this relation on log-log axes, straight lines were obtained, but with different slopes (Fig. 20). Constant impeller tip speed also does not correlate the results, although better results were obtained using the empirical relation N^3D_i. An important weakness in the interpretation of these data is that power uptake was not experimentally measured but calculated using the Joshi et al. equation.

In another series of experiments, using fermentors with total volumes of 45, 450, and 4500 L (25, 267, and 2500 L working volumes, respectively), power uptake was measured and runs were performed using a constant superficial gas velocity. As shown in Fig. 21, the curves of Ac/Bu as functions of N are similar in shape but shifted on the N axis.

In Fig. 22 the product ratio is plotted against the specific power uptake. It can be seen that the data for the smallest reactor lie significantly above the data for the largest reactors. Although there was a small difference in the stirrer geometry and Griot et al. [31] suggested that surface aeration might be important in small fermentors, their conclusion is that the explanation for this behavior is not known.

None of the scale-up criteria employed among the geometrically similar reactors was completely successful in correlating the observed product ratios. It was, however, possible to establish the empirical product ratio curve for each scale and accurately correlate the product ratio on this basis.

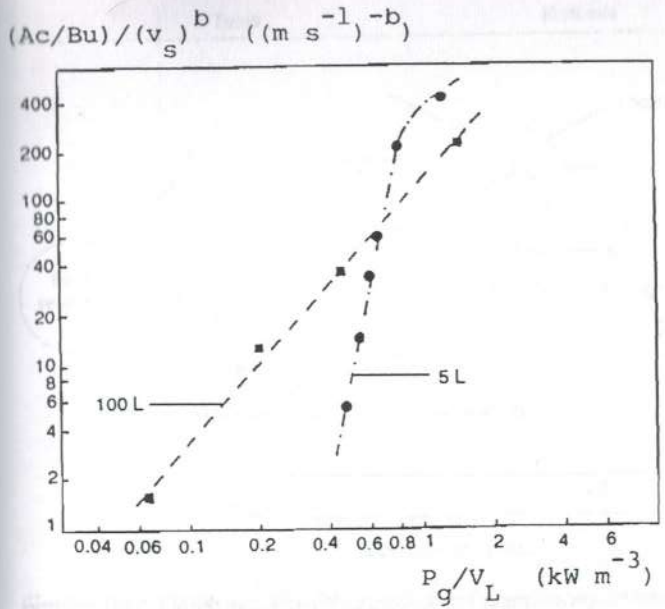


Figure 20 Calculated k_La as scale-up criterion. (From Ref. 29.)

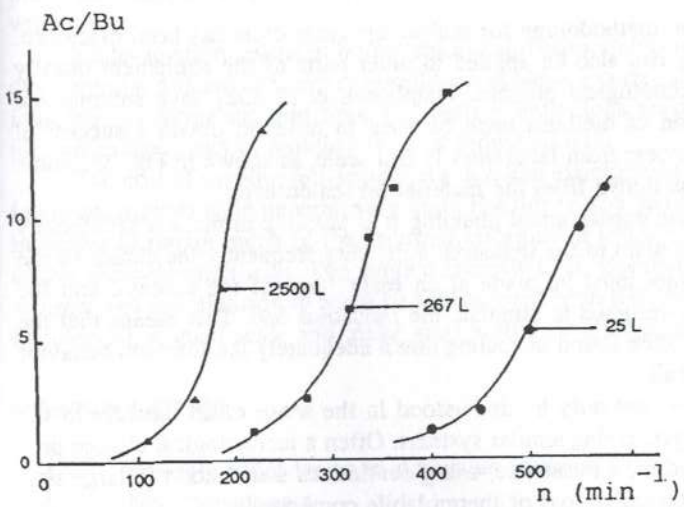


Figure 21 Product ratio as a function of stirrer speed (45, 450, and 4500 L total volume reactors). (From Ref. 29.)

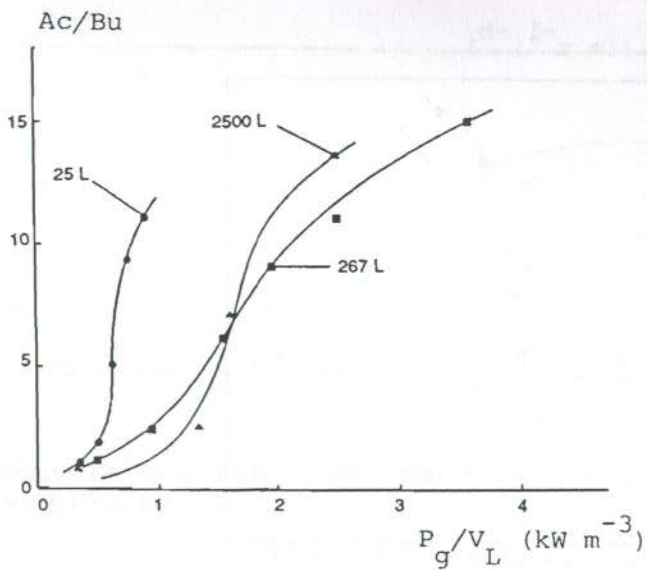


Figure 22 Power uptake as scale-up criterion for the 45, 450, and 4500 L total volume reactors. (From Ref. 29.)

VI. CONCLUSIONS

In this chapter, the methodology for scaling up bioreactors has been discussed. This methodology can also be applied to other parts of the equipment usually found in a biotechnological process. Trambouze et al. [32] have summarized how a combination of methods must be used in order to obtain a successful translation of a process from laboratory to full scale, as shown in Fig. 23. Some conclusions can be drawn from the material presented here:

With adequate experimental planning it is possible to make a satisfactory passage from pilot plant to the industrial unit. Very frequently the choice of the industrial technology must be made at an early stage of the process, and the pilot bioreactor is designed to simulate the industrial one. That means that the real problems are often found in scaling down adequately the foreseen behavior at the industrial scale.

Scale-up must not only be understood in the sense of an increase in the dimensions while preserving similar systems. Often a technological change provides a better solution, for instance, using continuous sterilization in large systems, preventing excessive loss of thermolabile compounds.

The development of mathematical models able to describe the key characteristics of the system is perhaps the most effective tool for successful scale-

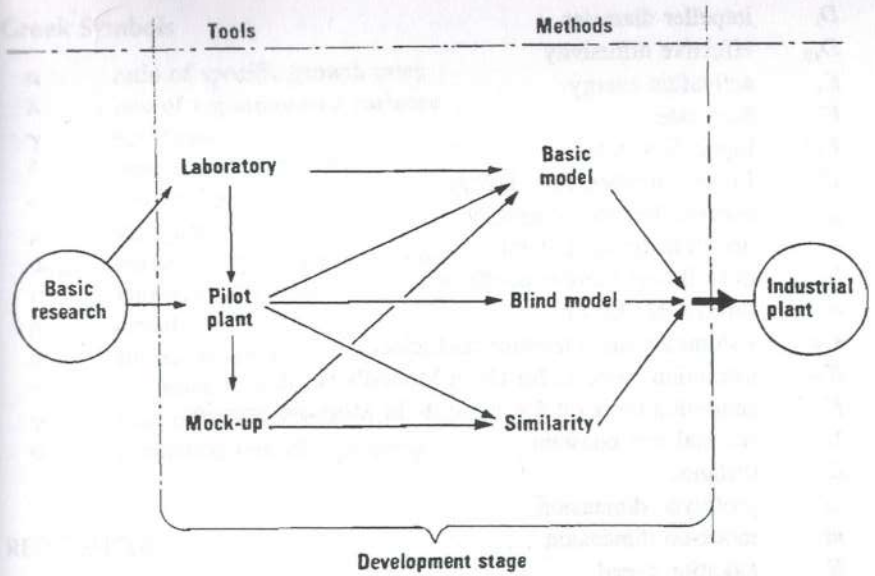


Figure 23 Combinations of means usable for scaling up a process. (From Ref. 32.)

up. However, a reasonable balance must be achieved between the model comprehensive character and the effort needed for its experimental calibration and validation.

Scale-up often results in both technical and economic compromises. When developing a process, it is never possible to achieve a perfect anticipation of its industrial performance, and time and budget limitations make it necessary to take calculated risks in building the first industrial unit.

The cost of running full-scale, long-duration experiments to develop a new industrial process is, in general, very high. For this reason there is a considerable incentive to obtain much of the required information using small-scale models and/or short-duration tests. The applied chemist L. H. Baeckeland (1863–1944) summarized this philosophy when he said (quoted from Ref. 33): "Commit your blunders on a small scale and make your profits on a large scale."

NOMENCLATURE

A	preexponential factor in Arrhenius equation
A	heat transfer area
C	concentration
C_p	activation energy

D_i	impeller diameter
D_{eff}	effective diffusivity
E_a	activation energy
F	flow rate
F_1	liquid flow rate
Fr	Froude number, $Fr = D_i N^2/g$
g	acceleration due to gravity
h	heat transfer coefficient
h_b	overall heat transfer coefficient
k	lineal scale factor
$k_L a$	volumetric mass transfer coefficient
K_{02}	saturation constant for O_2 in Monod's equation
K_s	saturation constant for substrate in Monod's equation
k_t	thermal rate constant
L	distance
m'	prototype dimension
m	mock-up dimension
N	agitation speed
N_a	aeration number, $N_a = Q/nD_i^5$
N_g	number of generations
N_p	power number, $N_p = P/\rho N^3 D_i^5$
OTR	oxygen transfer rate
OUR	oxygen uptake rate
p	pressure
P	power
P_G	gassed power
Q	gas flow rate
r_i^{max}	maximum consumption rate for species i
r_{HM}	heat production by the microorganisms
r_{HS}	heat production by stirring
Re	Reynolds number, $Re = D_i^2 N\rho/\mu$
t	time
T	temperature
U	fluid velocity
V	fermentor volume
V_L	liquid volume in fermentor
v_s	superficial gas velocity
We	Weber number, $We = N^2 D^2 d\rho/\sigma$
x	biomass concentration
X	number of cells

Greek Symbols

α	ratio of specific growth rates
λ	rate of appearance of variance
γ	shear rate
δ	generalized diffusivity
ϵ	gas holdup
μ	viscosity
μ_{max}	maximum specific growth rate
ν	kinematic viscosity
ρ	density
σ	surface tension
τ	residence time
ψ	propiety concentration
ψ_G	generation rate of a property

REFERENCES

1. A. Bisio, *Scale-up of Chemical Processes* (A. Bisio and R. L. Kabel, eds.), Wiley, New York, 1985, p. 3.
2. R. Van Sweringen, D. J. Cecchini, and R. P. Palluzi, *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed., vol. 17, Wiley, New York, 1982, p. 892.
3. B. L. Tarmy, *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed., vol. 19, Wiley, New York, 1982, p. 892.
4. S. Shelley, *Chem. Eng. 98*: 90 (1991).
5. D. M. Himmelblau and K. B. Bischoff, *Process Analysis and Simulation*, Wiley, New York, 1978.
6. E. Costa et al., *Ingeniería Química*, vol. 1, Alhambra, Madrid, 1983, p. 112.
7. R. B. Bird, W. E. Stewart, and E. N. Lightfoot, *Transport Phenomena*, Wiley, New York, 1968.
8. A. P. J. Sweere, K. Ch. A. M. Luyben, and N. W. F. Kossen, *Enzyme Microb. Technol. 9*: 386 (1987).
9. S. Y. Oldshue, *Biotechnol. Bioeng. 8*: 3 (1966).
10. R. I. Fox, *Proceedings, 1st European Congress of Biotechnology*, vol. 1, 1978, p. 80.
11. D. I. C. Wang, et al., *Fermentation and Enzyme Technology*, Wiley, New York, 1979.
12. M. Charles, *Trends Biotechnol. 3*: 134 (1985).
13. M. Charles, *Comprehensive Biotechnology* (M. Moo-Young, ed.), vol. 2, Pergamon, Oxford, 1985.
14. M. D. Lilly, *Bioactive Microbial Products 2. Development and Production* (L. J. Nisbert and D. J. Winstanley, eds.), Academic Press, London, 1983.
15. A. Fiechter, *Adv. Biochem. Eng. 30*: 7 (1984).
16. D. W. Hubbard, *Ann. N.Y. Acad. Sci. 506*, 600 (1987).

17. A. P. J. Sweere, K. Ch. A. M. Luyben, and N. W. F. Kossen, *Enzyme Microbial Technol. 9*: 386 (1987).
18. N. M. G. Oosterhuis, Ph.D. Thesis, Delft University of Technology, 1984.
19. D. E. Leng, *Chem. Eng. Prog. 87*: 23 (1991).
20. B. Kristiansen and B. McNeil, The design of a tubular loop reactor for scale-up and scale-down of fermentation processes, Proceedings of the International Conference on Bioreactors and Biotransformations, Gleneagles, Scotland, 1987, pp. 321–334.
21. P. B. Choi, *Chem. Eng. Prog. 86*: 32 (1990).
22. M. Y. Chisti, *Air-lift Bioreactors*, Elsevier, New York, 1989, p. 26.
23. M. Zlokarnik, *Dimensional Analysis and Scale-up in Chemical Engineering*, Springer-Verlag, Berlin, 1991, p. 49.
24. N. W. Geary and R. G. Rice, *AICHE J. 38*: 76 (1992).
25. G. H. Shoutens, R. P. Guit, G. J. Zieleman, K. Ch. A. M. Luyben, and N. W. F. Kossen, *J. Chem. Tech. Biotechnol. 36*: 335 (1986).
26. B. C. Buckland, *Bio/Technology 2*: 875 (1984).
27. D. Jain, and B. C. Buckland, *Bioprocess Eng. 3*: 31 (1988).
28. A. Humphrey. *Chemical Engineering Problems in Biotechnology* (M. L. Shuler, ed.), AIChE, New York, 1989, p. 333.
29. M. Griot, U. Saner, E. Heinzle, I. J. Dunn, and J. R. Bourne. *2nd International Conference on Bioreactor Fluid Dynamics* (R. King, ed.), Elsevier, Barking, 1988, p. 17.
30. J. B. Joshi, A. B. Pandit, and M. M. Sharma. *Chem. Eng. Sci. 34*: 813 (1982).
31. M. Griot, U. Saner, E. Heinzle, I. J. Dunn, and J. R. Bourne. *Chem. Eng. Sci. 43*: 1903 (1988).
32. P. Trambouze, H. van Landeghem, and J. P. Wauquier, *Chemical Reactor Design, Engineering, Operation*, Technip, Paris, 1988.
33. D. E. Rosner, *Transport Processes in Chemically Reacting Flow Systems*, Butterworths, Stonehem, 1986.

15
Sterilization and Containment

Andrew Sinclair and Michael H. J. Ashley
John Brown Engineers and Constructors Ltd.
Portsmouth, England

I. INTRODUCTION

Sterilization is the process of rendering materials to a condition which is totally devoid of living microorganisms. It is an absolute condition and, in practice, it is an ideal which is approached in terms of achieving an acceptable probability of the absence of viable organisms. In the context of bioreactor design, sterilization has three applications:

Presterilization of equipment
Sterilization of feedstocks
Maintenance of sterile operating conditions

In reality, this means that having ensured that all viable organisms are absent it is necessary to prevent entry of potentially infecting microorganisms during the entire period of plant operation.

The reason for specifying sterile operation is the key factor in determining the desired level of sterile integrity, and this will dictate batch process philosophy and design approach. The most frequent bioreactor operating requirement is for monoseptic conditions. This means operating equipment with a single strain of microorganism which is transferred to the bioreactor under strictly

controlled conditions and maintained in an infection-free environment during fermentation (bioconversion) processing.

Containment is the means by which materials within a process system are prevented from leaving. For practical process systems, materials must enter and leave, and they must do so in such a way that particular constituents are either retained or processed in such a way that they may be safely released. In the context of bioreactor design it is again the reason for specifying containment of microorganisms, which is the key issue. The most frequent requirement is for the safe utilization of a strain of a genetically modified microorganism within a process system. This to some extent requires a further assessment of the potential hazard should containment fail and in turn requires an assurance that the system is designed to allow less than an acceptably low probability of release of this organism or of fragments of cells which may have transgenic potential.

Sterile design requires keeping all but the intended microorganism out of the process system. Containment design requires keeping this microorganism in the system. Because both of these requirements entail imposition of an effective barrier, to some extent they require engineering solutions which are similar. Design specifications to suit the designed level of sterile integrity will often go a long way toward satisfying the required category of containment and vice versa.

One further issue is worthy of consideration before dealing with details of design techniques. This is the increasing need to operate bioreactor systems apyrogenically. Pyrogenic materials are fragments of cell walls of gram-negative bacteria which contain endotoxins and therefore promote allergenic responses. The elimination of pyrogenic fragments from process water and from defined medium ingredients places an additional constraint on the operation of feed sterilizers and on sterile design approach for bioreactor equipment. However, operation of a high-integrity sterile and pyrogen-free process can simplify specifications for containment because it may be possible to give a more accurate definition of the particles that must be retained or destroyed within the system.

II. STERILIZATION

A. Total and Consistent Design Discipline

The requirements for sterility—that is, in practice, a combination of presterilization of equipment, sterilization of feedstocks, and maintenance of a condition devoid of viable microorganisms or alternatively of monoseptic bioreactor conditions—vary from one application to another. For example, food processing systems, pharmaceutical manufacturing facilities, and power alcohol plants all have particular requirements related to sensitivity to infection of the microorganism which has been selected to effect bioconversion and also the level of

contamination which can be permitted in order to cost-effectively manufacture the desired quality of final product. There are no strict guidelines, and design solutions are dictated by both safety considerations and economic judgments. Every stage of the process must be safe, and because uncontrolled contamination must be avoided, design for hygienic operation is a minimum requirement. In some cases the infection of a fermentation system at very low levels can reduce conversion efficiency or cause unacceptable spoilage by introducing toxic materials which necessitate rejection of the final product. This may be after many costly downstream purification stages have been carried out.

Often yeast and fungal batch fermentations which demonstrate high growth rates and short cycle times are relatively insensitive to bacterial infection or can be protected with antibiotics. Conversely, fill-and-draw and continuous fermentation processes employing a bacterial strain require a more stringent approach to sterile integrity.

The cost of overdesign of sterile systems can limit profitability, but an inadequate design specification of only one critical item can completely prevent beneficial process operations. The overriding need is to assess the level of sterile integrity appropriate to plant requirements and to develop a comprehensive sterile engineering specification which must be applied consistently to every item of equipment, valve, pipe fitting, control instrument, etc. It is often one small item which constitutes the weakest link in the chain which ultimately determines the level of sterile integrity that is achieved during plant operation. With regard to the practical application of sterile engineering, there are no alternatives to experience, diligence, consistency, and attention to detail. Extra effort spent during the conceptual design stage can result in simplification of the plant design resulting in major capital cost benefits as well as improvements in operability. Extra effort spent during the detailed engineering phase of a project can save months of commissioning time by avoiding costly postconstruction modifications.

It is a common misconception that for sterile engineering design to be cost-effective it should be restricted to interaction between process and piping engineers. Process engineers provide a definition for all conditions of plant operation. Competent piping engineers ensure that the correct spatial configuration of all items and their interconnecting piping is suitable for sterile operations. Certainly, both are essential to successful plant design. However, all engineering disciplines must be closely involved at every stage of sterile engineering development. Sterile engineering requires active contributions of the plant commissioning engineer, vessel and heat exchanger designer, control systems and instrument engineer, process architect, and machinery engineer. Sterile engineering is a total and consistent interdisciplinary design approach requiring a coordinated engineering team dedicated to providing a unique and cost-effective solution for each application.

B. Defining Sterile Integrity

A high-growth-rate batch fermentation operating at low pH and utilizing small volumes of pure substrates with fresh inoculum for each batch should be relatively insensitive to infection and may require low sterile integrity. If the desired product from this fermentation is a parenteral therapeutic protein, the need to ensure monosepsis combined with the need to maintain low pyrogen levels will dictate a very high sterile integrity.

An animal feed biomass fermentation product such as fodder yeast does not impose a requirement for more than a simple robust hygienic standard of engineering, but a protein foodstuff for human consumption produced by a large-scale continuous fermentation which is prone to infection by common airborne spores will need particular attention for the sterile filtration of fermenter process air. Each case must be considered in relation to sensitivity to infection and challenge levels in the outside environment and in all feedstocks. Scale of operation, product specification, and the cost of process interruption due to infection are also relevant factors. Depending on particular process conditions, such as low pH in the presence of chlorides or proteinaceous materials which can foul hot surfaces, the appropriate materials of construction and surface finishes must be chosen.

Another result of defining sterile integrity is the choice of presterilization conditions in terms of a combination of sterilizing time with steam temperature and pressure for all equipment within the sterile boundary. Medium sterilizing conditions for batch or continuous operation should, if possible, use the same clean steam supply. Sterile integrity during process operations also requires the specification of an appropriate level of automatic sequencing to give a sufficient probability of continuity of monoseptic operation consistent with economic operation. Obviously, as the complexity of a sterile system increases, sequencing hardware and software become more extensive and this will further increase capital cost.

C. Sterilization by Thermal Deactivation

The most common method of rendering equipment and media sterile is by heating to deactivate microorganisms. Conventionally, death rate may be expressed as a first-order relationship:

$$\frac{dN}{dt} = kN \tag{1}$$

where N is the number of viable cells, t is time, and k is the specific death rate. If N_0 is the number of cells at zero time then $N = N_0 e^{-kt}$. Typical thermal destruction curves are shown in Fig. 1.

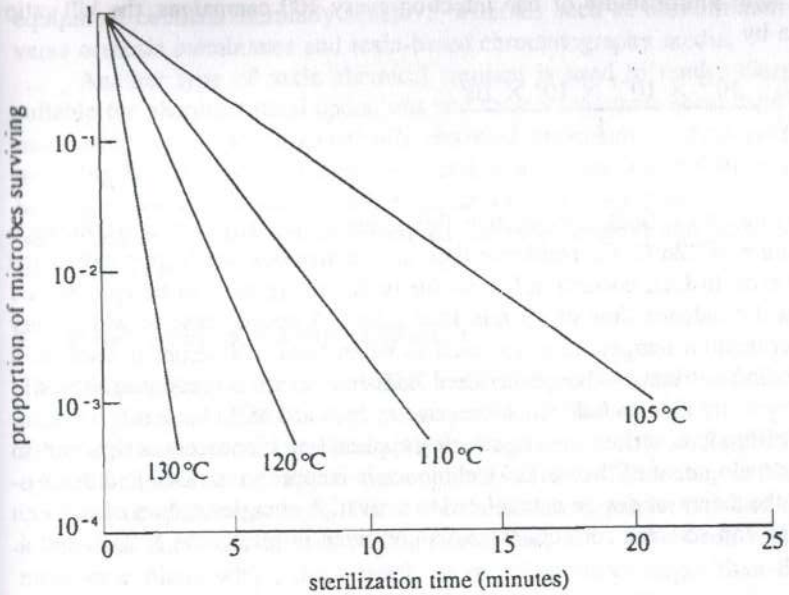


Figure 1 Typical thermal destruction curves for microbes.

Alternatively, spores may need to be held at a specific temperature for a transition period before becoming sensitive to deactivation giving rise to a sequential first-order relationship.

The specific death rate varies with temperature according to the Arrhenius equation $k = ae^{-AE/RT}$ where a is a constant, AE is the activation energy of death (deactivation energy), and T is the absolute temperature. Combining these two equations gives

$$\ln \frac{N_0}{N} = a_0 e^{t-AE/RT} dt \tag{2}$$

Bacillus stearothermophilus is the most commonly cited example of a thermally tolerant infectant. This has a deactivation energy of 70 kcal/gmol and a k value of 0.77/min in the presence of wet condensing steam at 121°C.

Using this relationship, the decimal reduction time (99% microorganisms killed) is calculated to be 3 min. At 130°C the decimal reduction time is reduced to 0.3 min.

The residence time to achieve a given probability of sterility may be calculated as follows. For a continuous process medium stream containing 10^6/ml viable cells ($10^{12}/m^3$) in a flow of 0.1 m^3/h and an operating campaign time of

1000 h with a probability of one infection every 100 campaigns, the kill ratio is given by

$$\frac{N_0}{N} = \frac{10^{12} \times 10^{-2} \times 10^{3} \times 10^{2}}{1}$$
$$= 10^{15}$$

and so $\ln(N_0/N) = 36.8$. Substituting this value in Eq. (2) with a sterilization temperature of 126°C, the residence time in a continuous sterilizer is calculated to be 8 min. In fact, correction factors for back mixing need to be applied and a practical residence time of 15 min is required. A special case in which very high sterilization temperatures are used is when fetal calf serum is used as a fermentation nutrient. Although certified BSE-free serum is mandatory it is still necessary to be able to hold the bioreactor system at 135°C for 2 h.

This book describes the engineering approach to bioprocess design and so further development of theoretical techniques is inappropriate. Detailed descriptions of the theory of design data related to activation energies, values of reaction constants, and selected correction factors are given in references 1, 2, 3, and 4.

D. Chemical Sterilization

A number of chemical reagents are lethal to microorganisms. Their destructive power may be related to inorganic chemical reactivity such as dehydration by concentrated sulfuric acid and lysis by caustic soda or, more commonly, it may be related to the toxic nature of the chemical, which can inhibit normal metabolic pathways and so deactivate cells.

In general, aggressive or toxic materials are not used to sterilize media because their destructive power would be carried through to the bioreactor and could also contaminate the product. Chemical sterilants are most often used for sanitization or presterilization of equipment, for decontaminating process operating areas which may be within a secondary containment boundary, and for deactivating the contents of a bioreactor before safe disposal.

It is essential to be able to remove or control the toxicity of these chemicals before process operations recommence in order to ensure safe operator working conditions and nontoxic process conditions.

Typical examples of chemical sterilants used within process equipment are dilute sodium hydroxide, dilute nitric acid, and sodium hypochlorite. These materials have a "searching" effect which may be augmented by the addition of surfactants and they are useful as cleaning, defouling, and depyrogenating agents. They are usually flushed away from the process equipment with water of the purity required in the production process before resumption of normal production operations. Chemical sterilants are the only choice when process

equipment contains thermally sensitive materials such as ultrafiltration and reverse osmosis membranes and resin-based chromatography media.

Another type of toxic chemical sterilant is used to render clean rooms suitable for pharmaceutical operations and to decontaminate secondary containment areas within which genetically modified organisms or fragments thereof are being handled. Ethylene oxide, peracetic acid, ozone, and formaldehyde have been used for such applications but special precautions are necessary to ensure safety and to specify resistant materials for all equipment and surfaces within the clean room.

E. Sterilization by Absolute Filtration

The design principle for conventional depth filters which are used in chemical process plants is based on impingement of solids throughout the depth of the filter in order to reduce the probability of transmission to a tolerable level of contamination. This technique was originally used for fermenter air filtration in the form of glass wool beds. These were difficult to presterilize and dry prior to process duty and, almost without exception, they have been replaced by membrane filters which theoretically have no apertures larger than their size rating and so are conventionally termed absolute filters. The components of an air filtration assembly is shown in Fig. 2.

Disposable cartridge membrane process air filter assemblies are available to suit all sizes of fermentors. Membrane materials such as P T F E are used for fermentor air. These are hydrophobic and are generally reverse flow presterilized with steam. Integrity testing of membrane filters is carried out by using a solvent-water mixture, which is gas pressurized, as a wetting fluid. Liquid surface tension maintains pressure and provided the filter has "integrity," i.e., there is no aperture larger than the specified size rating, the rate of gas diffusion can be predicted and should not be exceeded.

An absolute rating of 0.2 μm retains most monocellular organisms and spores. However, subjecting large volumes of air to filtration requires an appropriate choice of prefilters. This entails selection of filtration area (number of cartridges) for each stage. The optimum combination of stages and sizes should provide an acceptable total pressure drop and cartridge life. Capital and operating costs of the air compressor and the filter installation as well as cartridge replacement cost need to be optimized to ensure that the most economic system design is installed. The first in a series of filters may be a coalescing filter to remove water droplets from compressed air, and this may be followed by a prefilter to reduce solids loading further.

Sterile filters are also available for aqueous and nonaqueous fluids. These are particularly useful for solutions of very heat-sensitive materials or fluids with low boiling points which cannot be thermally sterilized.

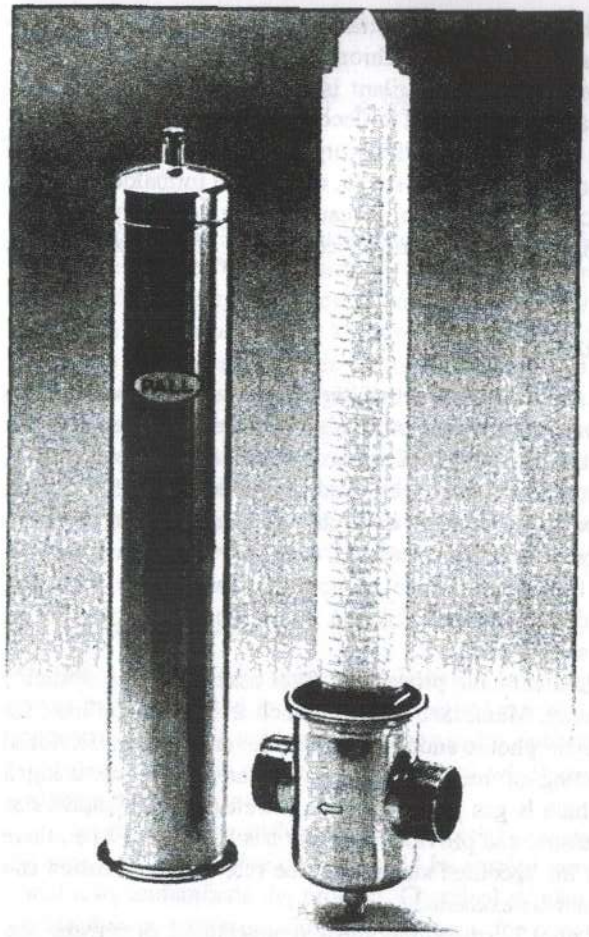


Figure 2 Structure of an air filtration cartridge.

F. Medium Preparation

Most fermentation media are heat sterilized but they often contain materials which are thermally degraded. Vitamins and amino acids have Arrhenius relationships with lower activation energies and lower inactivation rates than infecting organisms. The optimum solution to take advantage of these relative properties is dictated by the logarithmic relationship which allows sterilizers to operate at a *high temperature* provided the medium is retained for only a *short time* (HTST). Under these conditions infectants are destroyed at a high rate with

a considerably slower rate of medium degradation. Careful selection of plant operating conditions gives the required sterile integrity with minimum loss of nutrient quality.

Most fermentations require a carbon-containing substrate plus various nutrients and trace ingredients. The tendency is toward defined media which are much cleaner and pose few fouling and deactivation problems in thermal sterilizers. Nevertheless, it is still necessary to consider carefully which nutrients can be combined in order to avoid precipitation. For example, the calcium source must be kept away from sulfate and phosphate salts. Iron salts may form complexes with manganese salts which plate out in heat exchangers. It is usually more convenient to vaporize liquid ammonia into fermentor air and filter sterilize the mixture. In this way ammonia can be used as the nitrogen source and also for pH correction.

Dissolution and filtration of nutrient solutions precede thermal deactivation sterilizers and the water supply necessary to support the required dilution rate for fermentation must be apportioned to these flows. Continuous or batch sterilizers may be used, depending on the nature of the fermentation, i.e., batch, fed batch, or continuous.

The rates of addition of some nutrient streams may be too low to allow continuous sterilization, so a practical volume may be batch sterilized and then held for continuous transfer.

G. Presterilization Operations

Sterilize in place (SIP) techniques are employed for pipework and vessels which comprise most process systems handling organisms. Equipment is rendered in a state such that it will not be contaminated by the external environment. Design aspects which are normally addressed are finishes, pressure and temperature rating, and detailed configurational design. The areas of concern are complete drainage, location of steaming points, elimination of crevices, and reliable procedures for collapsing sterilizing steam. SIP may be carried out before or after cleaning; this depends on the nature of the operation.

The general procedure for thermal deactivation using condensing steam is

Open steam points
Vent air
Build up pressure
Hold
Collapse steam in a controlled manner
Start next step

The exact temperature-time profile employed depends on the selected organism, its susceptibility to infection, removal of pyrogens, and the detailed design of

equipment. Generally, thermal deactivation is designed for the inactivation of *B. stearothermophilus*.

H. Sterile Engineering

Design, detailed engineering, and fabrication of the entire section of process plant which constitutes a sterile boundary need special attention at all stages. Most bioprocess operations occur at modest temperatures and pressures, so it is presterilization which determines design conditions as the highest combinations of temperature and pressure and temperature with full vacuum. Generally, stainless steel is the material of construction for all plant and the extent and nature of fouling at gas-liquid interfaces must be assessed in order to choose the appropriate surface finish.

The specification of sterile plant should ensure that every item and every component of the complete system must be able to be drained without leaving substantial pools. All parts must be able to be freely vented during filling, leaving no gas pockets; all surfaces must be able to be cleaned by clean in place (CIP) fluids without any crevices or dead ends; and the entire system has to be brought to the sterilization temperature using saturated steam with special attention given to potential cold spots.

The design of piping and vessels without substantially horizontal surfaces so that they are self-draining and venting needs particular attention. All piping and valves should be welded and equipment should have a minimum of flange connections.

Special seals are required on flanges which provide a depth rather than line contact seal. Generally, double mechanical seals with a sterile barrier such as condensing steam between them are required for rotating elements such as fermentor agitators. A typical sterile seal for an agitator drive is shown in Fig. 3.

Instrument connections should, wherever possible, be noninvasive, such as pressure diaphragms mounted on pads, thermocouple pockets, and Coriolis-type flow-measuring elements. Sensors which enter the sterile environment, such as pH probes, should have double O-ring seals with steam connections for sterile withdrawal and similar piping configurations are needed for sample taking. Controlled steam collapse by sterile air pressurization is advisable and pressure relief should be via bursting disks with special flange seals. Transfer of material should be by sterile air pressure egging or by pumps situated outside the sterile barrier (i.e., upstream of the sterilizer). All process inlets and outlets, exhaust gas, and liquid drains connections should be via double block valves with steam supply and condensate drain connections.

To accomplish presterilization and maintain sterile conditions in bioreactors of modest size and above requires accurate manipulation of complex piping systems consisting of process lines, sterilizing steam supply, process drains,

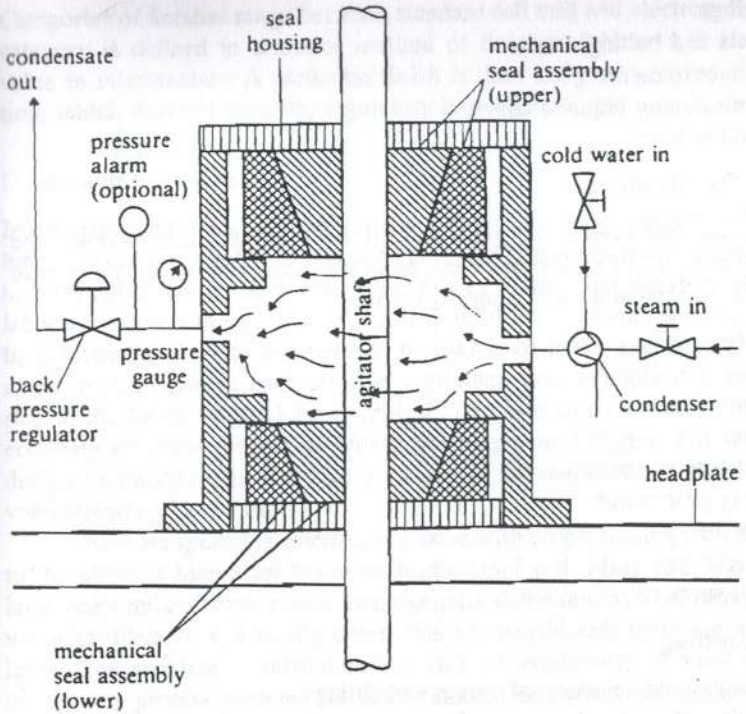


Figure 3 A sterile agitator seal (top drive).

condensate drains, vents, air transfer lines, and CIP supply and drain lines. Generally this is achieved with autosequenced valves which have limit switches giving feedback to a PLC sequencer. Temperature level and pressure sensors also send signals to the sequencer, which arranges for sections of the plant to be periodically cleaned, presterilized, and brought back into process operation.

1. Vessel Specification

The essential information for a vessel specification is contained on process and mechanical data sheets. This is backed up by a general specification for vessels for sterile and sanitary service. These documents cover

- Finishes
- Nozzles sizes and connection details
- Construction detail including welding
- Insulation requirements
- Documentation requirements

Openings
Gaskets and bolting details
Code requirements
Commissioning requirements
Inspection tests

2. Piping Specifications

Piping specifications need to be generated on a job-by-job basis to cover the specific requirements of each project and particular user preferences. For sterile pipework, the specification will typically cover

Tube type
Finish
Jointing
Gaskets
Clamp-type connections
Welding procedures
Valves
Traps
Special supports

with notes covering

Orientation and selection of valves and fittings
Protection of materials delivered to site
Passivation
Inspection

3. Gasket Materials

Both process and sterilizing duties will dictate suitable materials. For simple aqueous duties EPDM and silicone are acceptable and both can be supplied as FDA registered materials for cGMP facilities. Both these materials stand up to cyclic steam sterilization without creep and hence leakage. A conflict can arise where aggressive solvents are used within a steam-sterilizable system; here PTFE may be the only option, but this material has the disadvantage that after several sterilizations, leaks can develop.

4. Surface Finishes

A practical approach is to use a general specification on finishes which covers

Codes and standards
Surface finish
Fabrication
Inspection and acceptance procedure

Categories of finishes range between standard roll mill and electropolished. Each category is defined in terms of method of finish, minimum grit size, and Ra value in micrometers. A particular finish is then assigned to a specific application, which depends on duty, regulatory requirements, etc.

I. Design Scale-Up

In common with the chemical process industries, the development of manufacturing processes utilizing materials of biological origin or using microbial strains to carry out bioconversions entails a number of steps. Starting at the researcher's laboratory bench in equipment of a few milliliters volume, scale-up continues to industrial scale with hundreds or thousands of cubic meters capacity. Scale-up of design between each stage is important, but generally it is only the last step to the largest unit which is critical. This is because in most cases there is economy of scale and plants would get bigger and bigger if it were not for design limitations dependent on a variety of factors, the most important being volume/surface area ratio.

There are special problems associated with bioprocessing which are related to the ultimate bioreactor being each microbial cell. Mass and heat transfer at large scale must always ensure heterogeneity at the microscopic scale. However, when sterilization and sterile operations are considered, there are a few problems. One problem is related to the lack of availability of very small scale continuous process systems for semitechnical and pilot operation, which may result in mismatch of flows between unit operations. It may also be necessary to operate on a campaign basis with consequent extra demands on holding materials under sterile conditions. At the other extreme, very large vessels which are connected to steel structures for support have, at these points, substantial heat sinks which may need compensating steam coils to ensure that sterilization temperature is reached. Similarly, the supports for and connections to agitators may also constitute considerable heat sinks.

Large sterile vessels usually have limpet coils or jackets for cooling, and these need particular consideration during steam sterilization. A number of operating regimes may be employed, such as

Leaving the coils full and able to withstand steam pressure as the water heats up
Emptying the coils
Steaming coils at the same time as the vessel.

Each of these options presents particular mechanical design requirements concerning differential pressures and differential thermal expansion.

As scale increases it may be necessary to enlarge the sterile envelope to incorporate some equipment downstream from the bioreactor. Hold tanks with

agitators do not present design uncertainties, but cell separation systems such as centrifuges, rotary drum filters, and ultrafilters give rise to very difficult problems. These have to be addressed by specialist fabricators and are outside the scope of this book.

For large-scale medium sterilizers, particular attention must be paid to the design of the flow regime in the retention vessel. In general, the vessel should be very well insulated to avoid thermally induced convection currents and the Reynolds number should be sufficiently high to maintain substantially plug flow. This may be achieved in a rising process coil which is supported within a steam-filled vessel. Alternatively, very large flows may require a vertical limpet coiled vessel with full-length flow straighteners to ensure plug flow.

J. Design Optimization

The preceding section indicates that economy of scale encourages plant operators to select the largest sizes of equipment for each unit operation because scale-up of sterilizers in general leads to lower product-specific capital costs. However, in the case of thermal and filtration sterilizers it is important to consider a combination of capital and operating costs.

Thermal sterilizers often consist of a train of four units, namely interchanger, steam heater, retention vessel, and cooler; see Fig. 4. The larger (and so more costly) the interchanger, the lower the cost of steam and cooling water. A simple optimization equation can be solved for this case. However, the choice of sterilization temperature influences heat exchange area and retention vessel

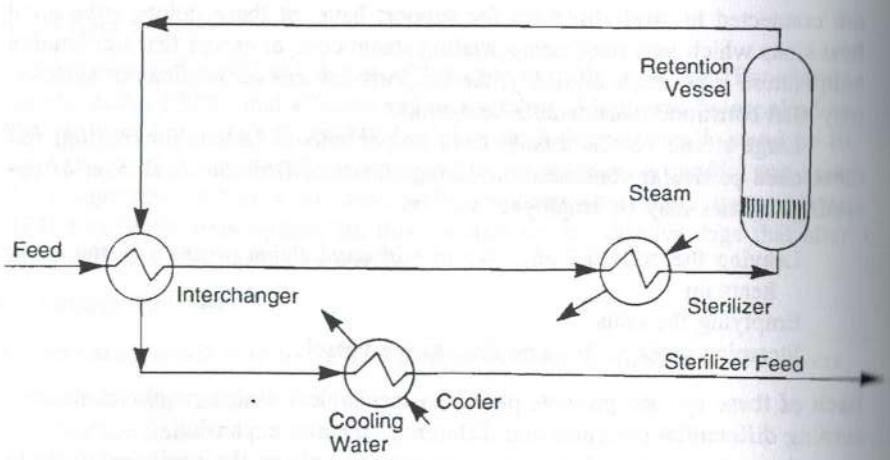


Figure 4 Continuous sterilizing system.

volume. This makes the overall cost optimization more complex. Reference 5 gives more detailed consideration of this problem.

Optimization of filter sterilizers is related to the number of cartridges which are installed in the filter vessel, which increases capital cost but reduces pressure drop and also reduces the frequency of changing a larger number of fouled cartridges, which in turn allows longer production campaigns to be operated. In fact, a series of filters is usually installed—for example, 10-μm coalescing filter, 1-μm prefilter, and 0.2-μm sterile filter. The filtration areas within each unit need to be optimized to suit local conditions in relation to airborne dust load and concentration of microbial spores. A low pressure drop across a series of filters requires less compression power, but a high overpressure requirement by the fermentor will have a more profound cost impact by increasing compressor power while decreasing fermentor agitator power. However, the actual volumetric air flow in the filters is reduced and therefore the pressure drop is lower for the same filtration area. If the filter size is reduced there would be no reduction in compressor operating cost but the capital cost of filter system is lower. A complex optimization is necessary, including capital costs of compressor, filters, and agitators, the power costs of compressor and agitator, and cartridge replacement costs. Again, reference 5 provides more detailed consideration of this case.

III. CONTAINMENT

A. Regulatory Issues

1. Global Issues and Background of Regulation

In the late 1970s a series of techniques known as genetic engineering were developed to allow manipulation of genetic material. Pharmaceutical companies were extremely interested in this technology, as it freed them from the constraints of conventional laborious breeding and screening programs required for the development and improvement of organisms. The new techniques also allowed the development of novel products by enabling the transfer of gene products from one organism to another. One of the earliest commercial examples of the application of these techniques is Eli Lilly's product Humulin, containing human insulin, produced by *Escherichia coli*. This product was traditionally available only from the human or animal pancreas prior to the development of the genetically engineered *E. coli*. Development work took place in the early 1980s, and in 1990 Humulin was one of the 50 top-selling pharmaceuticals. However, the ability to alter the heredity material of organisms and thereby generate unnatural products raised fears of developing a "Frankenstein" organism. Concern focused on the hazard both to the individual and to the environment.

The groundswell of these fears in the 1970s led to the first conference on biohazards, held at the Asilomar Conference Center in California in January 1973. This was followed 3 years later by the release of a pioneering set of guidelines from the National Institute of Health (NIH). These early days were characterized by reactionary behavior, which was later followed by an easing of the constraints embodied in the early guidelines. Now the emphasis is on a balanced assessment of the specific and general risks associated with each particular process.

The early responses to transgenic developments in biotechnology tended to distort risk assessment by overconcentration on the perceived risks of genetically engineered organisms. A correct balance between the various risk categories becomes more important as processes are scaled up. The aim is to reach a balanced appreciation of all the following hazards:

Pathogenic
Pharmacological activity
Allergenic
Oncogenic
Solvent hazards
Mechanical hazards

All operations involving the contained use of recombinant microorganisms must be carried out in a way which does not harm human health. When designing facilities for contained operations there are two stages: (1) defining control measures and (2) applying for approval.

1. The user must carry out a prior safety assessment and apply the principles of containment.
2. Notification of certain categories of work must be made to the competent national authority, which in the United Kingdom is the Health and Safety Executive (HSE). In certain cases consent is required before starting work.

In many countries there is no requirement to review the design with the competent national authority until a facility is about to be used. Nevertheless, it is prudent to do so because an informal review at an early stage of the design process can highlight potential problems, which can be corrected before the facility is built.

2. Future Trends

The main driving forces for the definition and development of regulations and guidelines on contained use of genetically manipulated organisms (GMOs) are the European Community (EC) and the United States.

In the United States there are specific guidelines [6] covering the contained use of GMOs. The main developments concern the definition of good large industrial scale practice (GILSP) in terms of engineering and facility features.

The EC enacted a directive [7] on contained use of GMOs in October 1990, but it is not yet fully implemented in member states. Some countries (Ireland, Greece) have not started to implement this directive, whereas others (Germany, UK) are well under way. For example, as a part of the enactment of the European directive on contained use of GMOs the UK's 1989 Genetic Manipulation Regulations (GMRs) are being replaced.

3. Equivalence with the United States

It is important to note that the differences in regulatory environments in the UK, the EC, and the United States can affect the design of a facility. A good example is seen in the regulatory framework for GMOs, which is different in the United States, particularly in the risk assessment process and application of containment categories. For example, in the United States, laboratory-scale containment category 1 is equivalent to large-scale containment category 1, whereas in the UK laboratory-scale containment category 2 is equivalent to large-scale containment category 1. However, direct numerical equivalence is often assumed when comparing U.S. and UK laboratory- and large-scale containment categories.

4. Deliberate Release

Much discussion has taken place in this area, where the real issues are concerned with the threat to the environment on the occasion of a particular release of viable numbers or reproducible fragments of a microorganism. The result will depend on the survivability and fitness of the microorganism. Survivability is the ability to remain potentially active outside the fermentor, and fitness is the factor that determines its ability to displace wild populations. Another factor is ability to exchange heritable material either within species or across species. Much of this type of information is not known and is not easily predicted.

There is a specific EC directive [8] which addresses the issue of deliberate release of recombinant microorganisms. There has been some debate to date on the meaning of what constitutes a deliberate release, particularly if it applies to facilities which operate contained processes.

B. Containment Design

In many countries only general guidelines and regulations exist for the specification of equipment. These general guidelines and regulations are largely based on the original OECD recommendations contained in the book *Recombinant DNA Safety Considerations* [9]. Table 1 reproduces the large-scale containment categories, which are extracted from the UK guidelines [10]. These mirror those

Table 1 Containment Requirements for Large-Scale Applications

		Containment Categories		
		1	2	3
Primary Containment				
1.	Viable organism should be handled in a system which physically separates the process from the workplace environment (closed system)	Yes	Yes	Yes
2.	Exhaust gases from closed system are to be treated so as to	Minimise Release	Prevent Release	Prevent Release
3.	Sample collection, addition of material to a closed system and transfer of viable organisms to another closed system should be performed so as to	Minimise Release	Prevent Release	Prevent Release
4.	Bulk culture fluids should not be removed from the closed system unless the viable microorganism have been	Inactivated by validated means	Inactivated by validated chemical or physical means	Inactivated by validated chemical or physical means
5.	Seals should be designed so as to	Minimise Release	Prevent Release	Prevent Release
Secondary Containment				
6.	Closed system to be located within a controlled area	Optional	Optional	Yes, purpose-built
a)	Biohazard signs posted	Optional	Yes	Yes
b)	Access should be restricted to nominated personnel only	Optional	Yes	Yes via airlock
c)	Personnel should wear protective clothing	Yes, work clothing	Yes	Yes. A complete change
d)	Decontamination and washing facilities should be provided for personnel	Yes	Yes	Yes
e)	Personnel should shower before leaving controlled area	No	Optional	Yes
f)	Effluent from Sinks and showers should be collected and inactivated before release	No	Optional	Yes
g)	The controlled area should be mechanically ventilated to minimise air contamination	Optional	Optional	Yes
h)	The controlled area to be maintained at an air pressure negative to atmosphere input	No	Optional	Yes
i)	Input air and extract air to the controlled area to be HEPA filtered	No	Optional	Yes
j)	The controlled area should be designed to contain spillage of the entire contents of the closed system	Optional	Yes	Yes
k)	The controlled area should be sealable to permit fumigation	No	Optional	Yes
7.	Effluent treatment before final discharge	Inactivated by validated means	Inactivated by validated chemical or physical means	Inactivated by validated chemical or physical means

of the EC directive. To interpret these general objectives for actual facilities containing equipment it is necessary to define key terms, such as

Minimization
Prevention
Best available technology not entailing excessive cost (BATNEEC)

For example, Table 1 states that seals for contained operation should be designed so as to

Minimize release for LSCC1
Prevent release for LSCC2
Prevent release for LSCC3

However, neither of the terms "minimize" or "prevent," is quantified. So for many countries there is no clear consensus on what engineering features are required to achieve the requirements of regulations. The only country where this situation differs is Germany, where the Chemical Industry Association has published specific engineering guidelines [11] describing the detail engineering features required at specific containment levels.

The requirements for contained operation are not the only constraints imposed on design. In practice, aseptic or sterile operation and the requirements of current good manufacturing practice usually define minimum required engineering features. For example, at LSCC1 the guidelines require that release from seals is *minimized.* For such a seal to leak at all would compromise aseptic operation. In practice, process requirements dictate that, in practice, seals specified for LSCC1 are designed to *prevent* release. Hence a distinction is not drawn between LSCC1 and LSCC2 in the physical specification of static seals. However, some relaxation of the specification would be permissible for commissioning, validation, quality assurance and procedures. Therefore, when defining design requirements, it is important to recognize that containment is only one of many constraints on design and should certainly not be considered in isolation from the proposed method of operation.

Currently, there is no commonality of approach in the UK and indeed the majority of the world as to what constitutes best practice in terms of engineering controls. This is a hindrance to industry, which needs basic quantitative data on hazard assessment based on the likelihood of failure for critical applications. This lack of data is not just restricted to biotechnology but includes both chemical and nuclear industries. In the UK this is understood by the HSE. Moreover, the HSE, together with industry, is making a start to address this situation by commissioning research to examine and generate key reliability data for components and systems.

However, it is recognized that the value of a reliability assessment lies not in the particular measurement of system reliability but in the determination of

ways to improve this figure. The objective is to minimize risk in a rational manner by cost-effective design. Many operating companies have developed a workable definition of the requirements for contained operation by taking into account the pharmaceutical industry's long experience in designing systems to operate aseptically. This means that there is a large body of qualitative knowledge concerning the selection of components which are suitable for plant operations and for which data are available on component integrity when subjected to multiple sterilization cycles. This experience has been gained in the UK, Europe, and the United States. It is this experience which, together with the many years of safe operation in the bioprocess-based industries, forms the basis of the principles described in this section.

1. Scale-Up

The design of containment features is described in qualitative terms in published guidelines. These guidelines nominally equate a particular organism [15] hazard category to a particular containment category. Hazard categorization is the same for pathogens, mammalian cell lines, and recombinant organisms. This procedure can be used to define nominal containment requirements when a process is transferred from the laboratory to either pilot plant or production using the relationships described in Table 2. However, a formal qualitative hazard assessment procedure is required to determine the containment category at the larger scale. Nominal hazard categorization is used only as guidance when assessing special containment requirements for recovery equipment.

2. Analysis of Failure

An important requirement of the design and operation of equipment for contained operation is to make due assessment of both the likelihood and consequences of failure of any part or function of the system that can give rise to a loss of containment. This requires special attention to critical aspects of operation in order to arrive at an overall assessment of the likelihood of failure in terms of loss of containment.

Table 2 Nominal Relationship Between Hazard Groups and Containment Categories

Hazard groups for organisms	ACGM Note 8 laboratory scale	ACGM Note 6 large scale
1	1	GILSP
2	2	1
3	3	2
4	4	3

To do such an exercise rigorously requires a detailed analysis of all mechanical components and an analysis of the operator's performance in terms of

Type of activity
Time available to do it
Operator qualities
Situation status
Ergonomic factors

This will allow an objective assessment of whether it is probable that the equipment can be operated without loss of containment. This should be considered, together with likelihood of mechanical failure, to arrive at an overall failure rate for the operation. This process is time consuming and requires key personnel to conduct the analysis. Accordingly, it should concentrate on critical items, these items being identified during formal safety reviews.

3. Common Mechanical Elements

This section sets out to examine principal common areas of concern regarding containment of equipment. To date, only a limited number of items of equipment have been developed with containment in mind and much equipment is only now evolving to meet the needs of users, in terms of both functionality and safe operation. A brief outline of the approach adopted in the design of some of the key elements that go into any facility is given below. The components are selected on the basis of

Robust design
Proven track record
Cleanability
Capability of being sterilized

The basic mechanical features of each component and assembly of any item of equipment should be critically assessed, particularly with regard to fabrication integrity, jointing, and moving seals. These joints, which by their nature have potential for leakage, comprise

Welded joints
Static sealing elements
Rotating seals

The basic areas to be considered for the maintenance of primary containment in any piece of equipment are illustrated in Fig. 5.

Valves. The valve types suitable for contained operation are usually limited for practical purposes to diaphragm valves. Vendors of these types of valves (Fig. 6) understand the issues involved, have test data, are able to give guar-

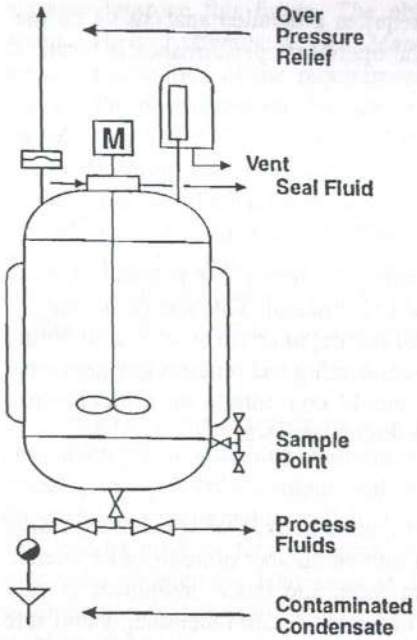


Figure 5 Typical process vessel.

antees on performance, and claim their valves are suitable for contained operation to category LSCC2.

Piping Connections. Process pipelines which will contain viable organisms are designed to provide a primary containment barrier. This is achieved by the adoption of aseptic design techniques and standard operating procedures (SOPs). In practice, piping systems designed for recent projects incorporate the following features, which ensure that they meet the requirements of contained operation:

- Material of construction is thin-walled stainless steel tube, grade 316L or better, subject to corrosion requirement.
- Joints are kept to a minimum and where required can be made using BS 4825 (stainless steel pipes and fittings for the food industry), Part 3 (clamp-type couplings), or equivalent (triclamp; see Fig. 7).
- Construction is generally all welded using orbital welding.
- A quality control procedure is used to inspect welds and ensure that fabrication meets the defined standards.

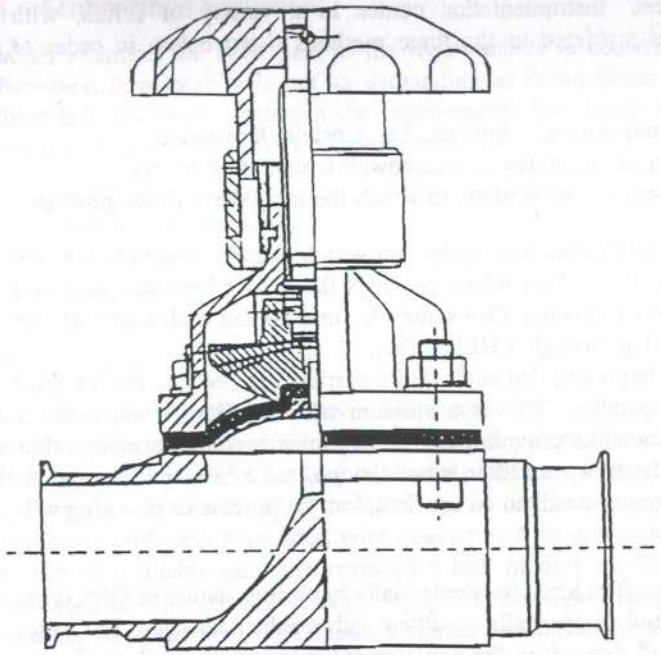


Figure 6 Diagram of a diaphragm valve.

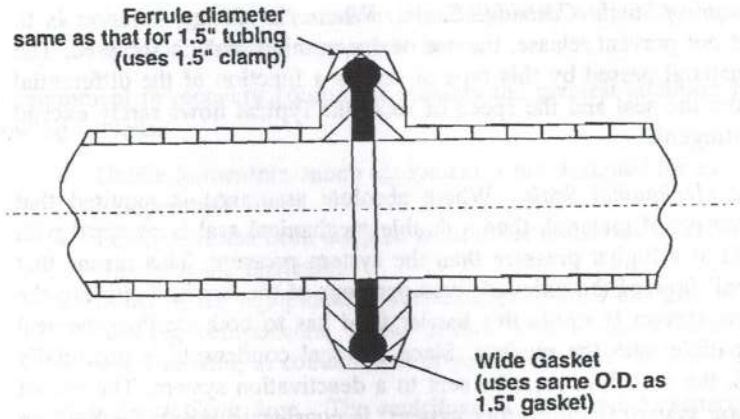


Figure 7 Triclamp detail.

Instrumentation. Instrumentation comes in a variety of forms, with process connections restricted to the three methods listed below in order of preference:

1. Use of nonintrusive techniques, i.e., Coriolis flowmeters
2. Use of surface-mounted or thermowell temperature probes
3. Use of triclamp connections to attach the instrument to the process

Vents. Gas vented from any equipment which contains viable organisms must pass through a sterile filter which provides the barrier between contained and noncontained environments. Gases may be manifolded and routed to the outside without passing through a HEPA filter.

It is therefore important that such sterile barriers are checked for integrity prior to contained operation. This is a problem where a filter is required for aseptic operation as well as providing the containment barrier. Normally, after sterilizing the integrity of a vent filter is not checked prior to aseptic operation. For contained operation it needs to be checked, but the process of checking will compromise sterility.

Rotating Seals. The hazardous/potentially hazardous nature of GMOs requires that agitator and pump shafts are fitted with mechanical seals. The exact seal type adopted will depend on the containment category. Typical configurations include

Dry-running single cartridge seals
Double mechanical seals—steam flushed
Double mechanical seals—thermosiphon

Dry-Running Single Cartridge Seals. Where the design criterion is to minimize but not prevent release, the use of dry-running seals is accepted. The amount of material passed by this type of seal is a function of the differential pressure across the seal and the speed of rotation. Typical flows rarely exceed 5 ml/h (as nitrogen).

Double Mechanical Seals. Where absolute assurance is required that there is no release of material, then a double mechanical seal is required with a barrier fluid at a higher pressure than the system pressure. This means that there is a small flow of the order of liters per hour of the barrier fluid into the product. If the system is sterile this barrier fluid has to both sterilize the seal and be compatible with the product. Since the seal condensate is potentially contaminated, the seal fluid must be sent to a deactivation system. The use of a thermosiphon system (recirculatory system) is considered where the load on the deactivation system has to be minimized.

4. Fermentation Equipment

Much attention has been paid to the requirements of contained fermentations. However, fermentation is not as demanding as downstream recovery because there is commonality between the requirements for aseptic and contained operations. The key areas which require attention are

Inoculum transfer
Agitator shaft seal
Exhaust gas filtration
Sampling
Broth transfer

5. Downstream Equipment

Generally, the principal objective before commencing downstream process operations is to inactivate viable organisms either within the fermentor or as close to it as possible, thereby limiting the extent of the process containment boundary. It is not always practicable to inactivate in the fermentor, and this presents particular problems associated with containment in downstream equipment. A survey of available literature revealed a lack of data on the containment performance of downstream recovery equipment for these hazardous duties. Only recently have research organizations started to examine containment in equipment for biologically hazardous duties. This has been stimulated by needs of industry and requirements set by the regulations. As the amount of information published concerning actual contained performance of equipment is limited, an alternative approach is suggested:

- Seek specific advice from the manufacturers of equipment in the recovery area.
- Where specific concerns exist, additionally utilize the experiences of users of particular equipment items.

Containment in recovery equipment requires the greatest attention for the following reasons:

- Unlike fermentors, much equipment is not designed for extended aseptic operation.
- Equipment has been adapted from other industries where the industry standards are different.
- Some of the equipment generates high internal energies or has reciprocating components. These factors have to be examined carefully when looking at containment or possible failure modes.

Disk Stack Centrifuge. The centrifuge is often used to separate the bulk solids from fermentation broth continuously as the first step of a recovery proc-

ess, and often contained operation is required. The operation is automatic once it has been correctly set up; solids are automatically discharged. At the end of a run the whole device is decontaminated by steam sterilization. These machines play a critical role in contained recovery operation. Manufacturers claim the following features:

Machine is capable of aseptic operation.
There is no release of viable organisms during operation.
The whole machine can be decontaminated with steam at the end of a run.

Independent assessments [12,13] of this type of machine with respect to containment requirements were good, although it is not certain that containment by the completed installation can be assured for long periods. Operator training is critical to ensure that human probabilities are kept within acceptable limits.

Homogenizer. Depending on process requirements, a cell disruption step may follow isolation of the cells using a centrifuge. This is done to release intercellular products. The homogenizer destroys cells by generating high pressures (up to 1000 bar) and dropping the pressure across a letdown valve. The high pressures are generated using reciprocating positive displacement pumps. The flow through the unit is continuous.

For contained operation of homogenizers it is necessary to contain losses. One method is to enclose the whole machine during operation. Where this is done, an effective decontamination method for the internal surfaces of the barrier used must be developed and validated.

Tubular Bowl Centrifuge. These devices have been used downstream of the harvesting operation, where difficult solid-liquid separations are encountered. They have been used for isolating intracellular granules, which is an imperfect operation in this instance where paste is contaminated with cell debris. The problems identified with operation of this centrifuge are

- During operation the shaft seal generates aerosols.
- The contaminated machine has to be dismantled to enable removal of the bowl in order to recover the product.
- To remove the product from the bowl the end cap is removed, and the product is pulled out together with its liner.

The key problem with this machine is that to gain access to the product it is necessary for the operator to handle contaminated equipment. Moreover, the operations are not simple, involving lifting and manipulation of a heavy bowl. For this reason it is not recommended that this device be used for contained operations above category LSCC1.

Tangential Flow Filtration Package. Tangential flow filtration packaged units can be specified to meet the requirements of contained operation. The units

are based on many of the standard elements described above. A typical package comprises the following elements:

A "low pressure" pump
Fixed pipework with static seals
Ceramic-membrane cartridge

The main area for special consideration is decontamination of membranes. Only a minority of the systems offer steam-sterilizable membranes. With ceramic units care is required to ensure that the rate of heating/cooling is controlled to prevent damage to the membranes.

6. Building Features

Items from Table 1 that specifically concern building features are as follows:

- Closed systems should be located within a controlled area.
- The controlled area should be mechanically ventilated to minimize workroom contamination.
- The controlled area should be maintained at an air pressure negative to atmosphere.
- Extract air from the controlled area should be HEPA filtered.
- Access should be restricted to nominated personnel only via a changing room.
- Input air to the controlled area should be HEPA filtered.
- The controlled area should be sealable to permit fumigation.

The Controlled Area. The purpose of the controlled area is to define the boundary limit between contained and noncontained operations. The actual boundaries are the surfaces of the walls, floors, and ceilings, except where two contained areas are separated by a common wall. The general principles for people and materials movements across the controlled area boundary ensure that access is controlled to the contained area and that no material that either contains or has the potential to contain viable organisms may leave the contained area.

Movement of Materials. A facility which is designed for operations at or above category LSCC2 ensures that equipment containing viable organisms does not cross into noncontained areas. This covers

Process transfer lines
Vessels containing process fluids
Process condensate
Process wastes
Sink and shower wastes
Any other fluids that are exposed to the controlled environment

The only fixed lines which enter or leave the area are utilities which service the contained area and inactivated wastes or products which leave it.

Movement of People. For contained operations at category LSCC2, people entering the contained area are controlled by key access or a similar system. The lobby is a physical break between the noncontained and contained areas. It will have the following features:

- Biohazard sign at the entrance
- Wash hand basins
- Emergency shower
- Changing facilities for laboratory coats or oversuits, with segregation of the two types of clothing
- Air pressure in the lobby positive with respect to the contained area but negative with respect to the noncontained areas
- Any sink wastes, shower wastes, and spillage to be treated
- Outer doors to be interlocked with the inner doors

Finishes. No specific guidance is given concerning finishes. For all facilities the finishes will typically be

Capable of being easily cleaned
Capable of being disinfected
Nonporous
Crack resistant

These criteria apply to walls, floors, ceiling, doors, ducting, piping, and any other exposed surface within the controlled area. The number of dust traps is minimized by limiting horizontal runs of pipes and ducts; and where they are unavoidable they should be accessible to allow cleaning.

7. Heating, Ventilating, and Air Conditioning

The topics which should be addressed are

Room pressures
Biosafety
Fume cabinets/extraction enclosures
HEPA filtration

Where there is differential room pressure between contained and noncontained areas, the controlled areas are placed at the lowest differential pressure relative to atmosphere. Ancillary support rooms such as change rooms and airlocks are operated at a differential pressure one grade above the controlled areas and may be positive relative to atmosphere. This philosophy of room pressures ensures that air flow is toward the area of highest risk.

C. Aspects of Contained Operation

1. Spillage Emergency Procedures

During contained operations in a controlled area it is a requirement that procedures and equipment are provided to deal with spillage of biologically contaminated materials. Typical procedures for a large spillage are given below. Note that any disinfectants or chemical sterilants which are used must be shown to be effective against the actual microorganisms under the conditions of use. Operators dealing with spills must be provided with adequate protection against biological agents and any chemicals used. This may require the provision of oversuits and mobile breathing apparatus in the case of large spills.

Large Spills

1. Move the liquid to a low point; this can be aided by a sloping floor.
2. Treat the liquid and the contaminated floor area with a chemical sterilant, and let it stand for the required period.
3. Pump out the low point, sending material to the deactivation system.
4. Wash down the area with water, again transferring the wash to the deactivation system.
5. Conduct swabs tests in predefined critical areas, testing for viable microbiological contaminants. If a positive result is recorded repeat 1 to 4. If positives are again recorded fumigate the area with a suitable gaseous agent.
6. Retest the room and fumigate again until no positives are recorded. At this stage the room can be released for normal use.

Treatment of Waste. For contained operation effluent is split into four categories listed in Table 3. The sources of waste from a facility may be as follows:

Fixed equipment
Recovery plant
Floors/sump pumps
Condensate
Sinks
Showers
Deactivation system
Autoclave

Small volumes (less than 10 L) may be treated either in a double entry decontamination autoclave or by use of a chemical sterilant in a manner that does not compromise containment.

In large volumes, only category W3 wastes are to be sent direct to the public sewer. Other wastes are either treated in situ or sent to a deactivation

Table 3 Waste Classification

Category	Description	Comments
W1	High-level waste	Normally containing viable organisms
W2	Low-level waste	Not normally containing viable organisms
W3	Treated waste	Either treated by a validated method or originating from noncontained operations
W4	Solvent contaminated	Waste containing viable organisms which are contaminated with solvents

tank. Where wastes are treated in situ routing direct to the public sewer is possible; the choice of routing is made via a lockable swing elbow or a crossover station. The routing choice is controlled by standard operating procedures and should require authorization by a supervisor.

The preferred method for decontaminating large volumes of waste is by heating using saturated steam in deactivation tanks which will handle all biological wastes. The exact temperature-time profile for decontamination has to be determined and validated for the particular organism.

2. Approvals and Validation

Validation of Decontamination Procedures. It is necessary to demonstrate that every item of equipment plus associated pipework which operates as a primary containment device can be effectively decontaminated before the primary containment is breached. Decontamination can be either by steam or by chemical means and includes the following items:

Fermentation equipment
Recovery equipment
Contaminated condensate
Drain to deactivation tank
Deactivation tank
Process lines
Treatment following accidental loss of primary containment
Local secondary containment barriers

Condensing steam is, where applicable, the preferred method for decontamination. It has the advantage that a general validation can be carried out at the end of commissioning.

Test of Containment. Before contained operations proceed, the integrity of critical items of equipment needs to be checked. After contained operations equipment is subjected to steam sterilization, disassembly, cleaning, and assembly. These routines are carried out to a predetermined standard operating procedure.

Equipment Integrity Testing. This can be as simple as a pressure hold test, as long as its effectiveness as an indicator of loss of containment is demonstrated. The pressure hold test is the one most commonly employed on routine basis in industry [14]. The lack of sensitivity of this procedure means that its use has to be carefully assessed on safety-critical applications where a high containment category is required. Two specific tests are used. The first uses extremely sensitive inert gas leak testing procedures based on helium or Halon and requires bottled gases and the appropriate analytical equipment. The second entails the pressure hold test for the normal test protocol to be used prior to contained operations. Various tests are employed, depending on sensitivity and on whether a release is being monitored directly or tested indirectly.

Vent Filter Integrity Testing. This is based on filter manufacturers' standard procedures. It requires the use of special integrity monitoring apparatus. All filters that constitute a containment barriera are integrity tested prior to contained operations. The test is carried out according to the filter manufacturer's validated procedure using the vendor's proprietary integrity test equipment. Where possible the test is conducted in situ, so that any leaks in the assembled filter area can be identified. If this is not possible (or not desirable), the filter membrane can be tested externally and a pressure hold test is used to detect any leaks in the filter housing once it is installed.

PROBLEMS

1. A medium in a fermentor is sterilized batchwise at 120°C. The temperature-time profile observed with a recorder attached to the fermentor is as follows:

t(min)	0	10	30	36	43	50	55	58	63	70	102	120	140
T(°C)	30	50	90	100	110	120	120	110	100	90	60	44	30

Assuming that the specific denaturation rate of contaminating bacterial spores is

$$k = 7.94 \times 10^{38} \exp\left(-\frac{68.7 \times 10^3}{RT}\right) \text{ min}^{-1}$$

and that the initial number of spores is $N_0 = 6 \times 10^{12}$, calculate the sterility level after the sterilization.

2. It is desired to sterilize a medium (60,000 kg, 40°C) within 40 min using in parallel two continuous sterilizers. The pipes of the reactor have inner diameter $d = 0.155$ m and length $L = 50$ m; the steam which is injected into the sterilizers elevates the temperature of the raw medium almost instantly. It is desired to estimate the tempreature of sterilization which will satisfy the following requirements.

Contamination level: $N_0 = 10^5$/ml
Sterility level: $1 - P = 10^{-3}(=N)$

The specific denaturation rate of bacterial spores is the same as assumed before. The physical properties of this medium at the temperature of sterilization are $c_{pp} = 1$ kcal/kg °C, $\rho = 10^3$ kg/m^3, and $\mu = 3.6$ kg/m h.

Solutions

1. Figure 8 shows the temperature–time profile and the values of k as a function of t. A graphical integration yields

$$\int_{t=34}^{t=64} k\,dt = 33.8$$

For the periods before $t = 34$ min and after $t = 64$ min, the values of k are small enough to be neglected.

$$1 - P = N_0 e^{-kt}$$
$$= 6 \times 10^{12} \times e^{-33.8}$$
$$= 1.5 \times 10^{-2}$$

2.
Sectional area A of each sterilizer:

$$A = \frac{\pi}{4}(1.55 \times 10^{-1})^2$$
$$= 1.88 \times 10^{-2} \text{ m}^2$$

Mean velocity $\bar{u}$ to satisfy $t = 2/3$ h:

$$\bar{u} = \frac{60/2}{At} = \frac{30}{(1.88 \times 10^{-2})(2/3)}$$

$$N_{Re} = \frac{d\bar{u}\rho}{\mu} = \frac{(1.55 \times 10^{-1})(2.39 \times 10^3)(10^3)}{3.6} = 10^5$$

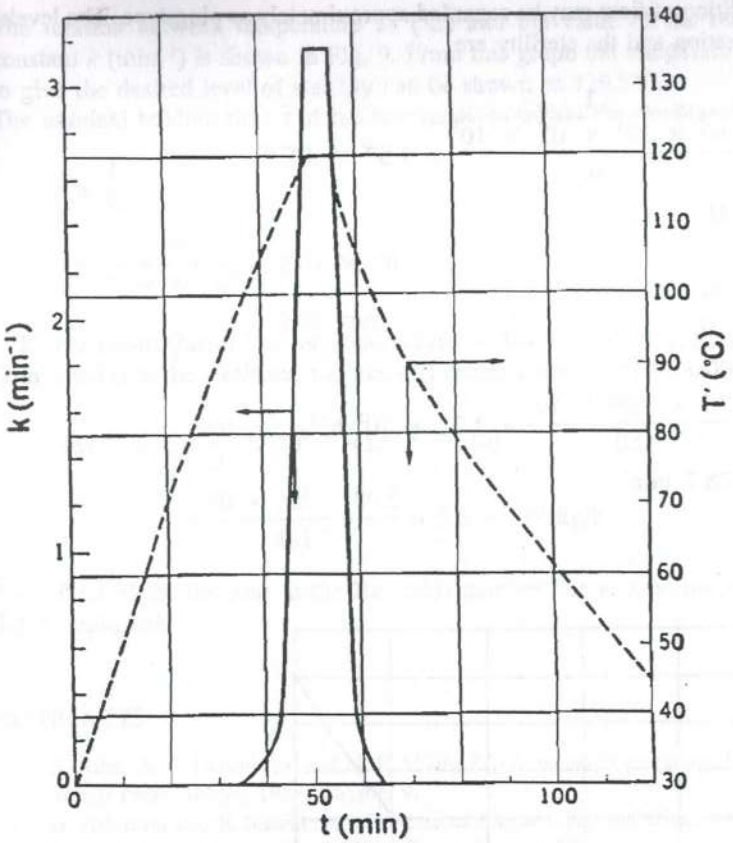


Figure 8 k (min^{-1}) and T' (°C) versus t (min).

From the data published by Levenspiel regarding $E_z/\bar{u}d$ versus N_{Re},

$$E_z/\bar{u}d = 0.2 \qquad (N_{Re} = 10^5)$$

Then

$$\text{PeB} = \frac{\bar{u}L}{E_z} = \frac{\bar{u}d}{E_z}\frac{L}{d}$$
$$= \frac{1}{0.2}\frac{50}{0.155} = 1610$$

The condition of flow may be regarded approximately as plug-type. The levels of contamination and the sterility are

$$\frac{N}{N_0} = \frac{1}{\dfrac{60 \times 10^3 \times 10^3 \times 10^3}{10^5}} = 1.67 \times 10^{-16}$$

$$N_t = \frac{kL}{\bar{u}}$$

$$= 36$$

$$k = \frac{N_t \bar{u}}{L}$$

$$= \frac{36 \times 2.39 \times 10^3}{50} = 1.72 \times 10^3 \text{ h}^{-1}$$

$$= 28.7 \text{ min}^{-1}$$

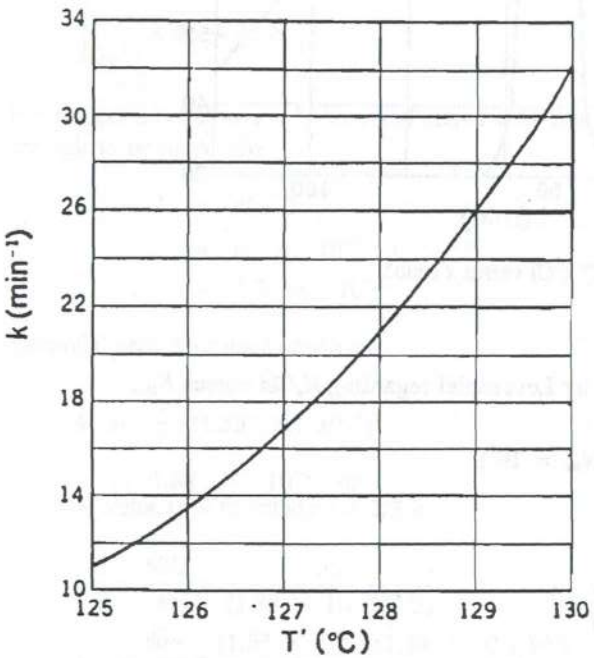


Figure 9 Values of the reaction rate constant k at different sterilization temperatures T'.

The relation between temperature T' (°C) and the value of the reaction rate constant k (min^{-1}) is shown in Fig. 9. From this graph the temperature required to give the desired level of sterility can be shown as 129.5°C.
The nominal holding time $\bar{t}$ of the raw medium within the sterilizer is

$$\bar{t} = \frac{L}{\bar{u}}$$

$$= \frac{50}{2.39 \times 10^3} = 2.09 \times 10^{-2} \text{ hr}$$

$$= 1.25 \text{ min}$$

If live steam (latent heat of vaporization = 481.7 kcal/kg) at 9 kg/cm^2 gage is injected into the sterilizer, the mass of steam s needed per hour is

$$481.7 \times s = \frac{60}{2} \times 10^3 \times (129.5 - 40)$$

$$s = \frac{30 \times 10^3 \times 89.5}{481.7} = 5.6 \times 10^3 \text{ kg/h}$$

The effect of the increase in the Reynolds number due to s on the value of $E_z/\bar{u}d$ is negligible.

REFERENCES

1. S. Aiba, A. E. Humphrey, and N. F. Millis, *Biochemical Engineering*, University of Tokyo Press, Tokyo, 1983, Chapter 9.
2. B. Atkinson and F. Mavituna, *Biochemical Engineering and Biotechnology Handbook*, 2nd ed., Macmillan, Stockton Press, Chapter 14.
3. D. I. C. Wang, C. L. Cooney, A. L. Demain, P. Dunnill, A. E. Humphrey, and M. D. Lilly, *Fermentation and Enzyme Technology*, Wiley, New York, Chapter 8, Section 2.
4. J. E. Bailey and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2nd ed., McGraw-Hill, New York, Chapter 7, Section 7.
5. M. H. J. Ashley, Continuous sterilisation of media, *Chemical Engineer*, February 1982.
6. 51 FR 16958, May 1985: Amended/NIH/52 FR 31848, August 24, 1987. *Guidelines for Research Involving Recombinant DNA Molecules*. Bureau of National Affairs Inc.
7. EC219/90, Council Directive on the Contained Use of Genetically Modified Micro-Organisms, Brussels, April 23, 1990.
8. Environmental Protection Act (November 1990). Part IV, Controls on the Containment and Release of Genetically Modified Organisms.
9. OECD. *Recombinant DNA Safety Considerations*, OECD Publications ISBN 92-64-12857-3, 1986.

10. ACGM. Guidelines for the Large-Scale Use of Genetically Manipulated Organisms, Advisory Committee on Genetic Manipulation - ACGM/HSE/Note 6. Health and Safety Executive, London, 1987.
11. Berufsgenossenschaft der Chemischen Industrie. Safe Operation Industrial Operation Equipment and Standard Practices, Guidelines, M 057e, 3/89, ZH 1/343e, BG Chemie, 1989.
12. The assessment of Risks in Scaled-Up Biotechnological Processes—(Progress Report of Activity 1). TNO Report, Ref. No. 89-371, February 1989.
13. R. Tinnes and M. Hoare, The biocontainment of a high speed disc bowl centrifuge, *Bioprocess Eng.* 8 (1992).
14. G. Leaver, Measuring and monitoring containment in bioprocessing equipment, *Hazards XI—New Directions in Process Safety*. Inst. Chem. Eng. Symposium Series No. 124. Hemisphere, Washington, DC, 1991, pp. 349–361.

16
Bioreactor System Supplies

T. M. Roberts, M. J. Kearns, and T. J. Latham

Foster Wheeler Energy Ltd.
Reading, England

I. INTRODUCTION

The purpose of a bioreactor is to create, control, and contain a suitable environment for the growth of living cells. To achieve this function, virtually all bioreactors must be provided with piped services of some sort. These system supplies may include steam for heating and sterilization, process water for medium preparation and cleaning, chilled water for temperature control, and process air, if the cells grow aerobically. The mechanical operation of the bioreactor requires electrical and instrument air supplies. The environment within the reactor is utterly dependent on the provision of these supplies in adequate quantity and to an acceptable quality.

Historically, the provision of bioreactor supplies was little different from that of any other industrial process. The standards of conventional process operations were applied. For some systems, such as cooling water, this is still true. For supply systems which can directly influence the quality of the final product, by virtue of entering or coming in contact with the process fluid itself, there has been an increasing need to define their chemical composition. This is true of process water, process air, and the steam used for sterilizing equipment by

direct injection. This need has arisen from the increasing use of biotechnology for pharmaceutical manufacturing. It has been driven by the regulatory requirements related to pharmaceutical good manufacturing practice (GMP). The aim has been to ensure an acceptable quality of the final pharmaceutical product by defining and controlling all aspects of its manufacture, including the service supply systems. The resulting high purity standards have led to the need, in some cases, for sophisticated treatment and distribution systems, capable of generating high-quality service supplies and maintaining them at that required quality up to the point of use.

This chapter largely concentrates on the bioreactor supply systems that have requirements which differentiate them from those of conventional process plants. The aim is to describe the required qualities of supplies, the means of generating and distributing them, and methods for calculating the quantities needed for bioreactor operations.

II. WATER

Water is used throughout the pharmaceutical manufacturing process. For bioreactors, water is used directly for medium makeup and in association with other additions (e.g., dilution of acid or alkaline solutions to appropriate concentrations) and indirectly for cleaning, for cooling, and for process steam generation. Water is used in relatively large quantities and in various qualities.

A. Process Water

Process water can be defined as the water used inside the bioreactor. Examples are medium makeup, in association with direct liquid additions, and for cleaning of the vessel interior and internals.

1. Process Water Quality

Requirements concerning the quality of process water in bioreactors vary depending on the process. Bioreactors used in animal cell fermentations require extremely high-quality water and tend to use water for injection (WFI) or sometimes European Pharmacopoeia/U.S. Pharmacopeia (EP/USP) water. Large-scale bulk pharmaceutical processes tend to utilize lower-grade softened or demineralized (DI) water. The requirement to use higher-quality water for animal cell processes is due to the inability to utilize steam sterilization of medium solutions; the higher contamination risk is due to slower growth rates plus potential chemical contaminants that can poison the cells.

Typically, the water types available fall into the categories listed in Table 1.

2. Water Pretreatment Operations

The water pretreatment operations required are determined by the source and quality of the feedwater. It is important to note that seasonal variations do occur,

Table 1 Water Quality Categories and Applications

Pharmacopoeia limits set by FDA:

1. Purified water—none specified	
2. Water for injection (WFI)	0.25 EU/ml
3. Sterile water for injection	0.25 EU/ml
4. Sterile water for irrigation	0.25 EU/ml
5. Sterile water for inhalation	0.5 EU/ml
6. Bacteriostatic water for injection	0.5 EU/ml

Applications of low-endotoxin water:

1. All applications 2 > 6 of Table 1—distillation obligatory under B.P./PhEur. regulations
2. Manufacture of reference or standard solutions (e.g., L.A.L. test blanks)—any suitable technology under GMP rules
3. Preparation of bulk process water for biotechnology (e.g., fermentation)—any suitable technology under GMP rules
4. Preparation of wash/CIP water for biotechnology applications—any suitable technology under GMP rules
5. Prerinsing of vials/packaging to contain injectable products—any suitable technology under GMP rules
6. Water for veterinary products—any suitable technology

for example, in organics load and colloids. The selected pretreatment operations must be capable of handling the worst-case water quality. The water pretreatment operations must be capable of handling any or all of the following contaminants or characteristics:

Suspended materials (organic and inorganic)
Colloids
Organics
Ionic charge
Metals
Bacteria
Temperature
pH
Pyrogens
Dissolved gases

Typical components of a water pretreatment operation are

- Filtration. Pre- and fine filtration can be used to improve the water quality by eliminating all material down to (μ) levels. A balance be-

tween flow rate and quality requirements with capital and operating costs must be undertaken for final equipment selection.

- Activated carbon beds are used for elimination of taste and odors and for dechlorination and removal of total organic carbon (TOC).
- Ion exchange beds are normally mixed or anionic resins are used. Removal of tertiary amines is a typical requirement.
- Reverse osmosis can be used in various arrangements with spirals and fibers constructed from polyamides and cellulose acetate. Depending on the permeability, can be used for pretreatment to reduce ionic charge and TOC. For high-quality water using a high-quality reverse osmosis (RO) system there is an opportunity to reduce pyrogens. Ionic charge and TOC can also be improved further.
- Ultrafiltration (UF) construction is from polysulfone or ceramics. UF is capable of totally removing bacteria and organics but is normally used for higher quality of water, since it is easily fouled.

A typical pretreatment system can consist of

Prefiltration (followed by)
Activated carbon bed
Softening and ion exchange (dependent on water hardness)
Reverse osmosis

Typically, water from such a system could be used for all applications other than for WFI final rinse.

3. Process Water Production Processes

Further improvements over the pretreatment operation can be made by use of the following systems.

Ultrafiltration. Ultrafiltration is a low-pressure membrane process which relies on differential rejection of impurities on the basis of their size. Membranes are available with pore sizes in the range 0.001 to 0.02 μm. (Use of membranes with larger pore sizes, up to 0.2 μm, is usually termed microfiltration.)

Ultrafiltration has two applications in water treatment. As pretreatment, membranes with a cutoff of 80,000 to 100,000 daltons can be used for removing colloidal material. More commonly, membranes with a cutoff of 6000 to 20,000 daltons are used for polishing pretreated water to remove bacteria and some pyrogens.

UF membranes can be polyacrylonitrile or polysulfone. Until recently these could not be steam sterilized. Instead, they had to be sanitized with agents such as sodium bisulfite or hypochlorite. This restricted their use in process water (PW) applications. Today, double-film membranes are available that can operate continuously at 80°C and can resist regular steam sterilization at 120°C.

UF is accepted by the Japanese Pharmacopoeia as a final production step for WFI. Results have shown that UF can produce filtrate (permeate) with an endotoxin concentration below the 0.25 EU/ml limit of WFI-USPXX1 from feedwater carrying 6.25 EU/ml. UF should be considered for bioreactor applications requiring low-pyrogen water.

Compared with RO, UF requires more pretreatment for PW applications. This is because UF does not alter the concentration of the low-molecular-weight solutes such as inorganic ions. It is usually preceded by a deionization stage. However, it has lower capital costs than RO, and because operating pressures are lower and there is less rejection of water, operating costs are also lower.

4. Water Storage and Distribution Systems

Storage. Most bioreactors operate on a batch basis and require a process water supply only intermittently. In some cases direct on-line treatment of water during batching is feasible, but the more common situation is that purified water is generated continuously and stored for use. The choice is determined by considerations of operability, equipment maintenance, sometimes available space, and by the balance of costs—high-capacity treatment equipment and simple distribution systems versus low-capacity treatment equipment and sophisticated storage and distribution systems.

Purified water must be stored in a way to preserve its quality. This requires prevention of leaks into the system and use of inert materials to preserve the chemical quality of the water. Because purified water is rarely specified as sterile, the maintenance of microbiological and pyrogen quality requires elaborate precautions against microbial proliferation within the storage system.

Figure 1 shows a typical storge tank for PW. The reasons behind some of the design features are as follows:

1. The vessel is built of 316L stainless steel for corrosion resistance. It is designed to allow steam sterilization at 121°C, 1 bar g and to resist poststerilization vacuum formation.
2. The vessel is hygienically designed internally for ease of cleaning, being free of crevices. The internal surfaces are polished for the same reason. The vessel is free-draining through a flush-mounted outlet valve, such that no residual cleaning fluids are retained after draining.
3. The vent to atmosphere is protected by a sterilizing filter. This is typically a 0.2-μm hydrophobic membrane filter, sized for two-way flow during tank filling and emptying. For hot-water storage, this filter may be electrically traced to limit condensation of water on the membrane, which that can cause blockage.
4. Instrumentation is present to allow operating control and monitoring and also validation. That shown is the minimum desirable—a level

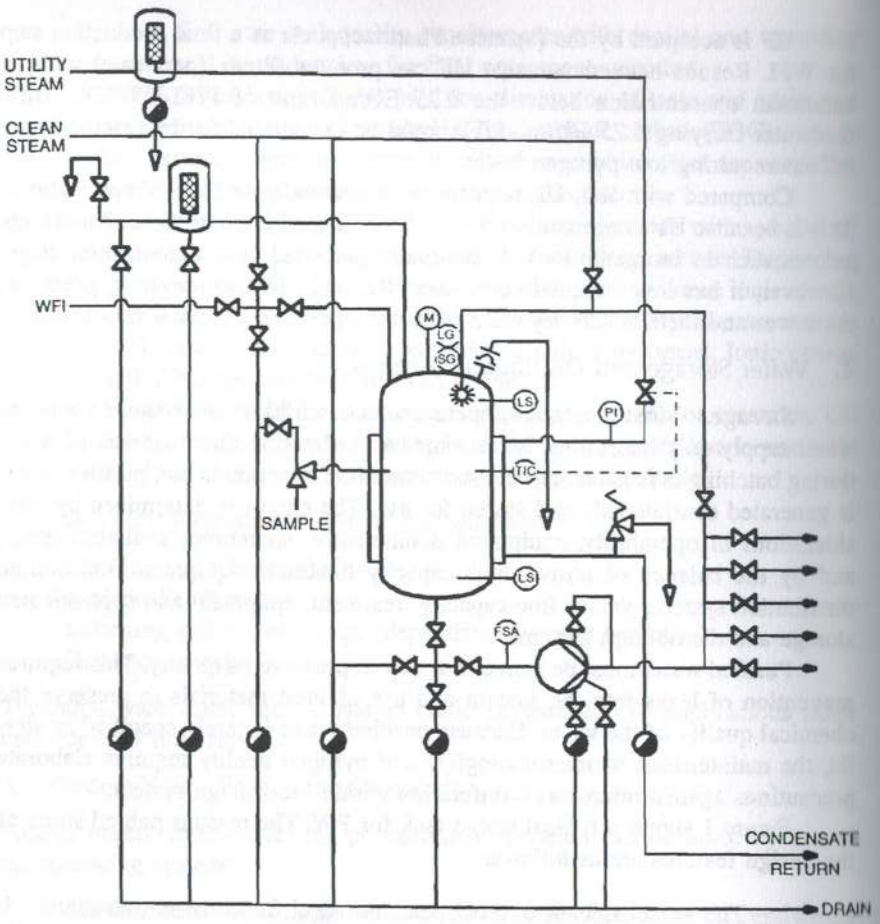


Figure 1 WFI storage.

gauge, pressure gauge, and bursting disk for pressure relief. A high level switch may also be added to prevent overfilling. These instruments must meet the same hygienic requirements as the rest of the system. Pressure gauges are diaphragm sealed. Differential pressure (DP) level gauges of similar type can be used, or gauges operating on a capacitance or ultrasonic principle. Air pressure control to prevent vacuum formation after sterilization is sometimes specified. Other instruments for PW systems are usually mounted away from the vessel, on outlet piping. They might include a temperature monitor

(for sterilization), a sampling valve, and pH and conductivity monitors.

5. Returning water to the vessel is often passed through a sprayball, causing the water to flush the tank interior above the water level. (A less common method of preventing microbial proliferation is to insert an ultraviolet lamp in the headspace.)
6. The tank has a manway and a light/sight glass for inspection.

Distribution. Preventing microbial proliferation within a PW distribution system requires that the water is not left to stagnate. Thus, distribution systems are continuously circulated through a system with minimum deadlegs. Because microbial contamination will occur, if only after maintenance activity, the systems are designed at least for periodic sanitization and often for steam sterilization. Some of the key features of these systems are

1. Deadlegs should be minimized wherever possible. They are unavoidable in some instances, such as at piping takeoffs. In these cases, the deadleg should be kept below six pipe diameters (measured from the valve face to the centerline of the distribution pipe and based on the diameter of the takeoff pipe).
2. To prevent cross-contamination, the system should be isolated from other distribution systems, especially those for lower-grade water and steam condensate. The system can be isolated at equipment either by disconnectable hoses or double block-and-bleed valve arrangements (Figs. 2 and 3).
3. The supply side of the distribution system should be designed for a flow velocity between 1.5 and 2.7 m/s (5 to 9 ft/s). At maximum system takeoff, the flow velocity in the return line should not fall below 0.9 m/s (3 ft/s). This ensures that turbulent flow is maintained and that deadlegs are continuously flushed.
4. The system materials should be inert and resistant to periodic sanitization (with disinfectants or hot water) and preferably also steam sterilization.
5. The system should be hygienically designed, such that sanitizing procedures can be effective. The requirements are that the piping and fittings have a smooth internal finish with minimum crevices and no undrained pockets. This determines the type of equipment that is usually used in such systems.

Valves. Diaphragm valves are commonly used, with ball, plug, and butterfly valves occasionally being specified. Where ball valves are selected, they are often of the encapsulated type to eliminate the dead space between the ball and the valve body. Now that diaphragm valve arrangements are being produced

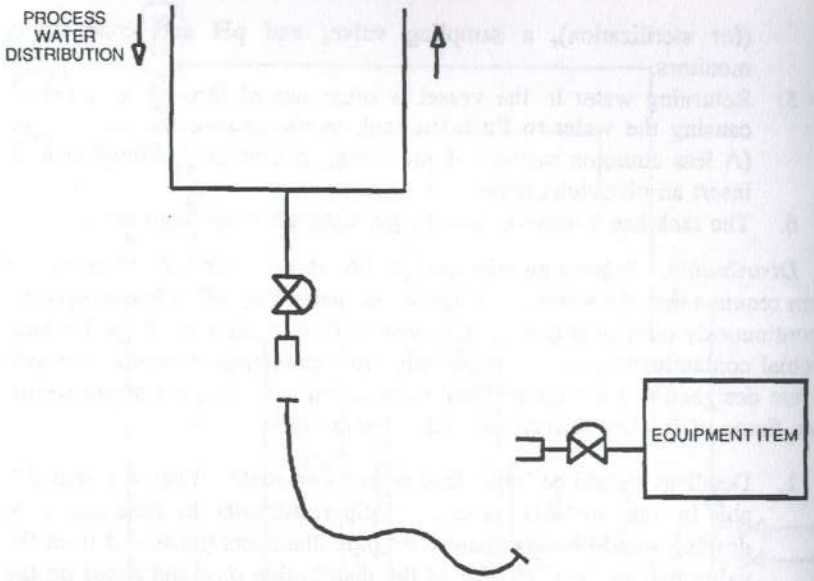


Figure 2 Disconnectable hoses.

which are specifically designed to minimize deadlegs at takeoffs, it can be expected that these will become the predominant selection. Valve bodies should be stainless steel and nonmetallic components should be inert—typically virgin polytetrafluoroethylene (PTFE), EPDM rubber, and silicone rubber are selected.

Piping/Tubing. Tubing is almost universally used for WFI systems, typically ANSI grade 316L ASTM 270 or equivalent. This is butt welded by automatic orbital welding wherever possible. For some PW systems Sch.10 pipe may be selected, and polyvinyl chloride (PVC) piping has been used for some process water systems where costs have had to be minimized.

Where pipe couplings have to be included, a variety of dairy fittings are suitable. Triclamp fittings appear to be most popular, especially in the United States, but IDF and DIN 11851 fittings have a cleaner external appearance and are not so easily interfered with. If disconnection is only occasional, flanges with a smooth-bore gasket can be acceptable. However, couplings should be limited to situations in which equipment items need to be removed. Wherever possible, valves, filter housings, and any other suitable item should be welded into the system.

Hoses are usually smooth-bore PTFE or silicone, braided with stainless steel for the larger diameters (above 1 inch). Any of the hygienic fittings already

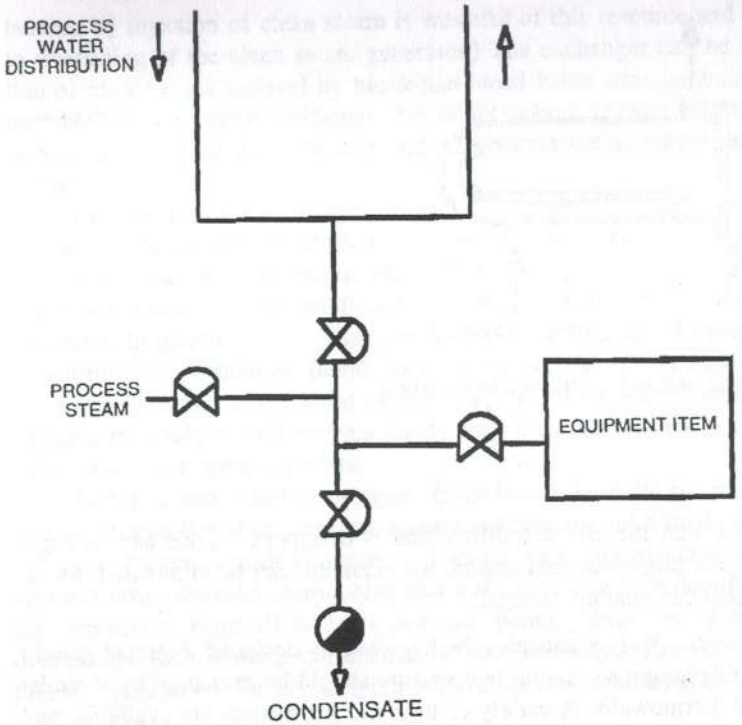


Figure 3 Double block and bleed valve arrangement.

mentioned may be used for the couplings, although specialized quick-disconnect couplings are now becoming available.

Pumps. Distribution through the water system should be by a centrifugal pump of hygienic design. The outlet port should be oriented to the low point to allow drainage, or a casing drain should be fitted. Packed gland seals accumulate contaminants, so mechanical seals should be selected. Double seals can be lubricated with the PW itself or by clean steam or steam condensate. Glandless magnetically driven pumps are available and remove the need for sealing water altogether.

Heat Exchangers. Heat exchangers are often found in WFI systems, principally in those that are run hot. To prevent contamination from the utility service, exchangers with double tube sheets or double pipes can be selected (Fig. 4). A more economical approach is to design a system such that the utility service is at a lower pressure than the water side. The system should be moni-

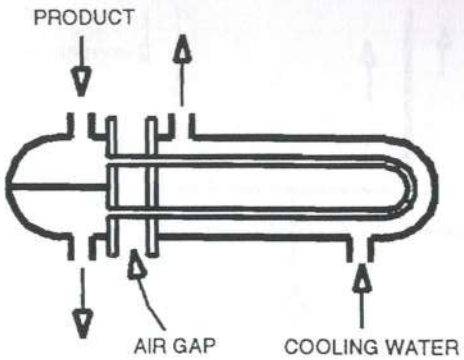


Figure 4 Heat exchanger with double tubesheet.

tored to ensure that the pressure differential is maintained. Thus any leakage, across gaskets in plate heat exchangers, for example, can be eliminated. Spiral heat exchangers can also be selected.

Instruments. Instruments must be hygienically designed. Pressure gauges should have diaphragm seals, and temperature should be measured by thermistors in sealed thermowells. A variety of hygienic flowmeters are available, and increasingly Coriolis mass flowmeters are being selected. Turbine and PD meters can be damaged by overrevving during steaming, ultrasonic meters tend to be inaccurate, and vortex meters have limited rangeability. Electromagnetic flowmeters are perfectly hygienic, but the low conductivity of PW systems usually precludes their use.

5. Sanitization of Water Systems

Water systems do become contaminated, most often by gram-negative bacteria, *Pseudomonas* sp. being a principal culprit. In such circumstances the system must be drained and sterilized. To prevent such extreme circumstances arising, a periodic sanitization procedure is often employed. This is designed to reduce the numbers of vegetative cells, but it will not necessarily produce a sterile condition or eliminate bacterial spores. Such sanitization methods may be either chemical or thermal. In pharmaceutical plants, where chemical residues must not be allowed to enter the process water, thermal methods are now becoming the favored option.

Thermal Sanitization. Hot-water sanitization can be accomplished by circulating the water through a heat exchanger and raising its temperature. (Direct

heating by injection of clean steam is wasteful of this resource and would lead to oversizing of the clean steam generator.) The exchanger can be mounted in line or on a bypass isolated by block-and-bleed valve arrangements or disconnectable hoses. A second exchanger can be included for postsanitization cooling, or a single exchanger can be supplied with steam and cooling water for dual service.

Hot-water sanitization may be carried out on a regular basis, typically weekly. On small plants operating on a day-shift basis, the water system can be maintained at a high temperature (85–90°C) overnight. It can be designed as an automated operation with minimum manual intervention and is therefore less disruptive to plant operation than steam sterilization. Many WFI systems used in pharmaceutical finishing plants are operated hot (60–85°C) continuously, the water being cooled at the point of use. The microbial quality requirements of bioreactors would not themselves justify such a method, which is expensive in both capital and operating costs.

Water systems must be designed for at least occasional steam sterilization. It is preferable that steam and condensate systems are not directly connected to the water system, as this is a potential source of cross-contamination. Disconnectable hoses are used, introducing steam at the system high point(s) and draining condensate from all takeoffs and low points. Since the system must be drained and then refilled, sterilization is more disruptive than thermal sanitization and is reserved for requalifying the system after maintenance or a contamination episode.

Chemical Sanitization. If chemicals are used for sanitization, their residues must be removed before the system is ready to return to operation. This may require two or three rinses with pure water, an operation that is both time consuming and wasteful of pure water. Historically, chemicals were used for sanitizing systems constructed of nonmetallic materials such as PVC. These are cheaper than stainless steel but not resistant to the high temperatures of thermal sanitization, let alone those of sterilization.

Sodium hypochlorite is one of the most commonly used disinfectants, its bactericidal activity largely resulting from its action in destroying cellular enzymes. It is used to produce active chlorine concentrations in the range 200 to 500 ppm, its effectiveness being related to exposure time. Hydrogen peroxide is also commonly used and disinfects by releasing free oxygen molecules. It is used as a 10 to 30% solution. One advantage is that it dissociates to innocuous oxygen and water, so it requires less postsanitizing rinsing than chemicals such as hypochlorite or sodium bisulfite.

Ozone is commonly used for sanitization in the United States. As with peroxide, its sanitizing effect comes from its oxidizing property, but it is effective at far lower concentrations. It, too, decomposes to oxygen, having a half-

life of about 20 min. The ozonation system would comprise a generator, passing gaseous ozone through a diffuser into a storage vessel or circulation system. A catalytic destructor is required on the vessel vent and external monitoring is recommended because ozone is toxic to humans at low concentrations. Ozonation can also be used on a continuous basis, in which case a UV sterilizer is installed after the ozone diffuser in the water circulation system. This is sized to destroy all ozone residue before the water reaches the distribution points. The principal disadvantages of ozonation are its relatively hazardous nature and the high capital cost of the generating equipment.

B. Cooling Water

There are no special quality requirements for cooling water in biological plants, provided the water remains contained and isolated from the process. Typically the water is river water or borehole water, sand filtered to remove silt and gross organic contamination. For small plants with limited water usage, a once-through (total loss) system can sometimes be justified, but economies tend to favor the use of cooling towers with recirculating systems. For very large bioreactors, such as those used for single-cell protein (SCP) processes, once-through seawater cooling has been used. This tends to be confined to reactors employing external cooling loops or those operated under nonaseptic conditions. The chloride content of seawater is incompatible with stainless steel equipment that is also subject to steam sterilization (due to its susceptibility to stress corrosion cracking under such conditions). Titanium plate heat exchangers have been used to overcome this problem.

In biological plants subject to GMP regulations, it is wise to consider the possibility of process contamination from the cooling water. If leakage in equipment is a potential problem, the cooling water should be supplied at as low a pressure as possible. This may avoid the need to use expensive equipment, such as a double-tubesheet exchanger on purified water cooling applications. The cooling water pressure can be monitored and alarmed to ensure that it remains below process pressures. Prevention of room contamination should also be considered and may require that equipment drains are discharged outside the process area or into a sealed sewer.

1. Cooling Water Systems

Cooling Water Temperature. The temperature of water delivered from a cooling tower is governed by the wet bulb temperature of the inlet air. This value varies throughout the year. In the UK design valves for air wet bulb temperature (i.e., those achieved during the summer months) vary from 18°C in the south to 15°C in the north and west. In the United States the values are

higher, ranging from 18 to 27°C. Cooling towers can be economically sized to produce water within 4 to 10°C of these temperatures. Therefore, at best, tower cooling water can be dependably produced at temperatures no lower than about 22°C in the UK—sometimes worse, depending on plant location and tower design. Allowing for a temperature differential at the equipment of 4°C minimum, this means that any process cooling to temperatures below about 26°C requires some form of chilled water supply.

Unadulterated water can be chilled as low as 4°C using refrigeration plant and is suitable for process temperatures down to about 8°C. Some bioreactor broths are chilled below this temperature prior to downstream processing (4 to 5°C is typical for many protein solutions). In such cases a coolant with antifreeze must be supplied. Ethylene glycol is most common and, at suitable concentration, can operate down to about −20°C. Ethanol solutions have sometimes been used, but they present a flammability hazard. Salt solutions, commonly used elsewhere in industry, should be avoided because of their corrosive effect on stainless steel. Such coolants are recirculated through refrigeration plant, the heat there being removed by air or cooling water. It is unlikely that very low temperature will be required for bioreactors, but there are specialized heat transfer fluids, such as Dowtherm J, which can operate down to about −70°C.

Cooling Water Distribution. Cooling water distribution in biological plants is conventional. The distribution system is usually carbon steel. Inside clean rooms the piping may be changed to stainless steel for reasons of hygienic external appearance. Alternatives are to epoxy paint the carbon steel pipe or cover it with a hygienic-finish insulation material. There is no economic justification for insulating cooling water systems against heat gain. However, in unventilated piping voids, where temperatures are uncontrolled and can rise substantially above those of the process areas, care should be taken that water does not condense from the air onto the pipe surface. This could generate a moisture pathway allowing contamination to spread from the void to the adjacent clean room. This problem can be avoided by specifying antisweat insulation.

Cooling equipment is sized on the basis of an economic temperature rise in the cooling water, and typically this is about 7°C. If the maximum supply temperature of the cooling water is 22°C, the return will not exceed 29°C. While a continuous plant can operate in this fashion, a batch plant can exceed these limits. In biological plants, the problem usually occurs with vessels being cooled after sterilization. While cooling from 121°C a bioreactor can return cooling water initially well above 60°C, and this may damage cooling tower internals if they are wood or plastic. If the hot water is not reduced in temperature by mixing with an adequate quantity of cool water from elsewhere on the distribution system, a control system must be devised to limit the quantity of hot water returned during the initial stages of vessel cooling.

III. STEAM

A. Plant Steam

Plant steam is provided to fermentation plants for general process heating. It supplies jacket heating to bioreactors, sterilizing vessels, and autoclaves and other process equipment. It is usually generated from a fired boiler at medium pressure, typically 5 to 8 bar g, and is distributed through carbon steel headers to the point of use.

In biopharmaceutical manufacturing plants, there is normally a need to inject steam into equipment and piping for sterilization purposes. The condensate from the injected steam cannot be completely removed prior to the processing operation, so any contaminants introduced with the steam can appear in the process fluids. Plant steam carries with it rust debris from the piping system and also chemicals from the boiler feedwater, such as anticorrosion and antiscaling agents. Although a sintered stainless steel filter can remove the debris, the volatile impurities will remain. Where steam is to be injected into pharmaceutical manufacturing plant, a separate "clean" steam supply, free of contaminants, is required. This must be generated from a source separate from that of the plant steam and distributed through its own header system.

B. Clean System

Clean steam is defined as steam that has been produced from boiler feedwater free of volatile additives such as amines or hydrazines. In fact, the boiler feedwater would normally be chemically equivalent to PW, and clean steam condensate would be chemically and microbiologically equivalent to PW. Where it is required that the condensate be pyrogen-free, equivalent to WFI, the steam is defined as pure steam. As with water standards, bioreactors rarely have an operational requirement for pure (pyrogen-free) steam themselves. They may be supplied with pure steam if subsequent operations in the plant demand this quality and the provision of two sepatate systems is uneconomic.

Clean steam is used for equipment and piping sterilization (by injection), steam blocks in piping around aseptic vessels, autoclaves, and clean room dehumidification.

1. Clean Steam Generators

Since the definition of clean steam precludes the use of boiler additives, clean steam generators are usually 316 stainless steel vessels, with either stainless steel or titanium tubesheets. They usually operate on a thermosiphon principle (Fig. 5), water cycling from the steam vessel through a heat exchanger and the clean steam being released into the headspace of the generating vessel. The heating medium is usually plant steam for the larger generators, which have outputs of

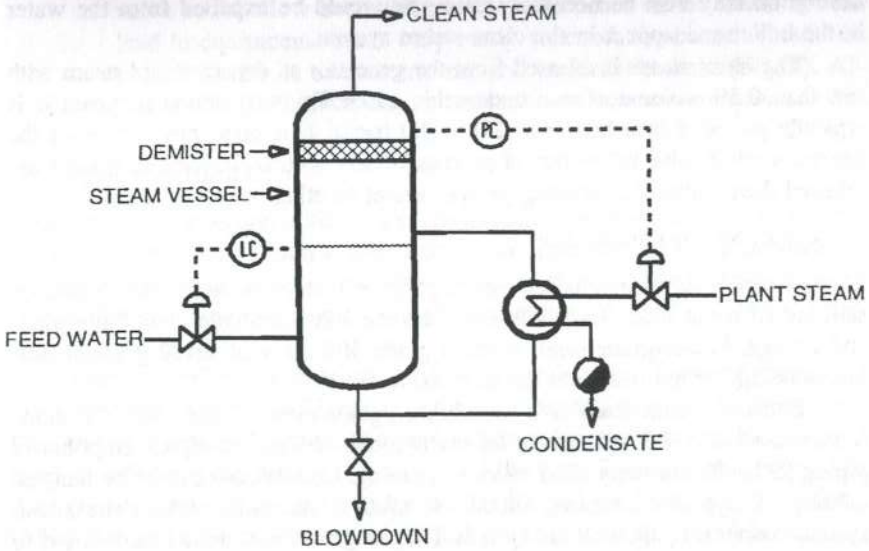


Figure 5 Clean steam generator—thermosiphon.

clean steam up to 4000 kg/h. Pressurized hot water can also be used for heating, and in the smaller units, 10 to 150 kg/h steam output, electrical heating can be used.

If plant steam is used as the heating medium, it should be at least at a pressure 2 bar greater than the desired clean steam pressure, and units are available which can operate with plant steam up to 10 bar g. As a rule, plant steam consumption tends to be about 30% greater than clean steam production. The highest efficiency is achieved with the highest plant steam pressures.

The quality of the clean steam is reduced if water droplets are carried over from the generator. Therefore the steam vessel is vertical, large in diameter, and relatively tall to reduce steam velocity and allow droplets to separate out. Usually a demister, baffles, or cyclone separator are incorporated in the design to capture any persistent droplets. To prevent contamination from the plant steam, the heat exchanger should be of the double-tubesheet type.

Clean steam generators operate satisfactorily only if the feedwater is of appropriate quality. Limits are placed on hardness, particulates, and silica concentration to minimize scaling problems, and on chlorine concentration to minimize corrosion. Softening is often an adequate pretreatment, especially if the generator can operate an intermittent blowdown, although demineralization or RO pretreatment is needed for some waters with high salts content. Feedwater

deaeration may also be necessary, since air would be expelled from the water in the boiler and appear in the clean steam system.

The clean steam is released from the generator as dry, saturated steam with less than 0.5% volume of noncondensable gases. For sterilization purposes it is typically provided in a header at 2.0 to 3.0 bar g. It is good practice to set the generator relief valve below that of process vessels, so that pressure excursions are relieved there rather than causing process vessel bursting disks to rupture.

2. Clean Steam Distribution

Clean steam and its condensate are highly corrosive, so materials should be selected to resist this. Grade 316 stainless steel is a suitable choice for pipes and fittings. Lower-grade steels such as grade 304 can rust, leading to contaminants being carried over into the process system.

Because steam itself is a sterilizing agent, there is not quite the same requirement as in PW systems to follow hygienic design principles. Unpolished piping (Sch.10) is usually used instead of tubing. Connections can be by flanges, although a hygienic coupling should be used at the points where the steam system connects to the process system. Diaphragm valves are not best suited to constant exposure to high temperatures (the diaphragm life is severely reduced). Ball valves are usually used, although plug, butterfly, and gate valves are also sometimes specified.

It is important for the efficiency of sterilization processes that clean steam is delivered dry and saturated at the point of use. The distribution system should be sloped to low points for condensate drainage, preferably in the direction of flow. Typical points of good engineering practice for clean steam distribution systems are as follows:

1. Steam pipes should have a minimum 1:100 fall in the direction of flow and should be properly supported to prevent sagging.
2. Steam traps should be located at all low points and at convenient points along any extended pipe length (approximately every 30 m) to ensure that the pipeheads are kept clear of condensate.
3. Valves, fittings, and steam traps should preferably be installed so that they are self-draining.
4. The clean steam system should be completely isolated from the plant steam system and that system's condensate collection system.
5. Condensate from clean steam systems should not be recovered for clean steam generation. It can, however, be collected for return to the plant steam system (provided it does not contain process debris or biological material). In some instances it may also be used in various process applications where clean water (not PW) is required, such as CIP prerinses.
6. During periods of equipment isolation, steam condenses and cools in the header. The accumulation of cold condensate behind a process valve could allow bacterial growth. If branches of the steam system lead to equipment that is only rarely steamed, the branch should be provided with its own steam trap and condensate drain (Fig. 6). Alternatively, the branch can be isolated by a hose connection.

C. Steam Consumption

Typically the process and utility steam consumption varies substantially throughout the process cycle within a bioreactor. Peak loads occur during sanitization and steam sterilization operations. The amount of steam utilized depends on the sanitization/sterilization operation undertaken; either "empty" or "full" operations can be used.

Empty sterilization or sanitization is undertaken on an empty vessel and requires direct injection of large quantities of process steam into the vessel. Condensate is removed through the vessel drain valve; the pressure in the vessel is set by adjusting the drain valve to control the flow. Process steam is injected through all overhead lines, typically the feed lines, air inlet, and vent lines. Utility steam is used on the bioreactor jacket to assist on the initial heating up.

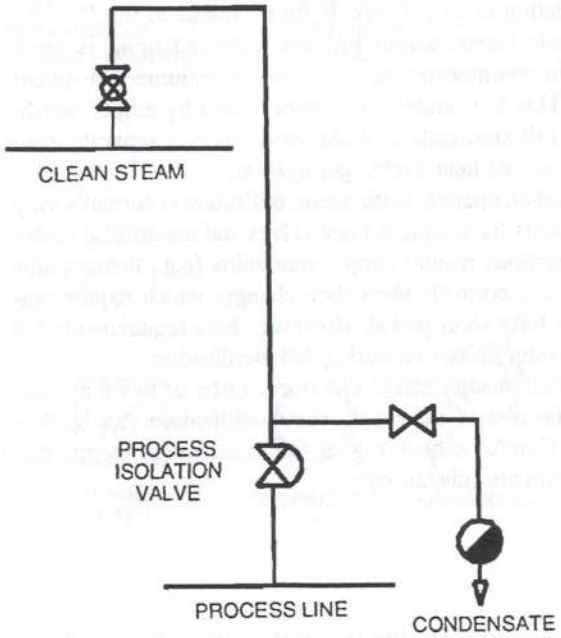


Figure 6 Trapping of clean steam system.

However, once the temperature has reached >100°C this steam is usually switched off. Total reliance is then on the directly injected process steam to maintain the sterilization temperature. This method is used to ensure good surface cleaning/sterilization of the inside of the bioreactor due to the scouring effect of the process steam flow as well as the temperature effect. This type of sterilization/sanitization operation is typically carried out on animal cell operations where the medium cannot be sterilized in situ or in production environments where schedule, hence turnaround, is critical.

Full sterilization is undertaken once the unsterilized medium is added to the bioreactor. This sterilization or sanitization operation (water used rather than medium in the vessel) requires substantially more utility steam to the bioreactor jacket. The utility steam to the jacket is the sole method of heat input and is used throughout the sterilization cycle. Process steam is required but only in relatively small quantities and for ancillary operations (e.g., sterilization of the sample valve, harvest valve).

Normally, the pressure within the bioreactor is controlled by the main vent control valve. The pressure control, however, is initiated only when the vessel and contents temperature has reached +95°C. This ensures that any air within the system is removed and thus effective sterilization is achieved.

The overhead lines are normally sterilized by steam produced from the bioreactor contents. The direction of steam flow is thus "inside to out."

During some larger-scale fermentations process steam sparging is used. This technique assists with the maintenance of the contents' volume and speeds up the sterilization process. This has tended to be superseded by empty sterilization of the bioreactor and full sterilization of the medium in a separate, specially designed sterilization tank or heat exchanger system.

During nonsteam sterilization operations the steam utilization is normally very low (e.g., only steam requirements for sample/harvest valves and mechanical seals). Occasionally fermentation operations require temperature shifts (e.g., thermophilic production of product). These are normally short-term changes which require reasonable amounts of steam, but for a short period. However, these requirements fall well within the demands for steam utilization during full sterilization.

Typically, steam sterilization/sanitization can occur once or twice a week and can occupy 2 to 6 h. The rest of the week, steam utilization can be less than 20% of the peak load. Careful scheduling of the sterilization events can thus reduce the steam requirements substantially.

IV. AIR AND GASES

A. Process Air

Process air is supplied to bioreactors primarily for aerating the culture medium. It is also often used, in smaller quantity, for performing pressure transfers and

breaking vacuum after steam sterilization. As a material that enters the process system, it has the potential to contaminate biopharmaceutical products. In such plants, its quality must therefore be defined, to a standard in excess of that normally required for process plants.

Table 2 gives some typical contaminants of compressed air. These contaminants are derived from the atmosphere, the compressor, and the piping system. In designing a process air supply system, steps may have to be taken to remove atmospheric contaminants and avoid contamination being introduced by the compression system itself.

Figure 7 shows a typical air compression system.

Table 2 Compressed Air Contamination

Contaminant	Source	Typical concentration
Dirt particles	Atmosphere	Up to 140×10^6 per m^3
Carbon	Burnt oil	Up to 10 mg/m^3
Water	Atmosphere	Up to 1100 mg/m^3
Rust	Pipework	Up to 4 mg/m^3
Oil	Compressor lubricant	5 to 50 mg/m^3
Oil/water emulsion	Mixture of oil and water	Up to 1100 mg/m^3
Vapor	Gaseous oil	0.05 to 0.5 mg/m^3
Microorganisms	Atmosphere	Up to 3850 per m^3
Unburnt hydrocarbon	Atmosphere	Up to 0.5 mg/m^3

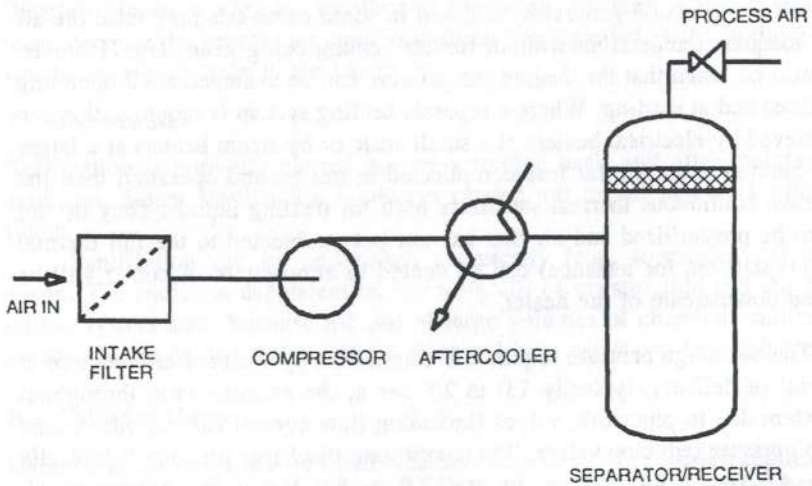


Figure 7 Air compression system.

1. Phage Control

Airborne bacteriophage are a concern in many biological operations. In conventional antibiotic processes, such as tetracycline fermentation, phage contamination can lead to a severe reduction in yield, that is, in processes using genetically modified bacteria there is a more fundamental concern. Infection of the production organism with foreign genetic material could alter the nature of the therapeutic protein being produced, perhaps making it ineffective or altering its activity. This potential loss of control of product quality is viewed seriously within the industry. To some extent conventional sterilizing filters, especially the depth type composed of packed borosilicate glass fibers, will absorb phage. However, this cannot guarantee complete removal or inactivation, and so sometimes specific phage inactivation processes are incorporated into the air treatment system.

As with other microorganisms, bacteriophage are susceptible to heat treatment. Using the conventional techniques of thermal sterilization, it is possible to design an effective phage inactivation system. A dry heat survival curve can be determined experimentally, and from this the thermal inactivation constant at various temperatures, can be calculated. Then, once the initial phage concentration in the incoming air has been determined and the desired reduction selected, a thermal treatment process can be specified. Because phage have less thermal resistance than bacterial spores, these processes are at relatively low temperature and require shorter holding times. Typically they are at 90 to 100°C for 2 to 5 s. Thus dry heat inactivation, impractical for air sterilization, is entirely feasible for phage removal.

Air compression generates heat, and in some cases this may raise the air to an adequate temperature without further heating being necessary. However, care must be taken that the desired temperature can be achieved at all operating conditions and at start-up. Where a separate heating system is required, this can be achieved by electrical heaters at a small scale or by steam heaters at a larger scale. Such systems are far less complicated in design and operation than the equivalent continuous thermal sterilizers used for treating liquids. They do not need to be presterilized and air that has not been subjected to the full thermal cycle (at start-up, for instance) can be vented to atmosphere. A cooler will be required downstream of the heater.

The discharge pressure required is determined by the pressure required at the point of delivery, typically 1.0 to 2.5 bar g, the pressure drop throughout the system due to pipework, valves (including flow control valves), filters, and system pressure reducing valves. The compressor discharge pressure is typically 3.0 to 4.5 bar g for process air and 7.0 to 8.0 bar g for instrument air requirements.

2. Storage and Distribution

Process air is typically supplied from a centralized site or building compressor area and is then distributed through pipelines to the various points of use.

Because demand varies, the compressors are normally sized for average rather than short-term peak loads. Air receivers are installed in the system to cope with the peak loads and short-term drops in supply due to compressor changeover or start-up of additional units. Typically, receivers are sized to meet 50% of the demand for a period of 5 to 10 min. Care should, however, be taken with facilities containing large numbers (e.g., 5+) of large bioreactors (e.g., 10 m^3+), since the sizing factors would result in an extremely large receiver. For this type of facility a commercial balance has to be made between increasing the compressor discharge pressure and hence the receiver design pressure but maintaining the receiver size with reduction of the period to 1 to 2 min for 50% of the average demand.

Receivers are normally located close to the generating compressor systems and can be located outside. They normally have pressure gauges, low-pressure alarms, pressure relief valves, and a pressure reducing valve assembly on the discharge line.

Receivers are constructed from mild steel, galvanized or stainless steel. The choice is dependent on air quality, environment at the receiver location (external to the elements or internal within a utility building), and corporate standards.

The distribution pipework is normally constructed from similar materials; however, the trend is to use more stainless steel, which allows line steam sterilization. However, after the sterilization filters the pipework is always stainless steel, because the process air sterilizing filters are regarded as the sterile barrier for the air supply train to the bioreactor.

3. Sanitization

Sanitization is normally carried out on a routine basis and after maintenance activities. Steam sterilization is always carried out on all pipework after the filters.

Sanitization can be undertaken chemically (e.g., formaldehyde) or by steam. The choice is dependent on the materials of construction and the distribution system size. Because the use of large volumes of chemical sanitization agents can create disposal concerns, the trend is to use steam for sanitization.

B. Cylinder Gases

Cylinder gases are used with small bioreactors, generally in research applications where defined composition or control of oxygen mass transfer is required. Carbon dioxide and ammonia are sometimes used for pH control.

Ammonia and carbon dioxide in cylinders are normally provided as liquids, at 34 and 53 bar g, respectively. A heated vaporizer may be needed, particularly at high flow rates and with carbon dioxide, to prevent freezing at the expansion valve on the cylinder outlet. Oxygen, nitrogen, and zero air (79% nitrogen, 21% oxygen) are provided as pressurized gases.

Gas cylinders are normally color coded and marked with the gas name. The cylinders should preferably be mounted vertically in a cylinder rack, in a secure, well-ventilated location away from heat sources. Nitrogen and carbon dioxide are asphyxiants, but ammonia offers the greater hazards of both toxicity and flammability. If ammonia cylinders are located in occupied or unventilated areas, it is advisable to have a detection and alarm system to monitor for any leakage.

Pressure reducers, flowmeters, pressure gauges, manual or automated mixing manifolds, and valving may be needed, and these can usually be provided by the gas supplier. Often cylinders are located near their point of use and supply only a single user. Distribution systems have been incorporated into some research and small-scale cell culture facilities. These operate at low pressure and tend to use stainless steel tubing for the distribution piping.

1. Quality

Cylinder gases are normally used in relatively small volumes, and as a consequence high-quality medical grade gases can be used without a substantial cost penalty.

2. Operations

Cylinder gases are typically used for enrichment (O_2) and pH control (CO_2 and NH_3). The gases are injected into the air supply to the sparge system to provide an effective dilution system and good mixing within the bioreactor. The gases are typically injected prior to the air sterilizing filters and the flow rates are controlled by rotometers and manually operated needle valves or small automated mass flowmeters. Nonreturn valves are normally provided to prevent backflow. Local pressure relief valves are typically installed in each gas supply line, because the gas supply is at a much higher pressure than the carrier air supply, usage is low, and the stagnant lines can overpressurize due to environmental heating of the line.

3. Production Processes

Cylinder gases are usually supplied by reputable suppliers and delivered for use with certificates indicating quality compliance.

4. Storage and Distribution

The cylinders are the primary storage. However, the cylinders themselves must be stored in a safe manner. There are regulations and standards related to the location of stored cylinders, cylinders in use, and empty cylinders dependant on the gas within the cylinder, the environment, and the site security.

5. Sanitization

The transfer lines are not normally sanitized regularly because the gases are effectively sterile. However, care is usually taken to ensure that the lines are degreased prior to use, following installation. This is important not only for contamination requirements but also for safety reason, particularly for oxygen, where explosive environments can occur.

C. Sizing for Process Air and Cylinder Gas Requirements

1. Process Air

Bioreactors used in microbial fermentations tend to operate at flow rates of air up to 1.5 to 2 VVM and typically at pressures up to 1.5 bar g. Bioreactors used in mammalian cell cultures typically operate at 1.0 to 1.5 VVH and typically at pressures up to 0.5 bar g.

Typically, the air flow requirements increase throughout the fermentation. Sizing of the supply and distribution system should take account of this, particularly in facilities with multiple bioreactors, and sizing should be based on an average load plus a safety factory rather than the short-term maximum load.

2. Cylinder Gas Requirements

These tend to be used largely on the mammalian cell fermentations and typically operate at flow rates up to 10% of the air flow rate, i.e., 0.1 to 0.15 VVH, and at pressures up to 1.0 bar g. The usage is usually intermittent but, because of the nature of the process, tends to be for a longer process period (i.e., 10 days+). The usage is normally low but reliability becomes a more important issue, so systems tend to be oversized and have multiple cylinders with auto-switch-over systems.

V. SUMMARY AND CONCLUSIONS

Regulatory demands are increasing the quality requirements expected from utility and service systems. Commercial constraints are ensuring that increased reliability continues to be an important issue.

The drift away from standard site utility systems to higher-quality, building-specific, service systems will continue. More of each system is likely to be constructed from stainless steel to ensure that sanitization and sterilization operations can be undertaken. The utilization of clean steam is anticipated to increase with the amount used increasing to cope with the additional systems and pipework.

Process water demands are likely to increase to cope with the increased process steam demands but also because of the use of higher-quality water for more applications which previously used standard town water or softened water.

Process air demands are anticipated to remain similar to present requirements. However, as this is an expensive service to provide, it is anticipated that greater attention will be paid to ensuring that the system is correctly sized with erosion of spare capacity. The present advantage of using cylinder gases, relatively small volume requirements, is likely to remain for small laboratory bioreactors and mammalian cell systems.

A general trend will be to improve reliability, and economic effectiveness will continue to be an increasing demand.

VI. TROUBLESHOOTING

Problems can be anticipated and minimized or removed by regular proactive maintenance and careful design. The following areas typically generate concerns.

System	Problem	Resolution
Process water	Reduced feedwater quality	Usually caused by seasonal quality variations. Ensure variations reviewed and process water pretreatment designed for worst case.
Process water	Contamination	Ensure design reduces potential for cross-contamination by use of isolation systems (double valve block and bleeds, piping manifold, etc.).
Process water	Reduced flow rate	Normally caused by water being drawn off elsewhere. Different water requirements reviewed in detail and SOP developed to ensure overutilization does not occur.
Process water	Leaks	Normally occur as a result of sterilization/sanitization temperature cycling or a failed component. Design using high-quality components and materials plus regular proactive maintenance.
General	Faulty measurement	Use tried and tested instrumentation systems and ensure systems are regularly calibrated and maintained. Use duplicate probes for critical parameters.
Cooling water	Cooling rate reduced	Commonly caused by fouling of heat exchange surface. Ensure systems are regularly cleaned and design anticipates a level of fouling.

Steam system	Reduced pressure	Normally caused by actual demand exceeding design. Ensure requirements carefully reviewed during design and SOP developed to ensure overutilization does not occur.
Steam system	Contamination	Normally caused by carryover or distribution line contamination. Ensure regular steam generator blowdown (can be automated) and distribution line components are appropriately selected during design.
Process air	Reduced quality	Usually caused by distribution system or compressor leakage. Ensure oil-free compressors used and regularly maintained. Ensure appropriate components selected during design.
Process air	Reduced flow rate	Blocked filters or leaks; ensure filters are regularly checked and changed. Air distribution system regularly checked and maintained, particularly blowoff/relief systems.
Process air	Wet air	Ensure knockout systems provided in the distribution.
Cylinder gases	Reduced quality	(As for process air)
Cylinder gases	Reduced flow rate	Leaks or faulty changeover systems. Ensure system regularly checked and maintained.

NOMENCLATURE

DI	Demineralized water
DP	Differential pressure
EP	European Pharmacopoeia
GMP	Good pharmaceutical manufacturing practice
PW	Process water
RO	Reverse osmosis
SCP	Single-cell protein
TOC	Total organic carbon
UF	Ultrafiltration
USP	United States Pharmacopeia
VVH	Volume of air per volume of bioreactor contents per hour
VVM	Volume of air per volume of bioreactor contents per minute
WFI	Water for injection

Index

Absorption spectrum, 443
Air
  compressed, 607
  contamination, 607
Air conditioning and containment, 580
Airlift
  bioreactors, 184–188, 265, 266
  for immobilized animal cells, 395–398
Algae
  batch and continuous culture, 451–454
  growth kinetics, 448–454
  light regime during growth, 455
  photosynthetic efficiency, 459
  productivity as function of dilution rate and light, 454
[Algae]
  yield and maintenance, 459
Animal cell
  banks, 24–27
  bioreactor operation and control, 401
  entrapment, 399
  growth and product formation, 402–404
  products, 19
  selection, 18
Animal cell concentration as function of power input, 391
Animal cell immobilization, advantages, 378
ATP generation and utilization scheme, 144

Bacteria, 50–53
classification, 52
Batch operation, basic equations, 484–485
Bioenergetic yields, *Chlorella vulgaris*, 459
Biological system identification, 2–4
Bioreactor design fundamentals, 139–197
Bioreactors (*see also* Design of Bioreactors)
airlift, 265–266
continuous operation, 493
design equations, 293–296
dialysis culture, 501
extractive culture, 501
filtration culture, 501
flow patterns, 275–279
gas phase characterization, 279
gas residence time, 281–284
industrial practice, 291–293
operation modes, 479–503
overview, 1–12
parameter distribution, 275–279
perfusion culture, 502
physical characterization, 269–270
repeated operations, 500
schematic of interactions, 196
selection, 5–6, 57–72
types, 7–8, 10
Bubble columns
bioreactors, 184
mass transfer, 184
Bubble velocities in bioreactors, 285, 288

Cell
compatibility with membranes, 327
confinement, 308–310
interaction with membranes, 327
population kinetics, 149–161
protection, 313–314
recycle in chemostat operations, 498–500
Chemical sterilization, 558
Chemostat, basic equations, 494–498
Circulating bed for immobilized microorganisms, 358
Clean steam
distribution, 604–605
generator, 603
Clean system, 602
Compartment models, 231–238
Containment
categories and hazard groups, 572
design, 569
regulatory issues, 567
requirements for large-scale applications, 570
Continuous operation
control indices, 493
with feedback control, 498

Data requirement for bioreactor design, 76–78
Deliberate release, 569
Design of bioreactors, 1–12, 293–296
basic elements, 9
data required, 76–78
Diffusion, transport in membrane reactors, 321
Distributed digital control of a bioreactor, 539
Droplet bioreactor for shoot cultures, 432

Effectiveness factor in immobilized biocatalysts, 363–367
Enzyme
compatibility with membranes, 327
confinement, 308–310
interaction with membranes, 327
protection, 313–314
Essential oil production, 435

Failure analysis, 572
Fed-batch culture and feedback control 490–492
Fed-batch operation, 485–488
classification, 489
Fermenter, 208
contained, 574, 577
impellers, 209–210

Finite volume element compartments, 243–247
Flow models in bioreactors, 258–269
Flow patterns in bioreactors, 275–279
Flow profiles in hollow-fiber reactors, 317, 320, 324
Fluid flow, effect on animal cells, 404–406
Foam, 68–70
Fungi, 53–54

Gas cylinders, 609
Gas phase in bioreactors, 279
Gas requirements, 611
Gas residence time in bioreactors, 279
Gas velocity in bioreactors, 286–287, 289
Glass fiber in packed-bed bioreactor, 392–393
Glucose oxidase, distribution in a dual-membrane reactor, 318
Growth factors, 95
Growth kinetics, 55–57, 148–180
Growth rate vs. light intensity, 445

Heat exchangers in WFI systems, 597
Heat transfer, 194–195
in bioreactors, 194–195
in bubble columns, 194–195
in stirred bioreactors, 218–219
Heterologous protein expression systems, 20–24
Hydroponic bioreactor, 432
Hyphol growth, 53–54

Immobilized animal cell bioreactors, 337–407
Immobilized microorganisms (*see also* Microbial immobilization)
apparatus for continuous interesterification, 354
bioreactors, 339–375
and fluidized bed reactors, 355
industrial applications, 340
mass transfer to, 357
and packed bed reactors, 353
[Immobilized microorganisms]
and reactor configuration, 351–357
and stirred tank reactors, 353
Impellers, 209–210
Inhibition, 161–162
Instrumentation, 576
Intraparticle mass transfer, 193–194

Kinetic models, 156–180
animal cells, 179–180
effects of temperature and pH, 162–165, 166
inhibition, 161–162
multiple substrates, 157–158
plasmid, 175–179
product formation, 158–161
recombinant microorganisms, 174, 179
structured and segregated, 167–174
Kinetics, 5, 149–180
cell population, 149–154
heat evolution, 165–167, 168, 169, 170

Length ratio in membrane reactors, 322
Light intensity, variation with distance, 446, 447
Light utilization, 442–448
Liquid circulation, and performance characteristics, 535
Liquid miting in animal cell bioreactors, 382
Long-term production in bioreactors, 315

Macromolecules, supply and removal in membrane reactors, 319
Mammalian cell media, 121–127
Mass and energy distribution in bioreactors, 230
Mass transfer
in bioreactors, 180–194, 211–218
convective in membrane reactors, 323
coupling with reaction, 359–367
effect on microbial activity, 191–193
effects of flowrate on $k_L a$, 369
fundamental approach, 188–191
gas-liquid 181–182, 211–218, 287–288

[Mass transfer]
in immobilized animal cells, 381
in immobilized biocatalyst particles, 360–367
liquid-microorganism, 191
in membrane reactors, 315–327
of metabolites, 319–320
mycelial pellets, 217–218
particle size and k_La, 368
in photobioreactors, 466
in scale-up, 524
in stirred tank bioreactors, 182–184, 215–216
of substrates, 319–320
Media suppliers, mammalian cells, 123
Medium
antibiotics, 118
biochemical mechanisms, 97–98
carbon and nitrogen sources, 102
cost, 100
design, 4–5, 89–127
development, steps involved, 92
formulation, 105–109
growth and product formation, 95–96, 111
ions, 99
macroelements, 93, 94, 114–115
microelements, 93, 112–114
plant cells, 109, 110, 121
processes, upstream and downstream, 98–99
yields, 103
Membrane, mechanical damage, 329
Membrane reactors, 305–338
Microbial immobilization
adsorption, 347
colonization, 348, 349–351
covalent bonding, 341, 342
gel entrapment, 342–347
methods, 341–351
Microbial products, 14
companies, 16–17
expression, 16–17
Microcarrier bioreactor for animal cells, 380–381, 388
Microfiltration, 306
Microorganisms
culture pH, 65–66
foam, 68–70
growth characteristics, 50–57
growth rate, 65
medium composition, 67–68
model, 48
safety, 71
sterility, 70–71
Microscopic balance equations, 518–519
Mixing in suspension cultures, 420
Model microorganisms, 48
Monoclonal antibodies
expression systems, 20–21
mouse myelomas, 21
Monod model
constants, 152
and mass transfer, 154–156
Mycelial pellets, mass transfer, 217–218

Neural networks, in bioreactors, 220–222, 224
Nonaqueous liquids in membrane reactors, 313
Number of generations, effect on variant strains fraction, 514–515
Nutritional requirements, media, 92–96

Operating boundaries for fermentation scale-up, 523
Optimization of sterilization, 506–507
Organism selection, 13–45
traditional processes, 14–18
Oxygen demand, 61–64
Oxygen-sensitive strain to test scale-up, 544
Oxygen supply to microorganisms, 213
Oxygen transfer, effect on diameter ratio in airlift bioreactors, 396

Permeability
effect of growth, 400

[Permeability]
effect on optimal diameter ratio in airlifts, 398
effect on oxygen transfer in fiber bed bioreactor, 397
Phage control, 608
Photobioreactor, 441–472
applications, 468–469
design, 462, 464–466
heat transfer, 466
operation and control, 467
pond culture, 462
shallow circulating systems, 463
Phototrophs
carbon source, 448
nutritional requirements, 442–444
Plant cell
bioreactors, 413–436
immobilization, 423–426
medium, 109–121
products, 414
suspension cultures, 419–423
Plant organ cultures, bioreactors for, 426
Plasmid
kinetic models, 175–179
Pneumatically agitated bioreactors, 257–296
Power consumption in bioreactors, 219–220
Power uptake as a criterion for scale-up, 546
Preservation
of animal cells, 24–26
of microorganisms, 24
Presterilization operations, 561
Process air, 606
requirements, 611
Process performance of bioreactors, 288–290
Product
characteristics, 75–76
formation, 158–161
inhibition, 75
synthesis, 75–76

Quantum yield in algal growth, 460

Radiation measurement, 444
Reactors
control, 330
hollow-fiber, 307
membrane, 305–338
monitoring, 330
stirred membrane, 326
Recirculation time models, 225–231, 229
Recombinant microorganism, 72–75
selection, 18–20
Recombinant proteins, 21–24
expression system, 22
system selection, 22
Regime analysis and scale-down, 529–532

Scale of operation consequences, 513–515
Scale-up
algorithm, 527–528
of bioreactors, 511–550
and circulation time, 537
columnar fermentors, 533–535
of containment, 572
criteria, 224
dependence of product ratio on stirrer speed, 545
gluconic acid, 533
methods, 521
and productivity, 538
of pullulan production, 535
and sterilization, 540–541, 565
Seals
double mechanical, 576
rotating, 576
Selection and design of bioreactors, 76–78
Selective supply and removal, 310–311
Shear, 195–197
in bioreactors, 195–197, 218–219
effect on kinetics, 196
effect on morphological changes, 62
Shoot cultures
submerged bioreactor, 431
transformed, 430–435

Similarity theory, 515–521
Steam, 602
Sterile agitator seal diagram, 563
Sterile engineering, 562
Sterile integrity, 556
Sterilization
  by absolute filtration, 559
  and containment, 553–587
  design discipline 554–555
  equipment specifications, 564
  in medium preparation, 560–561
Stirred tank bioreactor, 207–248
  basic equations, 482
  correlations, 215–216
  general scheme, 483
  mass transfer, 182–184
Stirring, mechanical, 182–184
Stoichiometry, 4–5, 140–148
  and bioenergetics in algal growth, 456–459
  overall equation, 140
  second law of thermodynamics, 143
Structured modeling of bioreactors, 222–225
Substrate
  degree of reductance, 143
  yield coefficient, 143
Suppliers of bioreactor systems, 589–613

Test
  of containment, 583
  of equipment integrity, 583
  of new filter integrity, 583
Thermal deactivation, 556–558
Time constants, for axial dispersion and circulation, 537
Transformed root cultures, 427–430
Transformed shoot cultures, 430–435
Transport across interphases, 22
Transport phenomena in bioreactors, 210, 180–197
Troubleshooting in system supplies, 612–613
Turbulence intensities in a stirred fermentor, 531
Turbulence models in bioreactors, 238–244

Ultrafiltration, 306

Validation, 582

Water
  for cooling, 600–601
  distribution, 595
  piping/tubing, 596
  production for process, 592
  sanitization of systems, 598
  storage and distribution, 593–595
  supply, 590–591

Yeast, 55
Yield
  anaerobic and aerobic, 146
  factors for aerobic growth, 147
  media, 93, 101, 103, 110