

Third Edition

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BIOCHEMICAL ENGINEERING

PRINCIPLES AND CONCEPTS

Syed Tanveer Ahmed Inamdar



Biochemical Engineering

Principles and Concepts

Third Edition

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BIOCHEMICAL ENGINEERING: Principles and Concepts, Third Edition
Syed Tanveer Ahmed Inamdar

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*To
the memory of my
Grandparents
&
Mother Naseem*

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Foreword

In this fast-changing world mankind is now exposed to an era where scientific and technical developments are taking place every day while making a deep impact on the living standards of human beings globally. The field of Biotechnology and Bioengineering has also witnessed a number of progressive advancements which are perceived to bring unprecedented benefits, especially towards human welfare, in the coming future.

Biochemical Engineering or Biotechnology is the buzz word today and is perceived by many as a career choice and more than a job. It is an invitation to participate in the development of newer products and processes that could improve the quality of human life and is a reflection of replacement of conventional high energy requiring chemical processes by the processes involving microorganisms or enzymes. In this context, the study of Biochemical engineering has become immensely valuable and relevant.

Taking the developments in the field of biotechnology into consideration, many chemical engineering departments are now offering Biochemical Engineering as a core course. In some of the universities, Chemical Engineering is modified as Biochemical Engineering, and Biochemical Engineers and Biotechnologists are much sought after professionals today.

This book, *Biochemical Engineering: Principles and Concepts*, now in its second edition, is a welcome effort and source for all those interested in acquiring a good exposure of the latest trends in the field. The book provides the course contents which are framed to meet specific objectives. It starts with an introduction to the microbial world, followed by biochemistry, enzyme technology and kinetics, biomass production in cell culture, bioreactors and details of the fermentation industry. The author has dwelt upon the emerging concepts in Biochemical Engineering. Though a number of books on this subject are available in the market, they lack the detailed description of the ongoing developments and requirements. Moreover, their orientation and presentation do not always suit the needs of the student community. This present book is written according to the curriculum for a first course at the undergraduate level of Chemical Engineering discipline.

The author's vast experience in industry and academics has helped him in this successful attempt. In this book, the author has provided the right and proportionate blend of life sciences and engineering aspects in order to make it an enjoyable teaching experience.

Finally, the author has made sure through comprehensive and extensive coverage of all the relevant topics, inclusive of detailed accounts on industrial practices, that the interests of young engineers working in this field are both sustained and taken care of. I am sure this book will be received well and make noteworthy contributions besides serving its purpose.

I commend and compliment the painstaking efforts of the author.



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Preface

The main objective of the Third Edition of this book continues to be the same as that of the previous editions: to cater to the curriculum for a first-course in biochemical engineering at the undergraduate level of chemical engineering discipline.

Keeping in mind the course curriculum of BTech Biotechnology and that offered at BSc Biotechnology, the book has been aptly revamped.

Indeed I have taken this opportunity to upgrade the text by providing elaborative explanations in certain selected topics. Chapter 7 on *Biological Reactors* is extended with the information on membrane bioreactors with concise description on their configurations and applications. Chapter 9 on *Downstream Processing* is given a new dimension by providing detailed explanations on selected topics, viz. cell disruption, supercritical fluid extraction, freeze drying, formulation, etc. and with some additions on microfiltration and nano filtration processes.

A paradigm shift in the question paper pattern has been observed these days especially by autonomous and deemed institutions by providing a due weightage of about 20–25% to the questions in the form of multiple choice, fill in the blanks, true/false, etc. in order to test the level of understanding of the student. Keeping this trend in mind, I have included the so-called “Self-Assessment Exercises” in Appendix D, which incorporates a variety of questions in different forms covering the entire syllabus. Self-Assessment as an exercise would really be useful for the students who have read the chapters and understood the concepts thoroughly.

Words shall not suffice to express my gratitude to my father, Dr. S.D Inamdar who has consistently stood behind me as a mentor in such endeavours. I also express my sincere gratitude to all my elders who have always stood by me at times whenever I needed. Many thanks go to my brothers Sayed Abdul Basith, Dr. Sayed Rehan Ahmar Inamdar and Dr. Sayed Afaque Hyder Inamdar, who have extended their help in preparing this manuscript for the third edition.

I am grateful to PHI Learning for publishing this book. Especially, my sincere thanks go to Babita Misra, Editorial Coordinator and

Mr. Bijendra Pandey, Copy Editor for helping me with their professional inputs to further improve the contents of the book.

Finally, I thank the Management of SDME Society, particularly the Principal and colleagues, SDM College of Engineering and Technology, Dharwad for their kind support.

Syed Tanveer Ahmed Inamdar

Preface to the First Edition

Biochemical engineering, a branch of chemical engineering, deals with the design and construction of unit processes and unit operations that involve biological organisms or molecules. Many chemical engineering departments offer biochemical engineering as a core course because of its diverse applications in the areas of pharmaceutical, biotechnology and water treatment industries. This book covers the curriculum for a first course in biochemical engineering at the undergraduate level of the chemical engineering discipline.

A biochemical process industry being a multidisciplinary one is incomplete without a chemical engineer, a microbiologist and a biochemist. All the three need to interact together in a biochemical industry. According to Bailey, chemical engineers should have a molecular insight into the problem-solving skills, along with the ability of both qualitative and quantitative logical interpretations to analyze and engineer a living cell. It is sufficient to surmise that chemical engineers should wholeheartedly embrace molecular biology.

This book, an outcome of my experience in industry and teaching, is based on my lecture notes and the material from conferences and symposiums that I participated in. It covers varied topics that are spread over ten chapters. Each chapter has significance in itself, starting with a preamble and ending with a summary, including graded exercises. Further to help the reader in exploring the topics, references are provided at the end of the book.

The subject matter is well organized and covers the concepts right from the fundamentals of microbiology and biological polymers such as proteins, carbohydrates, lipids, enzymes, etc. which are described in the first two chapters. Chapter 3 gives an insight into the significance of enzymes, their properties, types and their kinetics based on different models and theories, whereas Chapter 4 discusses industrial applications of enzymes. Chapter 5 emphasizes the techniques of immobilization of enzymes along with their advantages and applications. Chapter 6 focuses on cell growth and their kinetic aspects, while Chapter 7 deals with various types of biological reactors with emphasis on key engineering issues in bioreactor design and operation. In Chapter 8 the concepts and processes employed to manufacture traditional products of biotechnology are described. Chapter 9 elaborates on downstream processing and Chapter 10 discusses various aspects of controlling microorganisms.

Several important miscellaneous topics such as process kinetics, reactor analysis, bioenergetics, molecular genetics (with transcription and translation steps in the synthesis of proteins), and recombinant DNA technology with an elementary treatment on the concepts of DNA hybridization and DNA amplification and use of microbial sources in the treatment of effluents are treated in the appendices. The book also includes a glossary of important terms.

Well supported by plenty of figures and flow diagrams, the material of the book has been prepared with due consideration to the requirements of the students of chemical engineering and biotechnology. I do hope that the student community shall appreciate my efforts, and give suggestions for further improvement of the book.

I am deeply indebted to my parents, especially my father Dr. S.D. Inamdar for his consistent encouragement and moral support during this endeavour. I express my deep sense of gratitude to our President, Dr. Veerendra Heggade, SDME Society, Ujire, Karnataka. I owe thanks to Prof. Dr. H.V. Sudhaker Nayak, Principal, SDM College of Engineering & Technology, Dharwad for his support and guidance.

There are no words to express my appreciation to my brothers, Dr. Syed Rehan Ahmar Inamdar and Mr. Syed Afaque Hyder Inamdar, and to all my uncles for being a source of constant support and encouragement during preparation of this manuscript.

I am grateful to the publishers, PHI Learning and my sincere thanks to Mr. Sudarshan Das for his constructive suggestions during the editing of the manuscript.

Finally, I would like to acknowledge with thanks both teaching and non-teaching staff of the Department of Chemical Engineering, SDM College of Engineering & Technology for their support and cooperation in completing this endeavour.

Syed Tanveer Ahmed Inamdar

Biochemical Engineering

A Perspective

The roots of biochemical engineering can be traced back to brewing activities during the prehistoric era. The development of fermentation process of alcohol and leavening of bread by the Egyptians and Babylonians, the process of preparing cheese by ancient Greece, enzymes by Japan, tempeh by Indonesia, tofu by China, *idli* by Indians and porridge by Africans bear the testimony. The modern processes of biochemical engineering probably date back to the successful era of antibiotics. The isolation of penicillin in the 1940s from moulds and chemotherapeutic effects opened windows of opportunities for scientists and engineers working in pharmaceutical companies. Thus, successful interaction between microbiologists, biochemists and chemical engineers was initiated. The result was the production of more effective antibiotics, namely streptomycin, erythromycin, tetracycline as well as vitamin B₁₂.

In the early 1980s, James Bailey, a well-known biochemical engineer, foresaw a new horizon that helped bridge the gap between microbiologists, biochemists and chemical engineers. Till then, their relationship was based on a need-to-know basis. He believed that the unique combination of molecular insight, quantitative logic and problem-solving skills that allowed the chemical engineers to spearhead the analysis and engineering of naphtha cracking in the 1960s and 1970s could be equally useful for the analysis and engineering of a living cell. He also recognized that, to be effective at this new scientific interface, chemical engineers should wholeheartedly embrace molecular biology, which explains the complexities of life in molecular terms.

According to Bailey's school of thought, biochemical engineering is, and will be, what biochemical engineers do. It is also called the clever science of biology. The most recent definition states: Biochemical Engineering is the application of scientific and engineering principles to the processing of materials by biological agents (enzymes, cells or microorganisms) to provide new products and services.

The biochemical process industry has been at the forefront in developing target specific products with absolute purity and high efficacy. The following

trend of developments that have taken place gives us an insight into the biotechnology engineering industry.

- **Hierarchy with spotlights:** Hybrid antibiotics, recombinant DNA technology, enzyme immobilization, bioreactors, biofilters, biochips, and biosensors are some of the spotlights of biochemical engineering. Hepatitis-B vaccine, thaumatin (protein sweetener and flavour modifier), alkaloids, steroids, fuel alcohol, etc. are the recent developments.
- **The frontiers of biochemical engineering:** Conversion of CO_2 and N_2 into useful synthons, harnessing stem cells for regenerative medicine and metabolic engineering of complex human diseases.
- **Fields of application:** The present applications of biochemical engineering are in the areas of production of fine chemicals, alkaloids, steroids, hormones, fuel alcohol, vaccines, and a host of other fermentation products.

The principle on which biochemical engineering and biotechnology relies is: "Why trouble to make compounds yourself when a bug will do it for you?"

In biochemical engineering processes, living organisms are used for carrying out transformations. Since these living organisms are chemically complex, they are capable of carrying out transformations which can be manipulated by controlling their environment or by mutation.

Biochemical reactions are carried out at mild temperatures generally under 100°C and frequently below 50°C . Nowadays enzymes that are chemically proteins and water soluble are used as biocatalysts since they are powerful and action specific, although the cost for their isolation is exorbitant. Also, cells, microbes and enzymes are used in biochemical conversions.

Relationship between Biological Sciences, Processes and Chemical Engineering

We see from the following table that there exists a relationship between biological sciences, processes and chemical engineering that amalgamate to form a biochemical process industry. The interdisciplinary interaction taking place today between microbiologists, biochemists, and chemical engineers is also explained.

<i>Biological Sciences</i>	<i>Processes</i>	<i>Chemical Engineering</i>
Systematics	Culture choice	Reactor design
Genetics	Mass culture	Process control
Biochemistry	Cell response	Unit operations
Physiology	Process operation	Energy and material utilization
Chemistry	Product recovery	

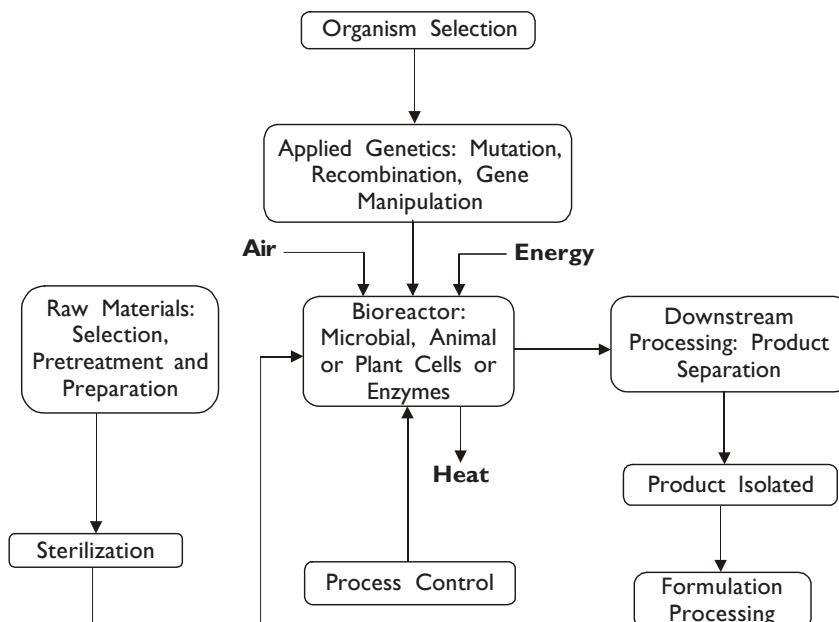
The various realms of biological sciences that are shown have significance in themselves. Systematics is one where a logical classification of microorganisms is made. Investigations made in terms of genetic makeup, gene transfer, characteristics and traits fall in genetics, while biochemistry is that part where the chemistry of a cell or microorganism describing the composition in terms of biological polymers like proteins, carbohydrates, lipids and fats, nucleic acid composition, etc. is studied. Physiology of a cell governs processes like digestion, excretion, etc.

In terms of the processes, namely culture choice, either a tissue culture or a cell culture, and its preparation to form a mass culture and investigating the cell response to these preparations or formulations, and also the response to various other surroundings form a combined job of a microbiologist and a biochemist.

Process operation and product recovery, also referred to as downstream processing, helps in isolation of product in a purer form where chemical engineers, who understand various separation processes (conventional or novel), dominate in a biochemical process industry. Of course, this is in addition to the usual design of reactors, process control and instrumentation, carrying out various unit operations, namely evaporation, adsorption, crystallization, drying, etc. Also chemical engineers are well versed in providing the material and energy balances for the effective utilization of energy and the materials.

Schematic Overview of a Biotechnological Process

The flow diagram given below illustrates the kind of activities that take place today in a typical biotechnological industry.



A typical biotechnological industry today incorporates the role of three different personnel at different times and at different locations within an industry.

A microbiologist is responsible for selecting microorganisms, providing the details with respect to genetics in terms of mutation that refers to a sudden change in genetic constitution due to exposure of cells of microorganisms to various extreme conditions during the process, recombination or more specifically recombinant DNA technology (also called genetic engineering with varied manipulations).

The selection of raw materials, their pretreatment and preparation, coupled with the sterilization of materials and the reactors to avoid process upsets and contaminations constitute more or less the responsibility of a chemical engineer.

The overall process within a bioreactor and the reactor control is spearheaded by the process control section of the industry. Finally, the recovery of products and purification along with isolation is a part of the industry, where the separation processes both conventional and novel are adopted. The present-day practices for separations use various synthetic membranes with different modules and high resolution techniques like gas-liquid chromatography, electrophoresis, etc. for carrying out the separation of proteins.

Product formulation is the final stage where different products with varied compositions and purity are manufactured.

It has thus been a long journey from brewing activities of prehistoric era to modern applications of biochemical engineering dedicated to discovering new products and services for the benefit of humankind.

Massive research and developments are taking place today in the field of biochemical engineering (also called bioprocess engineering or biotechnology) to cater to the needs of the hour.

There has been a proactive thinking and logical understanding, along with collaborative efforts between microbiologists, biochemists and chemical engineers, to see that this field harnesses the needs and requirements of common people.

Information technology was and is still in the limelight, but today biotechnology is much spoken of. The way the developments in this field are taking place is enough to say that this century is going to be exclusively for biochemical engineering or bioprocess engineering or biotechnology.

Chapter 1

Microbiology Fundamentals

Familiarity with the concepts of microbiology is essential to understand its theory, principles and its applications in the biochemical engineering process industry. Without knowledge of microbes, their types, features, characteristics, and their usage, one cannot proceed in the context of understanding the biochemical industry. The role of microbes is quite essential to signify the scenario of a biochemical industry.

In this chapter, are considered the aspects of microbiology beginning with the definition of a cell, types of cells and isolation of cells with a detailed study of types of microbes that are industrially useful and are much exploited. Emphasis is given on the types of microbes that are really significant to the applications to a biochemical process. Environmental and industrial microbiology is discussed briefly, and various biogeochemical cycles are also included.

I.I SCOPE OF MICROBIOLOGY

Microbiology is the study of living organisms of microscopic size, which include bacteria, fungi, algae and protozoa and also infectious agents at the borderline of life called viruses. The study is concerned with the form, structure, reproduction, physiology, metabolism and the classification. It indulges in the study of their distribution in the nature, their relationship to each other and to the other living organisms, their effects on human beings and on other animals and plants, their abilities to make physical and chemical changes in the environment, and their reactions to physical and chemical agents.

Certain microorganisms are beneficial and some are detrimental to the welfare of human beings. For example, microorganisms are used in the preparation of yoghurt, cheese, and wine; in the production of antibiotics like penicillin, tetracycline, etc; in the manufacture of alcohol, interferon, various hormones, vaccines, and steroids; and in the treatment of the industrial and domestic wastewater and wastes.

On the other hand, there are certain microbes which are harmful to human beings. For instance, these microbes can cause diseases, spoil food, and deteriorate materials like iron pipes, lenses, and wood pilings. On the large scale of the biosphere, which consists of all the regions of the earth containing life, microorganisms play an important role in capturing energy from the sun. Their biological activities also complete the critical segments of the cycles of carbon, oxygen, nitrogen, sulphur and other elements essential for life.

Most of the microorganisms are unicellular, i.e. they have a single cell and all the life processes are performed by it. In the so-called higher forms of life, i.e. multicellular organisms, cells are in plenty and are arranged in tissues and organs performing specific functions. Irrespective of the cell complexity of an organism, the cell is the basic structural unit of life. All the living cells are fundamentally similar.

It was Robert Hooke (1635–1703), an English, who first coined the word 'cell'. The concept of the cell as the structural unit of life was given by two Germans, Schleiden and Schwann in the year 1838–39.

The cell: It says:

I am the unit of biological activity;
Organized into the subcellular organelles;
Assigned to each other are the specific functions and duties;
Thus, I truly represent life!

All the living systems have the following characteristics in common:

1. The ability to reproduce.
2. The ability to ingest or assimilate food substances and metabolize them for energy and the growth.
3. The ability to excrete waste products.
4. The ability to react to the changes in their environment, sometimes called irritability.
5. The susceptibility to the sudden changes in the genetic constitution, called the mutation.

In microbiology, we can study microorganisms in great detail and observe their life processes while they are actively metabolizing, growing, reproducing, aging and dying.

By modifying their environment, we can alter metabolic activities, regulate growth, and even change some details of their genetic pattern—all without destroying the organisms.

Microorganisms have a wider range of physiological and biochemical potentialities than all other organisms combined. For example, some bacteria are able to utilize atmospheric nitrogen for the synthesis of proteins and other complex organic nitrogenous compounds. Other species require inorganic and organic nitrogen compounds as initial building blocks for their nitrogenous constituents. Some microorganisms synthesize all their vitamins, while others need to be furnished vitamins.

1.2 PLACE OF MICROORGANISMS IN THE LIVING WORLD

Classification means the orderly arrangement of units under study into groups of larger units. Present-day classification in biology was established by the work of Carolus Linnaeus (1707–1778), a Swedish botanist. He used nomenclature in microbiology, which was based on the principles established for the plant and animal kingdoms.

Until the 18th century, the classification of living organisms placed all organisms into two kingdoms, plant and animal. There are some organisms that are predominantly plant-like, others that are animal-like, and some that share characteristics common to both plants and animals.

There are also organisms that do not fall naturally into either the plant or the animal kingdom. In 1866, a German zoologist, E.H. Haeckel proposed a third kingdom and named it as *Protista*. This includes the unicellular organisms that are typically neither plants nor animals. For example, the organisms like bacteria, algae, fungi, and protozoa. Bacteria are referred to as the lower protists; the others—algae, fungi, and protozoa—are regarded as the higher protists. Viruses are not cellular organisms and therefore are not classified as protists.

1.3 WHITTAKER'S FIVE-KINGDOM CONCEPT

It is a more recent and comprehensive system of classification, proposed by R.H. Whittaker in the year 1969. It is based on the three levels of cellular organization which came up to accommodate three principal modes of nutrition: *photosynthesis*, *absorption*, and *ingestion*. The five kingdoms are:

1. Kingdom Monera that includes prokaryotes, which lack in the ingestive mode of nutrition, for example, bacteria and cyanobacteria.
2. Kingdom Protista that includes the unicellular eukaryotic organisms, where all three modes of nutrition can be seen.

The nutritional modes are continuous. In micro-algae, the mode is photosynthetic, in protozoa, it is ingestive, and in some other protists, it is absorptive with some overlap to the photosynthetic and ingestive modes.

3. Kingdom Plantae, which includes multicellular and multinucleate green plants and higher algae, where the nutrient uptake is mainly by photosynthesis.
4. Kingdom Fungi, which includes yeasts, moulds, and multinucleate higher fungi, where the nutrient uptake is mainly by absorption.
5. Kingdom Animalia includes multicellular animals, where the nutrient uptake is mainly by ingestion.

Figure 1.1 represents Whittaker's five-kingdom system.

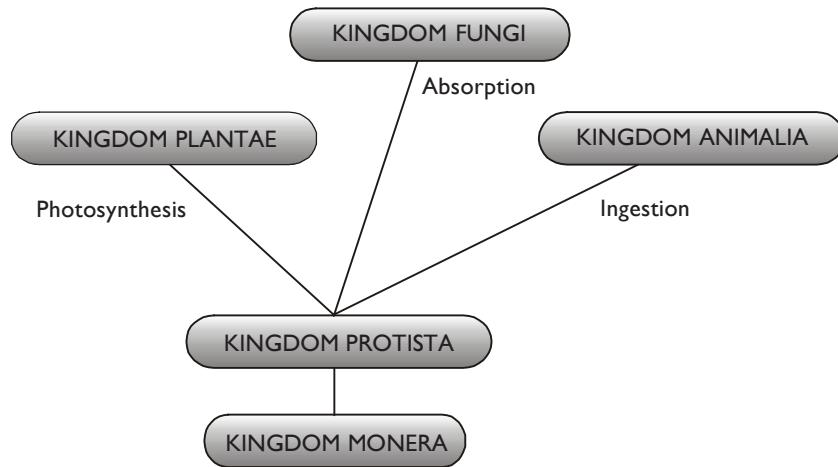


Figure 1.1 Simplified Whittaker's five-kingdom concept.

1.4 STRUCTURE OF CELLS

Observations under the electron microscope have revealed two markedly different kinds of cells: *prokaryotes* or *prokaryotic cells* and *eukaryotes* or *eukaryotic cells*. So far as it is known that all cells belong to either of these groups.

1.4.1 Prokaryotic Cells

Prokaryotic cells or prokaryotes have the following features:

1. They lack a well-defined nucleus, or a membrane-enclosed nucleus.
2. They possess a simple structure and are relatively small.
3. They usually exist alone, no association with other cells.
4. The size varies between 0.5–3.0 μm . A qualitative feel for such dimensions can be given as the size of a prokaryote relative to a human is approximately equal to the size of a human relative to the earth.
5. The volume is in the order of 10^{-12} ml per cell in which 50–80% is water and the mass is 10^{-12} grams.
6. They grow rapidly and are widespread in the nature.
7. They are versatile biochemically and are the choice for research and biochemical processing, e.g. bacteria and cyanobacteria (blue-green algae).

Prokaryotic cells are preferred for research and biochemical processing because of the following reasons:

1. They grow rapidly and are widespread in the biosphere.

2. They can accept a wide variety of nutrients and select the best among them.
3. They are versatile.

Description of a prokaryotic cell: Figure 1.2 shows a micrograph of a prokaryote.

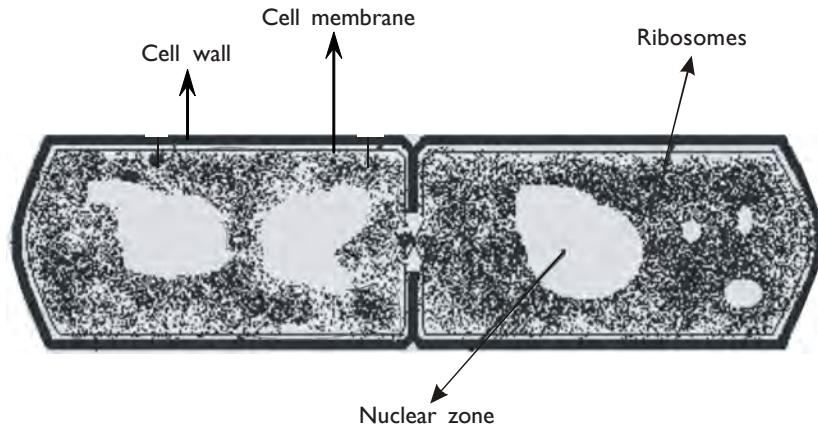


Figure 1.2 Electron micrograph of a prokaryote.

The components of a prokaryote are as follows:

Cell wall: It is a rigid wall surrounding the cell, which approximately measures 200 Å. The function of the cell wall is to lend structural strength to the cell thus preserving its integrity.

Cell membrane: It is present just inner to the cell wall and has a thickness of about 70 Å. It is also called *plasma membrane*. The cell membrane plays a very vital role in controlling the ingress and egress of nutrients.

Nuclear zone: It is a large, ill-defined structure. It acts as a dominant control centre for the cell operation.

Ribosomes: These are grainy dark spots inside the cell. They function as the sites for biochemical reactions. They are also called the *sites of protein synthesis*.

Cytoplasm: This is a fluid material occupying the rest of the space in the cell.

1.4.2 Eukaryotic Cells

Eukaryotic cells or eukaryotes constitute the major class of cell types. The human body is composed of about 10^{14} eukaryotic cells. Eukaryotic cells or eukaryotes have the following features:

1. They are the cells which possess a membrane-enclosed nucleus.
2. These cells are 1000 to 10,000 times bigger than the prokaryotes.
3. The cells of the higher organisms belong to this category.
4. The internal structure of eukaryotic cells is more complex, and there is a substantial degree of spatial organization and differentiation.
5. The size of a eukaryotic cell is about 10 to 100 μm in diameter.
6. The eukaryotic cell has well-defined subcellular organelles.

Description of an eukaryotic cell: Figure 1.3 shows the structure of an eukaryotic cell.

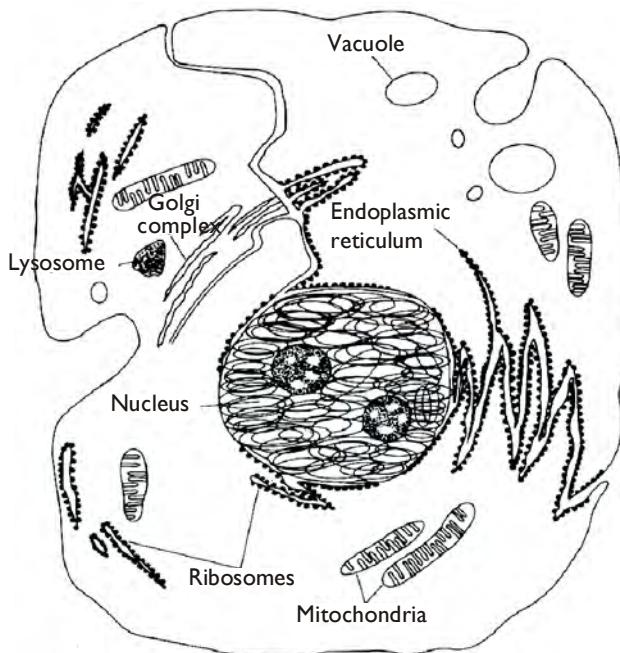


Figure 1.3 Eukaryotic cell structure.

Plasma membrane: This membrane is also called *unit membrane* and the nature of this outer covering depends on the particular cell, for example, the cells of higher organisms have a thin cell coat. The specialty of this membrane is that it possesses adhesive properties thus binding the like cells to form specialized tissues and organs.

Endoplasmic reticulum: It is a complex, convoluted membrane system that leads from the cell membrane into the cell and extends throughout the cytoplasm. The endoplasmic reticulum is present in two forms:

- *Rough endoplasmic reticulum:* It is the one which is studded with dark granules called *ribosomes*.

- *Smooth endoplasmic reticulum:* It is the one which is free from the dark granules, i.e. ribosomes.

The major function of the endoplasmic reticulum is to synthesize lipids like triacylglycerides, phospholipids, sterols, etc.

Nucleus: It is the largest cellular organelle that is surrounded by a double-layered nuclear envelope. The outer layer of the membrane is continuous with the membrane of endoplasmic reticulum. The two nuclear membranes or the nuclear pores (membrane is porous) have a diameter of 90 nm. The nucleus is composed of DNA, thus acting as the repository of genetic material. It is the control centre of the cell, controlling the catalytic activity at the ribosomes.

Ribosomes: These are the sites for the biochemical reactions and much of the ribosomes are embedded in the surface of endoplasmic reticulum. The major function of the ribosomes is to synthesize proteins. These are also called the *sites of protein synthesis*.

Mitochondria: These are rod-like or filamentous structures occupying about one-fifth of the total cell volume and measuring $1.0 \times 3.0 \mu\text{m}$. The major function of the mitochondria is to catalyze the reactions and produce enormous amount of energy in the form of ATP (adenosine triphosphate) thus, they are also known as the *powerhouses* of the cell.

Vacuoles, Golgi complex, lysosomes: These are the remaining organelles and in general, serve to isolate chemical reactions or certain chemical compounds from the cytoplasm.

1.4.3 Comparison between Prokaryotic and Eukaryotic Cells

The comparison between prokaryotic and eukaryotic cells is given in Table 1.1.

1.5 CELL FRACTIONATION

The process applied in the cell fractionation is called *differential centrifugation*.

Here an attempt is made to break the cells apart without significantly disturbing or disrupting the organelles within. As we know that any separation process is based upon exploitation of differences in the physical and chemical properties of the components to be isolated. Following are the steps in cell fractionation.

Step 1. Cell suspension (tissue homogenate) is homogenized in a special solution using a rotating pestle within a tube or by using ultrasonic sound waves.

Step 2. Cells are broken apart without disturbing the organelles within.

Step 3. Fractionation occurs successively.

Table 1.1 Comparison between prokaryotic and eukaryotic cells

Characteristic	Prokaryotic cell	Eukaryotic cell
Size	Small and are in the range of (1–10 μm) size	Large and are in the range of (10–100 μm) size
Cell membrane	Cell is enveloped by a rigid outer covering called cell wall.	Cell is covered by a flexible outer covering called plasma membrane.
Subcellular organelles	Absent	Distinct organelles are present.
Nucleus	It is not well defined, DNA appears as nucleoid with absence of histones.	Well defined, DNA is associated with histones.
Energy metabolism	Mitochondria absent, enzymes of metabolism are bound to the membrane.	Mitochondria present, and have enzymes in them.
Cell division	Occurs by fission or splitting of cells and absence of mitosis.	Occurs by mitosis.
Cytoplasm	Organelles and cytoskeleton called cell skeleton absent.	Contains organelles and cytoskeleton (a network of tubules and filaments).
DNA base ratios (C + G)	28–73% present	About 40%
Sexuality	Zygote is partially diploid called merozygotic.	Develops diploid zygote.
Ribosomes	They measure 70 S units and are distributed in cytoplasm.	They measure 80 S units and are arranged on the endoplasmic reticulum.

Note: S, Swedberg unit, the sedimentation coefficient of a particle in the ultracentrifuge.

When a centrifuge is spun at a high speed, particles suspended in the centrifuge tubes move away from the centrifuge axis. Since the rate of movement of these particles depends on their size, shape, and density, particles differing in these properties can be separated in a centrifuge. The following flow chart illustrates the overall process of differential centrifugation.

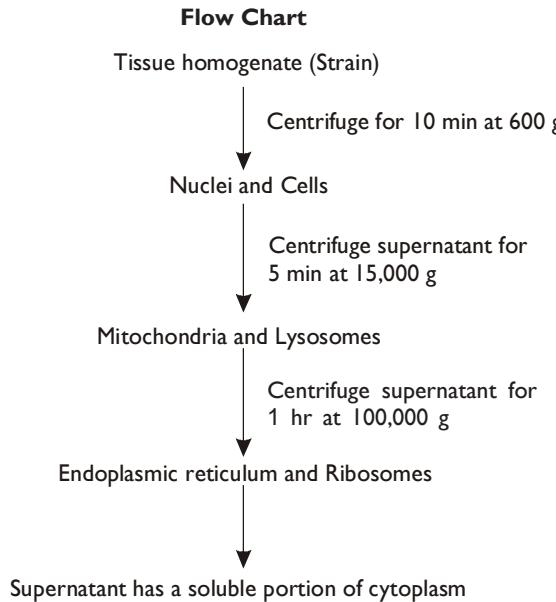


Figure 1.4 shows the process of differential centrifugation.

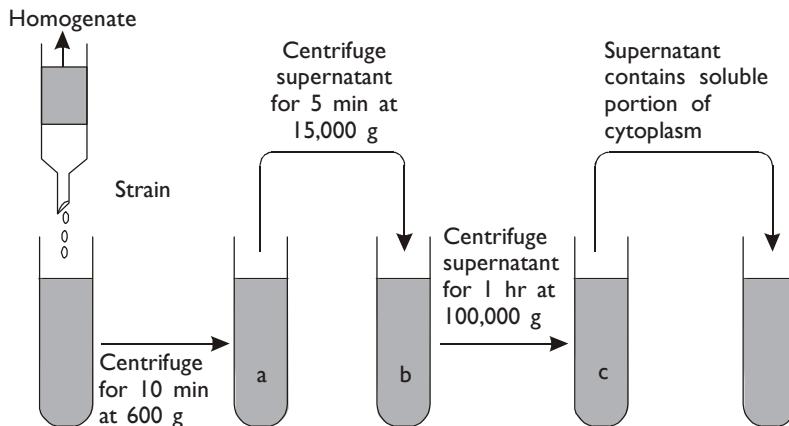


Figure 1.4 Differential centrifugation: (a) nuclei and broken cells, (b) mitochondria and lysosomes, and (c) endoplasmic reticulum and ribosomes.

1.6 CELL TYPES (COMMERCIALLY VIABLE)

The following are the cells or the microbes that are exploited commercially or industrially.

1.6.1 Protists

The kingdom protista consists of all the unicellular organisms and also the

organisms containing multiple cells which are of the same type. In short, it consists of all living things with simple biological organization.

Classification: The kingdom Protista has been classified taking into consideration the difference in the energy and nutritional needs, growth, product release rates, reproduction methods, capability of movements and of course the morphological factors. Refer to Figure 1.5.

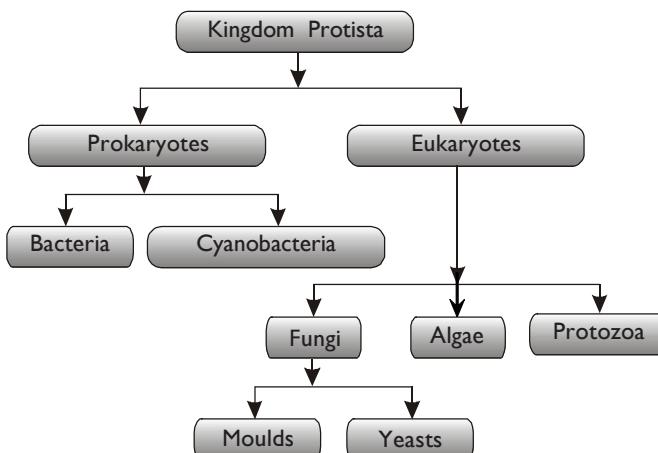


Figure 1.5 Classification of the kingdom Protista.

Morphology is the study of forms and external structures of the living organisms. This has its influence on the rate of nutrient mass transfer to it and also can profoundly influence the fluid mechanics of a suspension containing the organism.

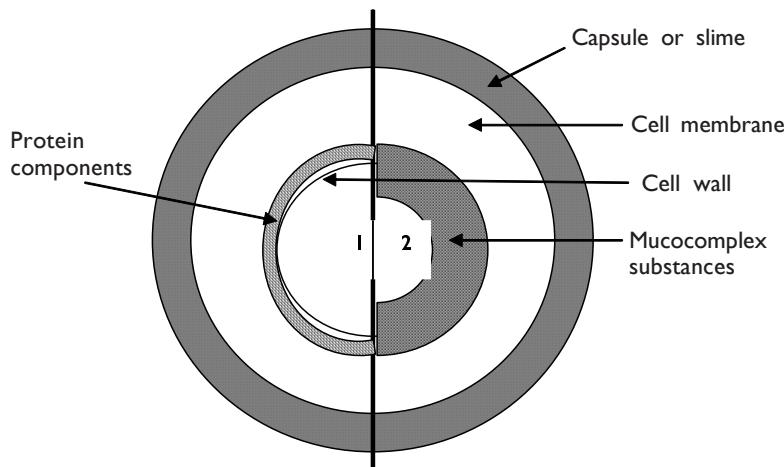
1.6.2 Bacteria

Bacteria are very small organisms which are enclosed in cell walls. The outer surface of the cell wall is covered by a slimy, gummy coating called the *capsule* or *slime layer*. These organisms are unicellular and motile. They reproduce by division process called the *binary fission*, through which the organisms divide into two daughter cells.

Gram reaction: It is the response of the bacteria to a rapid staining test. Here the cells are stained with a dye crystal violet, then treated with iodine solution and finally washed in alcohol.

Bacteria which retain blue crystal violet colour after the test are Gram positive and which lose colour after the test are Gram negative. Figure 1.6 depicts a summary of morphology of Gram-positive and Gram-negative bacteria.

Commercial usage: Commercial usage of bacteria is based on the oxygen supply, i.e. whether it is by aerobic or anaerobic process.



1. GRAM POSITIVE

Muralytic enzymes remove cell wall.
Cell membrane structurally and biochemically conspicuous.

2. GRAM NEGATIVE

Muralytic enzymes do not remove cell wall.
Cell wall multistructured, that is with many layers.

Figure 1.6 Summary of morphology of Gram-positive and Gram-negative bacteria.

Aerobic process: Here oxygen is supplied in the form of air to be used by the microbes. The manufacture of vinegar, antibiotics, animal feed supplements, etc. are done through this process.

Anaerobic process: The process takes place without the use of oxygen, production of some alcohols and digestion of organic wastes are done through this process.

In commercial practice, under adverse conditions, some bacteria are able to produce stable resting forms called *endospores*.

Endospores: These are dormant forms of cells which are capable of resisting heat, radiation and toxic chemicals.

When studied based on the biological activity of a cell, it is accepted that:

1. Spore forms are biologically inert (inactive) and when these inactive forms are sent back to the surroundings fit for normal cell function, spores germinate into normal cells. The spores need a high temperature to be killed (temperature of $>120^{\circ}\text{C}$). The most common endospores of this kind include aerobic *Bacillus* and *Clostridium*, which are Gram positive, non-photosynthetic, and found in soil.
2. Vegetative forms are biologically active and are rapidly killed at a temperature of 45°C . Examples include *Enteric* or *Coliform* bacteria, *E. coli*, etc. which are rod shaped and rest in the human intestine. They are Gram negative and non-photosynthetic. Lactic acid bacteria, *Lactobacillus*, *Streptococcus* and *Leuconostoc* are Gram positive, non-photosynthetic and found in plants.

Description of a bacterial cell: Figure 1.7 schematically shows the main constituents of a bacterial cell.

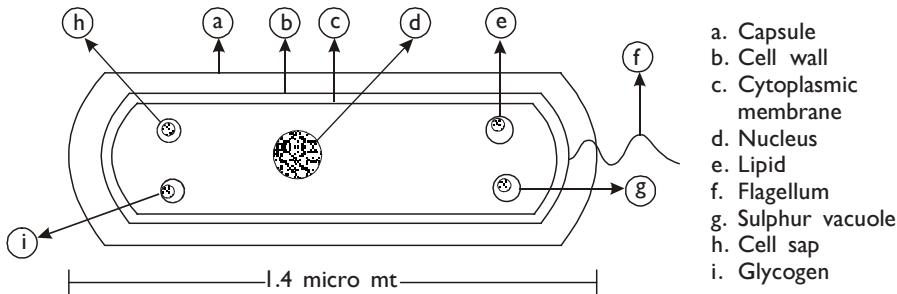


Figure 1.7 Main constituents of a bacterial cell.

Bacteria are the simplest form of plant life. They are single celled, use soluble food and are capable of reproduction. They are the fundamental organisms that stabilize organic wastes. Bacteria are seen in huge numbers in sewage (1 litre of sewage may contain 22–25 millions of bacteria). Majority of bacteria are harmless to human life and are used in many beneficial processes, for example, in conversion of complex organic compounds of sewage to stable, organic and mineral compounds resulting in purification. On the contrary some of the bacteria are pathogenic to human life.

Bacteria are also classified on the basis of their nutritive needs such as *heterotrophic* and *autotrophic* bacteria.

Heterotrophic: This class of bacteria use organic compounds and carbon for synthesis. There are four categories in it. They are aerobic which depends on free oxygen; anaerobic, which do not depend on free oxygen; facultative, that are flexible; and saprophytic, which depends on the dead organic matter and are found in the upper layers of soil.

Autotrophic: This class of bacteria use CO_2 as carbon source and oxidize inorganic compounds for energy. Nitrifying, sulphur and iron bacteria are autotrophic bacteria. These bacteria are responsible for corrosion in sewer systems.

Three forms of bacteria: As we know that bacteria are unicellular, but they exist in three basic morphological forms such as:

- *Spirilla* or Spirals
- *Cocci* or Spheres
- *Bacilli* or Rod like

Figure 1.8 shows these three forms of bacteria.

Note: Bergey's classification and other selective forms of bacteria are treated in Appendix C.

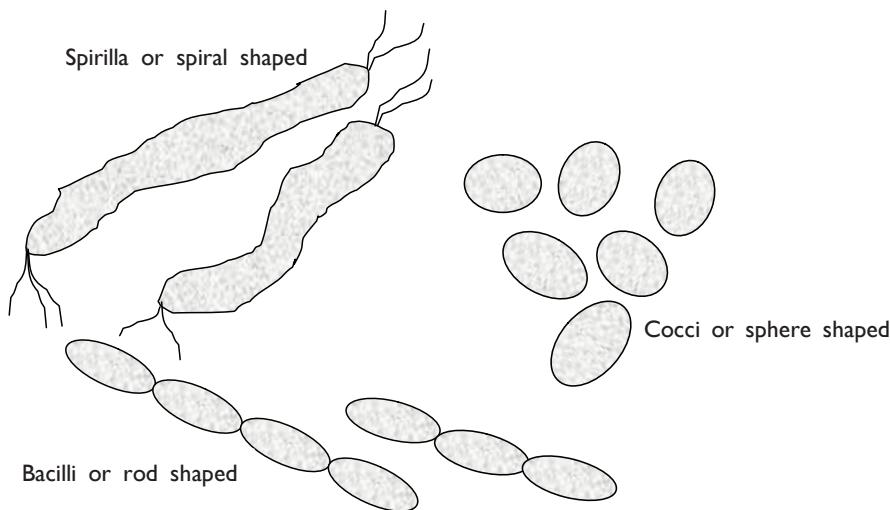


Figure 1.8 Shapes of bacteria.

1.6.3 Yeasts

Yeasts form an important group of fungi. They are widespread in nature, reside in soil of low humidity and cannot trap energy from sunlight.

They measure 5–30 μm long and 1–5 μm wide. The modes of reproduction seen in yeasts are *budding* (most common), *fission* and *sporulation*.

Asexual budding process of reproduction: In budding (Figure 1.9), the nucleus of a parent cell enlarges and then extends into a bud forming a dumb-

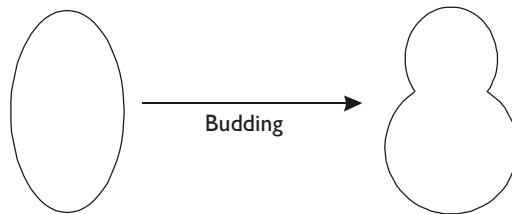


Figure 1.9 Asexual budding in yeasts.

bell shaped structure. Further, it divides giving rise to a nucleus in both the parent and daughter cells. The offspring grows on the side of the original cell.

On division an inactive surface scar remains on the mother cell surface. On further divisions of the mother cell, the cell surface is covered with a scar material and eventually leads to the death of the cell.

Fission process: This occurs by the division of a cell into two new cells (Figure 1.10).

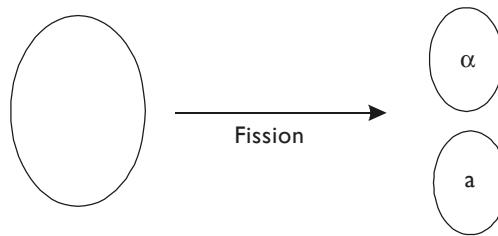


Figure 1.10 Fission process during reproduction of yeasts.

Sporulation process: It involves the formation of spores by either asexual means or by sexual means. Spores are called *Conidia* when formed asexually and *Ascospores* when formed sexually.

The nucleus in the diploid cell undergoes one or several divisions forming ascospores and each ascospore will form a new haploid cell (Figure 1.11).

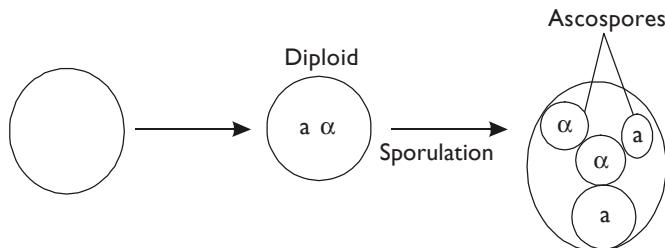


Figure 1.11 Sporulation process during the reproduction of yeasts.

The yeast cycle: Figure 1.12 depicts the life cycle of yeast.

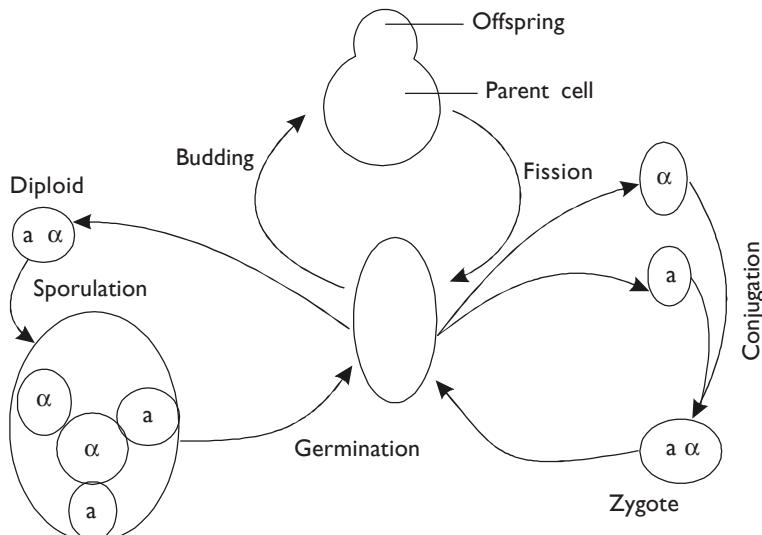


Figure 1.12 Life cycle of *Saccharomyces cerevisiae*.

Uses of yeasts: Following are the uses of yeasts:

1. Production of alcoholic beverages, beer, wine, etc.
2. Anaerobic yeasts produce industrial alcohol and glycerol.
3. Used for baking purpose.
4. As a protein supplement to animal feed.

1.6.4 Moulds

Moulds are a higher class of fungi with a vegetative structure known as *mycelium* (Figure 1.13).

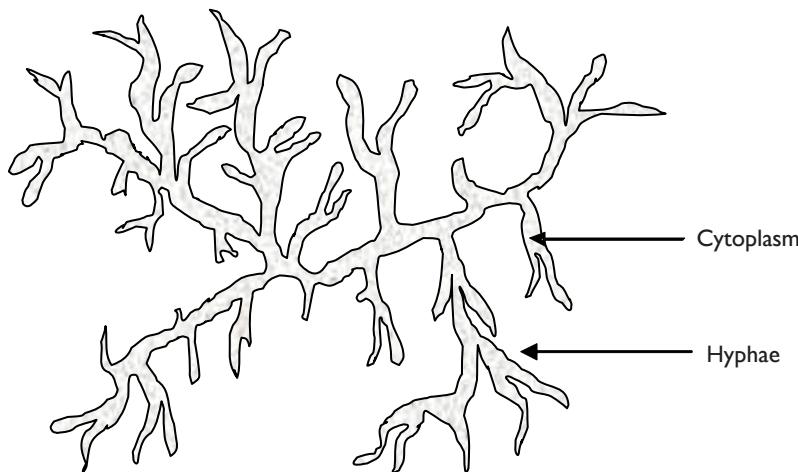


Figure 1.13 Mycelial structure of moulds.

A mycelium is a fine network with a system of tubes, which has a mobile mass of cytoplasm containing many nuclei. It has long, thin and slender filaments of cells known as *hyphae*, over which the asexual spores are present. *Moulds* lack chlorophyll and are non-motile. They reproduce by either asexual or sexual means. Two industrially important moulds are *Aspergillus niger* and *Penicillium*.

The hyphae of *Aspergillus* and *Penicillium* are shown in Figure 1.14.

The major useful products of *Aspergillus* and *Penicillium* are antibiotics, organic acids and biological catalysts. The strain *Aspergillus niger* produces oxalic acid.

Besides bacteria and fungi, one should not leave out *actinomycetes*, which are microbes with some properties of both fungi and bacteria. These are extremely important for the antibiotic manufacture. Though they are formally classified as bacteria, they resemble fungi in their formation of long-branched hyphae.

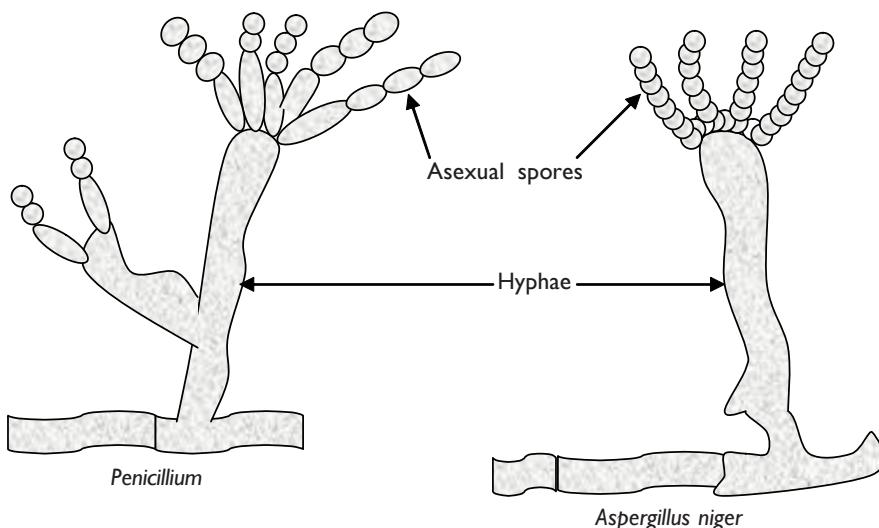
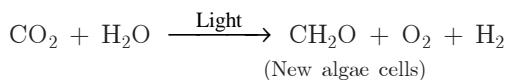


Figure 1.14 The hyphae of *Aspergillus* and *Penicillium*.

1.6.5 Algae, Protozoa and Viruses

Algae and protozoa both belong to the class of eukaryotes and both of them are used in the industrial fermentations, e.g. in production of glycerol, pigments and their derivatives.

Algae: They are autotrophic and photosynthetic plants and require light for growth. In the presence of light, algae convert inorganic materials in water into inorganic matter in the form of protoplasm through photosynthesis.



Algae are found usually in the oxidation ponds. The most common algae are *Chlorella* (green algae).

In Japan, algae food cultivation is done and are also used as foodstuffs and food supplements.

Protozoa: They are strictly aerobic, non-photosynthetic organisms which reproduce by fission process. They are found in oxidation ponds and are used in water treatment processes as trickling filters, activated sludge processes and also as biological control agents. Rhizopoda, Flagellate, Ciliate, etc. are common protozoa.

Viruses: Since viruses are acellular and possess both living and non-living characteristics, they are considered neither prokaryotic nor eukaryotic. They

are parasites or pathogens of plants, animals and bacteria as well as other protists. They are so small that they can be visualized only by the electron microscope and can be cultivated only in living cells.

1.6.6 Animal and Plant Cells

Many vaccines and other useful biochemicals are produced by growth of animal cells in process reactors, i.e. by *in vitro* cell propagation. Improvements in cultivation techniques for these tissue-derived cells and emerging methods for genetic manipulation of animal and plant cells offer great potential for expanded commercial utilization of these cells.

When a piece of animal tissue, after disruption to break the cells apart, is placed in an appropriate nutrient liquid, many cell types such as blood cells die within a few days, weeks or months, whereas other cells called *primary cell lines* multiply. These can be passaged by transfer to fresh medium after which further cell multiplication occurs giving a secondary cell line. Many cell lines have been developed from epithelial tissues (skin and tissues which cover organs and body cavities), connective tissues, and blood and lymph of several animals including man, hamster, monkey, and mouse.

It is also possible to grow certain plant cells in culture, either as a callus, a lump of undifferentiated plant tissue growing on the solid nutrient medium; or as aggregated cells in suspension. As plants produce many commercially important compounds including perfumes, dyes, medicinals, and opiates, there is significant potential for future applications of plant cell culture. Cultured plant cells can also catalyze highly specific useful transformations. They have certain applications in agriculture like whole plant regeneration.

1.7 ENVIRONMENTAL AND INDUSTRIAL MICROBIOLOGY

Environmental microbiology pertains to the study of microbiology of soil, microbiology of aquatic systems, microbiology of domestic water and wastewater and also microbiology of foods.

This study at different source levels provides an insight into life under extreme conditions, i.e. harsh environmental conditions. For example, surface water of the ocean contains many microorganisms, and it has long been thought that relatively a few bacteria exist on the deep ocean floor because of low temperature of the water, scarcity of nutrients and absence of light to support the growth of phototrophic organisms, and the enormous hydrostatic pressure due to the great depth of overlying water column.

This is true for most regions of the ocean floor, but a startling exception was found somewhere in the 1970s during the exploration of deep sea hydrothermal vents (hot submarine springs) located along submarine tectonic rifts and ridges of the ocean floor at a depth of 2500–2600 metres. In the areas surrounding these vents, living organisms were discovered in abundance right from bacteria to invertebrates. Life exists here because these hot springs

can produce geothermally hydrogen sulphide and other reduced inorganic compounds and release into the surrounding water, which can be a source of carbon and energy.

1.7.1 Microbiology of Soil

Seemingly the earth looks dead, but a paragraph in the article “A Microbiologist Digs in the Soil” by Charles Thom reveals some interesting facts:

Bacillus tetanus, amoebic dysentery, thermophilic spoilage, Actinomycosis and Botulism today are much spoken of. This is because the soil harbors millions of organisms and can be taken as “Lilliputian zoo” which infest in wound, clothing and food. Directly or indirectly the wastes of humans and other animals, their bodies and tissues of plants are dumped or buried in the soil. They all disappear somehow; transform into the substances that make up the soil.

It is the microbes that bring the transformations—the conversion of organic matter into simple inorganic substances that provide the nutrient material for the plant world.

The transformations are brought by the following cycles: carbon cycle, nitrogen cycle, sulphur cycle, etc. The microbial flora of the soil consists of bacteria, fungi, algae, protozoa, viruses, rhizospheres etc.

The biodegradation of herbicides, pesticides, etc. is also brought about by the microorganisms and the process can be called *soil/land reclamation*.

1.7.2 Aquatic Microbiology

Aquatic microbiology is the study of microorganisms and their activities in the fresh, estuarine, and marine waters, including the springs, lakes, rivers, bays and the seas. It is the study of microorganisms like viruses, bacteria, algae, protozoa and fungi which inhabit these natural waters.

Some microorganisms are indigenous to the natural bodies of water, others are transient, entering water from air, soil, or from industrial or domestic wastes. Wastewaters usually contain microorganisms which influence the activities of microorganisms already present in the receiving waters.

Aquatic microorganisms and their activities are of great importance. They affect the health of humans and other animal life. Occupy a key position in the food chain by providing the rich nourishment for the next higher level of aquatic life. They are instrumental in the chain of biochemical reactions which accomplish recycling of the elements, for example, in the process of mineralization. The aquatic environment is influenced by the following factors. They are temperature, hydrostatic pressure, light, salinity, turbidity, pH, inorganic and organic constituents.

The temperature of surface waters is 0°C in the polar regions and 30–40°C in the equatorial regions. More than 90% of the marine environment

is below 5°C, a condition favourable for psychrophilic microorganisms. Some microorganisms occur in the natural hot springs where temperature is as high as 75–80°C and these microorganisms are called *Thermus aquaticus*.

Recently microbiologists have reported extreme thermophilic microorganisms associated with the geothermal vents in the Pacific Ocean floor and they can grow at a temperature as high as 250°C and at a pressure of 265 atmospheres. The hydrostatic pressure affects the chemical equilibrium, which results in lowering the pH of sea water, resulting in a change in the solubility of nutrients such as bicarbonates, HCO_3^- , etc.

It also increases the boiling point of water, thereby, maintaining water in its liquid state at high temperatures and pressures.

Salinity ranges from zero of fresh water to saturation in salt lakes. The concentration of dissolved salts ranges from 33–37 grams per kilogram of water. The pH value of sea water is generally between 7.5 and 8.5, and the microorganisms can grow at a pH of 6.5 to 8.5.

1.7.3 Domestic Water and Wastewater Microbiology

The sources of domestic water are rivers, streams, lakes, etc. Rivers and streams get polluted particularly with the domestic and the industrial wastes and become the carrier of pathogenic microorganisms that cause diseases like infection of the intestinal tract, typhoid, dysentery (bacillary and amoebic) and enteric fever. The causative organisms of these diseases are present in the faeces or urine of an infected person, and when discharged may gain entrance into a body of water that ultimately serves as a source of drinking water.

Wastewater treatment procedures, both natural and artificial, are largely dependent upon microbial activity to eliminate or greatly reduce the development of hazardous or objectionable situations. Activated sludge processes, trickling filters, oxidation ponds, etc. are some of the processes used to treat the wastewaters from the industry.

Note: Elementary concepts on the above are discussed in Appendix C.

1.7.4 Microbiology of Foods

Microorganisms are also associated in several ways with all the food we eat. They influence the quality, availability, and the quantity of our food. One of the activities of microorganisms upon food is food spoilage. To avoid food spoilage, some preservation techniques are used. They are aseptic handling, high temperature, canning, pasteurization, sterilization, aseptic processing, and freezing. Lyophilization also known as *freeze drying* is done to remove moisture content of foodstuffs so that they can be safely stored for a longer period of time.

1.8 CYCLES OF LIFE

Cycles of life are also known as *biogeochemical cycles*. A biogeochemical cycle describes the movement of any chemical element or chemical compound among interrelated biologic and geologic systems. This means that the biologic processes such as respiration, photosynthesis and decomposition act alongside and in association with non-biologic processes such as weathering, soil formation and sedimentation in the cycling of chemical elements. This also means that the living organisms can be important storage reservoirs for some elements.

Nutrients are continuously exchanged between organisms and their physical environment and it is unlike to the energy flow that flows in one direction. The nutrient cycle involves storage and transfer of nutrients through the various components of the ecosystem so that they are repeatedly used. The nutrient cycles are of two types: the gaseous and the sedimentary. The reservoir of gaseous type of nutrient cycle is generally located in the atmosphere or the hydrosphere, e.g. the nitrogen reservoir in the atmosphere, whereas the sedimentary type, the reservoir exists in the earth's crust, e.g. phosphorus in the lithosphere.

The nutrient cycles involve the input of nutrients, output of nutrients and the internal nutrient cycling. Nutrients enter the ecosystem from the external sources as wet deposition or dry deposition. Nutrients gained from rainfall in dissolved state are called *wet deposition* and the nutrients obtained from dust are known as *dry deposition*. Nitrogen fixation through the symbiotic biological fixation represents an input. The weathering of parent rocks releases nutrients from the fixed state into the ecosystem also represents an input.

In an undisturbed ecosystem that is without human interference, the input of nutrients more or less balances the output, rendering the cycle to be balanced in nature. There are various cycles, viz. oxygen cycle, carbon cycle, sulphur cycle, phosphorus cycle, nitrogen cycle and calcium cycle that are of course the balanced cycles in nature.

1.8.1 Oxygen Cycle

Oxygen is an integral part of all the activities of living organisms. It is taken in either from the atmosphere or from water by microorganisms to oxidize their food to release energy.

The ultimate source of oxygen is the atmosphere (21% of air). Oxygen is used up by the organisms and in turn, carbon dioxide is released as a by-product. Carbon dioxide is used by autotrophic plants to release oxygen back into the atmosphere. Because of green plants the percentage of oxygen in the atmosphere is almost constant, even though all the organisms require oxygen. The combustion process of any fire or fuel uses oxygen and releases carbon dioxide. Figure 1.15 schematically represents the oxygen cycle.

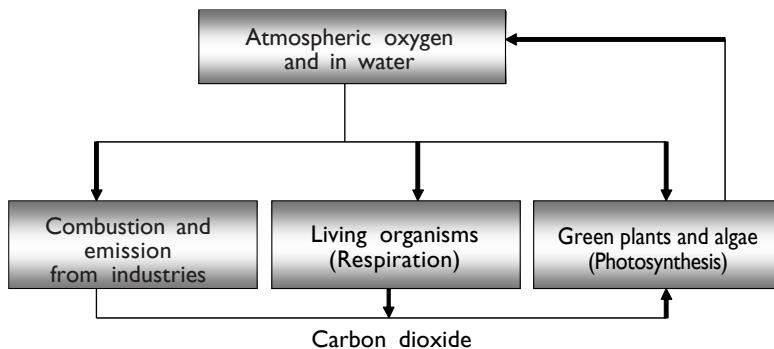


Figure 1.15 Oxygen cycle.

1.8.2 Carbon Cycle

Carbon is the fundamental building block virtually in all the molecules that make up living creatures in the biosphere; existing in rocks such as coal or limestone or in the atmosphere as a part of carbon dioxide gas. Carbon constantly cycles from one reservoir to another. To manufacture organic materials carbon must be fixed.

During photosynthesis, plants take in the atmospheric carbon dioxide from the atmosphere. Figure 1.16 depicts the carbon cycle.

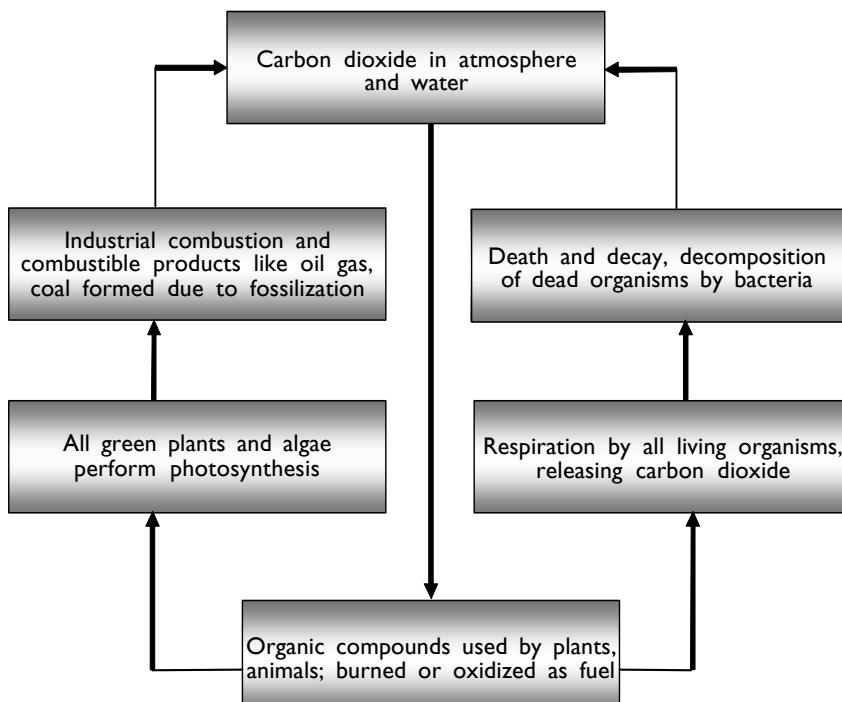


Figure 1.16 Carbon cycle.

When a plant is eaten by a primary consumer, the consumer obtains carbon atoms in the organic compounds of the plant. When the primary consumer is fed by a secondary consumer, there is another transfer of carbon atoms.

Note: The amount of carbon dioxide in the atmosphere is maintained by a balance between the processes which withdraw carbon dioxide from it (photosynthesis) and those which add carbon dioxide to it (respiration and combustion).

By the process of respiration, organic materials which contain carbon provide the living organism with energy and carbon dioxide as one of the waste products of such cellular metabolism. The decomposers also enter this cycle and they degrade the organic matter of dead plants and animals into carbon dioxide. Most of the carbon enters quickly to the atmosphere in the form of carbon dioxide by respiration and decomposition. There are some carbon-containing materials that do not enter this rapid recycling route and thus incompletely decomposed organic matter may be transformed and stored as fossil fuels like coal, oil, peat and natural gas.

Carbon may also be removed from the cycle for a longer or shorter period of time by the formation of limestone produced by the activities of organisms such as foraminifera and corals or by geological forces. These deposits of carbon are returned to the cycle when fossil fuels are burned and limestone weathers.

1.8.3 Sulphur Cycle

A large reservoir of sulphur is found in the soil (as iron sulphides) and sediments and a small reservoir in the atmosphere. Sulphate is the principal available form that is reduced by bacteria into sulphides and hydrogen sulphide.

Figure 1.17 schematically shows the sulphur cycle. Plants use sulphate for making proteins, as sulphur is an essential constituent of certain amino acids. Organic sulphur is transferred to animal bodies through food. During decomposition of plant and animal bodies, the sulphur in proteins is mineralized as sulphates by bacteria and fungi under aerobic conditions.

However, under anaerobic conditions, it may be reduced to sulphides and released to the atmosphere by some bacteria. Animal excretions are also a source of recycled sulphate. The burning of fossil fuels, especially coal, releases sulphur dioxide into the atmosphere and is increasingly becoming a bothersome component.

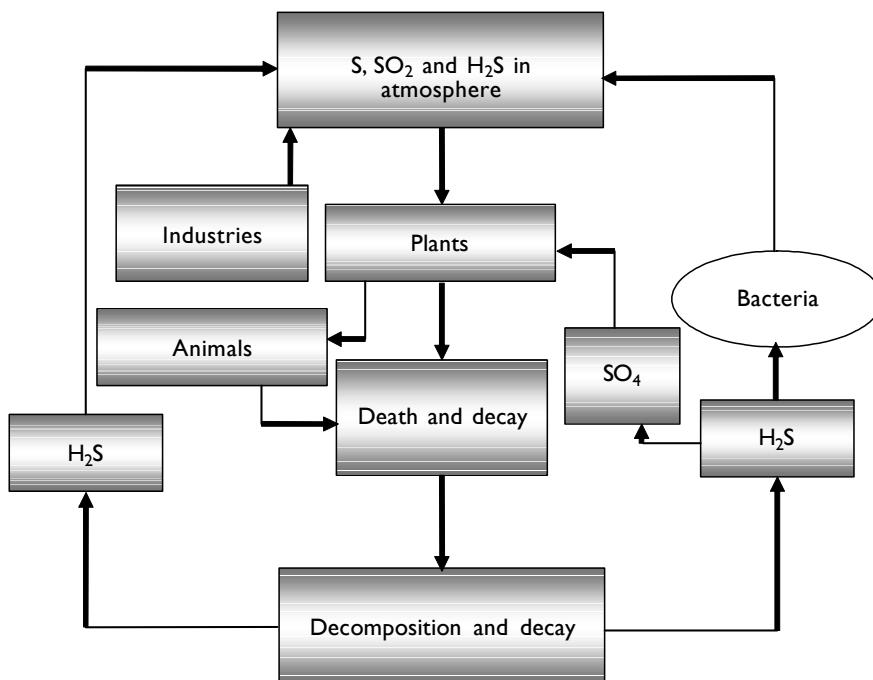


Figure 1.17 Sulphur cycle.

1.8.4 Phosphorus Cycle

Phosphorus is necessary for the formation of DNA, ATP, etc. which are the important molecules that control and govern the organic life processes for the synthesis of energy molecules.

Phosphorus is present in the environment in the form of organic mineral salts and chemical compounds. Rocks and other deposits formed in the past geological ages are the great reservoirs of phosphorus. Plants absorb phosphorus and utilize it in their metabolism. Animals feed on plants directly or indirectly and store phosphorus. When the dead bodies of plants and animals and their excreta are decomposed, the stored phosphorus is returned to the soil. Figure 1.18 depicts the phosphorus cycle.

Phosphorus-containing fertilizers add phosphorus to the soil. Microbes convert the phosphorus into orthophosphates and release inorganic phosphate. A water biome, especially the sea is enriched with phosphorus and the fish of it get them in their flesh. Birds that feed on these fish give back the phosphorus to the land through their excreta (Guano). However, the means of returning phosphorus to the cycle may be not enough to compensate for the loss of phosphate in sea sediments. There is no atmospheric phase of the phosphorus cycle.

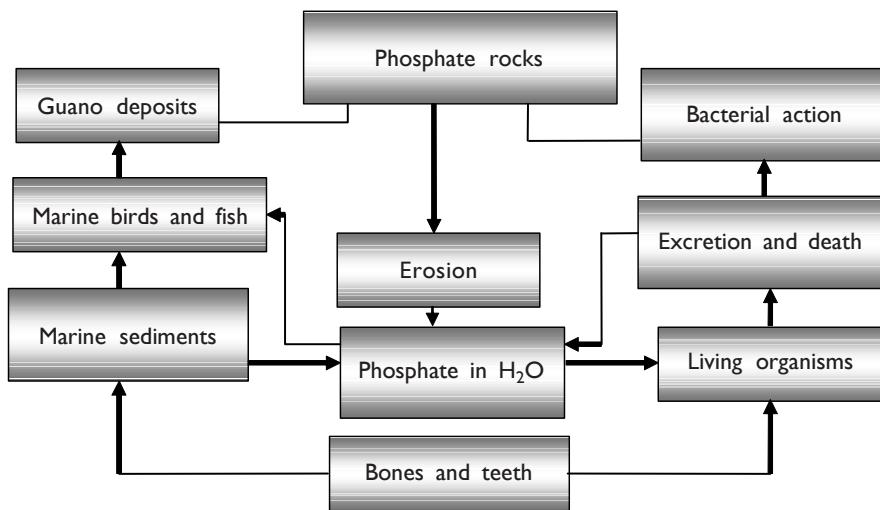


Figure 1.18 Phosphorus cycle.

1.8.5 Nitrogen Cycle

Nitrogen is an important constituent of all proteins and therefore essential for all forms of life. The key to understanding the nitrogen cycle is to understand how nitrogen moves among the four major reservoirs of the earth system—the atmosphere, biosphere, oceans and soil sediments. Figure 1.19 depicts the nitrogen cycle.

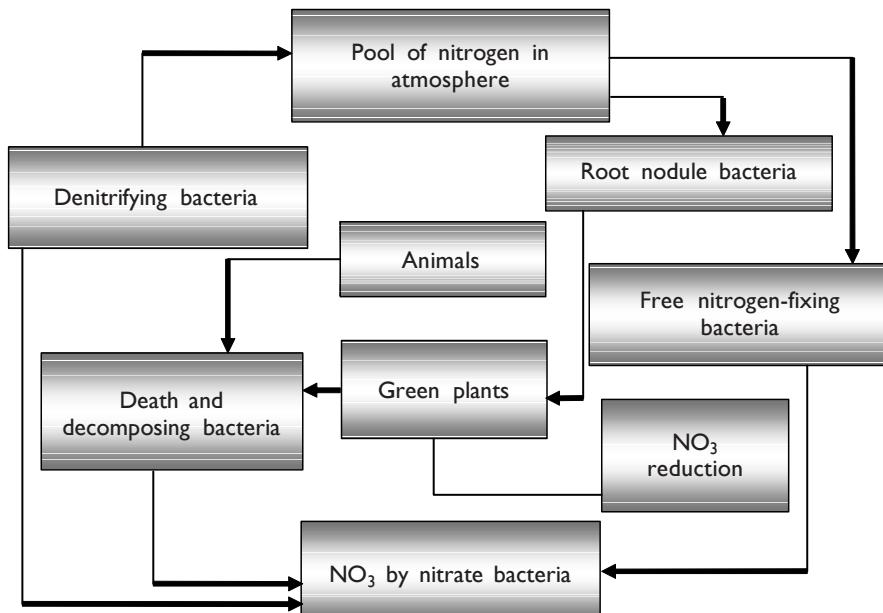


Figure 1.19 Nitrogen cycle.

Nitrogen cannot be used directly by organisms. Only in the reduced forms can nitrogen participate in the biochemical reactions.

The amount of nitrate available to plants is determined by the activities of decomposing bacteria together with the relative amount of nitrification and denitrification that take place in the soil. The amount of nitrogen in the atmosphere is maintained by a balance between the processes which withdraw nitrogen from it, nitrogen fixation and those which add nitrogen to it, denitrification. The organic nitrogen generated through nitrogen fixation and nitrate reduction enters the biomass as organic nitrogen like amino acids, purines, pyrimidines. In dead animals and plants this organic nitrogen is recycled by the process called *ammonification*.

It is carried out by many bacteria like *Bacillus ramosus*, *Bacillus vulgaris*, many fungi and detritivores. The ammonia produced by ammonification is either incorporated into the cell biomass directly or becomes the substrate for the next step in the nitrogen cycle.

1.8.6 Calcium Cycle

Calcium is a necessary mineral element required by living beings. For muscle action, blood clotting, and teeth and bone formation, calcium is required. Calcium is also needed for formation of shells of molluscs and middle lamellae of cell walls in plants.

Calcium is chiefly found in calcium-rich rocks. Figure 1.20 schematically shows the calcium cycle. Calcium mixes with water of the sea and rivers after the breakdown of calcium rocks. It then reaches higher organisms through the food chain. Finally, the calcium in the shells and bones of the organisms gets slowly recycled after decomposition and sedimentation.

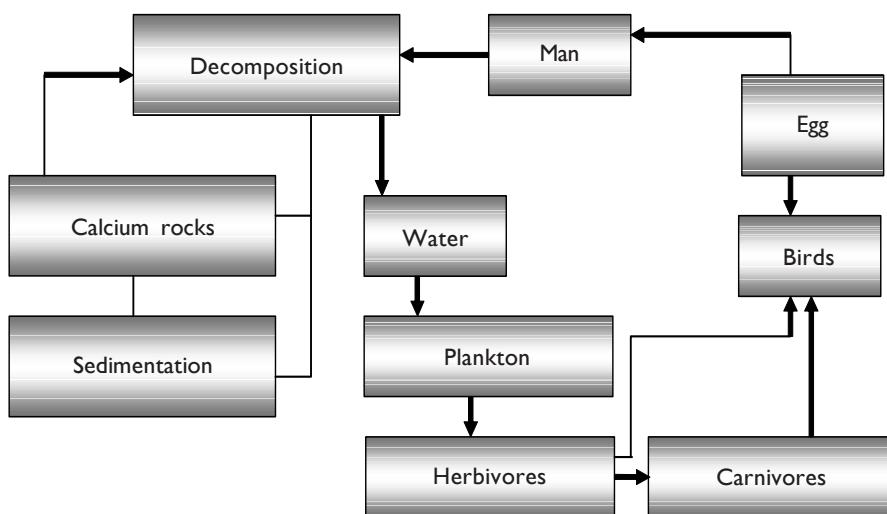


Figure 1.20 Calcium cycle.

SUMMARY

In this chapter, we learnt the following:

- The cell is a structural and functional unit of life. An eukaryotic cell consists of well-defined subcellular organelles, enveloped in a plasma membrane.
- The nucleus contains DNA, the repository of genetic information.
- Mitochondria are the centres for energy metabolism. They are the principal producers of ATP, which is required for cellular work.
- Endoplasmic reticulum is a network of membranous tubules that extend throughout the cytoplasm. Ribosomes are the sites of protein synthesis. Golgi complex is the cluster of membrane vesicles to which the newly synthesized proteins are handed over for further processing and export. Lysosomes are the digestive bodies of the cell, actively involved in the degradation of cellular compounds.
- Cell fractionation or cell differentiation is a method used for the isolation and separation of cell organelles based on the density differences using an ultracentrifuge.
- Gram staining test used for the identification of bacteria as Gram positive and Gram negative.
- Microbes can grow in extreme conditions: at temperatures above boiling and below freezing. Prokaryotes are primitive organisms and are usually single celled.
- Bacteria, yeasts, moulds, algae, and protozoa are the industrially exploited microorganisms.
- Endospores are the dormant forms of bacteria that can resist adverse conditions.
- Yeasts reproduce asexually by budding, fission, and sporulation.
- Nitrogen, carbon, sulphur, phosphorus, and calcium cycles are called *biogeochemical cycles*.

EXERCISES

- 1.1 What are the characteristics or features that all the living things have in common?
- 1.2 Compare prokaryotes and eukaryotes in terms of internal structure and functions.
- 1.3 Write on the Whittaker's five-kingdom concept.
- 1.4 Give a broader classification of microorganisms belonging to the kingdom of Protists.
- 1.5 Describe the internal structure of a bacterial cell.

- 1.6** With a neat diagram, explain all the sequences of steps involved in the process of cell fractionation or cell differentiation.
- 1.7** Describe the important cell types.
- 1.8** Explain briefly the following terms with their significance:
- (a) Cell wall
 - (b) Nucleus
 - (c) Chloroplast
 - (d) Plasma membrane
 - (e) Mitochondria
- 1.9** Describe the yeast cycle of reproduction.
- 1.10** What are the uses of yeasts?
- 1.11** Describe the mycelial structure of moulds and the applications of two important industrial moulds.
- 1.12** Brief the distinct features of actinomycetes and their important products.
- 1.13** Compare protozoa with algae in terms of their cellular structures and functions.
- 1.14** What are the three forms of bacteria? Explain the process of Gram staining test. How is it used in classifying the bacteria?
- 1.15** Explain the process of formation of endospores in bacteria.
- 1.16** Write notes on the following:
- (a) Industrial microbiology
 - (b) Environmental microbiology
- 1.17** Describe the following biogeochemical cycles:
- (a) Sulphur cycle
 - (b) Nitrogen cycle
 - (c) Phosphorus cycle
 - (d) Carbon cycle
 - (e) Calcium cycle
 - (f) Oxygen cycle

Chapter 2

Biological Polymers

Much of the chemistry of the cell is common to all living systems and is directed towards ensuring growth and cell multiplication, or at least the survival of the cell. Organisms also share various structural characteristics. They all contain genetic material, membranes, cytoplasm, etc. All these are supported by polymeric chemicals called *biopolymers*.

This chapter deals with predominant cell polymeric chemicals and smaller monomer units from which larger polymers are derived. It covers biopolymers, viz. proteins, carbohydrates, lipids and fats, nucleic acids, enzymes and to some extent, trace elements, micronutrients and macronutrients of the cell, vitamins, steroids, etc. The importance of these biochemicals is also discussed. At the end of the chapter is given the hierarchy of biological structure that signifies the proliferation right from the environmental precursors to the organism.

2.1 INTRODUCTION TO BIOLOGICAL POLYMERS

To live, an organism must synthesize all the chemicals needed to operate, maintain, and reproduce the cell. Within the cell, like a biological reactor, a network of reactions take place involving various reactants, products, catalysts and chemical controllers. Most important is the presence of predominant cell polymeric chemicals and their small monomers. The predominant cell lipids and fats, proteins, carbohydrates, and nucleic acids. Knowledge of these is essential to understand the cellular functions and in rational design of the processes which use cells.

All biological polymers are divided into two classes:

- Repetitive biological polymers (RBPs)
- Non-repetitive biological polymers (NRBPs)

2.1.1 Repetitive Biological Polymers

Repetitive biological polymers contain one kind of monomeric subunit. RBPs

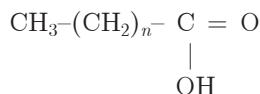
provide structures with desired mechanical strength, chemical inertness, permeability, etc. Also provide a means of nutrient storage.

2.1.2 Non-repetitive Biological Polymers

Non-repetitive biological polymers contain several (about 20) different monomeric subunits. The monomers are linked in a fixed genetically determined sequence. Each of these biological polymers has a fixed molecular weight and monomer composition.

2.2 LIPIDS

Lipids are regarded as the biological substances or compounds that are relatively insoluble in water and soluble in organic solvents such as alcohol, ether, benzene, chloroform, etc. They are related to the fatty acids and used by the living cells. Fatty acids (saturated) are simple lipids with the following general formula:



where $n = 12-20$.

The hydrocarbon chain has two identical carbon monomers and therefore are called the *non-informational biopolymers*. At the end there is a carboxylic group, for example, stearic acid for which the value of n is 16. Unsaturated fatty acids are formed by replacing of a saturated bond by a double bond, for example, oleic acid.

2.2.1 Classification of Lipids

The lipids are classified as follows: simple lipids, complex lipids, derived lipids and miscellaneous lipids.

Simple lipids: These are the esters of fatty acids with alcohols. Further, simple lipids are divided as fats and oils and waxes. Fats and oils are called *triacylglycerols*, which are the esters of fatty acids with glycerol. We find only the physical difference between the fats and oils, i.e. fats are generally solid at room temperature whereas oils are liquid.

Waxes are the esters of fatty acids (long chain) with alcohols, other than the glycerol. In waxes alcohols can be aliphatic or alicyclic. Cetyl alcohol is most commonly found in waxes.

Complex lipids (Compound lipids): These lipids are the esters of fatty acids with alcohols having additional groups as phosphates, nitrogenous base, carbohydrates, proteins, etc. For example:

1. Phospholipids, which are the fatty acids with alcohols and a phosphorus group. Examples: lecithin, cephalin, etc.
2. Glycolipids, which have a carbohydrate with a nitrogenous base. Examples: cerebrosides, gangliosides, etc.
3. Lipoproteins are the macromolecular complex of lipids with proteins. Example: amino lipids.

Derived lipids: These lipids are obtained by the hydrolysis of simple and complex lipids, for example, lipid soluble vitamins, steroids, etc.

Miscellaneous lipids: Carotenoids, squalene, hydrocarbon as pentacosane (in beeswax), terpenes, etc. all belong to the class of miscellaneous lipids.

Neutral lipids: These are the uncharged particles and behave neutral. Cholesterol is the best example.

2.2.2 Functions of Lipids

Lipids serve various functions. Some of the important ones are as follows:

1. Act as the concentrated fuel reserve of the body.
2. Act as the constituents of membrane structure and regulate permeability. For instance, phospholipids and cholesterol.
3. Act as a source of fat soluble vitamins (A, D, E and K).
4. Serve as cellular metabolic regulators (steroids).
5. Protect the internal organs, by serving as insulators.
6. Participate in the Electron Transport Chain (ETC).

2.2.3 Amphipathic Lipids

Generally, lipids are insoluble in water, but there are some lipids that have polar or hydrophilic groups, which tend to be soluble in water. The lipids that have both the hydrophobic and hydrophilic groups are called *amphipathic lipids*, for example, phospholipids, bile salts, cholesterol, fatty acids, etc. The general structure of these amphipathic lipids is shown in Figure 2.1.

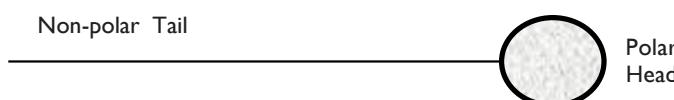


Figure 2.1 General structure of amphipathic lipids.

The amphipathic lipids exhibit certain orientations called the *stable configurations*, which are explained as follows.

2.2.4 Stable Configurations of Fatty Acids in Water

Fatty acids in contact with water can form some stable configurations that are discussed below:

Lipid monolayer: The hydrocarbon chain is nearly insoluble in water, but carboxyl group is hydrophilic and thus is soluble. Fatty acid when placed at an air–water interface, a small amount of acid forms an oriented monolayer, with polar group hydrated in water and the hydrocarbon tails on the air side.

For example, when soap mixes water, the soap lowers the air–water interfacial tension so that the ability of soap solution to wet and cleanse the confined regions increases.

Figure 2.2 schematically shows a lipid monolayer configuration.

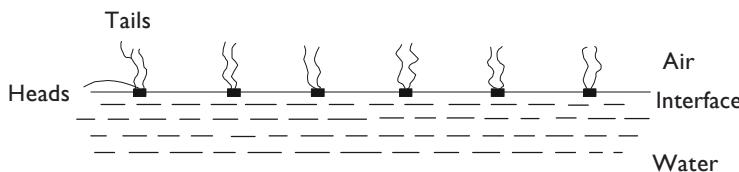


Figure 2.2 Lipid monolayer formation.

Lipid bilayer (Membrane bilayer): In biological membranes, a bilayer of lipids is formed orienting the polar heads to the outer aqueous phase on the either side and the non-polar tails into the interior (refer to Figure 2.3). It forms the basis for the membrane structure.

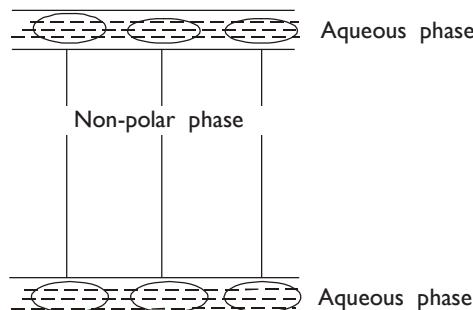


Figure 2.3 Lipid bilayers.

Lipid micelle: This stable configuration is due to the elevation in the concentration of solutions above the monomolecular solubility limit, which results in the condensation of excess solutes into the large ordered structures called *micelles*. It is a spontaneous process. The overall energy of the

resultant, i.e. micelle and the solution, is lower than that of the original solution.

This process is important in the digestion of lipids and absorption as well. The micelle formations increase the number of hydrophobic–hydrophobic and hydrophilic–hydrophilic interactions or contacts and subsequently decrease in the hydrophobic–hydrophilic associations. Figure 2.4 shows a lipid micelle.

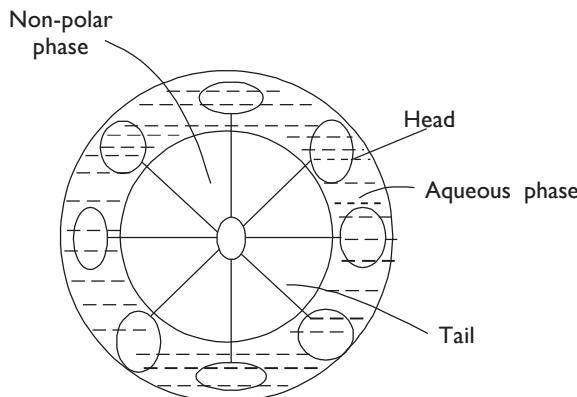


Figure 2.4 Lipid micelle.

2.2.5 Vitamins, Steroids and Other Lipids

Vitamins: Vitamins and minerals are needed for growth and maintenance of body structures. A living organism cannot synthesize minerals. The minerals the body needs in large amounts are calcium, magnesium, phosphorus, sodium, potassium, etc. The body also needs some other minerals in much smaller amounts, which are called *trace elements*. The trace elements include iron, copper, iodine, zinc, cobalt, etc. without these trace elements, a living organism cannot survive. Vitamins are generally divided into two categories: fat-soluble and water-soluble vitamins.

Water-soluble vitamins: By definition these are not lipids. They include thiamin, riboflavin, niacin (nicotinic acid), vitamin C, pantothenic acid, folic acid, biotin, etc.

Fat-soluble vitamins: These are water insoluble and are soluble in the organic solvents, for example, vitamins A, E, K, D, and beta carotene.

Note: The microbes synthesize certain vitamins. For example, ergosterol in yeasts, which acts as a precursor during the synthesis, undergoes a transformation in the presence of sunlight to produce vitamin D₂ also called *calciferol*. In the digestive tract of human beings and the animals, vitamin K is synthesized by the microbes (*E. coli*).

Steroids: Steroids are a class of compounds of lipids with the general structure as shown in Figure 2.5.

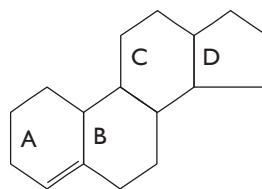


Figure 2.5 General steroid base.

Steroids contain a cyclic steroid nucleus called *perhydroxy cyclopentano phenanthrene*. The rings A, B, and C refer to the phenanthrene nucleus and the ring D is cyclopentane.

Cholesterol, bile acids, vitamin D, sex hormones, adrenocortical hormones, cardiac glycosides and alkaloids are some of the important steroids.

Microbes can synthesize steroids, for example the conversion of progesterone to cortisone is a two-step process, i.e. microbial and chemical. These lipids are associated with the disorders like obesity and atherosclerosis.

Other lipids (Poly β -hydroxy butyric acid (PHB) and cholesterol): PHB is an important food-storage polymer for some bacteria, including *Alcaligenes eutrophus*. It occurs as granules within the cells, is biodegradable and has properties adaptable to packaging. Cholesterol is found in membranes of animal tissues.

2.3 PROTEINS

Proteins (from the Greek word *proteios*, which means 'primary') are the most abundant organic molecules of the living system. They occur in every part of the cell and constitute about 50% of the cellular dry weight. Proteins are the high molecular weight substances, nitrogen rich, and present in all animals and plants. They are composed of 20 amino acids and are organized into primary, secondary, tertiary and quaternary structures.

2.3.1 Functions of Proteins

Proteins perform a wide variety of specialized and essential functions in the living cells. The functions can be categorized into (i) *static* or *structural* and (ii) *dynamic* functions.

Static functions: Certain proteins perform the 'bricks and mortar' roles and are responsible for the structure and strength of the body. For example, collagen and elastin are found in the bone matrix, vascular system and alpha-keratin in the epidermal tissues.

Dynamic functions: These are the most diversified in nature and include the proteins acting as enzymes, hormones, blood clotting factors, immunoglobulins, membrane receptors, storage proteins, in genetic control, muscle contraction, respiration, etc.

These proteins are regarded as the ‘working horses’ of the cell. The proteins and their expressions represent the functional form of DNA information.

2.3.2 Elemental Composition of Proteins

Proteins are predominantly constituted by five major elements such as:

- Carbon: 50–55%
- Hydrogen: 06–7.3%
- Oxygen: 19–24%
- Nitrogen: 13–19%
- Sulphur: 0–04%.

Besides these, there are present the other elements such as P, Fe, Cu, Mg, Mn, Zn, etc.

The content of nitrogen in the proteins on an average is 16%.

2.3.3 Classification of Proteins

The classification of proteins is based on their functions, chemical nature and solubility properties, and also on nutritional importance.

Functional classification: The functional classification includes the following proteins:

1. *Structural proteins:* Keratin of hair, nails, and collagen of bones
2. *Enzymes or catalytic proteins:* Hexokinase, pepsin, etc.
3. *Transport proteins:* Haemoglobin and serum albumin
4. *Hormonal proteins:* Insulin, growth hormones (ACTH), Adrenocorticotropic hormone
5. *Contractile proteins:* Actin and myosin
6. *Storage proteins:* Ovalbumin
7. *Genetic proteins:* Nucleoproteins
8. *Defense proteins:* Snake venom, immunoglobulins
9. *Receptor proteins:* For hormones, viruses

Classification based on chemical nature and solubility: The classification of proteins based on chemical nature and solubility is shown in Figure 2.6. It

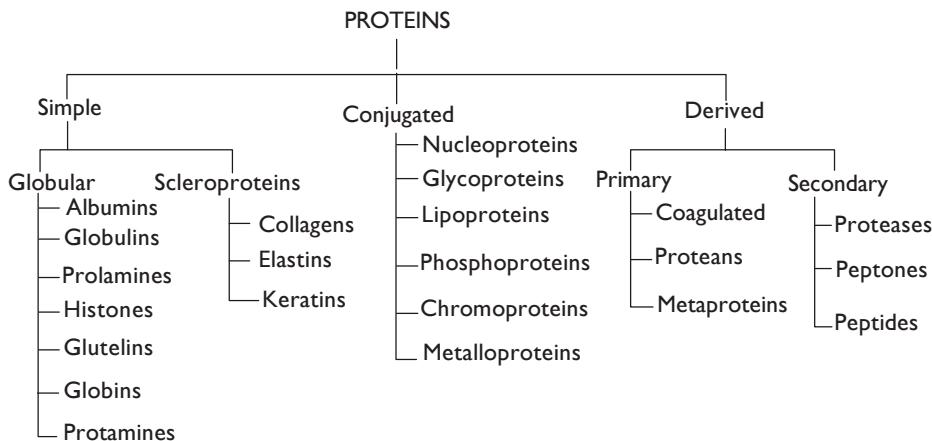


Figure 2.6 Classification of proteins based on chemical nature and solubility.

is a classification based on chemical composition (nature), which can have an influence on solubility. The broader classification is simple, conjugated and derived protein.

Simple proteins: They are made of chains of amino acid units only that are joined by amide linkages. On hydrolysis, these yield mixtures of amino acids and nothing else. Examples are subclasses as shown in Figure 2.6.

Conjugated proteins: These are made up of a simple protein, united covalently or non-covalently with a non-protein part, called *prosthetic group* or *cofactor*. Examples are subclasses as shown in Figure 2.6.

Derived proteins: These are obtained as degradation products due to the hydrolysis of proteins with acids, alkalis or enzymes. Examples are subclasses as shown in Figure 2.6.

Nutritional classification: Based on the composition of the essential amino acids, proteins are classified as shown below.

Complete proteins: Complete proteins have all the ten essential amino acids in the required proportions and are required for good growth. These proteins include egg albumin, milk casein, etc.

Partially incomplete proteins: These proteins partially lack one or more of the essential amino acids and are required for partial growth, for example, wheat and rice proteins.

Incomplete proteins: Incomplete proteins totally lack one or more of the essential amino acids, for example gelatin, maize proteins, etc.

Proteins are the polymers of L-alpha amino acids. On complete hydrolysis with the concentrated hydrochloric acid for several hours, yields L-alpha amino acids.

2.4 AMINO ACIDS

As many as 300 amino acids occur in the nature, of which only 20 are known, and they are called *standard amino acids*. These are isolated from plants, animals and microbes.

Amino acids are a group of organic compounds having two functional groups—amino ($-\text{NH}_2$) and the carboxyl ($-\text{COOH}$). The amino group is basic, and the carboxyl group is acidic.

The general structure of an amino acid is shown in Figure 2.7.

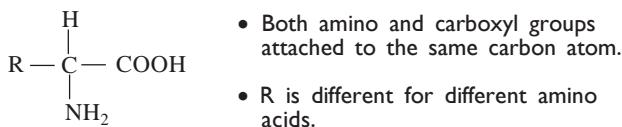


Figure 2.7 General structure of amino acid.

2.4.1 Optical Isomers of Amino Acids

Amino acids exhibit the optical isomerism as Laevo and Dextro amino acids. The structures are as shown in Figure 2.8.

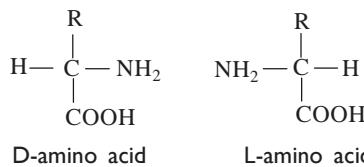


Figure 2.8 Optical rotations of amino acids.

2.4.2 Properties of Amino Acids

Amino acids differ in physical as well as chemical properties which ultimately determine the characteristic of the proteins.

Physical properties: The following are the physical properties of proteins:

Solubility: Most of the amino acids are soluble in water and insoluble in organic solvents.

Melting point: Proteins generally melt at higher temperatures—at temperatures greater than 200°C .

Taste: Amino acids can be sweet like glycine, alanine, valine, etc. They can be tasteless as leucine or can be bitter as arginine, isoleucine, etc.

Ampholytic nature: Amino acids since they have both the acidic and basic groups, they can donate or accept protons. Hence, they are called the *ampholytes*.

Zwitter nature: Each amino acid has a characteristic pH at which it carries both positive and negative charges and exists as a zwitterion.

Isoelectric pH: This is the pH at which a molecule exists as a zwitterion or the dipolar ion and carries no net charge. Thus, the molecule is electrically neutral.

Chemical properties: Proteins exhibit various chemical properties. Important ones are as follows:

With bases: Proteins form the salts ($-\text{COONa}$).

With alcohols: They form esters ($-\text{COOR}'$).

With ammonia: The carboxyl group of dicarboxylic amino acid reacts with ammonia to form amide. For example:



Reactions due to the amino group: Amino groups behave as bases and combine with acids to form the salts ($-\text{NH}_3^+ \text{Cl}^-$).

With ninhydrin: Alpha amino acids react with ninhydrin to form a purple, blue or pink colour complex called *Ruhemann's purple complex*.



Colour reactions: Amino acids undergo colour reactions, namely biuret reaction, ninhydrin reaction, xanthoproteic reaction and Milon's test reaction.

Biuret reaction: A dilute solution of copper sulphate when added to a protein solution mixed with sodium hydroxide, a violet colour appears. Violet colour is due to the formation of a coordination compound between Cu^{2+} and $>\text{C}=\text{O}$ and $-\text{NH}-$ groups of the peptide chain.

Ninhydrin reaction: Protein when boiled with a dilute solution of ninhydrin, a violet colour is developed. This test is shown by all proteins containing α -amino acids.

Xanthoproteic reaction: Protein solution on warming with a concentrated HNO_3 solution may turn yellow. This test is given by proteins having tyrosine and tryptophan (amino acids with benzene ring).

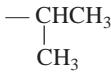
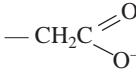
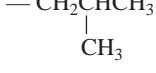
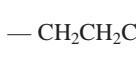
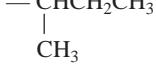
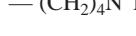
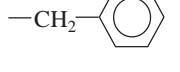
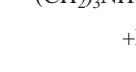
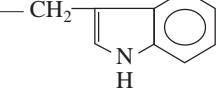
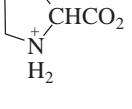
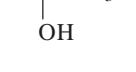
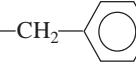
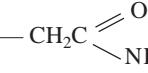
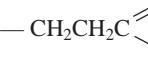
Milon's reaction: Milon's reagent containing mercurous and mercuric nitrate in nitric acid, when added to a protein solution, a white precipitate that turns brick-red on heating may be formed.

Transaminations: The transfer of an amino group from an amino acid to a keto acid results in a new amino acid.

Oxidative deamination: Proteins undergo oxidative deamination and liberate free ammonia, for example glutamate converts to NH₃ and alpha-ketoglutarate.

Table 2.1 shows the side chains of 20 amino acids commonly found in proteins.

Table 2.1 Side chains of 20 amino acids found in proteins

Non-polar side chains		Polar side chains	
– R	Amino acid (Symbol)	– R	Amino acid (Symbol)
— CH ₃	Alanine (Ala)		
	Valine (Val)		Aspartic acid (Asp) also called Aspartate
	Leucine (Leu)		Glutamic acid (Glu) also called Glutamate
	Isoleucine (Ile)		Lysine (Lys)
	Phenylalanine (Phe)		Arginine (Arg)
	Tryptophan (Trp)	— H	Glycine (Gly)
— CH ₂ CH ₂ —S—CH ₃	Methionine (Met)	— CH ₂ OH	Serine (Ser)
	Proline (Pro)		Threonine (Thr)
		— CH ₂ SH	Cysteine (Cys)
			Tyrosine (Tyr)
		— CH ₂ SH	Cysteine (Cys)
		— CH ₂ —N ₂ H	Histidine (His)
			Asparagine (Aspn)
			Glutamine (Gln)

2.5 PROTEIN STRUCTURES

The simple condensation between two amino acids to form one molecule yields a dipeptide (Figure 2.9). Polypeptides are thus large molecules of amino acids linked together via peptide bonds.



Figure 2.9 Dipeptide.

Simple proteins: These are composed of amino acids linked together by a peptide bond as shown in Figure 2.9. The molecule has an N-terminal end (free amino group) and a C-terminal end (free carboxylic group).

Conjugated proteins: Protein structures may be further complicated by the inclusion of materials such as metal ions and porphyrin rings, and by the addition of carbohydrates, lipids and nucleic acids. Such compounds are called *conjugated proteins*. The structure of proteins has various levels as, primary, secondary, tertiary and quaternary structures. But before this, let us take a look at the two major types of protein structure with variations which are shown in Figure 2.10.

Derived proteins: Derived proteins are degradation products obtained by hydrolysis of proteins with acids, alkalis or enzymes. They are produced when natural proteins are subjected to the action of enzymes and other hydrolytic agents. Examples include peptones, peptides, secondary proteases, etc.

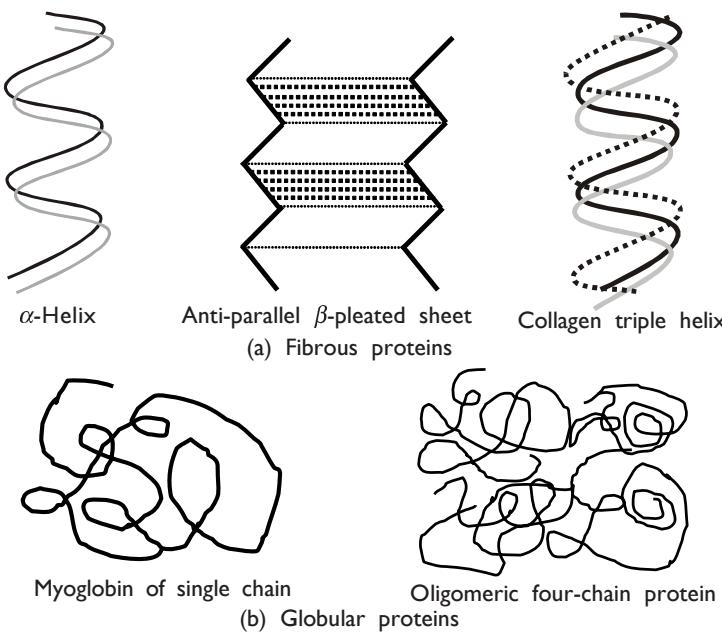


Figure 2.10 Major types of protein structures.

2.5.1 Primary Structure of Protein

The sequence of amino acid linked by peptide bonds forms a polypeptide chain. This is the primary structure. The first complete determination of amino acid sequence of a protein was done for the hormone insulin.

The primary structure of protein is important genetically as well as structurally; genetically because it indirectly suggests the sequence of nucleotides in DNA, and structurally because the primary structures form the basis of higher levels of organization of protein structure.

Figure 2.11 shows the protein primary structure.

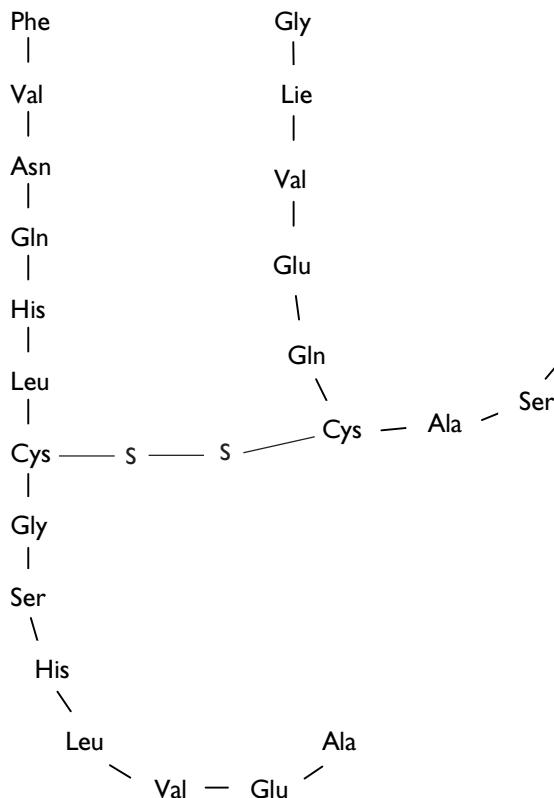


Figure 2.11 The amino acid sequence in the insulin molecule of oxen.

2.5.2 Secondary Structure of Protein

The secondary structure of protein arises from the way in which the primary structure is folded, maximizing the number of hydrogen bonds, and in effect

lowering the free energy of the molecules and their interactions with water. Generally, these interactions occur over relatively short range between different parts of the molecules.

Figure 2.12 illustrates the protein secondary structure with different configurations.

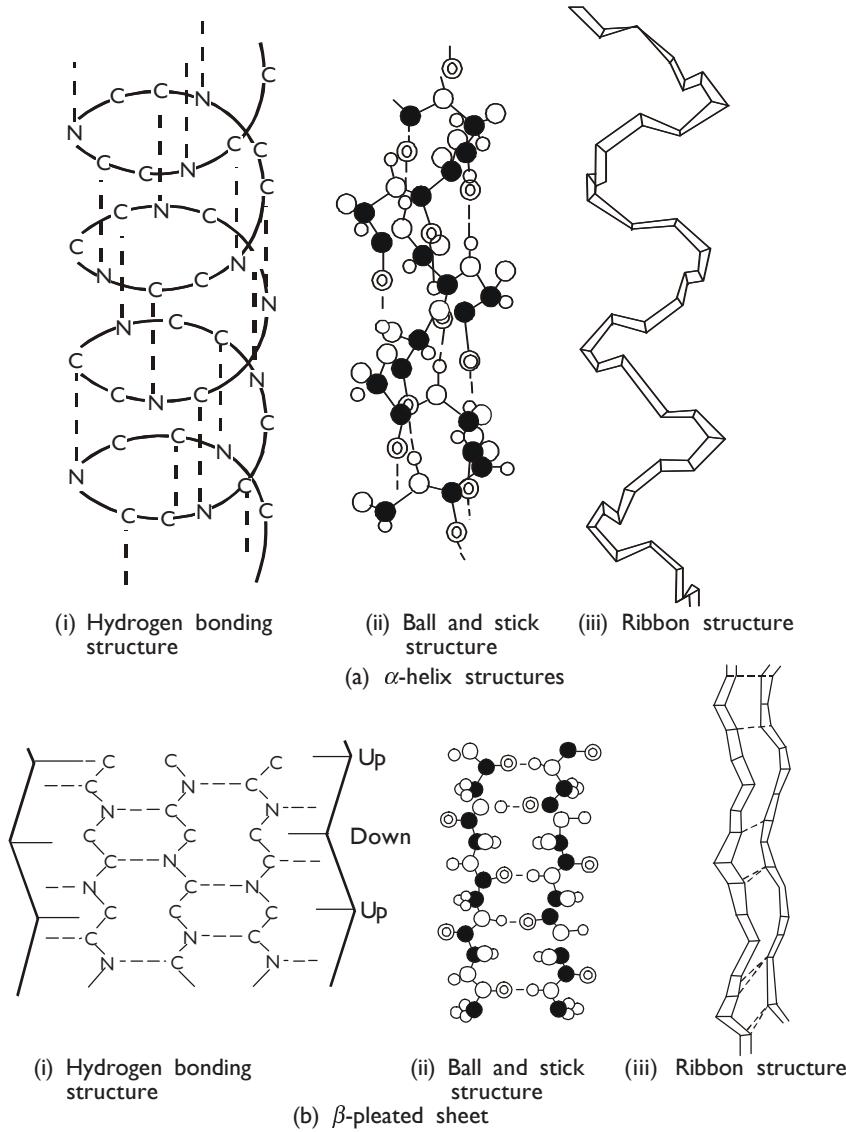


Figure 2.12 Secondary protein structure.

2.5.3 Tertiary Structure of Protein

A polypeptide chain with its secondary structure may be further folded and twisted about itself precisely because of the variety of amino acid present and

the different chemical properties of R-groups. For example, hydrophobic R-groups associate with one another and seek out the interior of the molecules.

Figure 2.13 indicates the forces that maintain tertiary structure of protein.

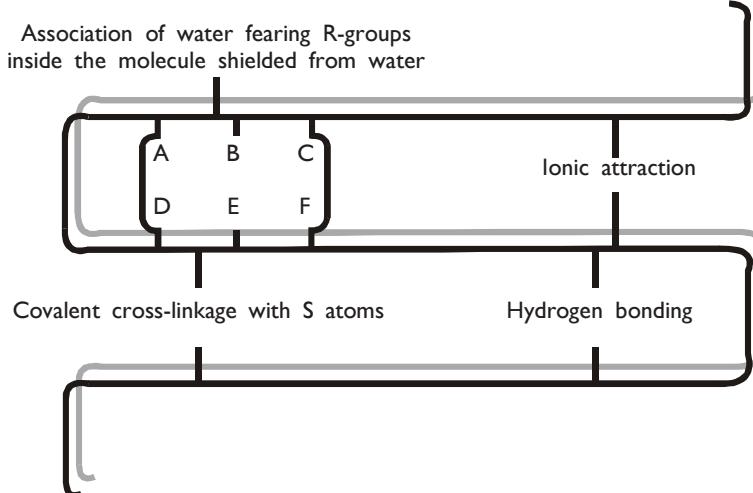


Figure 2.13 Forces responsible for tertiary structure of protein.

Although the steric effects and hydrogen-bonding maintain the secondary structure of proteins, the same factors also contribute to the stability of the tertiary structure. These involve long range interactions between the amino acids of the polymer. The tertiary structure plays a key role in the function of proteins, including enzymes in biological systems. The forces responsible for maintaining the tertiary structure of proteins are also called *weak forces*, consisting of van der Waals forces, hydrogen-bonding and weaker electrostatic interactions.

2.5.4 Quaternary Structure of Protein

The quaternary structure of a protein is the level of organization concerned with sub-unit interactions and assembly. Therefore, the quaternary structure applies only to multimeric proteins, i.e. proteins made up of more than one sub-unit. The polypeptides (which are multimeric) are quaternary in structure. Quaternary is a four-fold structure. Polypeptides which are quaternary, can have identical polypeptides or several different kinds of polypeptides altogether forming a quaternary. Examples include haemoglobin.

2.6 PROTEIN DENATURATION AND RENATURATION

Proteins in their functional confirmation are called native proteins. A specific protein structure is important for biological function. The loss of three-dimensional structure of a protein is termed *denaturation*.

Heat, extremes of pH, certain miscible organic solvents such as alcohol or acetone, and solutes like urea, detergents are all known to denature proteins.

After a protein has been denatured by exposure to an adverse environment, it will often return to native, biologically active confirmation following the restoration of suitable conditions. This result indicates that the primary protein structure determines the secondary and tertiary structures. Protein molecules in which the structural stabilizing bonds have been ruptured are said to be denatured. For example, when an egg is boiled, heat denatures the egg albumin and the various yolk proteins. It is usually impossible to reverse this process. However, some proteins can denature reversibly, for example the enzymes ribonuclease. When its denaturing agent mercapto-ethanol and urea are removed, the denatured enzymes snaps back to its original tertiary structure exhibiting catalytic activity.

This is called the *renaturation* of proteins (Figure 2.14).

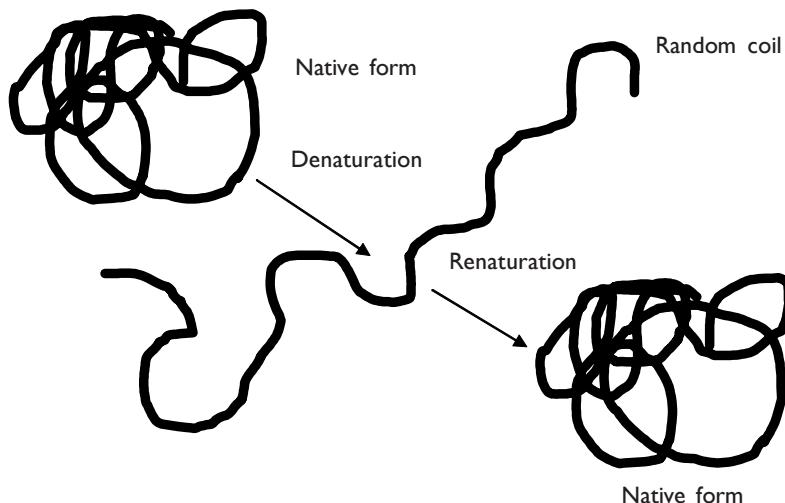


Figure 2.14 Protein denaturation and renaturation.

2.7 NUCLEIC ACIDS

There are two types of nucleic acids—deoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA). The nucleic acids are macromolecules and polymers of nucleotides consisting of a pentose sugar, nitrogen base and phosphoric acid. By partial hydrolysis, the nucleotide yields nucleosides containing nitrogen base attached to a pentose sugar. The sugar in DNA is deoxyribose, where hydrogen is attached to the second carbon. In the case of RNA, the sugar is ribose in which hydroxyl group is bound to that carbon.

The bases cytosine(C), adenine(A) and guanine(G) are common in both RNA and DNA. However, RNA molecules contain a unique base, uracil(U), while the unique DNA base is thymidine(T). These differences in the base

structure markedly affect the secondary structures of these polymers. There is a structural difference between the molecules of DNA and RNA. The RNA molecules are in the form of a single chain of nucleotides while the DNA molecules are normally in double chain, allowing specific hydrogen bonding between the bases to hold the two strands together. The two chains form a double helix.

Figure 2.15 represents the general structure of ribonucleotides and deoxyribonucleotides and five nitrogenous bases.

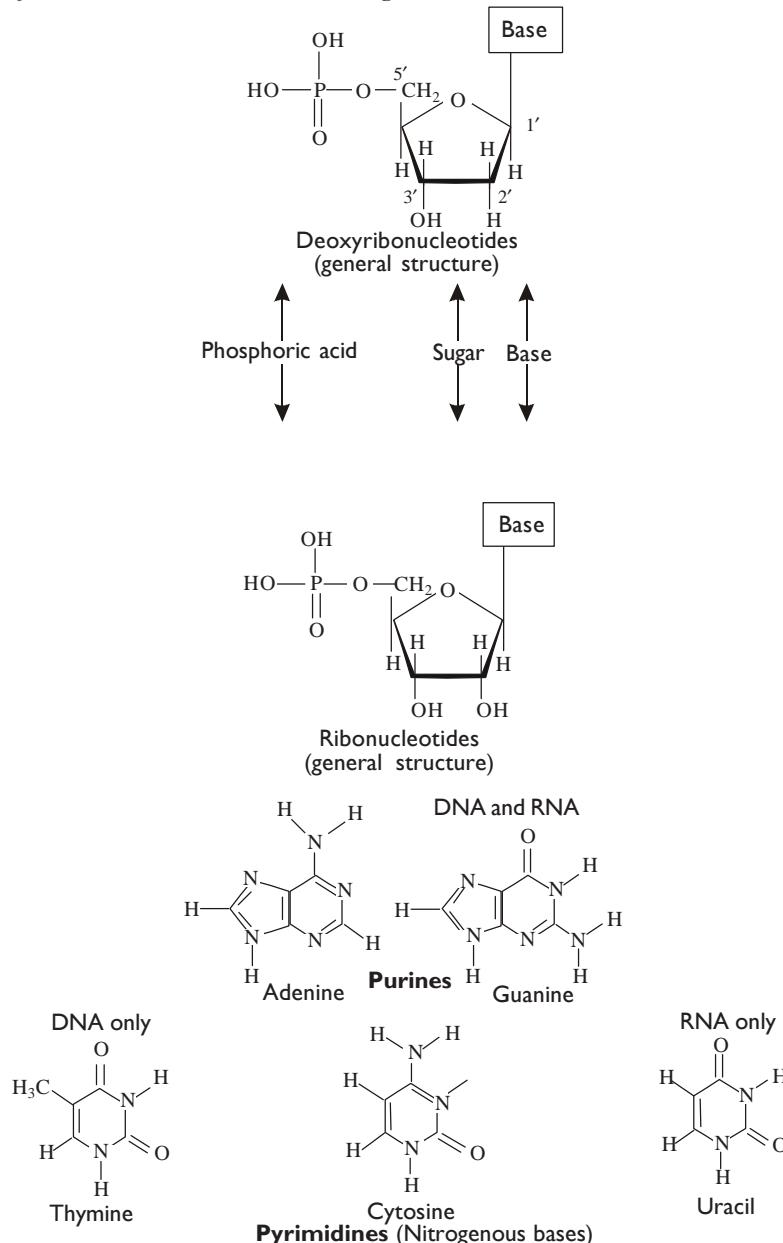


Figure 2.15 Structure of nucleotides and nitrogenous bases.

2.7.1 DNA Structure

Deoxyribonucleic acid (DNA) is a linear polymer of nucleotides linked via phosphodiester bonds which are formed between the 3' and 5' hydroxyl groups of the ribose moiety of the nucleotide. DNA is composed of four nucleotides—adenine(A), thymine(T), guanine(G) and cytosine(C). The primary structure of DNA is the linear sequence of nucleotide residues that consist of the polydeoxyribonucleotide chain.

DNA varies in length and in nucleotide composition, the shortest molecules being in the order of 10^3 bases in viruses, 10^6 in bacteria and 10^8 in human beings. Figure 2.16 represents the covalent backbone structure of nucleic acids.

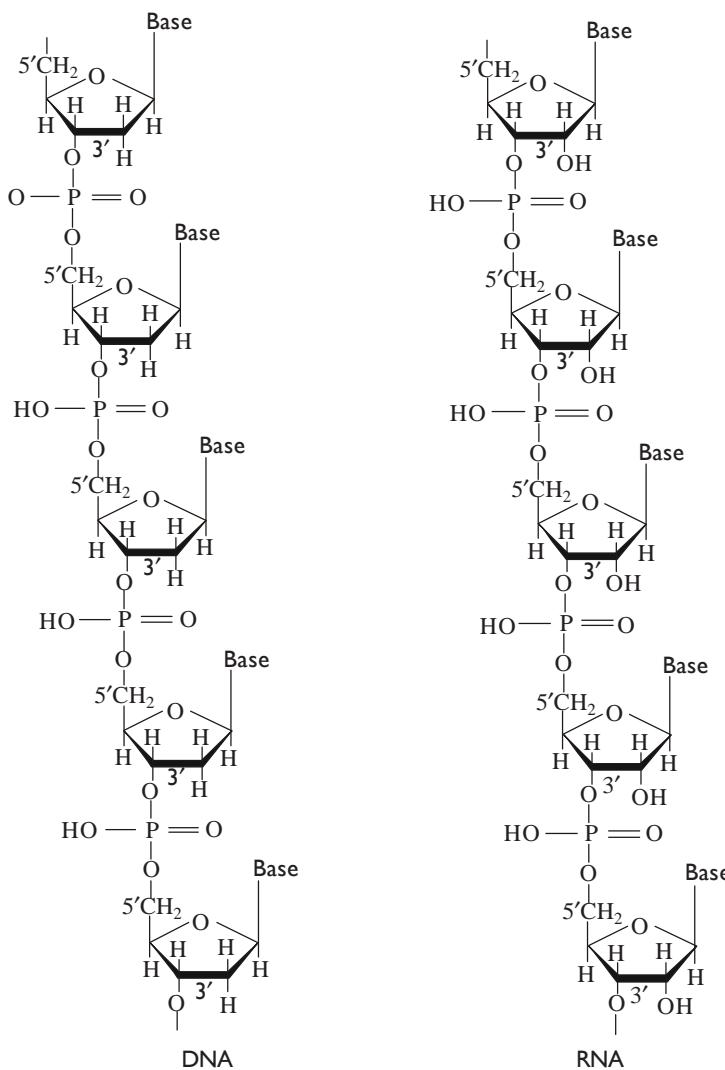


Figure 2.16 Covalent backbone of nucleic acids.

These polymers are found usually as an aggregated pair of strands that are held together by hydrogen bonding.

The nitrogenous base pairs that are held by hydrogen bonding is depicted in Figure 2.17. Adenine pairs with thymine by two hydrogen bonds (A = T) and guanine pairs with cytosine by three hydrogen bonds (C ≡ G) respectively.

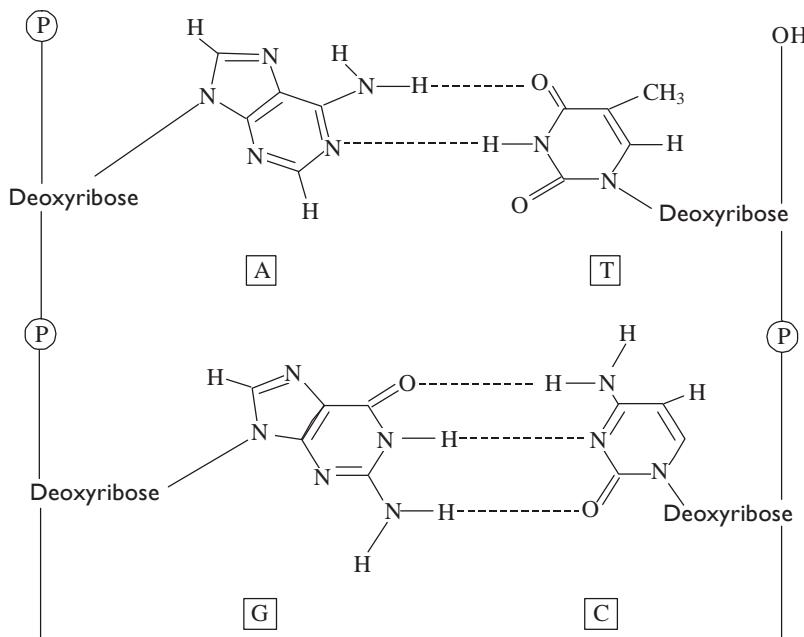


Figure 2.17 Base pairs held by hydrogen bonding.

2.7.2 Watson and Crick Model of DNA

James Watson and Francis Crick in the year 1953, put forward a possible explanation of the structure of DNA molecule which was a turning point for the concept of the gene. The key piece of data came from the results of their interpretation of some X-ray diffraction patterns of DNA crystals.

Watson and Crick conceived that DNA molecule as a twisted ladder, the upright arms composed of chains of alternating sugar and phosphate groups, and the cross bars containing nitrogen bases, thymine(T) and cytosine(C) as pyrimidine bases, and adenine(A) and guanine(G) as purine bases.

The cross bars can only be formed by linking purine bases with pyrimidine bases. The bases are held together by hydrogen bonds. The position of hydrogen atoms in relation to the shape of the molecule ensures that A can only link with T by two hydrogen bonds, and C with G by three hydrogen bonds.

The secondary structure of DNA consists of a double-stranded helix formed by the coiling of two linear polydeoxyribonucleotide strands around

an identical central axis. This form of DNA is believed to be predominant form present in living cells. The coils of the double-helix are regular and smoothly curved (Figure 2.18).

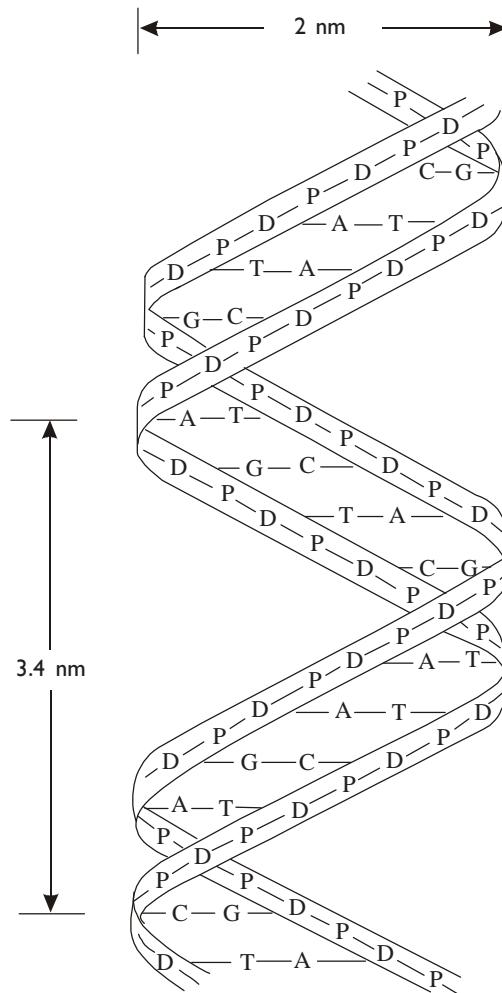


Figure 2.18 Double-helix structure of DNA.

Both the strands of the helix form right-handed coils, but they are antiparallel to each other, the 5' end of one strands facing the 3' end of the other and vice versa.

Chargaff's findings suggest that the number of adenine and guanine residues equals respectively those of thymine and cytosine residues in a DNA double-helix. It also follows that purines equal pyrimidines in number in the molecule, and that the two strands are complimentary to one another in base sequence.

The consecutive hydrogen-bonded base pairs lie in parallel planes at a distance of 0.34 nm from each other. There are ten base pairs in each turn of a helix. The pitch of the helix is 3.4 nm.

2.7.3 Ribonucleic Acid (RNA)

Ribonucleic acid (RNA) is the other important nucleic acid besides DNA. Some viruses like tobacco mosaic virus and foot-and-mouth virus, however, contain no DNA but only RNA in them. RNA, being the sole genetic molecule, carries the responsibilities of DNA. Such RNA is genetic RNA. In these cells in which the genetic substance is DNA, the RNA molecules are called *non-genetic* RNA.

There are three types of non-genetic RNAs, namely:

- Ribosomal RNA (rRNA)
- Transfer RNA (tRNA) and
- Messenger RNA (mRNA)

All the three types of RNA are synthesized inside the nucleus. The RNA differs from DNA in the following features:

1. RNA is mostly single stranded consisting of a single strand of ribonucleotides.
2. The sugar in the ribonucleotides is the ribose sugar.
3. The nitrogenous base thymine is replaced by uracil.

mRNA is the template used by ribosomes for the translation of genetic material into an amino acid sequence, and it is derived from a specific DNA sequence. The genetic code is made up of trinucleotide sequences, or codons, on mRNA. Each mRNA has a unique sequence coding for each protein (polypeptide chain).

tRNA is a single strand of RNA but it is in a highly folded configuration. The molecules are usually 70–95 ribonucleotides long (equivalent to 23,000–30,000 of molecular weight). Each of the 20 amino acids has one or more tRNAs to which it is able to bind. In this bound form the amino acids are transported into the ribosomes. tRNA molecules therefore serve as adapters for translating the genetic code or codons of the mRNA into the sequence of amino acids or proteins.

Each tRNA contains a trinucleotides sequence called an *anticodon sequence*, which is complementary to a codon, the trinucleotide sequence of mRNA that codes for one amino acid.

rRNAs are the major components of ribosomes and make up 65% by weight of the structure. The role of ribosomal RNA is very complex, but it is essential for protein synthesis which occurs within the ribosomes organelle. Each has a role in the biosynthesis of proteins.

2.7.4 Biological Information Storage and DNA Replication

DNA replication is a complex process requiring a number of enzymes and other proteins, and even the participation of RNA. The entire process of replication involves the following steps, and these are illustrated in Figure 2.19.

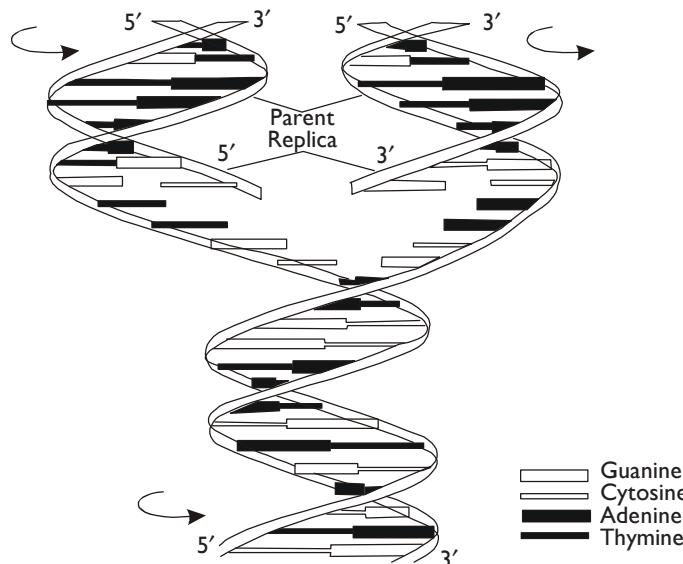


Figure 2.19 DNA replication.

It is important to see that the sequence of nucleotides has a direction or polarity, with one free 5'-end and the other terminus possessing a 3'-end not coupled to another nucleotide. The two strands of DNA have opposite polarity: they run 5'→3' in opposite directions. Combining this information with the base pairing rules, we can infer from the nucleotide sequence of a single deoxyribonucleotides chain, for example 5' CGAATCGTA 3', that the corresponding fragment of an intact, double-stranded DNA molecule looks like the following:

5' CGAATCGTA 3'
3' GCTTAGCAT 5'

Thus, in an informational sense, knowledge of one strand's sequence implies the sequence of the complimentary strand. Each strand is a template for the other. This feature of DNA provides directions for the synthesis of daughter DNA from a parent DNA molecule, and the process is called *DNA replication*. If the two complimentary strands are separated and double helices are constructed from each strand following the base pairing rules, the end products are two new molecules, each identical to the original double-stranded DNA, and each containing one new strand and one old strand.

Thus, the biological message is coded in the DNA nucleotide sequence. As the parent strands separate, complimentary strands are added to each parent, resulting in two daughter molecules identical to the parent.

Finally, each daughter molecule contains one strand from the parent. Regeneration of DNA from original DNA segments is known as DNA replication and it is semi-conservative as shown in Figure 2.20.

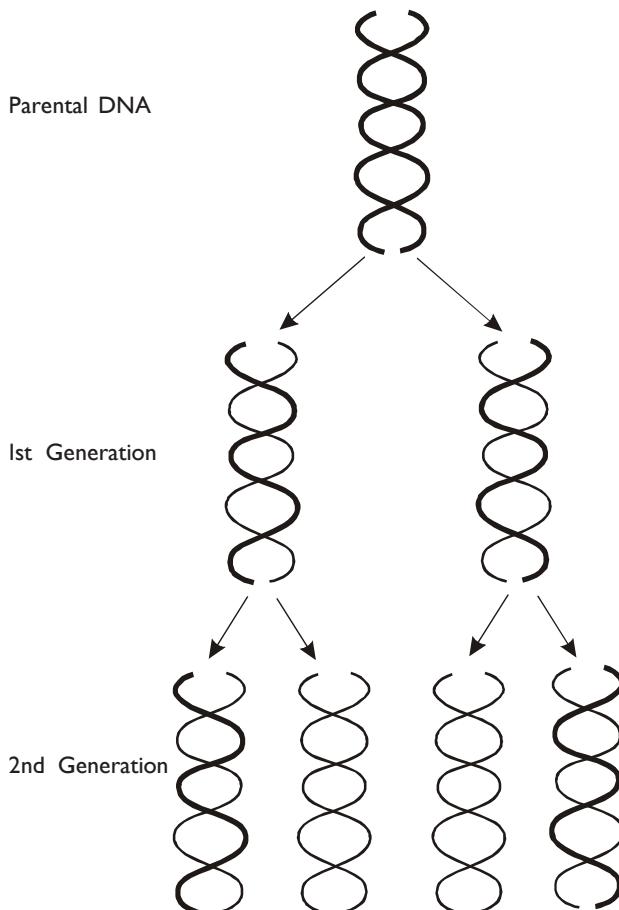


Figure 2.20 Semi-conservative replication of DNA.

Based on the findings of Watson and Crick, the central dogma of the molecular genetics is understood and the flow of information is seen to be essentially in one direction from DNA to the protein.

Three major steps are defined in the flow of information. They are replication, transcription and translation of the genetic material. The same is shown in Figure 2.21.

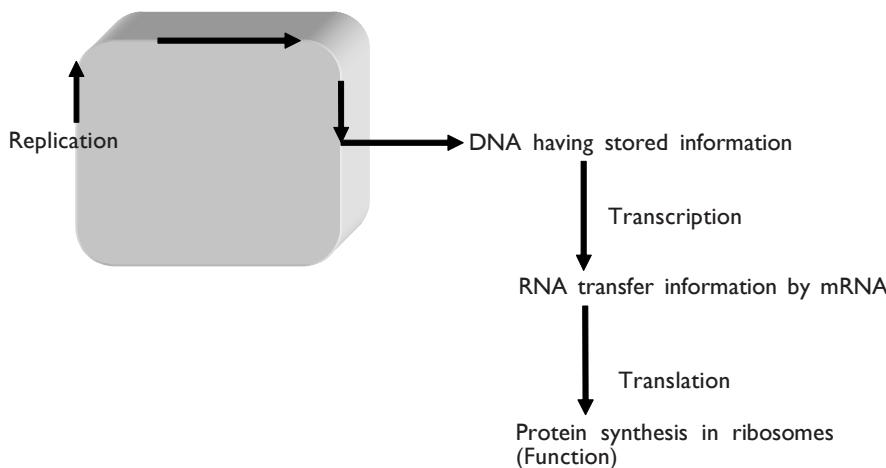


Figure 2.21 Information flow and storage in cell systems with major processes involved.

We have already discussed the replication of DNA. Once the replication occurs, the DNA has the information stored in it. Later on it undergoes a series of changes as follows.

Transcription: Transcription is the copying of a complementary messenger RNA strand on a DNA template (one of the strands of DNA on which mRNA is transcribed). The process of transcription requires the template, high-energy compounds like ATP, GTP, the enzyme RNA polymerase and divalent cations. The enzyme RNA polymerase consists of a core enzyme with subunits and sigma factor (σ). The sigma factor initiates the transcription of mRNA on the DNA template and the core enzyme continues transcription.

Translation: In this the genetic information present in mRNA directs the order of specific amino acids to form a polypeptide or a protein. The translation process consists of activation of amino acids, transfer of activated amino acids to tRNA, initiation of polypeptide chain, chain elongation and termination. These processes require ribosomes, mRNA and tRNA, initiation factors, termination factors, and high energy compounds like ATP, GTP, and the divalent ions like Mg^{2+} .

Separation of double-stranded DNA by heat is an important method in DNA characterization. Because AT base pairs involve two hydrogen bonds and GC base pairs have three, AT-rich region of DNA melt (i.e. the two strands separate) before GC-rich regions. The melting process is readily monitored by following absorbance of the DNA solution at 260 nm: single-stranded DNA absorbs more strongly, so that DNA melting is measured as an increase in overall absorbance. The melting temperature T_m is the temperature at which the absorbance is midway between the fully double-stranded and completely melted limits.

If a solution of melted DNA is cooled, the separated complementary strands will anneal to reform the double helix. Similarly, if two different single-stranded segments of DNA have complementary base pair sequences, these will hybridize to form a double-stranded segment. Also, double-stranded DNA in solution behaves hydrodynamically like a rigid rod while single-stranded DNA acts like a randomly coiled polymer.

2.8 CARBOHYDRATES

Carbohydrates are the most abundant organic substances in nature. Sugars, starches and cellulose found in green plants, glycogen in animal tissues, and glucose in the body fluids of animals are all examples of carbohydrates.

They occur in food, wood, paper and synthetic fibres. Carbohydrates have an empirical formula $C_n(H_2O)_n$ where $n \geq 3$. They are also called *saccharides* meaning sugars.

Carbohydrates play a key role as storage and structural compounds in the cell. They also play crucial roles in modulating aspects of chemical signalling in animals and plants. Carbohydrates are synthesized by plants through photosynthesis as illustrated in Figure 2.22.

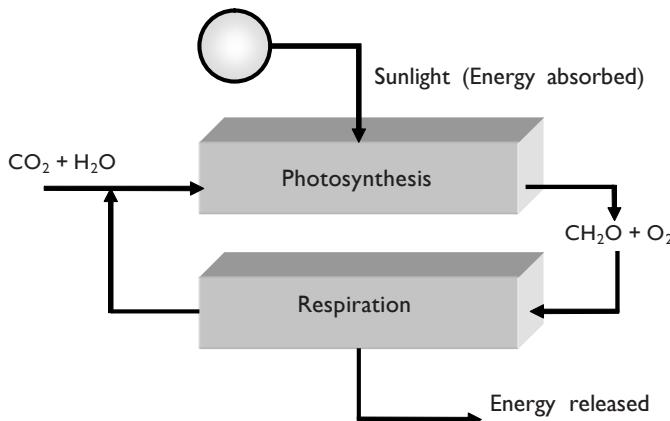


Figure 2.22 Synthesis of carbohydrates.

CO_2 and H_2O are converted through photosynthesis into sugars in the presence of sunlight and are then polymerized to yield polysaccharides.

Carbohydrates are classified into (a) monosaccharides, (b) disaccharides, and (c) polysaccharides.

2.8.1 Monosaccharides

Monosaccharides are the simplest and smallest carbohydrates and contain three to nine carbon atoms, and are the building blocks of complex carbohydrates. These cannot be hydrolyzed into a simpler sugar, for example, glucose which has a molecular formula of $C_6H_{12}O_6$.

Glucose may be present in the form of a linear or ring structure. In solution, D-glucose is in the form of a ring structure or pyranose. The L-form has a minor role in the biological systems.

Two conventional ways of representing the structure of glucose are as shown in Figure 2.23.

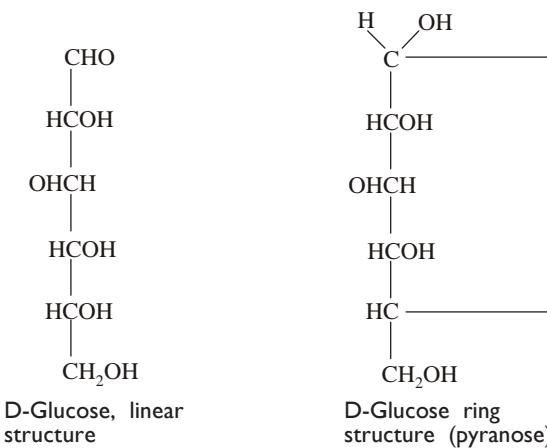


Figure 2.23 Structure of glucose.

The structures of α -D-glucose and β -D-glucose are as shown in Figure 2.24.

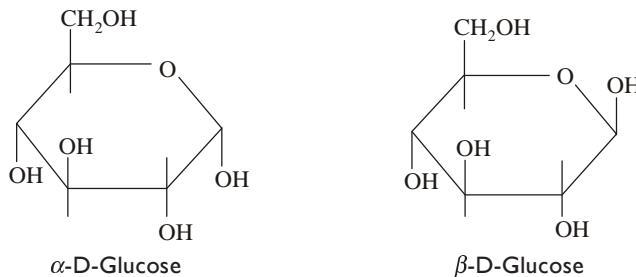


Figure 2.24 Structures of α -D-glucose and β -D-glucose.

According to the number of carbon atoms, the monosaccharides are classified into *trioses* (3C), *tetroses* (4C), *pentoses* (5C), and *hexoses* (6C).

Trioses (C₃H₆O₃): Trioses include glyceraldehydes and dihydroxyacetone. They are the intermediates in respiration during the cycle of glycolysis, photosynthesis (dark reaction) and other branches of carbohydrate metabolism.



Tetroses ($C_4H_8O_4$): Tetroses like erythrose are rare in nature and occur mainly in bacteria.

Pentoses ($C_5H_{10}O_5$): Pentoses include ribose, ribulose, etc. In the synthesis of nucleic acids, ribose is a constituent of RNA while deoxyribose in the case of DNA (Figure 2.25). Pentoses are involved in the synthesis of some coenzymes, e.g. NAD, NADP, coenzyme A, FAD, etc. and also in the synthesis of polysaccharides called *pentosans*.

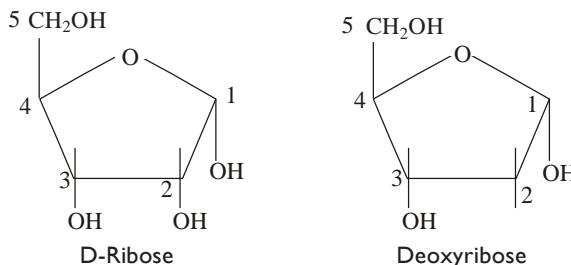


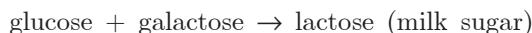
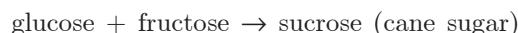
Figure 2.25 Structures of ribose and deoxyribose.

Hexoses ($C_6H_{12}O_6$): Glucose, fructose, galactose, mannose, etc. are some examples of hexoses. These are the sources of energy when oxidized in the process of respiration. Of these, glucose is the most common respiratory substrate monosaccharide, involved in the synthesis of disaccharides. If 2–10 monosaccharides are combined then these are termed as oligosaccharides.

Aldoses and ketoses are the other forms of monosaccharides. In monosaccharides, all the carbon atoms except one have a hydroxyl group attached. The remaining carbon atom is either part of an aldehyde group in which case the monosaccharide is called an *aldose* or *aldo sugar*, e.g. ribose, glucose, mannose, galactose, etc. or is part of a keto group, when it is called a *ketose* or *keto sugar*, e.g. ribulose, fructose, etc.

2.8.2 Disaccharides

These are formed by the condensation of two monosaccharides usually hexoses. On hydrolysis, a disaccharide yields the two respective monosaccharides.



Maltose is formed by the condensation of two glucose molecules via α -1,4 glycosidic linkage, which is shown in Figure 2.26.

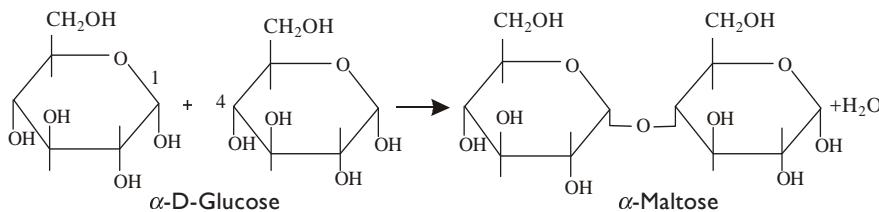


Figure 2.26 Formation of α -maltose.

In the same way, sucrose and lactose synthesis are also shown in Figure 2.27. Sucrose is a disaccharide of α -D-glucose, and β -D-fructose, and lactose is a disaccharide of β -D-glucose and β -D-galactose.

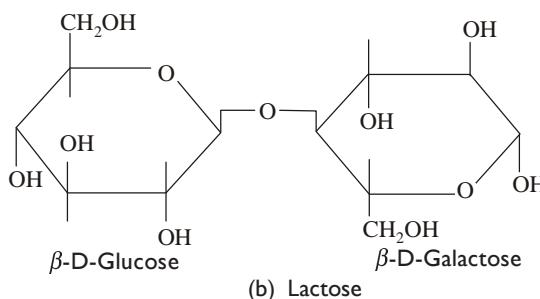
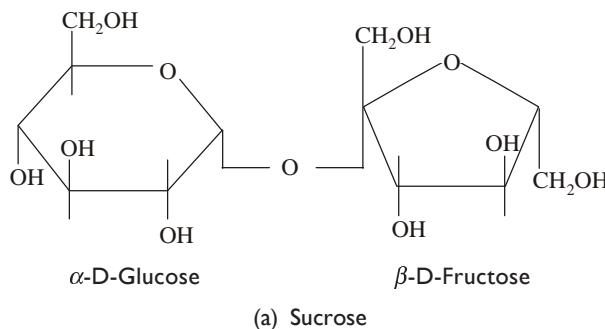


Figure 2.27 Synthesis of sucrose and lactose.

In general, monosaccharides and disaccharides are crystalline, water soluble, sweet to taste, and are therefore termed sugars.

2.8.3 Polysaccharides

Polysaccharides are normally amorphous, insoluble in water and are tasteless. These are referred to as non-sugars. Polysaccharides are composed of ten to many thousands of monosaccharides in their macromolecules, and their empirical formula is $(C_6H_{10}O_5)_n$.

Polysaccharides are formed by the condensation of more than two monosaccharides by glycosidic linkages or bonds. Thus, these are the

polymers of monosaccharides. Polymers of pentoses are called *pentosans*, polymers of hexoses are called *hexosans* and the polymers of glucose are *glucosans*. Chemically the polysaccharides are of two types—homopolysaccharides and heteropolysaccharides. Homopolysaccharides (homoglycans) are the ones that yield on hydrolysis, a single monosaccharide. Starch, inulin, pectin and chitin are homopolysaccharides. Heteropolysaccharides are the ones that produce a mixture of monosaccharides. Examples include hyaluronic acid, chondroitin sulphates, heparin, hemicellulose, some gums and mucilages.

Homopolysaccharides: Let us learn more about the following homopolysaccharides.

Starch: It occurs in grains, roots of plants, etc. Starch can be hydrolyzed into many monosaccharides molecules. The compact structure of a polysaccharide makes it ideal as a storage carbohydrate. Starch has two components, *amylose* and *amylopectin*. Amylose has a straight chain structure consisting of several thousands of glucose residues, though the chain coils helically into a more compact shape. Amylopectin is also compact as it has many branches formed by 1,6-glycosidic bonds. Starch deposits are usually about 10–30% amylose and 70–90% amylopectin. Figure 2.28 shows the glycosidic linkage in amylopectin.

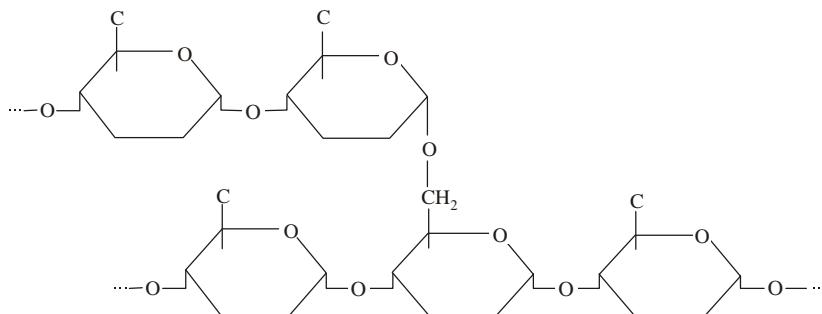


Figure 2.28 Glycosidic linkage in amylopectin.

Glycogen: It is the main polysaccharide occurring in the animal tissues, particularly in liver and muscle. It consists of a long profusely branched-chain of glucose molecules and is considered to be animal starch. Glycogens form branches similar to the branches seen in starch. The molecular weight of a typical glycogen molecule is less than 5×10^6 daltons.

Cellulose: This is a structural polysaccharide containing a large number of glucose units arranged in a linear fashion. It is unbranched chain of D-glucose with a molecular weight between 50,000 and 1 million daltons. The linkage between glucose monomers in cellulose is a β -1,4 glycosidic linkage (Figure 2.29).

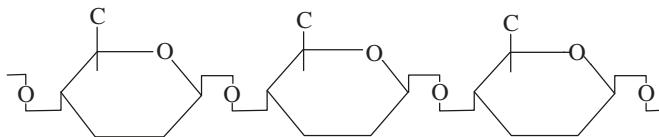


Figure 2.29 β -1, 4 glycosidic linkage.

Dextrans: These are produced by yeasts and bacteria and are made up of glucose residues linked mainly by $1 \rightarrow 6 \alpha$ glucosidic bonds. Branches are formed by occasional $1 \rightarrow 4 \alpha$ linkages and rarely through $1 \rightarrow 3 \alpha$ linkages. They have the property of absorbing water to form viscose colloidal solution. They are not metabolized by the tissues and hence are used for retaining water in circulation for a long period by administering it intravenously (plasma volume extender).

Inulin: Inulin is a low molecular weight polysaccharide present in tubers. It is a polymer of fructose. Not being utilizable, it is used in assessing the glomerular filtration rate (GFR) in the study of human kidney function.

Agar: Agar is found in sea weeds. It consists of two main components: agarose and agarpectin. Agarose is a non-sulphated linear polymer consisting of alternating residues of D-galactose and 3,6-anhydro-L-galactose. Agarpectin is a mixture of sulphated galactans, which may also contain glucoronic acid or pyruvic acid. It dissolves in hot water and sets to gel on cooling. It is extensively used for culturing the microbes.

Chitin: Chitin forms the characteristic exoskeleton of invertebrates like crab, lobster, prawns, etc. It is a polymer of acetylglucosamine.

Pectin: Pectins are present in apples, lemon and other fruits. In the middle lamella of cell wall, pectin is found as calcium pectate. Pectins consist of repeating units of galactose and galacturonic acid.

Heteropolysaccharides: Let us learn more about the following heteropolysaccharides:

Hemicellulose: It is found in association with cellulose in cell walls. The commonly found sugars in hemicellulose are D-xylose, L-arabinose, D-galactose, and D-glucoronic acid.

Gums: Gums are substances exuded by plants on mechanical injury or after bacterial, fungal or insect attack. The viscose substance helps to seal the wound and protects the plants. The polysaccharides in these are highly branched.

Hyaluronic acid: Hyaluronic acid is the simplest mucopolysaccharide and linear polymer of disaccharides which form repeating units. Each disaccharide is linked to the next by β -1, 4-glucosidic bonds. It consists of two monosaccharides D-glucoronic acid and N-acetyl-D-glucosamine. It is found in the skin, vitreous humour of the eye, the umbilical cord, as a coating around ovum and in certain bacteria.

Heparin: Heparin is an anti-coagulant secreted by most cells in the intestinal mucosa, liver, lung, spleen and kidney. It is a polymer of glucoronic acid and N-acetylglucosamine.

Chondroitin sulphates: These are predominant in cornea, cartilage, tendons, skin, heart valves and saliva. The repeating unit is a disaccharide of glucuronic acid linked to sulphite ester of N-acetylgalactosamine through a β -1,3-glycosidic bond.

2.9 MACRONUTRIENTS AND MICRONUTRIENTS

Macronutrients are needed in concentrations larger than 10^{-4} M. Carbon, nitrogen, oxygen, hydrogen, sulphur, phosphorus, Mg^{2+} and K^+ are the major macronutrients.

Micronutrients are needed in concentrations of less than 10^{-4} M. Trace elements such as Mo^{2+} , Zn^{2+} , Cu^{2+} , Ca^{2+} , Na^+ and Mn^{2+} , vitamins, growth hormones and the metabolic precursors are the micronutrients.

2.10 HYBRID BIOCHEMICALS

Hybrid biochemicals are the diverse compounds that comprise various combinations of lipid, sugar and amino acid building blocks. They provide several different significant functions. Table 2.2 lists some selected hybrid biochemicals with nomenclature, subunit composition and their locations and biological functions.

Table 2.2 Summary of hybrid biochemicals

Name	Building blocks	Location and function
Peptidoglycans	Disaccharides and peptides which are cross-linked	Found in bacterial cell walls and aid in forming the shape that is providing the morphology and protection to internal components.
Proteoglycans	Carbohydrates (95%) combined with proteins (5%)	Found in connective tissues and help in transport of materials in and out of the cells.
Glycoproteins	Carbohydrates (1–30%) with rest of proteins in major amounts.	Diversified location, well distributed in the outer surface of the cell. They are involved in defense functions of the cells. For example, antibodies.
Glycolipids	Lipids combined with sugar moieties	Found in membrane components of a cell. They serve in regulatory functions of membranes.
Lipoproteins	Lipids combined with proteins with varying contents (1–50%)	Found in membrane, blood plasma components. Helps in maintaining the liquidity of blood, i.e. liquid part of the blood.
Lipopolysaccharides	Lipids combined with a highly varying oligosaccharides content.	Found in outer portion of Gram-negative bacterial envelope. Its main function is to provide the protection to cell components.

2.10.1 Hierarchy of the Biological Structure

Figure 2.30 suggests the hierarchy of the biological structure, which means the build-up of or the proliferation taking place right from the use of environmental precursors upto the organs and the organism development.

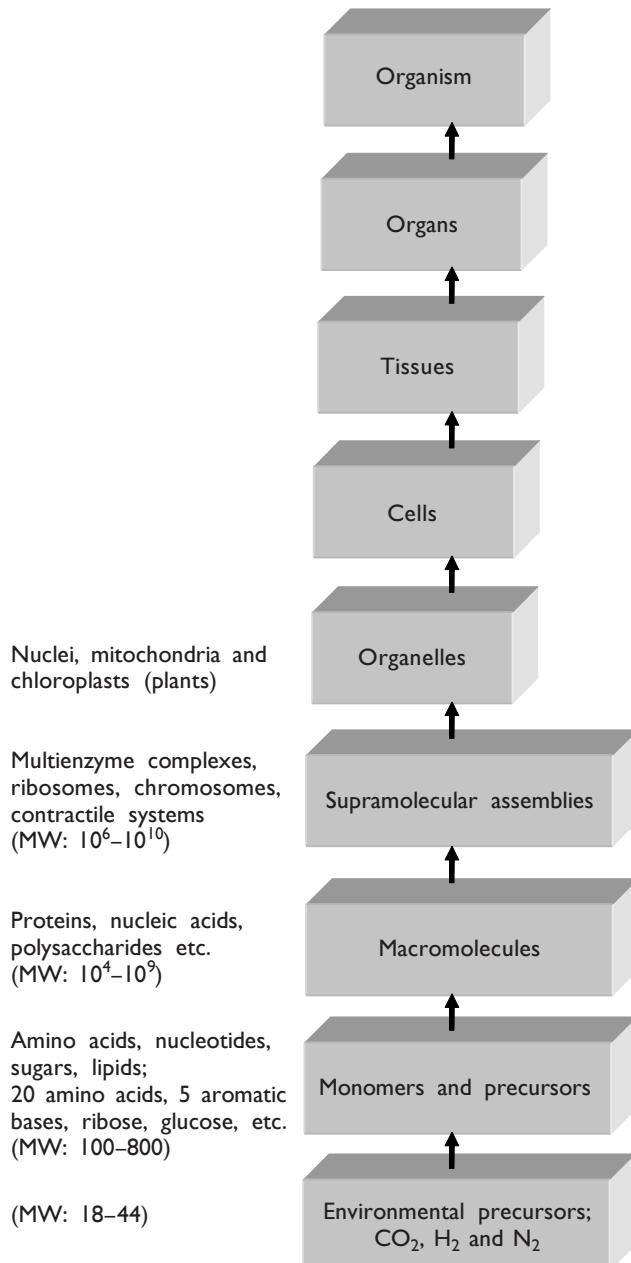


Figure 2.30 The hierarchy of the biological structure.

SUMMARY

In this chapter, we learnt the following:

- Lipids are the compounds of critical importance in the construction of cellular membranes.
- Fats serve as the reserves of energy and a number of growth factors or hormones involve lipid materials. Glycolipids and phospholipids are the primary components of the biological membranes.
- Lipids exhibit stable configurations like lipid monolayer, lipid bilayer and lipid micelle.
- Proteins perform structural and dynamic functions in the cells and organisms.
- Proteins are the polymers made up of L- α -amino acids which are 20 in number and are classified into different groups based on their structure, chemical nature, nutritional requirements and metabolism.
- Amino acids have two functional groups, namely carboxyl ($-COOH$) and the amino ($-NH_2$) and they exist as dipolar ions commonly called zwitterions.
- The structure of proteins is divided into four levels of organization, namely primary, secondary, tertiary and quaternary. The primary structure represents the linear sequence of amino acids, the secondary is the twisting and special arrangement of polypeptide chain, the tertiary is the three-dimensional structure of a functional protein, and the quaternary structure represents the assembly of similar or dissimilar subunits.
- The tertiary and the quaternary protein structures are stabilized by non-covalent hydrogen bonds, hydrophobic interactions and ionic bonds.
- DNA is the chemical basis of heredity organized into genes, the basic units of information.
- All the three RNAs are produced by DNA and carry out the synthesis of proteins.
- Nucleic acids are the polymers of nucleotides called polynucleotides, which are held by phosphodiester bridges. A nucleotide is a combination of base + sugar + phosphate.
- Nucleotides perform a wide variety of cellular functions as energy carriers, metabolic regulators, etc.
- The structure of DNA is a double helix (Watson and Crick model) composed of two anti-parallel strands of polydeoxyribonucleotides twisted around each other. The strands are held by the hydrogen bonds between the nitrogenous bases.

- DNA satisfies the Chargaff's rule that the content of A is equal to T and that of G equal to C.
- RNA is actively involved in the synthesis of proteins with a message coded by the DNA.
- Carbohydrates are classified into monosaccharides, disaccharides and polysaccharides.
- Monosaccharides are classified into trioses, tetroses, pentoses and hexoses.
- Disaccharides are formed by the condensation of two monosaccharides usually hexoses.
- Polysaccharides are referred to as non-sugars and are of types—homopolysaccharides and heteropolysaccharides.
- Hybrid biochemicals are the combinations of different biopolymers and have their own significance.

EXERCISES

- 2.1 What are repetitive and non-repetitive biological polymers? Give some examples for the same.
- 2.2 Explain saturated and unsaturated fatty acids. Discuss with neat sketches the stable configurations of fatty acids in water.
- 2.3 Enlist the functions of lipids and fats.
- 2.4 What is the general steroid base? Give its structure.
- 2.5 Write a note on fat soluble vitamins and steroids.
- 2.6 Describe the primary, secondary, tertiary and quaternary structures of proteins.
- 2.7 Describe the Watson and Crick model of DNA.
- 2.8 Write on the following:
 - (a) DNA replication
 - (b) Biological Information Storage
 - (c) Types of RNA and their functions
 - (d) Comparison between DNA and RNA.
- 2.9 Define carbohydrates. Explain the formation of these carbohydrates in nature.
- 2.10 What are simple sugars? With examples, explain the synthesis of the various types of monosaccharides.
- 2.11 What are the monosaccharides that are commonly found in the biological systems?

- 2.12** Describe the structures of various disaccharides and polysaccharides. Further explain the role of various polysaccharides.
- 2.13** Write on the following:
- (a) Properties of proteins
 - (b) Functions of proteins
 - (c) Hybrid biochemicals
 - (d) Macro and micronutrients.

Chapter 3

Enzymes and Enzyme Kinetics

A cell must be capable of performing a multitude of chemical changes in order to stay alive, grow and reproduce. It may have to alter complex nutrients in the medium before they can enter the cell. These nutrients are broken down chemically to provide energy for the cell and precursors for the synthesis of cell material. The chemical changes involved are exceedingly complex considering the diverse nature of materials used as food on one hand, and the varied type of substances synthesized into the cell constituent on the other. Enzymes present in the cell are minute and capable of speeding up the chemical reactions associated with the life processes. Any impairment of enzymes and their activity are reflected by some changes in the cell, or even by death. Hence, there can be no life without enzymes.

Enzymes function in sequences of reaction called pathways. For a cell to grow, normally it is essential that the flow of chemical substances or the metabolites through these pathways be under a high degree of regulation or control. This regulation ensures that no products are deficient or in excess exerted either on enzyme activity or on the enzyme synthesis.

This chapter gives an insight into the significance of enzymes, their properties, types, and the kinetics of reactions based on the different models and theories. Also given are the influence of various parameters on the activity of enzymes, their modes of regulation, modulation and applications at the different levels.

Enzymes are biological catalysts synthesized by the living cells. They are protein in nature, colloidal and specific in their action. Enzymes are thermo labile in character, for example the hydrolysis of proteins by a strong acid at 100°C may require a couple of days, but by using enzymes, the proteins are fully digested in the gastrointestinal tract at body temperature in just a few hours.

Also in a bacterial cell, hundreds of chemical reactions occur which lead to cell division in a few minutes, whereas the reactions taking place outside the cell will take a couple of days. The very existence of life is unimaginable without the presence of enzymes.

3.1 NOMENCLATURE AND CLASSIFICATION

Earlier enzymes were named in an arbitrary manner. For example, pepsin, trypsin, and chymotrypsin conveying no information about the function of the enzyme or the nature of the substrate on which they act. Later *-ase* was added to the substrate for naming the enzymes, for example *lipase*, *nuclease*, *lactase*, *protease*, etc. These are called the trivial names, which lack the complete information.

In most cases, the enzyme nomenclature derives from what the enzyme does rather than what the enzyme is.

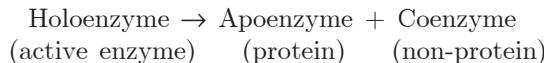
In the year 1961, the Enzyme Commission, IUB (International Union of Biochemists) proposed six major classes. Each class on its own represents the general type of reaction.

1. *Oxidoreductases*: They function in the oxidation-reduction reactions.
2. *Transferases*: They catalyze the transfer of the functional groups.
3. *Hydrolases*: They bring about the hydrolysis of compounds.
4. *Lyases*: They are specialized in the addition or removal of H_2O , NH_3 , CO_2 , etc.
5. *Isomerases*: They are used in the isomerization reactions.
6. *Ligases*: They catalyze the synthetic reactions where two molecules are joined together.

The other way of classifying is: *intracellular enzymes* and *extracellular enzymes*. The intracellular enzymes are functional within the cells where they are synthesized, whereas the extracellular enzymes are active outside the cells, for example, digestive enzymes like trypsin, pepsin, etc.

3.1.1 Chemical Nature and Properties of Enzymes

All the enzymes are invariably proteins. Each enzyme has its own tertiary structure and specific confirmation which is essential for its catalytic activity. The functional unit of enzyme is called *holoenzyme*, which is often made up of *apoenzyme* which is a protein part and a *coenzyme* which is a non-protein part.



The non-protein part is usually called *prosthetic group*, whenever it binds covalently with apoenzyme. Coenzyme is always separable by dialysis while apoenzyme is non-separable.

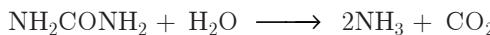
Monomeric enzymes: These are made up of a single polypeptide chain, e.g. ribonuclease trypsin.

Oligomeric enzymes: Oligomeric enzymes possess more than one polypeptide chain, e.g. lactate dehydrogenase.

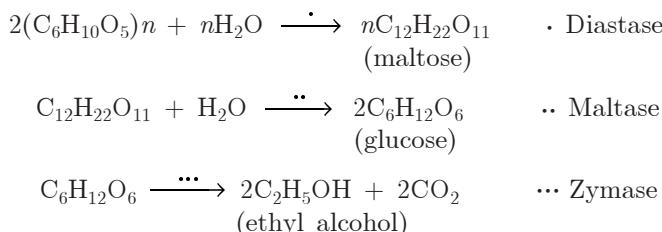
Multienzyme complexes: These complexes have the active sites to catalyze different reactions in a sequence, e.g. pyruvate dehydrogenase, fatty acid synthetase, etc.

3.1.2 Enzymes and Their Actions

1. *Urease*: It hydrolyzes urea into ammonia and carbon dioxide.



2. *Alcohol dehydrogenase*: It catalyzes the removal of hydrogen from alcohols.
3. *Lipase*: It acts on lipids and fats.
4. *Protease*: This enzyme hydrolyzes proteins and peptides.
5. *Carbohydrases*: They act on the carbohydrates. For example, diastase, maltase and zymase are the enzymes that act on starch, maltose and glucose, respectively, finally producing ethyl alcohol.



3.2 ENZYME KINETICS

1. *Ribozymes*: Ribozymes are the RNA molecules that have the catalytic properties.
2. *Isozymes*: Enzymes that occur in several different molecular forms, but catalyze the same reaction are called *isozymes*.
3. *Proximity effect*: In the multi-substrate–enzyme catalyzed reactions, E (enzyme) can bind S (substrate) in such a way that the reaction regions of S are close to each other and to the active site of E. It is called *proximity effect*. This eliminates randomness of collision in free solution.
4. *Orientation effect*: E can hold the S at certain positions and angles to improve the reaction rate. It is called *orientation effect*. This eliminates randomness of substrate orientation.

Note: For proper functioning, certain enzymes require coenzymes and cofactors.

3.2.1 Enzyme Catalysis

A catalyst is a substance which increases the rate of a chemical reaction

without itself undergoing a permanent chemical change. It only influences the rate of a chemical reaction; it does not affect the reaction equilibrium.

Comparison between uncatalyzed and the catalyzed reactions: Let us take an example of conversion of glucose 1-phosphate to glucose 6-phosphate. In the case of uncatalyzed reaction, as shown in Figure 3.1(a), conversion is achievable but requires a high energy of activation, E_a . The magnitude of this energy of activation is shown by the profile of the curve, which shows that for the conversion to take place, it has to surmount the mountain. In the case of catalyzed reaction, the energy of activation E_a is low. The magnitude of this is shown in Figure 3.1(b) as a tunnel.

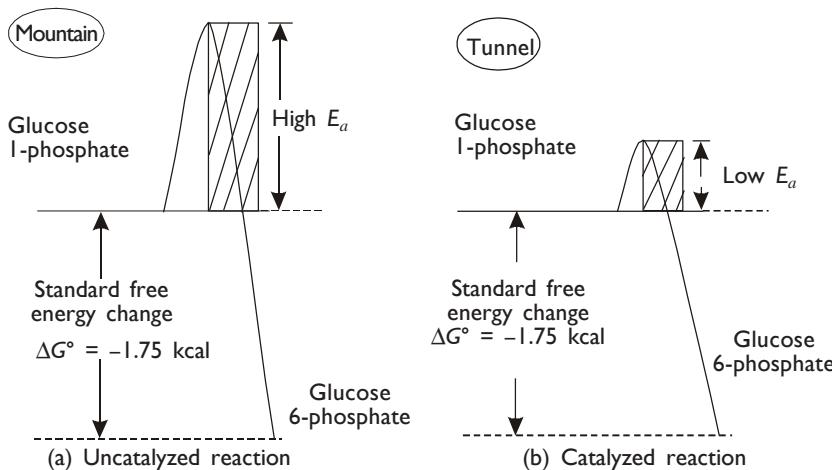


Figure 3.1 Comparison between uncatalyzed and catalyzed reactions.

Overall it is seen that catalytic reactions are much faster than the non-catalytic reactions. The standard free energy change remains unchanged in both the cases.

These mechanisms are well explained by the Arrhenius equation, i.e.

$$K = A e^{-E_a/RT} \quad (3.1)$$

where A = Arrhenius constant

E_a = energy of activation

R = gas constant

T = temperature

3.2.2 Order of Reaction

Consider a non-enzymatic reaction,



where S = substrate and P = product.

The rate or velocity of a reaction (v), varies directly with concentration of the reactants, whether substrate or catalyst. The rate is given as follows:

$$\frac{\Delta[P]}{\Delta t} = v = k[S] \quad (3.2)$$

where k is the rate constant that indicates the speed or the efficiency of a reaction. If we look at the graph of v vs $[S]$, it is linear (Figure 3.2).

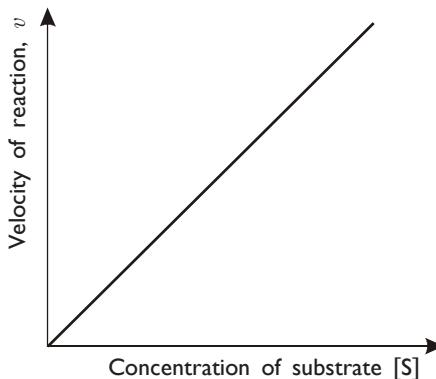
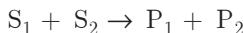


Figure 3.2 Velocity of the reaction versus the concentration of the substrate.

The overall kinetic order of a reaction is the sum of the exponents in the equation, i.e. as to how many molecules are reacting in the rate-determining step. Therefore, $S \rightarrow P$ is a first order reaction.

Now, consider



It is a single step reaction. In this case, the rate is found by knowing the concentrations of both the reactants, i.e. the substrates. If S_1 and S_2 are present in similar concentrations, the rate equation is

$$v = k[S_1]^1[S_2]^1$$

With respect to each reactant, the reaction is the first order and overall it is a second order or a bimolecular reaction.

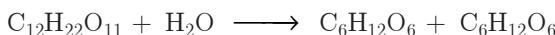
If $[S_2] \gg [S_1]$, so that it remains essentially constant, the reaction is a zero order reaction with respect to that reactant, i.e. $[S_2]$. So $[S_2]$ is unchanging and can be eliminated. Therefore,

$$v = k[S_1]^1[S_2]^0$$

or

$$v = k'[S_1]$$

For example, in the non-enzymatic hydrolysis of sucrose in the aqueous acid solutions,



Here $[H_2O] \gg [S_1]$ and $[H_2O]$ is constant. So the rate depends only on the concentration of sucrose, i.e. $[S_1]$.

Now let us consider purely enzymatic reactions and the various mechanisms of interaction of substrates and formation of products.

Generally, an enzymatic reaction is given as follows:



where

E = enzyme

S = substrate

ES = enzyme–substrate complex

P = product

The prime requisite for the enzyme catalysis is that S must combine with E at the active site to form ES , the enzyme–substrate complex. Once combined, after sometime form the products with the liberation of E .

More specifically, it is represented as



which shows that the formation of ES complex is reversible and the formation of the products is irreversible. The idea of ES complex formation was first conceived by Michaelis–Menten in the year 1913 and has been widely accepted. A few theories have been proposed to explain the formation of ES complex.

Emil Fischer proposed that E is a rigid template and S is a matching key. The rate of enzymatic reactions depends on the concentration of both the substrate and enzyme. When the amount of S is high enough or when the amount of E is much less than the S , then the reaction is the pseudo first order, i.e.

$$v = k[E]^1[S]^0$$

or

$$v = k[E]^1$$

or

$$v = k'[E]$$

It shows that the more is enzyme present, the faster is the reaction. Figure 3.3 shows that with increase in the concentration of enzymes the rate increases.

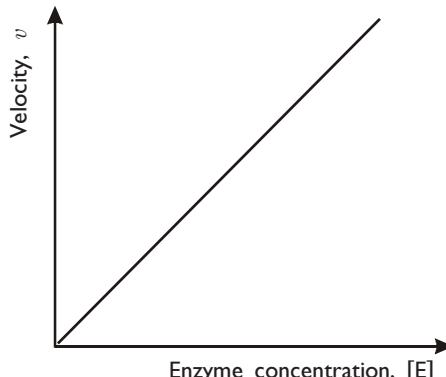


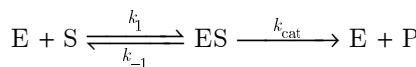
Figure 3.3 Velocity of the reaction versus the enzyme concentration.

Note: $[S]$ is high and fixed, i.e. at saturation throughout the reaction.

Pseudo first order reactions are used in the analysis called *enzyme assays* that determine the concentration of enzymes.

3.2.3 Progress of a Reaction

At the beginning of a reaction $E + S \rightarrow ES \rightarrow E + P$, the amount of product P formed is very low and so it is negligible. More specifically now the reaction is represented as



where the rate constants

k_1 = rate of association of E with S

k_{-1} = rate of dissociation of S from E

k_{cat} = catalytic constant

k_{cat} is also called Turn Over Number (TON). The TON is defined as the number of substrate molecules that can be converted by a molecule of an enzyme in a minute. Also defined as the number of catalytic events per second per enzyme molecule. The TON varies from 100 to 3×10^6 .

Note: The conversion of ES complex to $E + P$ is shown by a single one way arrow. During the initial stages when the measurements are made, little product is formed so the rate of reverse reaction is negligible. The velocity measured during this period, which is in fact very short, is called initial velocity, v_0 . The formation of ES complex and the dissociation of ES complex are in fact rapid whereas the conversion of S to product is usually slow. Hence, it is the rate limiting step. In this step, the substrate is chemically altered.

Figure 3.4 shows the curves for $[P]$ vs time (t) for different enzyme

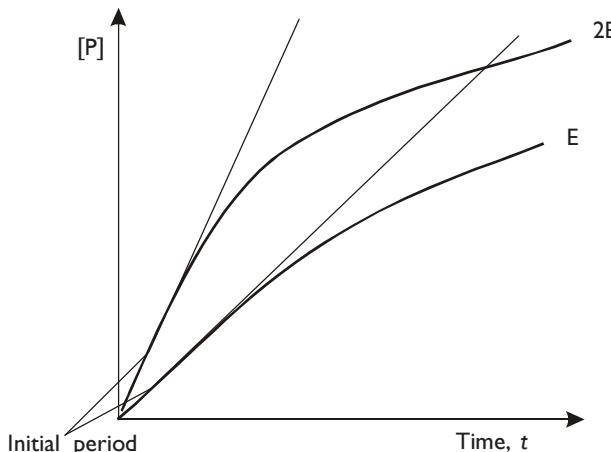


Figure 3.4 Product concentration versus the time factor.

concentrations. The lines drawn to the curve indicate the initial period, which is at the lower portion of the curve and given as

$$v_0 = \Delta[P]/\Delta t \text{ (slope of the curve)} \quad (3.3)$$

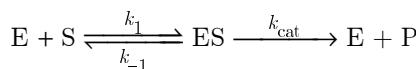
As the reaction proceeds, $[P]$ increases. The rate of reaction doubles when twice as much enzyme ($2E$) is added to another identical reaction mixture.

3.3 MECHANISM AND KINETICS OF ENZYMATIC REACTIONS

In 1913, L. Michaelis and M. Menten proposed the mechanism for the kinetics of the enzyme catalyzed reactions, which envisages the following steps:

Step 1. Formation of ES complex, which is a fast step.

Step 2. Decomposition of the complex.



The overall reaction is



Here the enzyme is consumed in step 1 and regenerated in step 2.

Before we take up the actual kinetics, let us try to envisage the various models that explain the ES complex formation. Various theories have been proposed to explain this ES complex formation such as:

- Lock and Key model or Emil Fischer's template theory
- Induced fit theory or Koshland's model
- Substrate strain theory

Each one of these theories has their own significance in explaining the mechanism of the ES complex formation and at the same time they all have certain limitations as discussed in section 3.3.1.

Let us make use of a common configuration that can illustrate the mechanism of ES complex formation (Figure 3.5).

3.3.1 Lock and Key Model or Fischer's Template Theory

The lock and key model was the very first model proposed to explain the mechanism of ES complex formation. It says that the structure of E is rigid, and the substrate fits to the binding site (now active site) just as a key fits into a proper lock or a hand into a proper glove. The active site of E is rigid and pre-shaped template where only a specific substrate can bind.

It does not give any scope for the flexible nature of the enzyme; hence the model fails to explain many facts of enzymatic reactions.

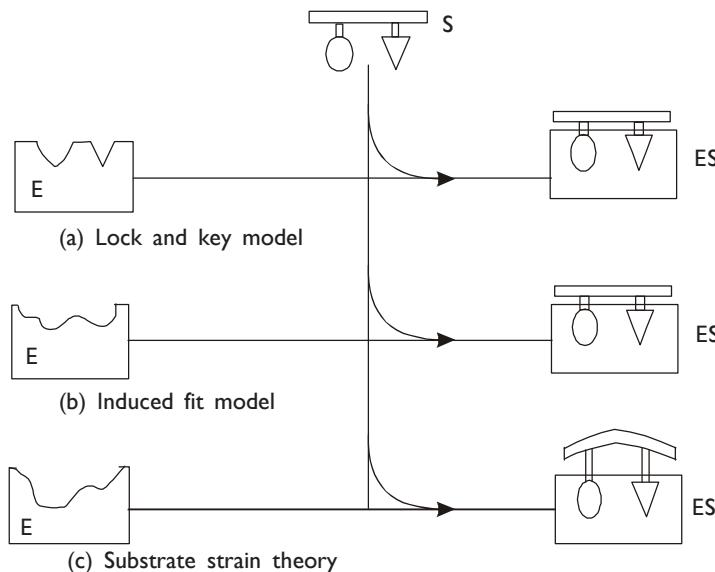


Figure 3.5 Common configuration for ES complex formation for depicting the three theories.

3.3.2 Induced Fit Theory or Koshland's Model

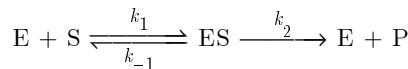
The induced fit was proposed in the year 1958. It is considered as the more realistic and acceptable model. As per this theory, the active site is not rigid and pre-shaped. The essential feature of the substrate binding site is present at the nascent active site. The interaction of S with E induces a fit or a conformational change in E which results in the formation of a strong binding site. Due to the induced fit, appropriate amino acids are repositioned to form the active site and bring about catalysis.

3.3.3 Substrate Strain Theory

A substrate is strained due to the induced confirmation change in an enzyme E. When S binds to the pre-formed active site, the E induces a strain to the substrate. The strained S leads to the formation of the product. In fact, a combination of Induced Fit and Substrate Strain is considered to be operative in the enzyme action.

The problem of the enzyme-catalyzed reactions can be treated by using any one of the following approaches: *Steady-State Approximation* (SSA) also called *Briggs Haldane Approach* and *Rapid Equilibrium Approach* (REA).

Steady-state approximation (SSA): The experiments reveal that a true equilibrium is never achieved in the fast step, i.e. $E + S \rightleftharpoons ES$ (fast) as the subsequent step $ES \longrightarrow E + P$ (slow) is constantly removing the intermediate ES complex. Generally, the concentration of E is far less than the concentration of S, i.e. $[S] \gg [E]$ so that $[S] \gg [ES]$. Hence, we can use the SSA for the intermediate complex ES. For



where $k_2 = k_{\text{cat}}$. According to the slow rate determining step, the rate of reaction is given as

$$r = \frac{-d[S]}{dt} = \frac{+d[P]}{dt} = k_2[ES] \quad (3.4)$$

Applying the steady-state approximation for the ES, we have

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] = 0 \quad (3.5)$$

Since we cannot measure the free enzyme concentration experimentally; the equilibrium between the free enzyme concentration and the bound enzyme concentration is given as,

$$[E]_0 = [E] + [ES] \quad (3.6)$$

where $[E]_0$ is the total enzyme concentration which is measurable, $[E]$ is free enzyme concentration and $[ES]$ is the bound enzyme concentration.

From Eq. (3.6), we get

$$[E] = [E]_0 - [ES] \quad (3.7)$$

Substituting Eq. (3.7) in Eq. (3.5) and further simplifying,

$$[ES] = \frac{k_1[E]_0[S]}{k_{-1} + k_2 + k_1[S]} \quad (3.8)$$

Now substituting Eq. (3.8) in Eq. (3.4), we get

$$r = \frac{k_1 k_2 [E]_0 [S]}{k_{-1} + k_2 + k_1 [S]} \quad (3.9)$$

Dividing the numerator and the denominator of Eq. (3.9) by k_1 and writing K_m as $(k_{-1} + k_2)/k_1$, we get

$$r = \frac{k_2 [E]_0 [S]}{K_m + [S]} \quad (3.10)$$

Equation (3.10) is called Michaelis–Menten equation, and K_m is called the Michaelis–Menten constant. Note that K_m is not an equilibrium constant.

Further simplification says that when all the enzymes have reacted with the substrate at high concentrations, the reaction will be going at the maximum rate. So free enzyme concentration is not present. i.e. $[E] = 0$.

Hence, $[E]_0 = [ES]$ from $[E]_0 = [E] + [ES]$. So the Michaelis–Menten expression reduces to

$$r = \frac{k_2 [ES] [S]}{K_m + [S]} \quad (3.11)$$

As the $[S]$ becomes very large, the $+\frac{d[P]}{dt}$ is commonly referred to as the maximal velocity of reaction (v_{\max}). Hence, Eq. (3.4) can be written as

$$\frac{+d[P]}{dt} = v_{\max} = k_2[ES]$$

Therefore, Eq. (3.11) can be written as

$$r = \frac{v_{\max}[S]}{K_m + [S]} \quad (3.12)$$

which is also known as the Michaelis–Menten equation.

Three cases arise in using the above equation. We will discuss one by one.

Case 1. When $K_m \gg [S]$, $[S]$ can be ignored. So Eq. (3.12) becomes

$$r = \frac{v_{\max}[S]}{K_m}$$

or

$$r = k'[S] \quad (\text{first order reaction})$$

Case 2. When $[S] \gg K_m$, K_m can be ignored. Hence, Eq. (3.12) becomes

$$r = v_{\max} \quad (\text{zero order reaction})$$

which is a constant.

Case 3. When $[S] = K_m$, Eq. (3.12) becomes

$$r = \frac{v_{\max}}{2}$$

This means the rate of product formation is half the maximum velocity.

All these can be represented on a graph of r vs $[S]$ as shown in Figure 3.6.

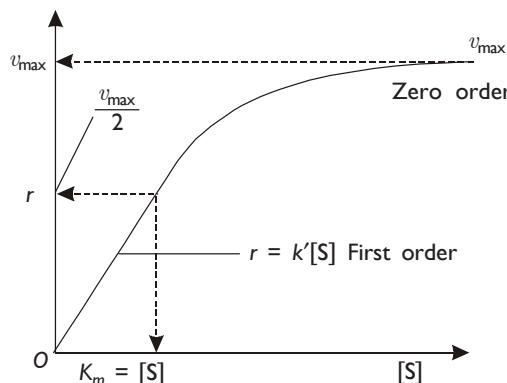
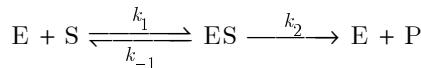


Figure 3.6 Reaction rate vs the substrate concentration.

Rapid equilibrium approach (REA): Once again for the reaction



the overall reaction is $S \rightarrow P$.

The rate of reaction is

$$r = \frac{-d[S]}{dt} = \frac{+d[P]}{dt} = k_2 [ES] \quad [\text{from Eq. (3.4)}]$$

Calling for a rapid equilibrium in the fast step between E and S to form the ES complex, we can use the equilibrium coefficient to express $[ES]$ in terms of $[S]$.

The equilibrium constant

$$K'_m = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]} \quad (3.13)$$

Now, substituting Eq. (3.7) into Eq. (3.13) and rearranging, we get

$$[ES] = \frac{[E]_0 [S]}{K'_m + [S]} \quad (3.14)$$

where $K'_m = \frac{k_{-1}}{k_1}$ is called the *dissociation constant*.

Now, substituting Eq. (3.13) into Eq. (3.4), we get

$$r = \frac{k_2 [E]_0 [S]}{K'_m + [S]}$$

or
$$r = \frac{v_{\max} [S]}{K'_m + [S]} \quad (3.15)$$

which is also called the *Michaelis–Menten equation*.

Note: K'_m is also Michaelis–Menten constant. As it is derived assuming the rapid equilibrium approach, a prime is used over K_m .

We have seen that the rate of reaction changes from the first order to the zero order whenever the first two cases are considered. To explain this we shall make use of the physical interpretation. Each molecule of an enzyme has one or several active centres or the sites onto which the substrate molecule must bind for a reaction to take place.

1. At low substrate concentrations, most of the enzyme active centres remain unoccupied at any point of time. So the rate of reaction is proportional to the substrate concentration, which is the first order.
2. As the substrate concentration increases, the active centres are fully occupied and hence the rate of reaction also increases, which is the zero order.

However, at very high concentrations of the substrate, virtually all the sites are occupied at any point of time, so that at these concentrations, will not increase further the formation of ES complex. At this point we say that the concentration of substrate is saturated.

3.3.4 Integral Michaelis-Menten Equation

For the reaction, $S \rightarrow P$; we can write

$$r = \frac{-d[S]}{dt} = \frac{+d[P]}{dt} = \frac{v_{\max}[S]}{K_m + [S]}$$

We can also write

$$\frac{-d[S]}{dt} = \frac{v_{\max}[S]}{K_m + [S]} \quad (3.16)$$

$$\{K_m + [S]\} d[S] = -v_{\max}[S] dt$$

Dividing this equation by $[S]$ throughout so that we get in the form

$$\frac{K_m d[S]}{[S]} + d[S] = -v_{\max} dt \quad (3.17)$$

Integrating the above equation between the limits for $S = S_0$ at $t = 0$ and $S = S$ at $t = t$; i.e.

$$K_m \int \frac{d[S]}{[S]} + \int d[S] = -v_{\max} \int dt$$

we get

$$K_m \ln[S_0/S] + (S_0 - S) = v_{\max} t \quad (3.18)$$

Dividing Eq. (3.18) by $\ln[S_0/S]$ and by rearrangement, we get

$$\frac{(S_0 - S)}{\ln(S_0/S)} = \frac{v_{\max} t}{\ln(S_0/S)} - K_m \quad (3.19)$$

This is called the integral Michaelis-Menten equation.

Further, plotting the graph of $(S_0 - S)/\ln(S_0/S)$ vs $t/\ln(S_0/S)$; we can evaluate the required parameters. It is valid when $S_0 \neq S$ and $S \neq 0$.

Example 3.1 For an enzyme-substrate complex obeying Michaelis-Menten mechanism, the rate of the product formation when the substrate concentration is very large has a limiting value of 0.02 mol-dm^{-3} . At a substrate concentration of 250 mg-dm^{-3} , the rate is half this value. Calculate k_1/k_{-1} assuming k_2 is less than k_1 .

Solution

We know that when $[S] \gg K_m$, Eq. (3.12) becomes

$$\begin{aligned} r &= v_{\max} \\ &= k_2[E]_0 \quad (\because [ES] = [E]_0 \text{ when } [E] = 0) \end{aligned}$$

When $K_m = [S]$,

$$r = \frac{v_{\max}}{2}$$

We know that

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

Therefore,

$$\frac{k_{-1} + k_2}{k_1} = [S]$$

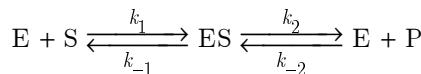
As $k_2 \ll k_1$,

$$\frac{k_{-1}}{k_1} = [S]$$

Now substituting values,

$$\frac{k_1}{k_{-1}} = \frac{1}{250} = 0.004 \text{ dm}^3/\text{mg}$$

Example 3.2 For the enzyme catalysis, the following mechanism is shown with usual notations. Using the steady-state approximation for the complex ES, derive the rate equation for the formation of the product during the initial stages.

**Solution**

Using the SSA, write the equation for $d[ES]/dt$ and equate it to 0. Further, use the enzyme conservation equation and eliminate for concentration of E. Substitute for [E] in $d[ES]/dt = 0$. Further, simplify to get the value of [ES].

The rate of product formation in the initial stages can be written as

$$r = \frac{-d[S]}{dt} = \frac{+d[P]}{dt} = k_2[ES] - k_{-2}[E][P]$$

Put $[P] = 0$ (initial stages). Therefore,

$$r = k_2[ES]$$

On simplification, finally we get

$$r = \frac{v_{\max} [S]}{K_m + [S]}$$

which is the Michaelis–Menten equation.

Example 3.3 The following data have been obtained on the enzyme catalyzed reaction at various substrate concentrations:

[S], mM	0.4	0.6	1.0	1.5	2.0	3.0	4.0	5.0	10.0
Rate, r (arbitrary units)	2.41	3.33	4.78	6.17	7.41	8.70	9.52	11.50	12.50

Plot r vs $[S]$ values and calculate the value of Michaelis–Menten constant.

Solution

As the rate of reaction also depends on the concentration of substrates, we can draw a graph of rate vs substrate concentrations to obtain the profile shown in Figure 3.7.

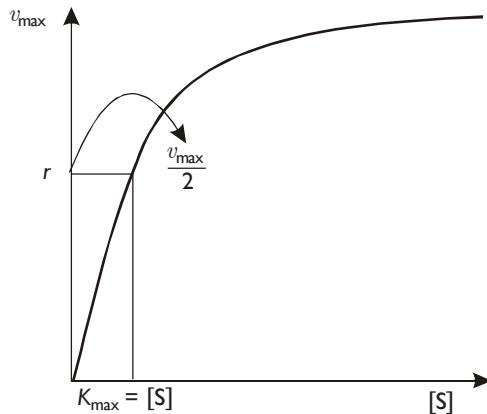


Figure 3.7 Example 3.3.

Using Eq. (3.15), from the graph find v_{\max} . Then taking $[S] = 10.0$ at which $r = 12.50$, we get $K_m = 2.0$ mM.

Example 3.4 Calculate the ratio of the substrate concentration required for 75% of v_{\max} to the concentration required for 25% of v_{\max} .

Solution

According to the Michaelis–Menten expression given in Eq. (3.15), we get

$$\frac{r}{v_{\max}} = \frac{0.75}{1.0} = \frac{[S]_{75}}{K_m + [S]_{75}}$$

or

$$[S]_{75} = 3 K_m$$

Similarly,

$$\frac{r}{v_{\max}} = \frac{0.25}{1.0} = \frac{[S]_{25}}{K_m + [S]_{25}}$$

or

$$[S]_{25} = 0.333 K_m$$

The ratio

$$\frac{[S]_{75}}{[S]_{25}} = \frac{3.0}{0.333} = 9.00$$

3.4 EVALUATION OF KINETIC PARAMETERS

The evaluation of kinetic parameters basically involves the application of the transformation of the Michaelis–Menten Equation (MME). The transformations involve the rearrangement of the MME by using the best known three methods.

- The Lineweaver–Burk method
- The Eadie–Hofstee method and
- The Hanes–Woolf method

All these methods help us evaluate the kinetic parameters.

3.4.1 The Lineweaver–Burk Method

It is also called the *double reciprocal method* and is comparatively simple. Originally, we have the MME as follows:

$$r = \frac{v_{\max}[S]}{K_m + [S]}$$

Writing the reciprocal for it as

$$\frac{1}{r} = \frac{K_m + [S]}{v_{\max}[S]}$$

$$\frac{1}{r} = \frac{K_m}{v_{\max}[S]} + \frac{1}{v_{\max}} \quad (3.20)$$

Now plot a graph of $1/r$ vs $1/[S]$ as shown in Figure 3.8.

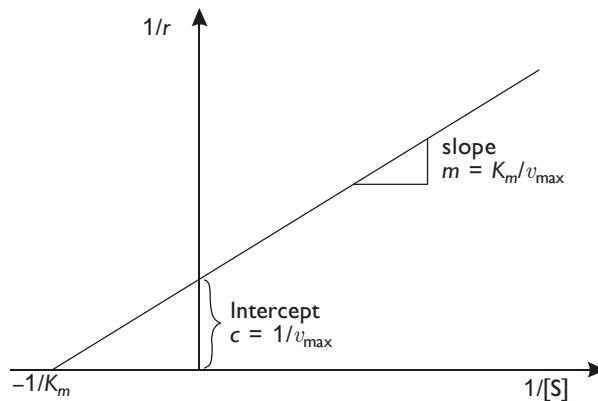


Figure 3.8 Lineweaver–Burk plot.

A double reciprocal plot gives a good estimate on v_{\max} , but not necessarily on K_m . Data points at low substrate concentrations influence the slope and intercept more than those at high substrate concentrations.

3.4.2 The Eadie–Hofstee Method

Multiplying the reciprocal of the MME [Eq. (3.20)] by v_{\max} and rearranging, we get

$$\frac{v_{\max}}{r} = \frac{K_m}{[S]} + 1$$

or

$$v_{\max} = \frac{rK_m}{[S]} + r$$

or

$$r = \frac{-rK_m}{[S]} + v_{\max} \quad (3.21)$$

Plot a graph of r vs $r/[S]$ to get the slope and the intercept as shown in Figure 3.9.

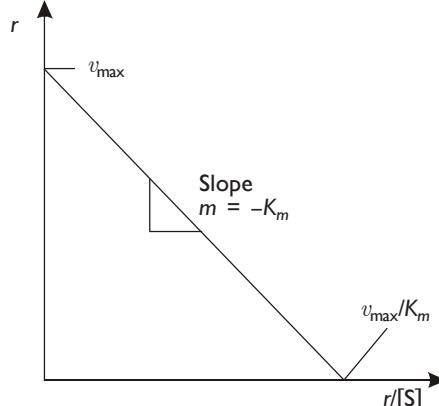


Figure 3.9 Eadie–Hofstee plot.

Eadie–Hofstee plots can be subjected to large errors since both coordinates contain r , but there is less bias on the points at low $[S]$.

3.4.3 The Hanes–Woolf Method

Once again consider the MME, i.e.

$$r = \frac{v_{\max}[S]}{K_m + [S]}$$

Take the double reciprocal and multiply throughout by $[S]$. We get

$$\frac{[S]}{r} = \frac{K_m}{v_{\max}} + \frac{[S]}{v_{\max}} \quad (3.22)$$

Plot a graph of $[S]/r$ vs $[S]$, and get the profile as shown in Figure 3.10. This plot is used to determine v_{\max} more accurately.

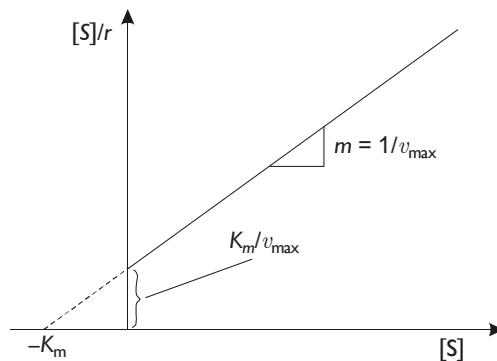


Figure 3.10 Hanes–Woolf plot.

Example 3.5 The following data have been obtained for two different initial enzyme concentrations for an enzyme-catalyzed reaction.

$[E]_0 = 0.015 \text{ g/L}$								
$r = v \text{ (g/L-min)}$	1.14	0.87	0.70	0.59	0.50	0.44	0.39	0.35
$[S] \text{ (g/L)}$	20.0	10.0	6.7	5.0	4.0	3.3	2.9	2.5
$[E]_0 = 0.00875 \text{ g/L}$								
$r = v \text{ (g/L-min)}$	0.67	0.51	0.41	0.34	0.29	–	–	–

Find the following by using the Hanes–Woolf method:

- K_m
- v_{\max} for $[E]_0 = 0.015 \text{ g/L}$
- v_{\max} for $[E]_0 = 0.00875 \text{ g/L}$
- k_2

Solution

Using Eq. 3.22, find $[S]/r$ for both initial concentrations of enzymes. As $[S]$ is g/L and r is g/L-min, $[S]/r$ is in minutes.

Tabulate the $[S]/r$ values for both initial concentrations of enzymes.

For $[E]_0 = 0.015$ g/L, plot $[S]/r$ vs $[S]$ values (Figure 3.11).

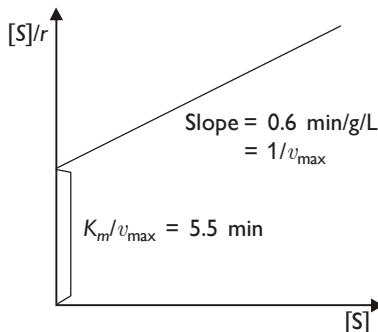


Figure 3.11 Example 3.5 (part 1).

From the above graph, we get

$$1/v_{\max} = 0.6$$

Therefore,

$$v_{\max} = 1.66 \text{ g/min-L.}$$

The intercept

$$K_m/v_{\max} = 5.5 \text{ minutes}$$

Therefore,

$$K_m = 5.5 \times 1.66 = 9.16 \text{ g}[S]/\text{L}$$

$$v_{\max} = k_2 [E]_0$$

or

$$k_2 = \frac{v_{\max}}{[E]_0} = \frac{1.66}{0.015} = 110.66 \text{ g/g (enzyme) min}$$

Similarly, for $[E]_0 = 0.00875$ g/L, plot $[S]/r$ vs $[S]$ values (Figure 3.12).

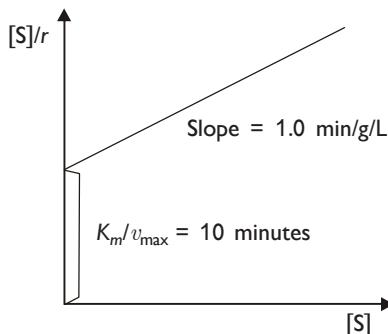


Figure 3.12 Example 3.5 (part 2).

From the above graph, we get

$$v_{\max} = 1 \text{ g/L-min}$$

and

$$K_m/v_{\max} = 10 \text{ minutes}$$

Therefore,

$$\begin{aligned} k_2 &= v_{\max}/[E]_0 \\ &= 1.0/0.00875 \\ &= 114.28 \text{ g/g (enzyme) min} \end{aligned}$$

Example 3.6 The following data on the substrate removal rate and the substrate concentration is available for some waste treatment.

[S] (mol)	0.002	0.005	0.02	0.04	0.06	0.08	0.10
Rate, r (mol/min)	0.045	0.115	0.285	0.38	0.46	0.475	0.505

Estimate the value of substrate removal rate constant K_m and the value of v_{\max} .

Solution

From the tabulated values, find $1/r$ and $1/[S]$ so that these can be used in terms of the double reciprocal method or Lineweaver–Burk method.

The reciprocals are tabulated as under:

$1/r$	22.22	8.695	3.509	2.63	2.17	2.105	1.98
$1/[S]$	500	200	50	25	16.67	12.5	10.0

From the above values plot $1/r$ vs $1/[S]$ and from which determine the slope and the intercept.

The slope from the graph is

$$K_m/v_{\max} = 0.0366$$

and the intercept on the y -axis

$$1/v_{\max} = 1.3 \text{ min/mol}$$

Therefore,

$$\begin{aligned} K_m &= 0.0366 \times 1.3 \\ &= 0.0476 \text{ min}^{-1} \end{aligned}$$

3.5 ENZYME ACTIVITY AND FACTORS AFFECTING ENZYME ACTIVITY

In this particular section, how the enzyme activity is influenced by various factors is explained.

3.5.1 pH Effects

Certain enzymes have ionic groups on their active sites and these ionic groups must be in a suitable form (acid or base form) for the enzymes to function. Variations in the pH values of the medium results in changes in the ionic form of the active site, and changes in the activity of an enzyme and hence the reaction rates. The changes in the pH values may also alter the three dimensional shape of an enzyme. For these reasons, enzymes are only active over a certain pH range. The pH of the medium may affect the maximum reaction rate v_{\max} , K_m and the stability of an enzyme. In some cases, the substrates may contain ionic groups, and the pH of the medium affects the affinity of the substrate to the enzyme. Figure 3.13 shows a graph of per cent maximal activity vs pH.

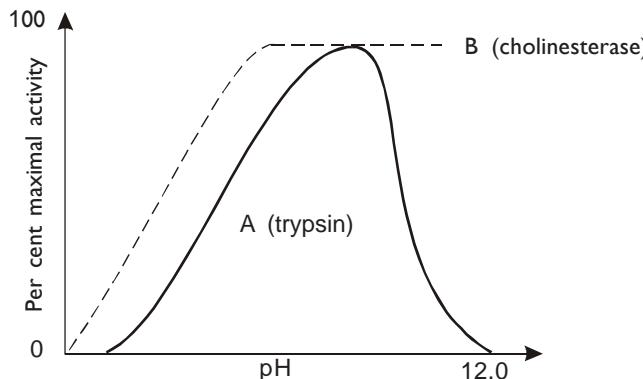


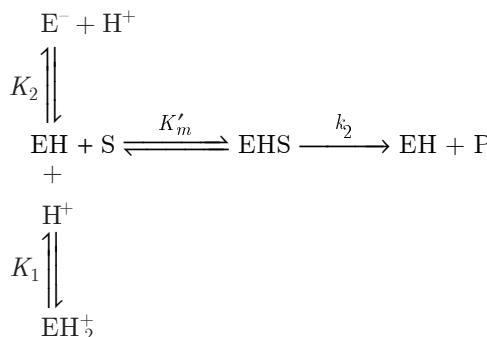
Figure 3.13 Per cent maximal activity vs pH.

The profiles shown in the figure are in particular for two different enzymes:

A: Approximate activity for trypsin

B: Approximate activity for cholinesterase

Consider the following scheme:



From the mechanism shown above,

$$K'_m = \frac{[EH][S]}{[EHS]} \quad (3.23)$$

$$K_1 = \frac{[EH][H^+]}{[EH_2^+]} \quad (3.24)$$

$$K_2 = \frac{[E^-][H^+]}{[EH]} \quad (3.25)$$

$$r = v = k_2 [EHS] \quad (3.26)$$

The enzyme conservation equation is given by

$$[E]_0 = [E^-] + [EH] + [EH_2^+] + [EHS] \quad (3.27)$$

Substituting Eqs. (3.24) and (3.25) in the above equation,

$$[E]_0 = \frac{K_2[EH]}{[H^+]} + [EH] + \frac{[EH][H^+]}{[K_1]} + [EHS]$$

or $[E]_0 = [EH] \left(1 + \frac{[H^+]}{K_1} + \frac{[K_2]}{[H^+]} \right) + [EHS]$

or $[EH] = \frac{[E]_0 - [EHS]}{\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} \right)} \quad (3.28)$

Substituting Eq. (3.28) in Eq. (3.23), we get

$$K'_m = \frac{[E]_0 - [EHS]}{\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} \right)} \times \frac{[S]}{[EHS]}$$

or $K'_m \left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} \right) [EHS] + [EHS][S] = [E]_0[S]$

$$[EHS] = \frac{[E]_0[S]}{K'_m \left(1 + \frac{H^+}{K_1} + \frac{K_2}{H^+} \right) + [S]} \quad (3.29)$$

Substituting Eq. (3.28) in Eq. (3.26),

$$r = \frac{k_2 [E]_0 [S]}{K'_m \left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} \right) + [S]}$$

or

$$r = \frac{v_{\max} [S]}{K'_m \left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} \right) + [S]} \quad (3.30)$$

or

$$r = \frac{v_{\max} [S]}{K'_{m(\text{app})} + [S]} \quad (3.31)$$

where

$$K'_{m(\text{app})} = K'_m \left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} \right)$$

As a result of this, the pH optimum of the enzyme is between pK_1 and pK_2 . In general, increase in pH considerably influences the enzyme activity. Each enzyme has an optimum pH at which the velocity is maximum. Below and above this pH, the enzyme activity is much lower and at the extreme pH, the enzyme is totally inactive. Most of the enzymes (higher organisms) show the optimum activity at a pH of 6.0–8.0. But there are some like pepsin that show optimum activity at pH 1–2, acid phosphatase at pH 4–5, and alkaline phosphatase at pH 10–11. In reality, the theoretical prediction of the optimum pH of enzymes requires knowledge of the active site characteristics of enzymes which is very difficult.

3.5.2 Temperature Effects

The rate of enzyme-catalyzed reaction increases with temperature up to a certain limit. Above a certain temperature, the activity decreases with temperature because of enzyme denaturation (Figure 3.14).

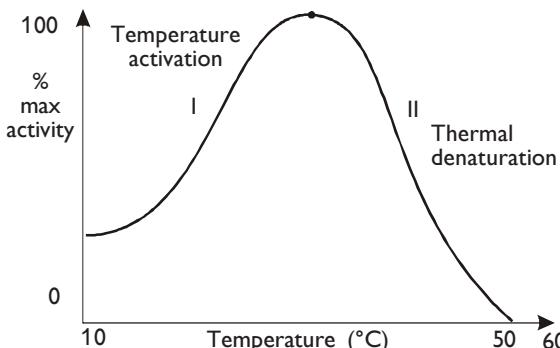


Figure 3.14 Effect of temperature on enzyme activity.

There are two parts in Figure 3.14—part I: temperature activation and part II: thermal denaturation.

Temperature activation: The rate varies as per the Arrhenius equation.

$$v = k_2[E]$$

Here

$$k_2 = A e^{-E_a/RT}$$

where E_a = activation energy (kcal/mol)

$[E]$ = active enzyme concentration (mol/L)

Thermal denaturation: Kinetics of thermal denaturation can be expressed as follows:

$$\begin{aligned} [E] &\rightarrow E_{de} \\ -d[E]/dt &= k_{de} [E] \\ [E] &= [E]_0 e^{-k_{de} t} \end{aligned} \quad (3.32)$$

A rise in temperature from 30°C to 40°C results in a 1.8-fold increase in activity but a 41-fold increase in enzyme denaturation.

Variations in temperature may affect both K_m and v_{max} values. In general, the reaction velocity increases with increase in the temperature up to a maximum and then declines, resulting in a bell-shaped curve. Temperature coefficient or Q_{10} is the increase in the enzyme velocity when the temperature is increased by 10°C. For most of the enzymes, Q_{10} is 2, between 0°C and 40°C. The optimum temperature for most of the enzymes is between 40°C to 45°C. Some plant enzymes like urease have the optimum activity at around 60°C. Enzymes like venom phosphokinases are active even at 100°C.

Overall, when enzymes are exposed to a temperature above 50°C, denaturation starts leading to the derangement in the native structure of protein and the active site. A majority of enzymes become inactive at higher temperatures greater than 70°C.

3.5.3 Concentration Effects

With increase in the concentration of enzymes, the velocity of reaction increases proportionately.

Increase in the concentration of substrates gradually increases the velocity of enzyme reaction within the limited range of substrate levels.

3.5.4 Activators

Metals act as activators of enzyme velocity through various mechanisms. They combine with the substrate to form a temporary Metal-Enzyme-Substrate (MES) complex, directly participate in the reaction and bring about a conformational change in the enzyme. For example, inorganic

metallic ions basically cations like, Mg^{2+} , Mn^{2+} , Zn^{2+} , Ca^{2+} , Co^{2+} etc. activate the enzymes and rarely anions like Cl^- activate the enzymes like amylases.

3.5.5 Product Concentration

The accumulation of reaction products generally decreases enzyme activity. For some enzymes, products combine with the active sites of enzymes and form a loose complex and then inhibit the enzyme activity.

3.5.6 Time

‘Always under’ ideal conditions, the time required for the enzyme reaction is less.

3.5.7 Radiations

Certain enzymes become inactive due to exposure to UV, beta, gamma and X-rays. The active sites are lost due to the loss or damage of the tertiary structure of the enzyme molecule.

3.6 FEATURES OF ACTIVE SITES

Active sites are the small regions on the surface of enzymes at which the substrate S binds and participates in the process of catalysis. These are due to the tertiary structures of the protein. Active sites or centres are the clefts or crevices or pockets that occupy a small region in a big enzyme molecule. These are not rigid in the structure and shape and are rather flexible. Active sites have a substrate binding site and a catalytic site.

Of 20 amino acids, only some are repeatedly found at the active sites, for example arginine, glutamic acid, etc.

3.7 INHIBITORS AND INHIBITION KINETICS

An enzyme inhibitor is a substance which binds with the enzyme and brings about decrease in the catalytic activity of the enzyme. An inhibitor may be organic or inorganic in nature. Enzyme inhibitions may be reversible or irreversible.

Irreversible inhibitors such as heavy metals like Pb , Cd , Hg , etc. form a stable complex with the enzyme and reduce the activity. Such enzyme inhibition may be reversed only by using the chelating agents such as EDTA, ethylene diamine tetraacetic acid and citrate.

Three categories of inhibitions are observed:

- Reversible inhibition
- Irreversible inhibition
- Allosteric inhibition

3.7.1 Reversible Inhibition

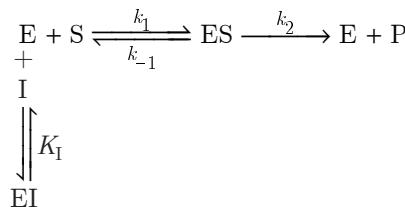
The features of reversible inhibition are as follows:

1. The inhibitor I binds non-covalently with the enzyme E to form EI.
2. It can be reversed if I is removed.

Reversible inhibition is further subdivided into three classes:

- (i) Reversible competitive, (ii) Reversible non-competitive, and (iii) Reversible uncompetitive inhibition.

Reversible competitive inhibition: The inhibitor which closely binds the real substrate is regarded as the substrate analogue. In this I competes with S and binds at the active site of the enzyme but do not undergo any catalysis. As long as the competitive inhibitor holds the active site, the enzyme is not available for S to bind. The mechanism of reversible competitive inhibition is shown as follows:



The relative concentration of S and I and their affinity towards E determines the degree of competitive inhibition. Refer to Figures 3.15 and 3.16.

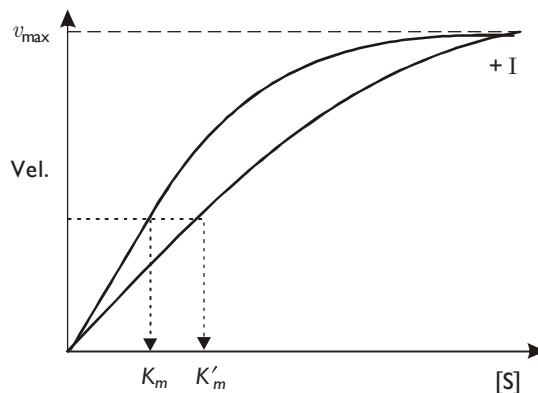


Figure 3.15 Enzyme velocities vs concentration of substrates.

We find from Figure 3.15 that v_{\max} remains unchanged. However, in Figure 3.16, K_m value increases. These can be seen on Lineweaver-Burk plot.

Assuming a rapid equilibrium for the step $E + S$ giving ES , we can write for K'_m as

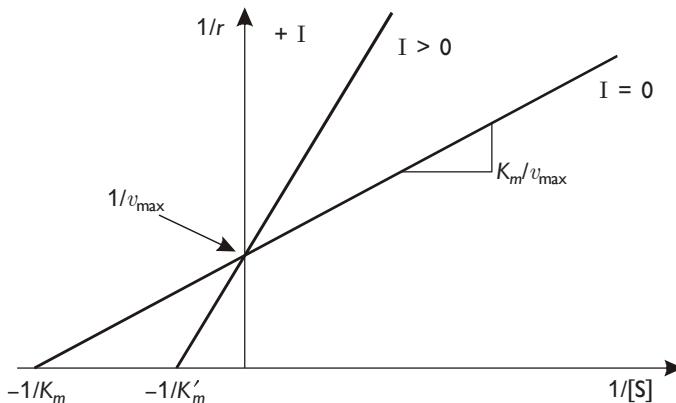


Figure 3.16 Lineweaver–Burk plots for reversible competitive inhibition.

$$K'_m = \frac{[E][S]}{[ES]} \quad (3.33)$$

and for the step $E + I$ giving EI , we can write for K_I as

$$K_I = \frac{[E][I]}{[EI]} \quad (3.34)$$

Further, the rate

$$r = k_2[ES]$$

Next the enzyme conservation equation is given in the presence of inhibitor as

$$[E]_0 = [E] + [ES] + [EI] \quad (3.35)$$

Substituting Eq. (3.34) into Eq. (3.35),

$$[E]_0 = [E] + [ES] + \frac{[E][I]}{K_I} \quad (3.36)$$

or

$$[E]_0 = [E] \frac{(1 + [I])}{K_I} + [ES]$$

or

$$[E] = \frac{[E]_0 - [ES]}{(1 + [I]/K_I)} \quad (3.37)$$

Substituting Eq. (3.37) in Eq. (3.33), we get

$$K'_m = \frac{([E]_0 - [ES])[S]}{\left(1 + \frac{[I]}{K_I}\right)} \times \frac{1}{[ES]}$$

or

$$K'_m \times \left(1 + \frac{[I]}{K_I} \right) = \frac{[E]_0[S]}{[ES]} - \frac{[ES][S]}{[ES]} \\ = \frac{[E]_0[S]}{[ES]} - [S]$$

or

$$[ES] = \frac{[E]_0[S]}{[S] + K'_m \left(1 + \frac{[I]}{K_I} \right)} \quad (3.38)$$

Substituting Eq. (3.38) in $r = k_2 [ES]$, we get

$$r = k_2 \frac{[E]_0[S]}{[S] + K'_m \left(1 + \frac{[I]}{K_I} \right)}$$

$$r = \frac{v_{\max} [S]}{[S] + K'_{m(\text{app})}}$$

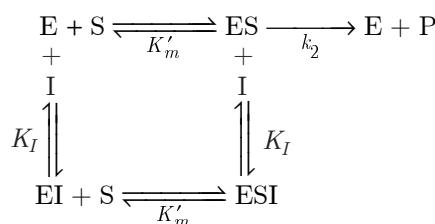
where

$$K'_{m(\text{app})} = K'_m \left(1 + \frac{[I]}{K_I} \right)$$

The net effect of competitive inhibition is an increased value of $K'_{m,\text{app}}$ and therefore reduced reaction rate. This type of inhibition can be overcome by high concentrations of substrate.

Reversible non-competitive inhibition: The inhibitor I binds at a site other than the active site on the E surface. This impairs the enzyme function. The inhibitor has no structural resemblance with the substrate. However, there exists usually a strong affinity for I to bind at the second site. There is no interference with E-S binding, but catalysis is prevented possibly due to distortion in enzyme conformation.

I generally binds with E as well as ES complex. The mechanism of reversible non-competitive inhibition is given as follows:



Refer to Figures 3.17 and 3.18. From these we find that:

1. K_m value remains unchanged.
2. v_{\max} is lowered.
3. Heavy metal ions (Ag^{2+} , Pb^{2+} , Hg^{2+} , etc.) act as non-competitive inhibitors.

From the mechanism, we get

$$K'_m = \frac{[E][S]}{[ES]} = \frac{[EI][S]}{[ESI]} \quad (3.39)$$

$$K_I = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[ESI]} \quad (3.40)$$

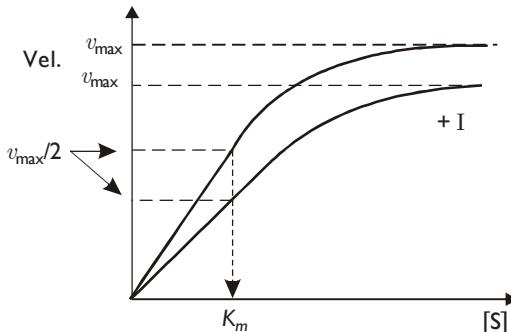


Figure 3.17 Enzyme velocities vs substrate concentration.

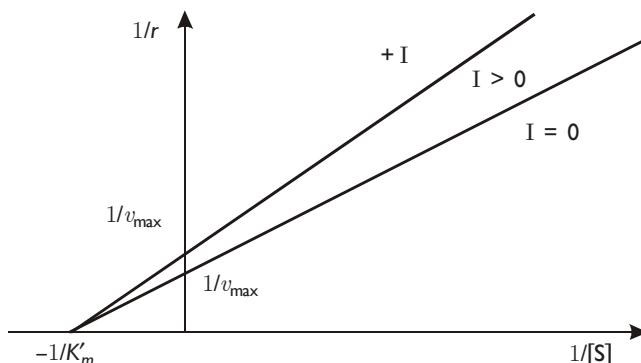


Figure 3.18 Lineweaver-Burk plots for reversible non-competitive inhibition.

or

$$[EI] = \frac{[E][I]}{K_I}; \quad [ESI] = \frac{[ES][I]}{K_I} \quad (3.41)$$

$$r = k_2[ES]$$

We know that

$$[E]_0 = [E] + [ES] + [EI] + [ESI] \quad (3.42)$$

Substituting Eq. (3.40) into Eq. (3.41), we get

$$[E]_0 = [E] + [ES] + \frac{[E][I]}{K_I} + \frac{[ES][I]}{K_I} \quad (3.43)$$

or

$$[E]_0 = [E] \left(\frac{1 + [I]}{K_I} \right) + [ES] \left(\frac{1 + [I]}{K_I} \right)$$

or

$$[E] \left(\frac{1 + [I]}{K_I} \right) = [E]_0 - [ES] \left(\frac{1 + [I]}{K_I} \right)$$

or

$$[E] = \frac{[E]_0 - [ES] \left(\frac{1 + [I]}{K_I} \right)}{\left(\frac{1 + [I]}{K_I} \right)} \quad (3.44)$$

Substituting the above equation in Eq. (3.39), we have

$$K'_m = \left\{ \frac{[E]_0 - [ES] \left(\frac{1 + [I]}{K_I} \right) [S]}{\left(\frac{1 + [I]}{K_I} \right) [ES]} \right\}$$

or

$$K'_m \left(1 + \frac{[I]}{K_I} \right) = \frac{\{ [E]_0 - [ES] (1 + [I]/K_I) \} [S]}{[ES]} \\ = \frac{[E]_0 [S]}{[ES]} - \left(1 + \frac{[I]}{K_I} \right) [S]$$

or

$$\frac{[E]_0 [S]}{[ES]} = K'_m \left(1 + \frac{[I]}{K_I} \right) + \left(1 + \frac{[I]}{K_I} \right) [S]$$

or

$$[ES] = \frac{[E]_0 [S]}{\left(1 + \frac{[I]}{K_I} \right) (K'_m + [S])}$$

Substituting the above equation in $r = k_2 [\text{ES}]$, we get

$$r = \frac{k_2 [E]_0 [S]}{\left(1 + \frac{[I]}{K_I}\right) \left(K'_m + [S]\right)}$$

or

$$r = \frac{v_{\max}[\mathbf{S}]}{\left(1 + \frac{[\mathbf{I}]}{K_I}\right)\left(K'_m + [\mathbf{S}]\right)}$$

Dividing the numerator and denominator of the above equation by $[S]$, we get

$$r = \frac{v_{\max}}{\left(1 + \frac{[I]}{K_I}\right)\left(1 + \frac{K'_m}{[S]}\right)} \quad (3.44a)$$

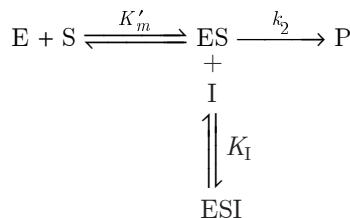
$$r = \frac{v_{\max(\text{app})}}{1 + \frac{K'_m}{[\text{S}]}} \quad (3.44\text{b})$$

where

$$v_{\max(\text{app})} = \frac{v_{\max}}{1 + \frac{[I]}{K_I}}$$

The net effect of non-competitive inhibition is a reduction in v_{max} . High substrate concentrations would not overcome this type of inhibition. Extra reagents need to be added to block the binding of the inhibitor to the enzyme.

Reversible uncompetitive inhibition: It is an uncommon kind of reversible inhibition. The inhibitor I does not bind with E but only binds with ES complex. The mechanism of reversible uncompetitive inhibition is shown as follows:



Now refer to Figures 3.19 and 3.20. From these we observe that:

1. K_m value decreases
 2. v_{\max} value decreases

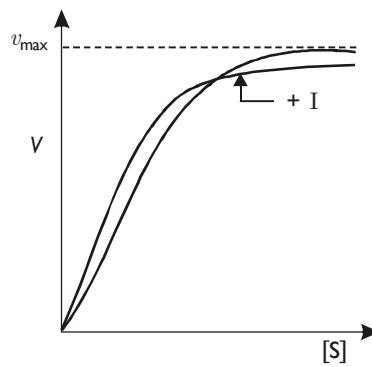


Figure 3.19 Effect of I on enzyme velocity.

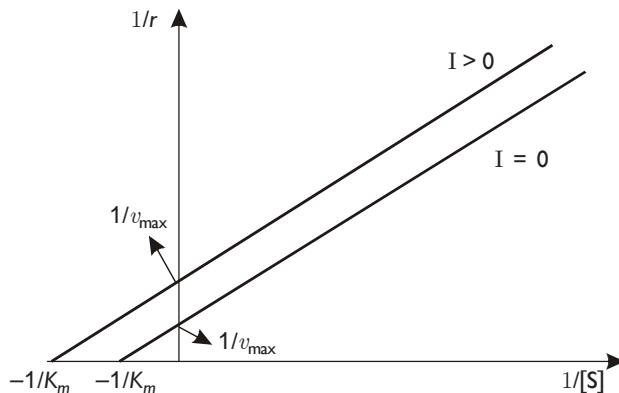


Figure 3.20 Lineweaver-Burk plots for reversible uncompetitive inhibition.

From the mechanism shown above, we get $K_m' = \frac{[E][S]}{[ES]}$

$$K_I = \frac{[ES][I]}{[ESI]}$$

or $[ESI] = \frac{[ES][I]}{K_I}$ (3.45)

The enzyme conservation equation is given by

$$[E]_0 = [E] + [ES] + [ESI] \quad (3.46)$$

Substituting Eq. (3.45) into Eq. (3.46)

$$[E]_0 = [E] + [ES] + \frac{[ES][I]}{K_I}$$

or

$$[E]_0 = [E] + [ES] \left(\frac{1 + [I]}{K_I} \right)$$

or

$$[E] = [E]_0 - [ES] \left(1 + \frac{[I]}{K_I} \right) \quad (3.47)$$

We have

$$K'_m = \frac{[E][S]}{[ES]}$$

Now substituting Eq. (3.47) in the above equation,

$$\begin{aligned} K'_m &= \frac{[E]_0 - [ES](1 + [I]/K_I)[S]}{[ES]} \\ &= \frac{[E]_0[S] - [ES][S](1 + [I]/K_I)}{[ES]} \\ &= \frac{[E]_0[S]}{[ES]} - \frac{[ES][S](1 + [I]/K_I)}{[ES]} \end{aligned}$$

or

$$K'_m = \frac{[E]_0[S]}{[ES]} - [S] \left(1 + \frac{[I]}{K_I} \right)$$

or

$$\frac{[E]_0[S]}{[ES]} = K'_m + [S] \left(1 + \frac{[I]}{K_I} \right)$$

or

$$[ES] = \frac{[E]_0[S]}{K'_m + [S] \left(1 + \frac{[I]}{K_I} \right)} \quad (3.48)$$

We know that

$$r = k_2 [ES]$$

Now, substituting Eq. (3.48) in the above equation, we get

$$r = \frac{k_2 [E]_0 [S]}{K'_m + [S] \left(1 + \frac{[I]}{K_I} \right)}$$

or

$$r = \frac{v_{\max} [S]}{K'_m + [S] \left(1 + \frac{[I]}{K_I} \right)}$$

Dividing the numerator and denominator of the above equation by $(1 + [I]/K_I)$, we get

$$r = \frac{v_{\max}[S] \left/ \left(1 + \frac{[I]}{K_I} \right) \right.}{\left\{ K'_m \left/ \left(1 + \frac{[I]}{K_I} \right) \right. \right\} + [S]}$$

or

$$r = \frac{v_{\max(\text{app})}[S]}{K'_{m(\text{app})} + [S]} \quad (3.49)$$

where

$$v_{\max(\text{app})} = v_{\max} \left/ \left(1 + \frac{[I]}{K_I} \right) \right.$$

and

$$K'_{m(\text{app})} = K'_m \left/ \left(1 + \frac{[I]}{K_I} \right) \right.$$

The net effect of uncompetitive inhibition is a reduction in both v_{\max} and K'_m values. The reduction in the v_{\max} value has a more profound effect than the reduction in K'_m is, and the net result is a reduction in reaction rate.

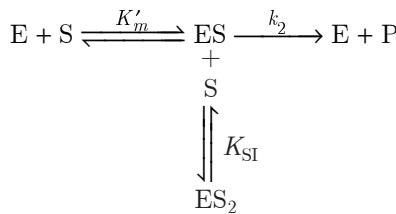
3.7.2 Substrate Inhibition

Usually an increase in substrate concentration increases the velocity of enzyme reaction. Some enzymes however display the phenomenon of excess substrate inhibition which refers to the large amounts of substrate that can influence oppositely and later slow down the reaction. For example, an enzyme invertase or β -fructofuranosidase which is responsible for hydrolyzing the disaccharide sucrose into glucose and fructose.

It is also seen that the substrate inhibition occurs with this enzyme when two substrate molecules bind to the active site at the same time. As long as the substrate molecules are attached to the active site, the enzyme molecule is effectively inactive and hence gets inhibited. For this process to take place the second substrate should approach the active site very fast after the first substrate otherwise the first substrate would quickly attain the correct catalytic placement.

The collision between the enzyme and the substrate are totally random and this is likely to occur at high substrate concentration when the frequency of random collisions is greatly increased, so inhibition is only seen in the presence of excess substrates.

High substrate concentrations may cause inhibition in enzymatic reactions, called *substrate inhibition*. The mechanism of substrate inhibition is shown as follows:



Refer to Figure 3.21 (the Lineweaver–Burk plot). It is no longer linear. However, towards the $1/v_{\max}$ axis, which is the high substrate end, the line curves upwards to show the reduction in velocity.

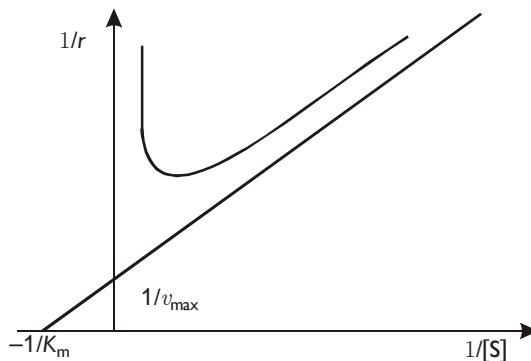


Figure 3.21 Profiles for the substrate inhibition ($1/r$ vs $1/[S]$).

Substrate inhibition is graphically presented in Figure 3.22. At high substrate levels, the curve falls as velocity is reduced by the inhibitory effect.

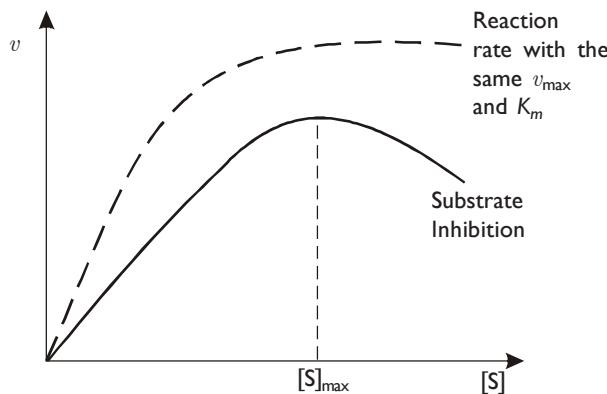


Figure 3.22 Comparison of substrate inhibition and pure enzymatic reactions.

From the mechanism shown above, we can define the following equations:

$$K'_m = \frac{[E][S]}{[ES]} \quad (3.50)$$

$$K_{SI} = \frac{[ES][S]}{[ES_2]} \quad (3.51)$$

or $[ES_2] = \frac{[ES][S]}{K_{SI}}$ (3.52)

and

$$r = k_2[ES]$$

The enzyme conservation equation is given by

$$[E]_0 = [E] + [ES] + [ES_2]$$

Substituting Eq. (3.52) in the above equation, we get

$$[E]_0 = [E] + [ES] + \frac{[ES][S]}{K_{SI}}$$

or $[E]_0 = [E] + [ES] \left(1 + \frac{[S]}{K_{SI}} \right)$

or $[E] = [E]_0 - [ES] \left(1 + \frac{[S]}{K_{SI}} \right)$ (3.53)

Substituting Eq. (3.53) in Eq. (3.50), we get

Use $K'_m = \frac{[E][S]}{[ES]} = \frac{\left[[E]_0 - [ES] \left(1 + \frac{[S]}{K_{SI}} \right) \right] [S]}{[ES]}$
 $= \frac{[E]_0 [S] - [ES] [S] \left(1 + \frac{[S]}{K_{SI}} \right)}{[ES]}$

or $K'_m = \frac{[E]_0 [S]}{[ES]} - [S] \left(1 + \frac{[S]}{K_{SI}} \right)$

or $\frac{[E]_0 [S]}{[ES]} = K'_m + [S] \left(1 + \frac{[S]}{K_{SI}} \right)$

or $[ES] = \frac{[E]_0 [S]}{K'_m + [S] \left(1 + \frac{[S]}{K_{SI}} \right)}$

or

$$[ES] = \frac{[E]_0[S]}{K'_m + [S] + \frac{[S]^2}{K_{SI}}}$$

Substituting the above equation in, $r = k_2 [ES]$, we get

$$r = \frac{k_2 [E]_0 [S]}{K'_m + [S] + \frac{[S]^2}{K_{SI}}}$$

or

$$r = \frac{v_{\max} [S]}{K'_m + [S] + \frac{[S]^2}{K_{SI}}} \quad (3.54a)$$

or

$$r = \frac{v_{\max}}{\frac{K'_m}{[S]} + 1 + \frac{[S]}{K_{SI}}} \quad (3.54b)$$

Case 1. At low substrate concentrations, inhibition is not seen. So

$$\frac{[S]^2}{K_{SI}} \ll 1.0$$

Therefore, Eq. (3.54a) becomes as follows:

$$r = \frac{v_{\max} [S]}{K'_m + [S]} = \frac{v_{\max}}{\left(1 + \frac{K'_m}{[S]}\right)}$$

Case 2. At high substrate concentrations, inhibition is dominant. So

$$\frac{K'_m}{[S]} \ll 1.0$$

Therefore, Eq. (3.54b) becomes as follows:

$$r = \frac{v_{\max}}{1 + \frac{[S]}{K_{SI}}}$$

The substrate concentration resulting in the maximum reaction rate can be determined by setting $\frac{dr}{d[S]} = 0$.

Then

$$[S]_{\max} = \sqrt{K'_m \cdot K_{SI}}$$

3.7.3 Irreversible Inhibition

The features of irreversible inhibition are as follows:

1. Inhibitors bind covalently with enzymes and inactivate them which are irreversible.
2. Inhibitors are usually toxic substances, natural or man-made.

For example, iodoacetate is an irreversible inhibitor for enzymes like papain, glyceraldehyde 3-phosphate dehydrogenase, etc. Organophosphorous insecticides like malathion are toxic to animals (including human beings) as they block the activity of acetaldehyde esterase (nerve conduction) resulting in paralysis of vital body functions.

3.7.4 Allosteric Inhibition

Before we discuss the allosteric inhibition, we need to know some basic terminologies related to allosteric. An *allosteric protein* is a protein containing two or more topologically distinct binding sites which interact functionally with each other.

This refers to the fact that there are at least two sites in different positions and configurations capable of binding the substrate or inhibitors. The binding of one either a substrate or an inhibitor at one site shall alter the properties of others. Majority of allosteric proteins are allosteric enzymes which are capable of catalyzing the reactions but some are simply the binding proteins, such as haemoglobin, the protein pigment present in the blood.

Cooperativity is the modification of the binding constant of the protein for a small molecule by the prior binding of another small molecule.

The definition of cooperativity means that binding of one ligand to the protein, which either increases or decreases the ability of the protein to bind a second ligand molecule. In case the binding ability or affinity increases, it is called *positive cooperativity*, and if the ability decreases it is called *negative cooperativity*.

Allosteric inhibitors and activators which can be collectively referred to as the **allosteric effectors** are one of the main reasons for the importance of allosteric enzymes. The alteration of speed of enzyme catalyzed reactions by inhibition and activation is the key factor of metabolic controlling strategies.

Figure 3.23 depicts the influence of increasing activators and increasing inhibitors on the kinetic plot of a typical allosteric enzyme which shows positive substrate cooperativity.

The core curve or the profile shows a typical sigmoid plot in the absence of any effector. The presence of an activator increases the reaction velocity at any given substrate concentration, while an inhibitor decreases the reaction velocity. This signifies that the allosteric inhibitor increases the level of substrate cooperativity while an activator decreases it.

In allosteric systems we come across the *K*-systems and the *V*-systems.

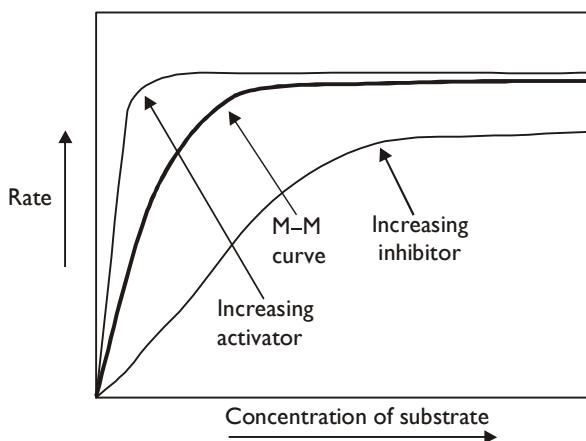


Figure 3.23 Effect of increasing activators and increasing inhibitors.

From Figure 3.23, it is apparent that all the lines are heading towards the same V_{\max} value which means that the effector has functioned by changing the substrate binding which will be seen as a change in the K_m value. Such systems are called K -systems while some enzymes have effectors which function by altering the V_{\max} values and are called V -systems.

Some enzymes like regulatory enzymes have more than one substrate binding site. The binding of one substrate to the enzyme facilitates binding of other substrate molecules. This behaviour is called allostericity ('allo' means 'other' or different) or cooperative binding.

The rate expression of allosteric inhibition is as follows:

$$r = \frac{-d[S]}{dt} = \frac{v_{\max}[S]^n}{K'_m + [S]^n} \quad (3.55)$$

where n = cooperative coefficient. If $n > 1$, it signifies positive cooperativity.

The plot of r vs $[S]$ given in Figure 3.24 shows the comparison between the Michaelis-Menten (MM) and allosteric kinetics.

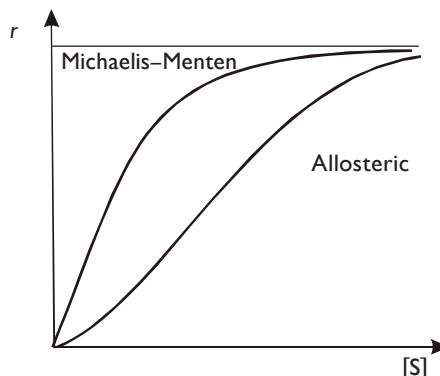


Figure 3.24 Comparison between Michaelis-Menten and allosteric kinetics.

3.7.5 Product Inhibition

We know that the final reaction in the enzymes reaction sequence is:

$EP \rightleftharpoons E + P$ and is often neglected. But it is an essential component of the process.

The product is bound to the active site by the same bonds which bind the substrates. Therefore, this reaction is similar to the substrate, binding the enzyme E and further it is very fast and reversible.

Consequently, the product molecules are capable of binding free enzymes to form the EP complex. Since in a single-substrate enzyme, the product and the substrate both attempt to occupy the site, they cannot both bind at the same time.

Therefore, prior binding of the product P prevents the enzyme from binding the substrate S and the product effectively acts as an inhibitor.

3.7.6 The Inhibitor Constant K_I

We have already seen the importance of the affinity of an enzyme for its substrate in determining how fast an enzyme can carry out its reaction. Also we know that dissociation constant of E-S complex is a measure of this affinity, while K_m is a reasonable estimate.

Enzyme inhibitor affinity is also important, as an inhibitor with a high affinity for the enzyme is more likely to bind to it and shall therefore be a more powerful inhibitor.

The inhibition or inhibitor constant K_I is a measure of this affinity. It is the dissociation constant of the enzyme-inhibitor complex and is directly comparable with the dissociation constant of E-S complex. Therefore, a large value of K_I is an indication of low affinity and vice versa.

To estimate the value of K_I , we can use three different ways.

1. Usual calculations which has been dealt with in the preceding discussions.
2. Use of primary and secondary plots which is also dealt with by using Lineweaver-Burk method.
3. Dixon method.

In the succeeding paragraphs, we find the Dixon method employed to determine the inhibitor constant K_I .

The Dixon plot to find the Inhibitor constant: The Dixon plot is a convenient method of calculating the inhibitor constant K_I . It has certain constraints as well, as it cannot be used to calculate the K_m , v_{max} etc.

The reaction velocity is measured at a fixed concentration of substrate but at varied inhibitor concentrations. Figure 3.25 shows the reciprocal of velocity against inhibitor concentration values. Indeed the plot is shown for three different substrate concentrations for non-competitive inhibition process.

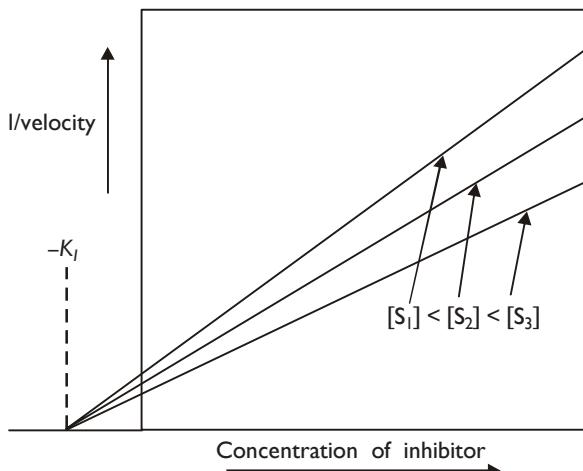


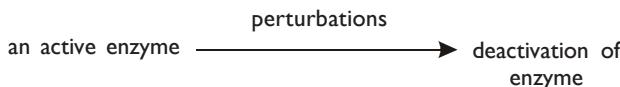
Figure 3.25 Reciprocal of velocity against inhibitor concentration value.

We find here a vertical line dropped from the point where the lines are intersecting down to the inhibitor axis finally giving $-K_I$. For a classical non-competitive inhibitor, the intersection would be on the axis so the constant is read off directly. For competitive or mixed inhibition, the intersection will be some way above the axis.

While for uncompetitive inhibition, the lines would be parallel and there is no intersection as K_I is irrelevant to these inhibitors.

3.8 ENZYME DEACTIVATION

As we know an enzyme is basically a protein. Its structure is stabilized by a series of weak forces and these give rise to the properties that are functionally important. Enzyme, when used as a biocatalyst within a bioreactor, comes across a multitude of physical and chemical parameters that cause perturbations in the native protein geometry and structure and finally deactivation of enzyme.



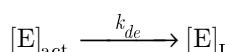
3.8.1 Enzyme Deactivation Model

Consider the following conversion:



where $[E]_{\text{act}}$ is activated enzyme and $[E]_I$ is inhibitory enzyme.

This conversion is described by a rate constant of first order form. Therefore,



where k_{de} is deactivated rate.

$$\frac{-d[E]_{act}}{dt} = k_{de}[E]_{act} \quad (3.56)$$

or

$$\frac{d[E]_{act}}{[E]_{act}} = -k_{de} \times dt$$

Integrating the above equation at $t = 0$; $[E]_{act} = [E]_{act,0}$ and $t = t$;

$$[E]_{act} = [E]_{act, t}$$

$$\int_0^t \frac{d[E]_{act}}{[E]_{act}} = -k_{de} \int_0^t dt$$

or

$$\frac{\ln[E]_{act,t}}{[E]_{act,0}} = -k_{de} \times t$$

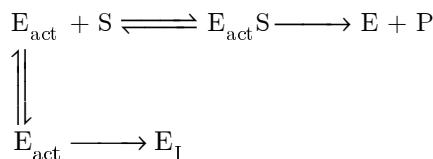
or

$$[E]_{act,t} = [E]_{act,0} \times e^{-k_{de} \times t} \quad (3.57)$$

Note: The deactivation rates are determined in the absence of substrate. The enzyme deactivation rates can be modified by the presence of substrate and other materials.

3.8.2 Combination of Deactivation Model with Simple Catalytic Sequence of Michaelis-Menten Equation

Look at the mechanism of the reaction.



Assume that the deactivation process is slow and that free E will deactivate faster than the enzyme in the bound state, i.e. ES complex.

We can write:

$$\frac{d[E]_{act}}{dt} = -[E]k_{de} \quad (3.58)$$

We know that

$$[E]_0 = [E] + [ES]$$

Replacing $[E]_0$ with $[E]_{act}$,

$$[E]_{act} = [E] + [ES]$$

or

$$[E] = [E]_{act} - [ES] \quad (3.59)$$

Substituting Eq. (3.59) into Eq. (3.58), we get

$$\frac{d[E]_{act}}{dt} = -k_{de}([E]_{act} - [ES]) \quad (3.60)$$

Also from the simple catalytic sequence,

$$[ES] = \frac{[E]_0[S]}{K_m + [S]} \quad (3.61)$$

Substituting the above equation in Eq. (3.60)

$$\frac{d[E]_{act}}{dt} = -k_{de} \left([E]_{act} - \frac{[E]_0[S]}{K_m + [S]} \right)$$

Replacing $[E]_0$ with $[E]_{act}$, the above equation becomes

$$\begin{aligned} \frac{d[E]_{act}}{dt} &= -k_{de} \left([E]_{act} - \frac{[E]_{act}[S]}{K_m + [S]} \right) \\ &= -k_{de} \times [E]_{act} \left(1 - \frac{[S]}{K_m + [S]} \right) \\ &= -k_{de} \times [E]_{act} \left(\frac{K_m + [S] - [S]}{K_m + [S]} \right) \\ &= -k_{de} \times [E]_{act} \left(\frac{K_m}{K_m + [S]} \right) \end{aligned}$$

Dividing the numerator and denominator by K_m , we get

$$\frac{d[E]_{act}}{dt} = -k_{de} \times [E]_{act} \left(\frac{1}{1 + \frac{[S]}{K_m}} \right) \quad (3.62)$$

The above expression links the rates of enzyme deactivation and substrate conversions.

3.8.3 Half-life Period

We can calculate half-life period by setting the enzyme activity at half the initial value.

$$[E]_{act,t} = \frac{[E]_{act,0}}{2} \text{ at } t = t_{1/2}$$

$$[E]_{act,t} = [E]_{act,0} \times e^{-k_{de} \times t}$$

$$\frac{[E]_{act,0}}{2} = [E]_{act,0} \times e^{-k_{de} \times t_{1/2}}$$

or

$$\frac{1}{2} = e^{-k_{de} \times t_{1/2}}$$

or

$$t_{1/2} = t_{0.5} = \frac{\ln 2.0}{k_{de}} = \frac{0.693}{k_{de}}$$

The above expression relates the deactivation constant and the time factor.

3.9 KINETICS OF MULTISUBSTRATE SYSTEMS

So far in the earlier topics, we have discussed the kinetics of enzymatic reactions on the basis that an enzymatic reaction involves a single substrate and subsequently formation of one product. In fact, such enzymes are very rare.

Typically, in certain cases an enzyme is involved in a reaction to convert two substrates to two products as shown below.



The above shown sequence incorporates slow steps which are the rate limiting and decide the rate of reaction. These are:



For such cases, the rate expressions are:

$$r_1 = \frac{-d[S_1]}{dt} = \frac{k_1[E]_0[S_1]/K_1}{1 + [S_1]/K_1 + [S_2]/K_2} \quad (3.67)$$

and

$$r_2 = \frac{-d[S_2]}{dt} = \frac{k_2[E]_0[S_2]/K_2}{1 + [S_1]/K_1 + [S_2]/K_2} \quad (3.68)$$

where $[E]_0 = [E] + [ES]$, the total enzyme concentration.

The above equations indicate that if two reactions are catalyzed by the same enzyme, the individual velocities are slower in the presence of both substrates than in the absence of one of the two substrates.

Let us say that if $[S_{\text{Total}}]$ is the total concentration of substrates present in the system i.e. $[S_{\text{Total}}] = [S_1] + [S_2]$ (3.69)

Then the overall rate of disappearance of the total substrates is given as

$$r_{\text{Total}} = \frac{-d[S_{\text{Total}}]}{dt} = -\left(\frac{d[S_1]}{dt} + \frac{d[S_2]}{dt} \right)$$

On substitution and rearrangement, we get the following equation given below.

$$r_{\text{Total}} = [E]_0 \left(\frac{k_1[S_1]/K_1 + k_2[S_2]/K_2}{1 + [S_1]/K_1 + [S_2]/K_2} \right) \quad (3.70)$$

Note: Conversion of two substrates to two products, three substrates to products are quite common while four is occasional.

In studying an enzyme with more than one substrate, the usual approach is to keep all the substrates, but one at a constant concentration.

The remaining substrate is varied in the assays and valuable information can be gained about the kinetic relationship between the substrates, enzymes and inhibitors.

(Inhibitors and inhibition kinetics is dealt with previously in section 3.7)

In other sense, we are treating as the apparent single substrate-enzyme system.

There are various terms and symbols to be understood to study the multi-substrate kinetics.

In the subsequent lines we signify here the terms and symbols.

Substrates: S_1, S_2, S_3, S_4 a four-substrate system.

Products: P_1, P_2, P_3, P_4 a four-product system.

Enzymes: Frequently we are dealing with one enzyme at a time, but in multisubstrate enzyme, different forms of the enzyme can be used. E is the usual form called free enzyme.

During the course of reaction in multisubstrate systems, we find that certain complexes are produced which are briefed in the following discussion.

Transitory complexes: The binding of S and P to E to form the complexes is very well understood. The complexes so formed are called *transitory*

complexes which are unstable and prone to undergo the transformations during the course of reactions. Also these transitory complexes stay or exist for a very brief period of time. Later they either break down again as the bonds holding them are weak or they may undergo a covalent change to convert sites into P or vice versa.

In multisubstrate enzyme there are two or more types of transitory complexes which are generated.

An enzyme having two substrates (S_1, S_2) and two products (P_1, P_2), there are three possible E-S complexes as ES_1 , ES_2 and ES_1S_2 and three possible E-P complexes as EP_1 , EP_2 and EP_1P_2 .

As well in such systems, there is every possibility of forming the combined E-S-P complexes as ES_1P_1 and ES_2P_2 . All these are called *transitory complexes*.

Central complexes: In a single S system the active site of an enzyme is effectively or fully occupied once one substrate has bound. Therefore, there is no vacancy for any other substrate, a product or an inhibitor to enter.

While in multisubstrate enzyme that can bind more than one substrate at the same time, binding of one substrate will not effectively occupy the active site. A central complex is a transitory complex which is full. It may be represented by enclosing it in the parentheses.

For example, enzyme binding two S and two P at the same site as ES_1 , ES_2 , EP_1 , and EP_2 are not central complexes while (ES_1S_2) and (EP_1P_2) would be.

Several kinetic mechanisms have been suggested to critically evaluate the kinetics of multisubstrate enzyme systems. These are:

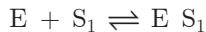
1. The ordered sequence mechanism
2. The random sequence mechanism
3. The ping-pong or double displacement mechanism.

The brief explanation of these mechanisms is given in the succeeding lines.

3.9.1 The Ordered Sequence Mechanism

According to this, the substrates bind to the enzyme in an orderly fashion and the products are released later in a defined sequence.

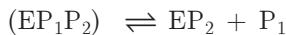
It is depicted below.



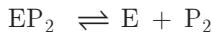
Later, this complex converts into the EP complex as shown



Consequently, the products now released are:



and further we get enzyme E which is regenerated.



The Cleland approach: This is rather used to explain elaborately on the ordered sequence phenomena.

It is actually a simple mechanism, but the sequence of reactions can get more complex, specifically in enzymes with more than two substrates and products.

This is a diagrammatic approach and is self-explanatory of the reaction sequence. In this, the horizontal line represents the enzymes; the arrows represent the arrival and departure of substrates and products while transitory complexes are shown below the enzyme line (Figure 3.26).

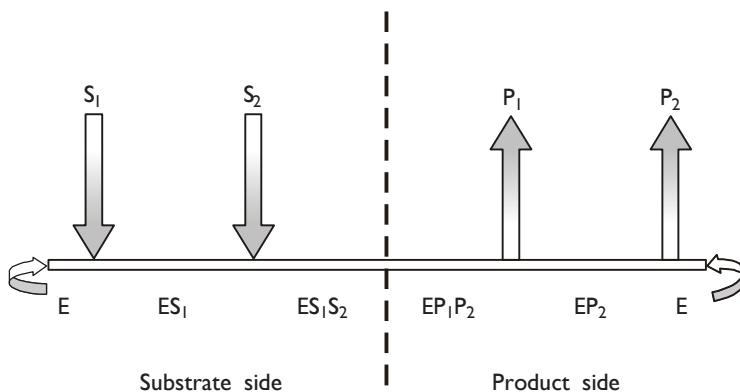
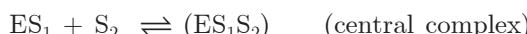
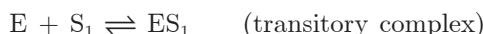


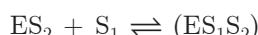
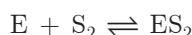
Figure 3.26 Diagrammatic representation of Cleland approach.

3.9.2 The Random Sequence Mechanism

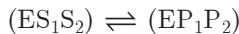
In this, we see that the enzymes work in a similar fashion to ordered sequence mechanism with the exception that there is no specific order for S to bind and products to get released as shown below. Therefore, this makes the reaction sequence more complex.



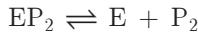
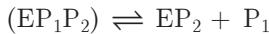
or



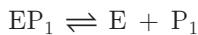
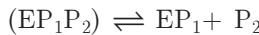
We find that the central complex is identical in both the cases while the subsequent step shown below is same with an ordered mechanism.



From the above, we have now a choice of sequence of product release as shown below:



or



The Cleland approach for the above is as shown (Figure 3.27).

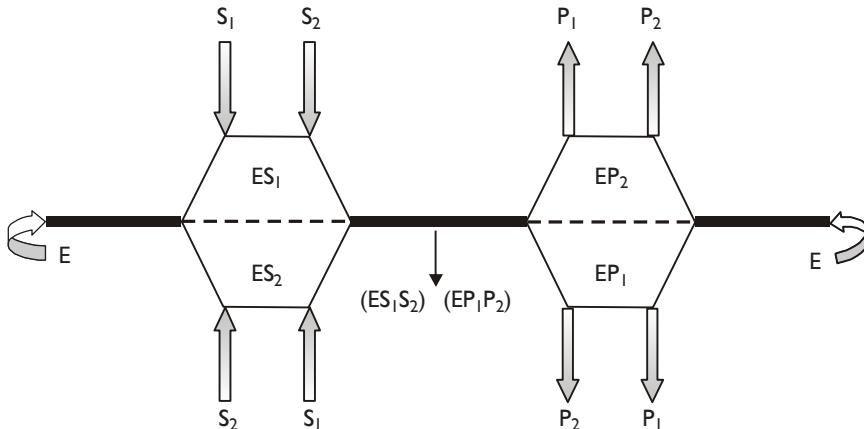


Figure 3.27 Diagrammatic representation of Cleland approach.

3.9.3 The Ping-Pong or Double Displacement Mechanism

In this, the process starts by binding of enzyme to the first substrate in a usual way.



Before the other substrate gets bound to the enzyme, at least one product is released from enzyme E. This seems to be unlikely, but is a common mechanism.

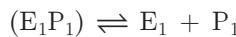
Now the active site is full as this substrate S_1 will be converted to the product before S_2 can bind. The active site has no room for S_2 .



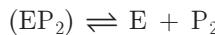
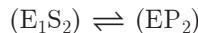
The above mechanism is a key to the entire process.

In the above reaction, a part of the substrate is removed from the substrate S_1 converting it to the product P_1 .

The removed section has covalently bound to the enzyme to create a new form of E i.e. E_1 . The first product of the reaction is now released and the second substrate binds.



Now the stored section of the first substrate is transferred to the second substrate to create the second product P_2 which is then released.



The Cleland approach for the above is as shown in Figure 3.28.

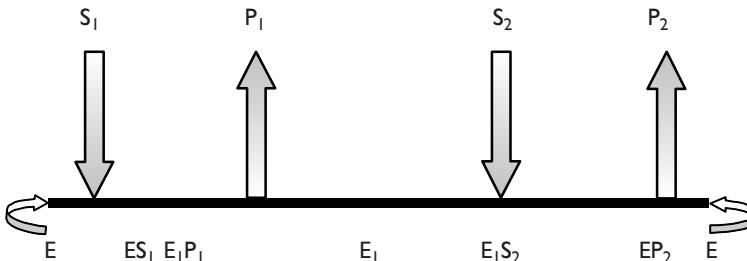


Figure 3.28 Diagrammatic representation of Cleland approach.

It is quite distinctive for ping-pong mechanism because we see that the first upward arrow for product P_1 is to the left of the first down arrow for the substrate S_2 .

3.10 MODULATION AND REGULATION OF ENZYME ACTIVITY

It has been realized that chemical species other than the substrates will combine with enzymes. This combination perhaps is seen during the reaction when certain chemical constituents enter and force themselves to combine with enzyme active sites. As a result, it is found that the catalytic activity of enzymes is altered or modulated.

Such substances that can modulate the activity of enzymes are called *modulators* or *effectors*. Of course, these may be normal constituents of cells. In certain other cases, they also enter from the cell's environment or act on isolated enzymes. Overall, it is seen that combination of an enzyme with a modulator or effector is itself a chemical reaction and possibly would be fully reversible, partially reversible or essentially irreversible.

The mechanism of reversibility (both partial and full) is understood in the preceding discussions. The 'poisons' such as cyanides and their ions which specifically deactivate Xanthine Oxidase and so-called *nerve gases*; a group of chemicals which deactivate 'choline esterases'. These choline esterases are enzymes that form an integral part of nerve transmission and thus of motor ability, overall belonging to nervous system.

Irreversible inhibition increases with time as more and more enzyme molecules are getting gradually deactivated. There are several other cases, more difficult to detect involving partial deactivation of enzyme molecule. In such cases, the inhibited enzyme retains its catalytic activity but at a level certainly reduced from the pure form.

Reversible modulation of enzyme activity is one control mechanism employed by the cell to make use of nutrients effectively.

In the regulation of enzyme activity, it is a well-accepted fact that a living cell contains much more than a thousand different enzymes, each of which can serve as an effective catalyst for certain specific chemical reactions. While these enzymes act together in a coordinated manner so that all the chemical activities in a living cell are integrated with one another.

As a result of this enzyme coordination, a living cell synthesizes and degrades the materials of various forms as required for normal growth, metabolism and functioning. Regulation of enzymes influences the control of cell metabolism and later the normal growth. For instance, in a bacterial cell the very existence of cellular regulatory mechanisms is far more important because of the absence of supracellular controls, such as neural controls and hormonal controls, as compared to tissues of higher organisms wherein such controls are very much present and are established.

Fortunately, microorganisms have evolved a variety of enzyme regulatory mechanisms that accommodate and adjust to the changing needs of a cell in a changing habitat and surroundings.

3.10.1 Enzyme Regulation Mechanisms

Though diversity exists between and among the organisms, but within the cells, there are observed two different regulatory mechanisms. These are:

1. Regulation of enzyme activity
2. Regulation of enzyme synthesis

Also, it is seen that both these mechanisms share certain properties as mentioned below.

- (i) These are mediated and governed by low molecular weight compounds. Low molecular weight compounds influence the mechanisms stated above, while formation of low molecular weight compounds is during cell metabolism or present in the environment.

- (ii) Involve the participation of a special class of control proteins. These special class of control proteins are mediators of changes taking place during metabolism. We also find that there are two types of control proteins, namely allosteric enzymes and regulatory proteins.

In the succeeding paragraphs, is given the outline of allosteric enzymes and regulatory proteins briefly.

Allosteric enzymes as discussed in the preceding sections are the enzymes that have allosteric sites, i.e. the sites other than the active sites on the surface, while this allosteric site regulates the activity of enzyme molecule.

The activities of allosteric enzymes are enhanced or inhibited by combination with their effector molecules. These effector molecules act on the allosteric sites, and not on the catalytic site.

With the presence of allosteric sites besides catalytic sites, these allosteric enzymes are generally larger and more complex than the simple enzyme molecules.

On the contrary, regulatory proteins have no catalytic activity, but can modulate the biosynthesis of enzymes. This is possible by attaching to the bacterial chromosome at specific sites and thus regulates expression of a gene.

3.10.2 Regulation of Enzyme Activity

All biosynthesis processes are controlled by modulation of enzyme activity, rather than by control of enzyme synthesis. We know that all such biosynthesis processes do require certain precursor substances that carry forward and are the building blocks, are formed during the cell metabolic processes. These metabolic processes supply energy and as mentioned above, also the precursors and certain other reduced compounds for other biosynthesis processes.

Of course, all such activities are controlled by enzyme activity modulation. However, it is not that all the enzymes in a pathway needs to be controlled but there can be one enzyme which can be controlled and this sets the control over the other enzymes in a sequence and further regulating the pathway.

Three modes of control are suggested. These are as follows:

Energy link control: This type of control involves the use of energy carriers that serve as effectors or modulators. Adenylates such as adenosine triphosphate (ATP), other purine or pyrimidine nucleotides are the effector molecules.

These are certain enzymes that show sensitivity to the absolute concentrations of ATP, ADP and even AMP; others seem to react to the ratio of a given two of these nucleotides.

Certain other compounds are Guanosine triphosphate (GTP), Uridine

triphosphate (UTP), Cytidine triphosphate (CTP), etc. that serve as energy carriers or effectors.

In general, it is realized that the enzymes responsible for energy production get inhibited by energy charge, for example very high concentration of ATP. In other sense, high concentrations produced or generated shall inhibit the reaction processes.

On the other hand, certain important enzymes which are biosynthetic get stimulated with high concentrations of ATP.

This regulation is very important in balancing the production of energy and its utilization.

Feedback inhibition: This is perhaps the other method of regulation of enzyme activity. In a biosynthetic pathway such as the one leading to formation of one major end product, say amino acid, control is exerted by the final concentration of this amino acid produced in the cell.

This product, so called the effector molecule, shall typically inhibit the enzyme activity in the first reaction of the pathway.

This method of regulation called the *feedback inhibition* is also termed as the *end-product inhibition*. It is due to this, microorganisms and other cells prevent the overproduction of low-molecular weight intermediates as amino acids and purine and pyrimidine nucleotides.

For example, glutamine synthetase is influenced by feedback inhibition from nine different compounds produced.

In the succeeding explanation is the feedback inhibition system, in which the activity of enzyme L-threonine deaminase is reduced by the presence of the end-product, L-isoleucine.

It is a five-step sequence for the biosynthesis of the amino acid i.e. L-isoleucine and the regulation is achieved by feedback inhibition, a closed-loop system. Figure 3.29 depicts the same.

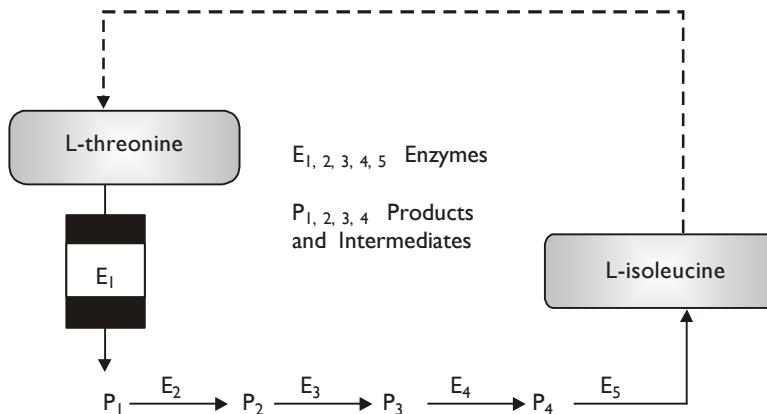
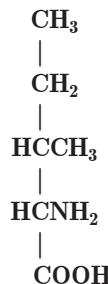


Figure 3.29 Feedback inhibition.

In Figure 3.29, is shown a five-step sequence for biosynthesis of amino acid L-isoleucine, the molecular formula for which is,

Molecular Formula of L-isoleucine:



The regulation of this sequence is achieved by feedback inhibition; the final product inhibits the activity of the first enzyme E_1 in the path. If the concentration of the final product builds up, the process of biosynthesis process will come to a halt.

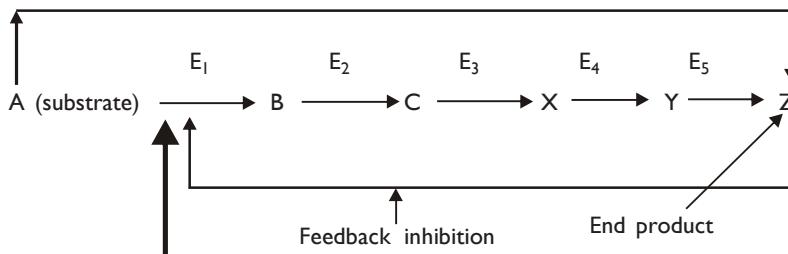
As the reaction catalyzed by E_2 through E_5 are essentially, at equilibrium and the first reaction being irreversible under cellular conditions, the response is especially fast. And this is a general feature of most of regulatory enzymes that they catalyze 'irreversible' reactions.

But it is evident that natural enzyme modulations should be necessarily reversible although spoken in terms of a biological sense.

Further, if L-isoleucine is depleted by its use in protein synthesis, inhibition of enzyme E_1 must be fast relaxed so that the required supply of amino acid is restored.

Precursor activation: In certain cases, the precursor or the first metabolite of a pathway is the effector molecule. This activates or stimulates the activity of the last or later enzyme in the sequence of reaction in a pathway.

For example, E_5 is activated by this precursor that carries forward the gene sequence in a pathway. This type of regulation of enzyme activity is called precursor activation; an example for the above is cited in the section 4.2.1 of Chapter 4. Figure 3.30 represents all the three modes of regulation of enzyme activity.



Energy-link control (AMP, ADP, ATP, etc.)
are controllers.

Figure 3.30 Three modes of regulation of enzyme activity.

SUMMARY

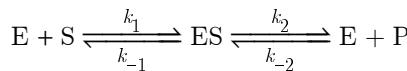
In this chapter, we learnt the following:

- Enzymes are protein biocatalysts synthesized by living cells. They are classified into six major classes—oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.
- An enzyme is specific in action, possessing active centres, where the substrate binds to form the enzyme–substrate complex, before the product is formed.
- Concentration of enzyme, concentration of substrate, temperature, pH, etc. are the factors that influence the activity of an enzyme. The substrate concentration to produce half-maximal activity is called Michaelis constant.
- The mechanism of enzyme action is explained by lock and key model, induced fit theory and the substrate strain theory.
- Most of the enzymes require the presence of non-protein substances called cofactors and coenzymes for their action.
- The kinetic parameters can be evaluated by using Lineweaver–Burk, Eadie–Hofstee and Hanes–Woolf methods.
- Enzyme activities are inhibited by reversible (competitive, non-competitive and uncompetitive), irreversible and allosteric manner.
- Reversible inhibition is of three types: reversible competitive, reversible non-competitive and reversible uncompetitive inhibition.
- Substrate and product inhibitions also occur along with irreversible inhibition and allosteric inhibition.
- Microorganisms have evolved a variety of enzyme regulatory mechanisms that accommodate and adjust to the changing needs of a cell in a changing habitat and surroundings.
- The three modes of regulation of enzyme activity are: energy link control, feedback inhibition and precursor activation.

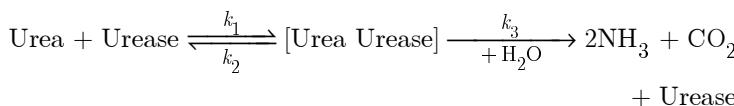
EXERCISES

- 3.1** List the six major classes of enzymes as proposed by the Enzyme Commission, International Union of Biochemists, in the year 1961.
- 3.2** Define the following terms:
(a) Holoenzyme, (b) Apoenzyme, (c) Coenzyme, (d) Cofactors, (e) Monomeric and Oligomeric enzymes, and (f) Multienzyme complexes.
- 3.3** Brief the following:
(a) Proximity effect, (b) Orientation effect, (c) Isozymes and Ribozymes, and (d) Turn over number.

- 3.4** Taking an example, compare the enzyme catalyzed and the uncatalyzed reactions.
- 3.5** How do you study the enzyme kinetics experimentally based on initial rate data?
- 3.6** Discuss the following theories to explain the formation of ES complex:
 (a) Lock and Key Model
 (b) Induced Fit Theory
 (c) Substrate Strain Theory
- 3.7** Derive the Michaelis–Menten expression for a single substrate enzyme mediated biochemical reaction stating the assumptions.
- 3.8** Discuss the influence of pH, temperature, fluid forces and chemical agents on enzyme activity.
- 3.9** Explain the effect of activators, product concentration, time and the radiations on enzyme activity.
- 3.10** What are allosteric effectors and allosteric enzymes? How do you classify the allosteric enzymes?
- 3.11** Derive an equation for the half-life period and enzyme deactivation.
- 3.12** What is enzyme inhibition? Discuss all the types of inhibitions in detail.
- 3.13** Derive the integral Michaelis–Menten equation. Further discuss the transformations of Michaelis–Menten equation.
- 3.14** For the enzyme catalysis, the following mechanism is shown with usual notations. Using the steady-state approximation for the complex ES, derive the rate equation for the formation of the product during the initial stages.



- 3.15** For an enzyme–substrate system obeying the MM mechanism, the rate of the product formation when the substrate concentration is very large has a limiting value of $0.003 \text{ mol}/\text{dm}^3$. At a substrate concentration of $285 \text{ mg}/\text{dm}^3$, the rate is half this value. Calculate the ratio k_1/k_2 assuming $k_2 \ll k_1$.
- 3.16** Determine the MM parameters v_{\max} and K_m for the reaction:



The rate of reaction is a function of urea concentration as shown in the following table:

[C] urea (kmol/m^3)	0.20	0.02	0.01	0.005	0.002
$-r$ urea ($\text{kmol}/\text{m}^3\text{-s}$)	1.08	0.55	0.38	0.2	0.09

Finally, express the rate equation for the above.

- 3.17** The following data have been obtained for two different initial enzymes concentrations for an enzyme-catalyzed reaction.

$[E]_0 = 0.021 \text{ g/L}$								
$r = v \text{ (g/L-min)}$	1.14	0.87	0.70	0.59	0.50	0.44	0.39	0.35
$[S] \text{ (g/L)}$	20.0	10.0	6.7	5.0	4.0	3.3	2.9	2.5
$[E]_0 = 0.00935 \text{ g/L}$								
$r = v \text{ (g/L-min)}$	0.67	0.51	0.41	0.34	0.29	—	—	—

Find the following: (a) K_m (b) v_{\max} for $[E]_0 = 0.021 \text{ g/L}$ (c) v_{\max} for $[E]_0 = 0.00935 \text{ g/L}$ and (d) k_2 by using Hanes–Wolf method of transformation.

- 3.18** During the hydrolysis of urea, which shows inhibition, the following data is obtained.

$[S] = 0.2 \text{ M}$		$[S] = 0.02 \text{ M}$	
$1/v$	$[I]$	$1/v$	$[I]$
0.22	0	0.68	0
0.33	0.0012	1.02	0.0012
0.51	0.0027	1.50	0.0022
0.76	0.0041	1.83	0.0032
0.88	0.0061	2.04	0.0037
1.10	0.0080	2.72	0.0044
1.15	0.0093	3.46	0.0059

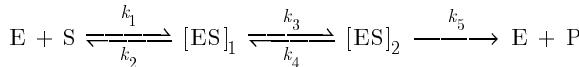
Where $v = \text{mol/L-min}$, and $[I]$ is inhibitor molar concentration.

- (a) Determine the value of K'_m for this reaction.
 (b) What type of inhibition is this? Substantiate the answer.
 (c) Based on the answer (b), what is the value of K_I ?
- 3.19** Eadie has measured the initial rate of hydrolysis of acetylcholine substrate by dog serum which is a source of enzyme in the absence and presence of prostigmine, an inhibitor of $1.5 \times 10^{-7} \text{ mol/L}$ and obtained the following data:

Substrate concentration (mol/L)	Initial reaction rate	
	$\left(\frac{\text{mol}}{\text{L-min}} \right)$	$\left(\frac{\text{mol}}{\text{L-min}} \right)$
	(Absence)	(Presence)
0.0032	0.111	0.059
0.0049	0.148	0.071
0.0062	0.143	0.091
0.0080	0.166	0.111
0.0095	0.200	0.125

- (a) Is prostigmine competitive or non-competitive?
 (b) Evaluate the Michaelis–Menten parameters in the presence of inhibitor.

- 3.20** Consider the following reaction sequence:



Develop a suitable rate expression for the product formation $[v = k_5[ES]_2]$ by using the Michaelis–Menten equation approach and the quasi steady state approach.

Hint: Michaelis–Menten equation approach:

Step 1: Find $r = k_5[ES]_2$

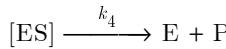
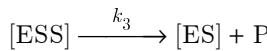
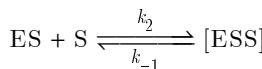
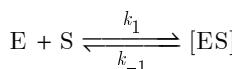
Step 2: $[E]_0 = [E] + [ES]_1 + [ES]_2$

Step 3: Substitute $[E]_0$ in step 1

Step 4: $r = +d[P]/dt = -d[S]/dt = k_3[ES]$

Step 5: Substitute $[ES]$ from step 3.

- 3.21** Suppose that enzyme has two active sites so that the substrate is converted to product with the reaction sequence as shown:



Derive a rate expression for product formation assuming quasi state for $[ES]$ and $[ESS]$.

Hint: Consider $d[ES]/dt = 0$; $d[ESS]/dt = 0$

$$[E]_0 = [E] + [ES] + [ESS]$$

Find $[ES]$, $[ESS]$. Equate to $d[P]/dt = k_3[ESS] + k_4[ES]$ then from MM equation find $r = +d[P]/dt$.

- 3.22** Calculate the time required for the conversion of 80% urea to ammonia and carbon dioxide in a 0.5 dm^3 vessel batch reactor. The initial concentration of urea is $0.1 \text{ mol}/\text{dm}^3$ and urease concentration is $0.001 \text{ M}/\text{dm}^3$.

The reaction is carried out isothermally at which $K_m = 0.0266 \text{ g/mol}/\text{dm}^3$.

Hint: Use equation $t = K_m/v_{\max} \ln 1/(1 - x) + S_0 \cdot x/v_{\max}$

Solve for v_{\max} , K_m and get t .

- 3.23** The following data have been obtained on an enzyme catalyzed reaction at various substrate concentrations:

[S], (mM)	7.5	12.5	20.0	32.5	62.5	155.0	203
Rate, r							
(μ mol/L-S)	0.067	0.095	0.119	0.149	0.185	0.191	0.195

Plot r vs $[S]$ and calculate the value of K_m and the limiting rate.

- 3.24** Michaelis-Menten constant of an enzyme hexokinase for glucose substrate is 0.15 mM and for fructose substrate is 1.5 mM.

Assuming that v_{\max} is same for both the substrates, calculate the values for the rate as v_{\max} per cent for each substrate, when $[S] = 0.15$ mM, 1.5 mM and 15 mM. Further, show that which substrate does hexokinase show a greater affinity for?

Hint: According to the Michaelis-Menten equation as per 3.12 or 3.15, we have

$$r = \frac{v_{\max}[S]}{K_m + [S]}$$

It is given that v_{\max} is same for the substrates and $K_m = 0.15$ mM for glucose and $K_m = 1.5$ mM for fructose.

For the concentration of substrates as given in the problem, $[S] = 0.15$ mM, for glucose and fructose, we can find out the values for respective substrates as

For glucose, at $[S] = 0.15$ mM, the value of r or v is 50% v_{\max} , since $[S] = K_m$.

Similarly, for fructose, at the above substrate concentration, the value of r or v is 9.1% v_{\max} .

In the same way, we can find out the values for other concentrations of substrate.

For $[S] = 1.5$ mM, for glucose, r or v is 91% v_{\max} ; for fructose it is 50% v_{\max} , since $[S] = K_m$.

For $[S] = 15$ mM, for glucose, it is 99% v_{\max} ; and for fructose it is 91% v_{\max} .

From the above estimations, it should be clear that hexokinase has more affinity towards glucose than fructose.

- 3.25** The following data is obtained in an enzyme catalyzed reaction. Using the same determine the values for Michaelis-Menten constant K_m , and the maximum velocity of the reaction.

Data:

S,
mol/lit. 4.1×10^{-3} 9.5×10^{-4} 5.2×10^{-4} 1.03×10^{-4} 4.96×10^{-5} 1.05×10^{-5}
 $r \times 10^6$

mol/lit.	177	173	125	106	80	67
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Hint: Using the data given above, determine the reciprocal values of S and r for each value given i.e. find out the values of $1/S$ and $1/r$ to get the linear profile on the ordinates of $1/r$ vs. $1/S$.

Next, plot all the values of $1/r$ vs. $1/S$ to get a straight line. Make use of Lineweaver-Burk method as shown in Figure 3.25.

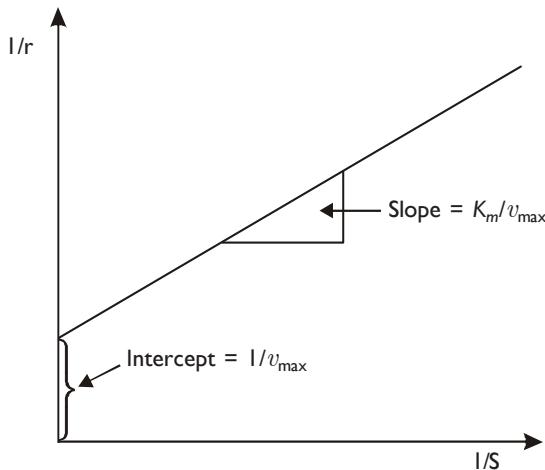


Figure Exercise 3.25.

From the graph, find out $1/v_{\max}$ and the slope of the line is K_m/v_{\max} . Then finally find out K_m and v_{\max} .

3.26 An antibiotic, Penicillin is hydrolyzed and thereby rendered inactive by an enzyme Penicillinase, usually present in the resistant bacteria, *Staphylococcus aurens*.

The mass of this enzyme measured is 29.6 k Daltons. The amount of penicillin hydrolyzed in 1 minute in a 10 ml solution containing 10^{-9} grams of purified Penicillinase was measured as a function of concentration of Penicillin.

Assume that the concentration of Penicillin doesn't change appreciably during the assay. Using the data given below,

Data:

Penicillin						
$\times 10^5$ M	0.1	0.3	0.5	1.0	3.0	5.0
Amount hydrolyzed,						
moles $\times 10^9$	0.11	0.25	0.34	0.45	0.58	0.61

Investigate the following:

- Does Penicillinase appear to obey the Michaelis-Menten kinetics? If so, find out the values for K_m and v_{\max} .
- The turn over number (TON) of Penicillinase under these experimental conditions. Further, assume one active site per enzyme molecule.

Hint: As per the given data, it is found that Penicillin is a substrate and the amount hydrolyzed is the rate. Therefore, for the above-mentioned values find out the reciprocals for each and represent in terms of $1/r$ and $1/S$.

Plot the graph of $1/r$ vs. $1/S$ to get the straight line as shown in Figure 3.26.

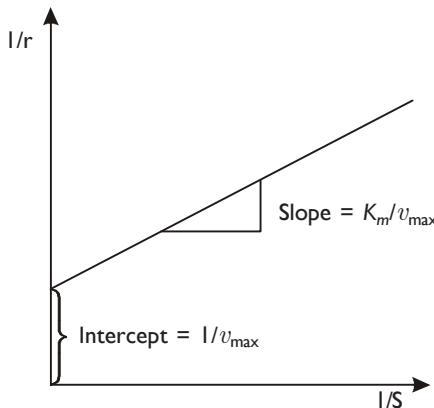


Figure Exercise 3.26

Find K_m and v_{\max} from the graph. The values obtained are the solution for (i).

In the second part of the problem, to find the TON:

Molecular weight = 29.6 grams/gram-mole = 1 Dalton.

29.6 grams implies 1 gram-mole

$$1 \text{ gram implies } \frac{1}{29.6 \times 10^3} \text{ gram-mole.}$$

Amount of Penicillinase present = 1×10^{-9} grams. (Given)

Now converting into gram-moles implies

$$(1 \times 10^{-9}) \left\{ \frac{1}{29.6 \times 10^{-3}} \right\}$$

The concentration of Penicillinase, an enzyme is $[E] = 0.03378 \times 10^{-12}$ moles/m³.

Using the relation:

$$v_{\max} = k_2 [E_0]$$

$$k_2 = v_{\max} / [E_0]$$

Here k_2 is the TON. Substitute the values of v_{\max} and $[E_0]$ and find k_2 .

- 3.27** A pesticide inhibits the activity of a particular enzyme E, which can therefore be employed in assay to determine the presence of inhibitor in an unknown sample.

During the assay in the laboratory, the following data is obtained. Data:

S, mol/lit:	3.3×10^{-4}	5.0×10^{-4}	6.7×10^{-4}	1.65×10^{-3}	2.21×10^{-3}
r, mol/lit-min:					
No Inhibitor-	56	71	88	129	149
With Inhibitor-	37	47	61	103	125

Investigate the following:

- Is the pesticide competitive or non-competitive? Further, find the values of K_I , v_{max} and K_m .
- Suppose after 50 ml of the same enzyme solution in Part (i) is mixed with 50 ml of 8×10^{-4} M substrate and 25 ml of sample, the initial rate observed is 43 μ moles/min. what concentration of pesticide is available in the unknown sample, assuming that no other inhibitor and substrate are present in the sample.

Hint: Using the data, write the reciprocal values for S and r (both for no inhibitor and with inhibitor). After getting the values for $1/[S]$ and $1/r$ respectively, plot the graph $1/r$ vs. $1/[S]$.

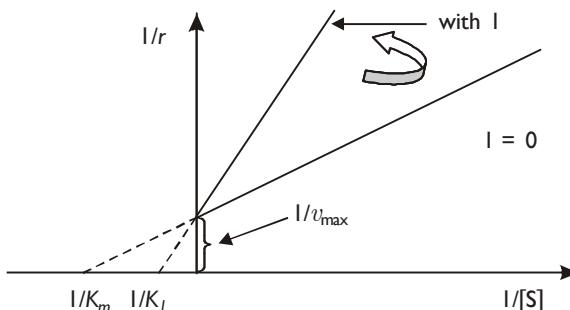


Figure Exercise 3.27

From Figure 3.27, calculate K_m , v_{max} and K_I for both competitive and non-competitive inhibition, although the inhibition is competitive.

- To find the pesticide or inhibitor concentration, $[I]$:

Enzyme solution is 50 ml

Substrate solution is 50 ml and sample is 25 ml. (Given)

Total solution is 125 ml.

The concentration of substrate is $\frac{8 \times 10^{-4}}{125} = 6.4 \times 10^{-6}$ M/ml. = $[S]$

Finally, using the equation for competitive inhibition, find $[I]$.

- 3.28** For the initial substrate concentration, the initial rate of reaction with and without inhibitor is shown below.

Data:

Substrate, $\mu\text{m}/\text{lit.}$	6.7	3.5	1.7	0.3
Initial reaction rate, $\mu\text{m}/\text{min.}$				
$I = 0$	0.30	0.25	0.16	—
$I = 146 \mu\text{m}$	0.11	0.08	0.06	—

Identify which type of inhibition holds good for the above and also find all the rate constants.

Hint: From the above data, write the reciprocals for $[S]$ and r for both without inhibitor and with inhibitor, i.e. $1/[S]$ and $1/r$ respectively. Plot the graph $1/[S]$ vs. $1/r$ using the above values.

Based on the nature of graph, predict the type of inhibition.

Using the intercept, slope values find out the values for K_m , K_I , and v_{\max} , etc.

- 3.29** In the study of enzyme kinetics, the following results were obtained.

[S], mol/lit.:	1.0	0.75	0.60	0.50	0.40	0.33	0.25
r , mol/lit-min:							
$[I] = 0:$	2.50	2.44	2.08	1.89	1.67	1.39	1.02
$[I] = 1.26:$	2.17	1.82	1.41	1.30	1.09	—	—

Using the above information, identify which type of inhibition is valid and further determine the kinetic parameters using double-reciprocal method.

- 3.30** The following data were obtained on an enzyme-catalyzed reaction as shown below:

[S], (mol/L)	0.002	0.005	0.020	0.040	0.060	0.080	0.100
r , (min)	0.045	0.115	0.285	0.380	0.460	0.475	0.505

Evaluate K_m using (a) the r vs $[S]$ plot and (b) the Lineweaver–Burk plot.

Chapter 4

Industrial Enzymes and Applications

All enzymes are derived from three sources—animal, plant and microbial sources. Animal and plant sources have been found to be relatively expensive, for example *rennin* which is derived from calf's stomach and *papain* derived from unripe papaya. On the contrary, the enzymes derived from microbial sources are comparatively cheap and the process can be scaled up easily. As microbes have rapid doubling time, these are exploited both commercially and industrially to meet the current market demands. Only those microbes or microorganisms certified as safe are used to synthesize enzymes particularly to be used in food and pharmaceutical industry.

In this chapter we shall survey some of the enzymes which are utilized in the absence of life. Such biological catalysts include extracellular enzymes secreted by cells in order to degrade polymeric nutrients into molecules small enough to permeate cell walls. Applications of various enzymes like hydrolytic, proteolytic, non-hydrolytic, etc. are discussed. In addition to these, the enzyme market and the commercial production of enzymes are also included.

Recombinant DNA Technology also known as Genetic Engineering is a much heard concept in the present era. This is also utilized in synthesizing newer enzymes from the microbial sources in order to meet the current market demands.

Certain applications demand the use of a pure extract, for example an enzyme, glucose oxidase, which is required in the desugaring of eggs, should be free of any protein splitting enzymes so that the protein part of an egg is essentially not destroyed. In the same way, the proteolytic enzymes that are used in the process of meat tenderization, to be injected before slaughtering, should be free of any compound which can cause serious physiological changes. Undoubtedly, these enzymes are also used in the clinical and medical diagnosis.

Basically, enzymes are intracellular and extracellular. Intracellular enzymes are present in cells. In order to synthesize, the cells require grinding,

mashing, lysing or otherwise killing and splitting whole cells that open frees the intracellular enzymes. While extracellular enzymes, which are utilized in the absence of life, degrade the polymeric nutrients into small monomers which can permeate the cell walls.

4.1 HYDROLYTIC ENZYMES

Hydrolytic enzymes are not only important in macroscopic degradations such as food spoilage, starch thinning and waste treatment, but also in the chemistry of ripening of picked green fruit, self-lysis of dead whole cells (autolysis), desirable aging of meat, curing cheese, preventing beer haze, texturizing candies, treating wounds and desizing textiles.

The three groups of enzymes those involved in the hydrolysis of ester, glycosidic, and various nitrogen bonds are esterases, carbohydrases and enzymes for nitrogen carrying compounds. Table 4.1 will illustrate these hydrolytic enzymes with the substrate on which they act upon and the product released.

Table 4.1 Hydrolytic enzymes

Enzyme	Substrate	Products of hydrolysis
Esterases		
Lipases	Act upon glycerides (fats)	Liberate glycerol and fatty acids (Glycerol + Fatty acids)
Phosphatases		
Lecithinase	Act upon ² lecithin	Liberate choline, phosphoric acid with some amount of fat (¹ Choline + H ₃ PO ₄ + Fat)
Pectin esterase	Act upon pectin methyl ester	Liberate methanol with polygalacturonic acid (Methanol + Polygalacturonic acid)
Carbohydrases		
Fructosidases	Act upon sucrose, cane sugar	Liberate fructose with glucose (Fructose + Glucose)
Maltase	Act upon maltose, malt sugar	Produce two molecules of glucose (Glucose + Glucose)
Lactase	Act upon lactose, milk sugar	Liberate galactose with glucose (Galactose + Glucose)
Amylases	Act upon polysaccharide, starch	Produce oligosaccharides with glucose (Glucose + Oligosaccharides)
Nitrogen carrying compounds		
Proteinases	Hydrolyze proteins	Yielding polypeptides (Many peptides)
Polypeptidases	Act upon proteins	Liberating monomers of proteins called amino acids
Desaminases		
Urease	Hydrolyze urea	Liberation of carbon dioxide and ammonia (CO ₂ + NH ₃) [NH ₂ CONH ₂ → CO ₂ + NH ₃] Aspartic acid with ammonia (Aspartic acid + NH ₃)
Asparaginase	Asparagine	Liberating ammonia with organic acids (Ammonia + Organic acids)
Desaminases	Act upon amino acids	

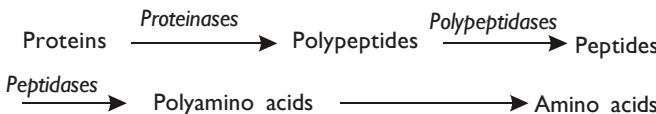
¹ *Choline*: Choline is an organic base and a component of some fats, egg yolk, etc. It belongs to a group of vitamin B.

² *Lecithin*: Lecithin is a naturally occurring lipid having glycerides, nitrogen and phosphorus.

4.2 PROTEOLYTIC ENZYMES

Proteolytic enzymes attack nitrogen-carrying compounds, i.e. proteins. If the attack is on the terminal group of polyamino acids they are called *exopeptidases*. Whereas, if the attack is on the internal linkages they are called *endopeptidases*.

Proteins on degradation yield amino acids which are shown as follows:



These protein-splitting enzymes are synthesized initially in an inactive form because an inactive form is suitable for storage or for transport from the site of synthesis to the site of activity. Table 4.2 gives the list of enzymes in inactive and active forms.

Table 4.2 Enzymes in inactive and active forms

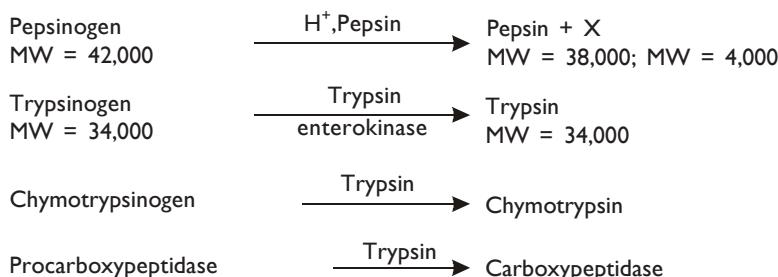
Inactive form	Active form
Pepsinogen (42,000)*	Pepsin (38,000)*; activates in the presence of a proton with a reduction in the molecular weight (MW).
Trypsinogen (34,000)*	Trypsin (34,000)*; activates in the presence of enterokinase and retains the same MW.
Chymotrypsinogen	Chymotrypsin; activates in the presence of trypsin
Procarboxypeptidase	Carboxypeptidase; activates in the presence of trypsin

Note: *indicates the figures in terms of molecular weights (MW).

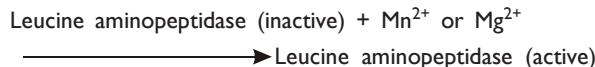
4.2.1 Methods of Activation

Proteolytic enzymes are activated by using the following methods:

From a precursor: The activation of pepsin and trypsin is autocatalytic; the inactive enzyme precursor is a substrate for the active form of the enzyme, the reaction product being more of the activated enzyme. Proteolytic attack is not observed after the initial conversion of inactive trypsin or pepsin to the active form.



By presence of a metal ion: It requires several specific metal ions for activation. Dialysis of the enzyme containing solution with resultant loss of metal ions is a standard form of determining specific metal ion requirements of enzyme.



Proteolytic enzymes are also known as *proteolytic enzymes* and they have the power of decomposing or hydrolyzing proteins.

Proteases also known as *proteinases* are capable of breaking up of proteins into amino acids and these are used during protein metabolism.

4.2.2 Applications of Proteolytic Enzymes

Different forms find use in several applications:

1. In meat processing industry, proteolytic enzymes are used for tenderization and aging of meat. The tenderization of individual meat pieces by commercial tenderizer products depends on proteolytic action of relatively inexpensive and non-heat labile plant proteases such as papain and bromelain. Aging of whole meat carcasses before cutting and packaging is normally accomplished by controlled partial self-digestion (autolysis) of the bled meat at about 15°C in the presence of UV radiations, which act as a germicidal agent thus preventing the surface growth of undesirable microorganisms.
2. In tanning industry, various ground pancreas preparations from different animal sources that contain all the digestive proteases, including trypsin, are useful in dehairing of animal hides and for simultaneous removal of other non-collagen proteins from the hides. Pepsin itself attacks collagen, the fibrous skin protein which is converted into leather.
3. In dairy industry, rennin or rennet is used and acts by removing glycopeptides from soluble calcium casein to yield a relatively insoluble calcium paracaseinate, which precipitates to form desired curd.
4. In clinical and medicinal, proteolytic enzymes act as digestive aids and cleansers of serious wounds. Trypsin reduces inflammation and swelling associated with the internal injuries and infections by dissolving blood clots and extracellular-protein precipitates, by locally activating other body defenses which do the same thing, or both. Severe lung infections are also reduced by administering proteolytic enzymes.

5. The other applications include laundry aids (dry-cleaning), as detergents, in the recovery of silver from photographic films and in the digestive aids.

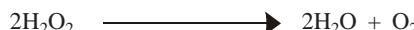
4.3 NON-HYDROLYTIC ENZYMES

Non-hydrolytic enzymes are in demand in current and developing industrial technology. For example, glucose oxidase, a very useful enzyme, finds applications whenever glucose or oxygen removal is desirable.

Glucose oxidase is used in the desugaring of eggs. Dried egg powder undergoes an undesirable darkening due to reaction between glucose and protein. This reaction, commonly called a *Browning reaction* or a *Maillard reaction*, can be prevented by addition of glucose oxidase.

In the production and storage of orange soft drinks, canned beverages, dried food powders, mayonnaise, salad dressing, and cheese slices, the presence of oxygen is usually avoided by addition of glucose oxidase and, in the case of cheese wrapping, glucose itself. Such enzyme additions increase the shelf life of food products by continually removing the oxygen that diffuses through the food packaging.

Hydrogen peroxide produced in the glucose oxidase-catalyzed reaction has an anti-bacterial action; if the presence of hydrogen peroxide is undesirable in the product, catalase is added, which catalyzes the reaction.



4.3.1 Recent Applications of Non-hydrolytic Enzymes

The following list of applications will describe the recent trend of non-hydrolytic enzymes.

1. L-malate is produced from fumarate by using fumarase.
2. ATP is produced from adenine by using enzymes from microbial sources.
3. NAD is produced from adenine and nicotinamide by using microbial enzymes.
4. Fructose is produced from glucose by isomerization employing glucose isomerase.
5. Fructose is also produced from sorbitol by using sorbitol dehydrogenase.
6. L-dopa, a derivative in the production of methyl dopa, an antidepressant is produced from α -keto acid by using transaminase.
7. DL-amino acids (dextro and laevo) are produced by enzyme called aminoacylase which is immobilized.
8. Transformation of fatty acids to poly unsaturated fatty acids is obtained by employing fatty acid desaturase.
9. Galactono lactose is produced from whey by using glucose oxidase and galactose oxidase.
10. Using an enzyme, aspartase; L-aspartate is produced from fumarate.

4.4 ENZYMES OF INDUSTRIAL IMPORTANCE

Table 4.3 provides a list of enzymes and their sources and applications.

Table 4.3 Enzymes of industrial importance

Name	Source	Application
Starch liquefying amylases		
Diastase	Extracted from malt	Used in digestion as digestive aid, supplement to bread and as syrup
Taka diastase	<i>Aspergillus oryzae</i>	Used in digestion as digestive aid, supplement to bread and as syrup
Amylase	<i>Bacillus subtilis</i>	Used in desizing of textiles, as syrup and in alcohol manufacture
Acid resistance amylase	<i>Aspergillus niger</i>	Used as digestive aid
Animal and vegetable proteases		
Trypsin	Extracted from animal pancreas	Used in medical field, meat industry, and beer haze removal
Pepsin	Extracted from animal stomach	Used as digestive aid and in the tenderization of meat before slaughtering of animals
Rennet, Papain, Bromelain, Ficin	Extracted from Calf's stomach, unripe papaya, pineapple, fig respectively	Used in cheese manufacture, tenderization of meat and also in beer haze removal
Microbial proteases		
Protease	<i>Aspergillus oryzae</i>	Used in flavouring industry, beer haze removal, as a feed supplement, and digestive aid
Protease	<i>Aspergillus niger</i>	Used as a feed supplement, and digestive aid
Protease	<i>Bacillus subtilis</i>	Used in the manufacture of detergents, recovery of silver from photographic films, recovery of gelatin and in fish solubles
Varidase	<i>Streptococcus spirilla</i>	Used in medical field
Other commercial enzymes		
Cytochrome C	Yeast (<i>Candida</i>)	Used in medical field
Invertase	<i>S. cerevisiae</i>	Used in confectionaries of sugar to prevent crystallization
Pectinase	<i>Sclerotina libertina</i>	Used to increase the yield and in the clarification of canejuice
Laccase	<i>Coriolus versicolor</i>	Used in Lacquer drying

4.5 ENZYME MARKET

In 1996, the US sales of industrial enzymes were \$372 million and sales are projected to grow to \$686 million by 2006 (Table 4.4).

Table 4.4 Industrial enzyme market

Application	1996 sales (US \$ in millions)	2006 projected sales (US \$ in millions)
Food	170	214
Detergents	160	414
Textiles	27	32
Leather	11	13
Paper and Pulp	01	05
Others	03	08
Total	372	686

Source: *Bioprocess Engineering: Basic Concepts*, Shuler and Kargi, 2005. Prentice-Hall of India, New Delhi.

The products made in enzyme processes are worth billions of dollars. For example, proteases which hydrolyze proteins to peptides constitute a large group (about 60%) of enzyme market.

Industrial proteases are obtained from bacteria (*Bacillus*), moulds (*Aspergillus*, *Rhizopus* and *Mico*), animal pancreas and plants.

4.6 PRODUCTION OF ENZYMES ON A COMMERCIAL SCALE

On a commercial scale, enzymes are produced by using the overproducing strains of certain organisms. Among the various enzymes produced are proteases like subtilisin, rennet, etc., hydrolases like pectinase, lipase, lactase, etc. and isomerases which includes glucose isomerases.

Overall the production of enzymes involves the following steps discussed in detail under separate subheadings:

4.6.1 Isolation of Microorganisms, Strain Development and Preparation of Inoculum

The first and the foremost step is the isolation of microorganisms. This is done on the culture media using certain microbiological techniques. The objectives of isolation of a suitable microorganism are:

- To produce the product enzyme in higher amounts or the yield to be high enough and subsequently producing low amount of metabolites.
- To complete the fermentation process in a very short period of time.
- To utilize the microorganisms of low cost culture.

Once a suitable microorganism is obtained its enzyme producing ability is optimized by rendering the conditions like pH and temperature to improvise the strains and formulate the culture medium.

The strains of microorganisms are developed by employing the mutagens i.e. agents like chemicals which are mutagenic in nature and also by using ultraviolet light.

Later, the inoculum of enzyme producing strains developed after treatment of mutagens is synthesized by multiplying its spores and mycelia on the liquid broth.

4.6.2 Medium Formulation and Preparation

Medium formulation has to be carried out carefully to see that it suits the conditions for good amount of enzyme production than the microbial growth.

In this context, the culture medium is formulated by providing all the necessary nutrients supporting the enzyme production. An ideal medium must have a cheap source of carbon, nitrogen, amino acids, growth promoters, trace elements and some amount of salts and even buffer.

All the precautions are taken to see that pH is maintained during fermentation process. It is a usual practice that for a specific microbe, pH, temperature and formulation of culture medium is optimized before inoculation. For this, experience based on a number of trials is needed.

The production of enzymes increases with the concentration of culture medium.

Typically a culture medium is made of carbohydrate sources like molasses, barley, corn, wheat, etc. and also the protein sources like meals of soybean, cotton seed, whey, corn steep liquor, etc.

4.6.3 Sterilization and Inoculation of Medium, Maintenance of Culture and Fluid Filtration

The inoculation medium needs sterilization and is carried out batchwise in a large-scale fermenter. But these days continuous sterilization is gaining a lot of importance to avoid certain constraints that are seen in the batch processes.

Once the medium is sterilized, inoculation with a sufficient amount of inoculum is done to start the fermentation process. Earlier, surface culture technique, so called a traditional method where the inoculum remains on the upper surface of the broth was adopted during the fermentation processes.

At present, it is the submerged culture practised in the fermentation processes. This has certainly an advantage over the former one as the probability of infection and getting contaminated is minimized to the maximum extent. Consequently, the yield of enzymes is more in submerged culture techniques.

During the fermentation process, the growth conditions viz. pH, temperature and oxygen are maintained at the optimum level. In fact, these

conditions differ from microbe to microbe and even in the same species of a microbe. There is every chance of foaming taking place inside the fermenter during the process. To control this, certain amount of oil is added.

After a period of 30 to 120 hours of incubation, extracellular enzymes are produced by the inoculated microbe in the culture medium. It is seen that most of the enzymes are produced actually when the exponential growth phase is complete, but in certain cases the enzymes are produced during the exponential phase.

During fermentation process, it is also observed that metabolites around 10–15% are getting produced besides the desired enzymes as products and for which the first step mentioned above is carried out carefully to see that the amount of metabolites formed is less compared to the amount of enzymes. Metabolites produced are removed by adopting the purification steps.

Once the fermentation process is complete, the broth is retained at 5°C to avoid contamination. Later the recovery steps are adopted to get the enzymes. It is also a fact that the recovery of enzymes from a bacterial broth is more difficult than the filamentous broth as fungal broth can be directly filtered or centrifuged after pH adjustment.

Therefore, the bacterial broth is first treated with calcium salts to precipitate calcium phosphate that aids in separation of bacterial cells and colloids. Subsequent to this, it is then filtered and centrifuged to remove the cell debris and recover the enzymes. This is followed by the purification step as discussed below.

4.6.4 Enzyme Purification

This is the last step and indeed a very complex process. The important steps employed are:

- Vacuum evaporation, to concentrate the solution at low temperatures or even achieved by ultrafiltration.
- Clarification of concentrated enzyme by a polishing filtration to remove other microbe if appeared by chance during the process.
- Addition of preservatives or stabilizers viz. sodium benzoate, calcium salts, etc.
- Precipitation of enzymes with acetone, alcohols or inorganic salts, e.g. sodium sulphate and ammonium sulphate.
- Freeze drying or vacuum drying or spray drying to dry the enzymes consisting precipitate.
- Packaging and storage or transportation for commercial supply.

Note: Certain techniques mentioned above are discussed in the Chapter 9, Downstream processing.

Table 4.5 gives a list of microorganisms producing enzymes.

Table 4.5 Organisms producing enzymes

<i>Microorganisms</i>	<i>Enzymes produced</i>
Bacterial origin:	
<i>Bacillus cereus</i>	Penicillinase
<i>B. coagulans</i>	α -amylase
<i>B. licheniformis</i>	Protease and α -amylase
<i>E. coli</i>	Penicillin acylase and β -galactosidase
<i>Klebsiella pneumoniae</i>	Pullulanase
Actinomycetes:	
<i>Actinoplanes sp.</i>	Glucose isomerase
<i>Microorganisms</i>	<i>Enzymes produced</i>
Fungal origin:	
<i>Aspergillus niger</i>	Amylases, proteases, pectinase, etc.
<i>A. oryzae</i>	Lipases, amylases, etc.
<i>Candida lipolytica</i>	Lipase
<i>Trichoderma viride</i>	Cellulase
<i>S. cerevisiae</i>	Invertase

SUMMARY

In this chapter, we learnt the following:

- Enzymes are classified as hydrolytic, proteolytic and non-hydrolytic.
- Esterases, carbohydrases and phosphatases are hydrolytic enzymes.
- Proteolytic enzymes are divided into exopeptidases and endopeptidases.
- Proteolytic enzymes are activated from a precursor and metal ions. They are also called proteolytic enzymes, and hydrolyze proteins.
- Enzymes can be intracellular and extracellular and are derived from three sources: animal, plant and microbial sources.
- Glucose oxidase is a non-hydrolytic enzyme and finds use in current and developing industrial technology.
- Glucose oxidase is added to remove oxygen which otherwise would react with proteins, producing dark brown colour and further affects the flavour and the taste of the product.
- The steps in the commercial production of enzymes are:
 - Isolation of microorganisms, strain development and preparation of inoculum.
 - Formulation of medium and preparation.

- (c) Sterilization and inoculation of medium, maintenance of culture and fluid filtration.
- (d) Enzyme purification.

EXERCISES

- 4.1** Give a detailed classification of enzymes with their applications.
- 4.2** What are proteolytic enzymes? Write on the methods of their activation.
- 4.3** What are hydrolytic enzymes? Give a detailed classification with respect to substrate on which they act upon and the product released.
- 4.4** Enlist the applications of non-hydrolytic enzymes.
- 4.5** Enlist some of the enzymes of industrial importance with respect to their sources and uses.
- 4.6** How does glucose oxidase act in the preservation of canned food products?
- 4.7** Describe all the steps incorporated in the production of enzymes on a commercial scale.

Chapter 5

Immobilized-Enzyme Technology

The remarkable catalytic properties of enzymes make them very attractive for use in processes where mild chemical conditions and high specificity are required. To this end, biocatalysts that have been immobilized have been developed where the original soluble enzyme has been modified to produce an insoluble material which can be easily recovered from the reaction mixture.

An immobilized enzyme refers to an enzyme that has been confined or localized so that it can be reused continuously. As we shall investigate in the succeeding discussions as to why immobilization is desirable, it has made this concept very attractive whenever a large throughput is required. Moreover, the ability to confine an enzyme in a well-defined, predetermined space provides opportunities for applications unique to immobilized enzymes.

This chapter emphasizes the techniques of immobilization of enzymes with their advantages and applications. Various strategies to immobilize, materials for immobilization, methods of immobilization and limitations are also covered. Towards the end, is discussed the diffusional mechanisms and limitations in immobilized enzyme systems, immobilized cell systems and methods, also a brief discussion on the transport across cell membranes and finally typical immobilized cell reactor configurations are also covered.

5.1 ENZYME STABILIZATION

In the previous discussion, we have come across the factors that deactivate enzymes (enzyme deactivation). The factors that cause the deactivation are pH, temperature, fluid forces, chemical agents like urea and hydrogen peroxide, irradiations, etc. The declination of enzyme activity is a critical characteristic, and this has to be avoided. Of course, this is valid when long-term use of an enzyme in a continuous flow reactor is considered.

Some strategies are required to stabilize enzymes called *enzyme stabilization*. The following are the strategies adopted in stabilizing the activity of enzymes.

1. Addition of stabilizing compounds to the storage medium, if the enzyme is to be stored or to the reaction medium if the enzyme is to be used for the reaction
2. Chemically modifying soluble protein
3. Immobilizing protein on or within an insoluble solid or the matrix

Table 5.1 provides some examples of enzyme stabilization.

Table 5.1 Enzyme stabilization

<i>Enzyme</i>	<i>Method of stabilization</i>	<i>Effect</i>
Chymotrypsin	Stabilized by the addition of 50–90% glycerol	Improves resistance to proteolysis, i.e. withstands hydrolysis of proteins
Trypsin	Stabilized by the addition of polyalanyl to amino groups of proteins	Improves resistance to heat, i.e. thermal resistance and to proteolysis
Papain	Stabilized by using glutaraldehyde and structures cross-linked	Imparts better thermal stability
α -Amylase	Stabilized by the addition of 50–70% sorbitol	Imparts better storage property and thermal stability
Glucoamylase	Stabilized by the addition of substrate analogs, glucose and gluconolactone	Imparts better storage property and thermal stability

5.2 IMMOBILIZATION

Immobilization refers to the confinement or localization of enzymes, so that they can be reused continuously. There are certainly some reasons as to why the process of immobilization is desirable. They are as follows:

1. For processing with isolated enzymes
2. Retaining inside a reactor to avoid the loss and consequent replacement
3. In retaining the activity for longer periods of time rather than the enzymes in solution.
4. Increases catalytic efficiency for the multi-step conversion
5. To get the large throughput of the substrate.
6. To avoid wastage of enzymes as they are costly and expensive.

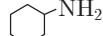
5.2.1 Adsorption Immobilization

To immobilize an enzyme, a support is required on which enzymes get adsorbed. The selection of a support for adsorption immobilization depends on its surface properties. In selecting the support, the following questions should be successfully answered.

1. Will the enzyme adsorb on the surface?
2. Does the material possess functional groups?
3. If native surface is not ideal, can it be chemically modified or coated to facilitate the enzyme attachment?

Table 5.2 illustrates several materials which have been employed for the covalent enzyme immobilization and some of their interesting surface functional groups.

Table 5.2 Insoluble materials/supports for covalent enzyme attachment to immobilize them and their functional groups

<i>Natural supports</i>	<i>Synthetic supports</i>
Cellulose ($-\text{OH}$); consists of hydroxyl group for attachment	Polyacrylamide derivates act as synthetic supports
Carboxymethyl cellulose ($-\text{COOH}$); consists of carboxyl group, also used in ceramic industry to impart viscosity in glaze preparation	Biogel, enzacryl (aromatic amino) 
Agarose also termed sepharose ($-\text{OH}$); consists of hydroxyl group	Polyaminopolystyrene having amino group ($-\text{NH}_2$)
Dextran also termed sephadex ($-\text{OH}$); consists of hydroxyl group	Maleic anhydride copolymers

Other materials which have been used as supports include ceramics, glass, and the metal oxides. When considering the selection of a support and adjustment of its surface properties, one must consider the interaction between the support surface and the reaction mixture. This interaction may cause the fluid environment adjacent to the surface in contact with the enzyme to differ substantially from the bulk fluid environment surrounding the immobilized enzyme catalyst.

Another important role of the support surface is in defining, directly or indirectly, the molecular environment of the enzyme. That is, to some degree the enzyme will contact, at the molecular level, the support surface. The enzyme molecule is structured so as to assume the proper configuration and corresponding activity in its native biological environment. The physical and mechanical properties of the support are also important. The pore size distribution and the porosity of a solid material determines the quantity of enzyme which can be immobilized in the support and the accessibility of the substrate to the enzyme attached to the internal surfaces.

The mechanical strength of the support influences significantly the catalyst suitability for different reactor configurations.

Enzymes can also be adsorbed on a variety of carriers which help practically in regeneration of enzymes. Regeneration is also an important parameter to avoid removal of deactivated enzyme and reloading with a fresh active catalyst.

Table 5.3 lists various carriers for immobilization by adsorption.

Table 5.3 Carriers for immobilization by adsorption

<i>Interaction</i>	<i>Adsorbents employed</i>
Physical adsorption: Weaker interactions observed due to hydrogen bonding and van der Waals forces involved	Activated carbon, silica gel, alumina, starch, clay, etc.
Ionic binding: Involves binding due to exchange of ions. Adsorbents are resinous in nature.	Modified materials: Modified structurally like Tannin-aminohexyl cellulose, Concanavalin, A-Sepharose. Cation exchangers: CMC, Carboxymethyl cellulose, amberlite, catalyst grade CG-50, Dowex 50 Anion exchangers: DEAE (diethyl amino ethyl) cellulose, DEAE sephadex, etc. [Most of the exchangers used in water-softening process]

5.3 METHODS OF IMMOBILIZATION

Basically, there are two methods used for the immobilization of the enzymes:

- Chemical methods
- Physical methods

The method to be adopted greatly influences the property of the resulting biocatalyst. Whether to go for chemical methods or physical methods depends upon the process specifications for the catalyst including such parameters like overall catalytic activity, effectiveness of the catalyst utilization, deactivation, regeneration characteristics and of course cost.

Also the toxicity of immobilization reagents should be considered in connection with the immobilization process of waste disposal and the intended application of the immobilized enzyme catalyst.

5.3.1 Chemical Methods

In chemical methods, covalent bonds are formed with enzymes and are comparatively stronger than physical methods.

There are three categories in the chemical methods of immobilization:

- Enzyme attachment to the matrix by covalent bonds
- Enzyme cross-linking by multifunctional reagents
- Cross-linked enzyme matrix

Covalent binding to facilitate the enzyme attachment is the retention of enzymes on the support surfaces by covalent bond formation. Enzyme molecules bind to support material via some functional groups, such as amino, carboxyl, hydroxyl and sulphhydryl groups. These functional groups must not be in the active site.

One usual way to block the active site is by flooding enzyme solution with a competitive inhibitor prior to covalent binding. The functional groups on the support material are usually activated by using chemical reagents such as cyanogens bromide, carbodiimide and glutaraldehyde.

Figure 5.1 illustrates the three chemical methods.

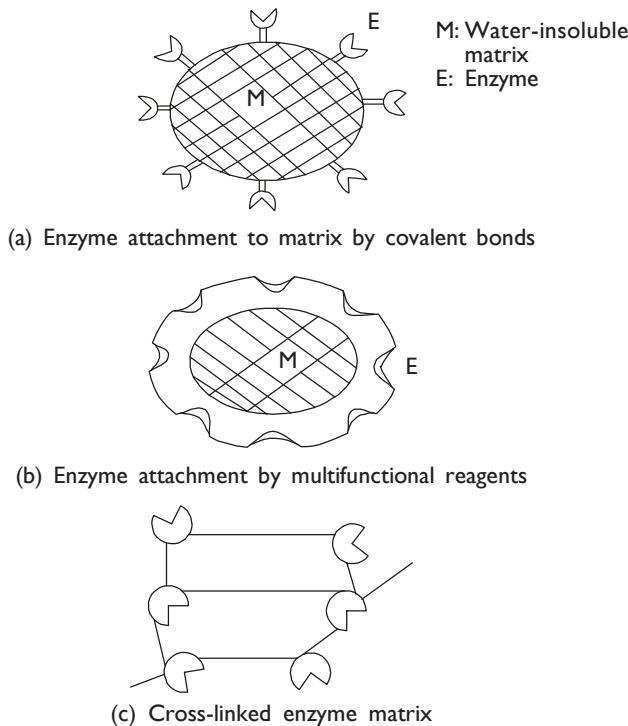


Figure 5.1 Chemical methods of immobilization.

Note: The bonding or the attachment with others should be indicated not into the groove, but outside it. Because the groove indicates the active site of an enzyme.

The cross-linking of enzyme molecules with each other using agents like glutaraldehyde, *bis*-diazobenzidine and 2, 2-disulphonic acid is another way of enzyme immobilization.

Cross-linking can be achieved in several different ways. Enzymes can be cross-linked with glutaraldehyde to form an insoluble aggregate, adsorbed enzymes may be cross-linked, or cross-linking may take place following the impregnation of porous support material with enzyme solution.

The process may cause significant changes in the active site of enzymes, and also severe diffusion limitations may result.

5.3.2 Physical Methods

There are four categories in the physical methods of immobilization:

- Entrapment in porous hollow fibres
- Entrapment in spun fibres
- Entrapment within insoluble gel matrix
- Entrapment within a microcapsule (microencapsulation)

Matrices used for enzyme immobilization are usually polymeric materials such as Ca-alginate, agar, k-carrageenan, polyacrylamide and collagen. Also there are some solid matrices such as activated carbon, porous ceramic and diatomaceous earth that can be used for this purpose. The matrix can be a particle, membrane or a fibre.

Matrix entrapment and membrane entrapment, including micro-encapsulation are the two major methods of entrapment.

Membrane entrapment of enzymes is possible for example, hollow fibre units have been used to entrap an enzyme solution between thin, semipermeable membranes. Membranes of nylon, cellulose, polysulphone, and polyacrylate are commonly used.

Figures 5.2 shows the physical methods.

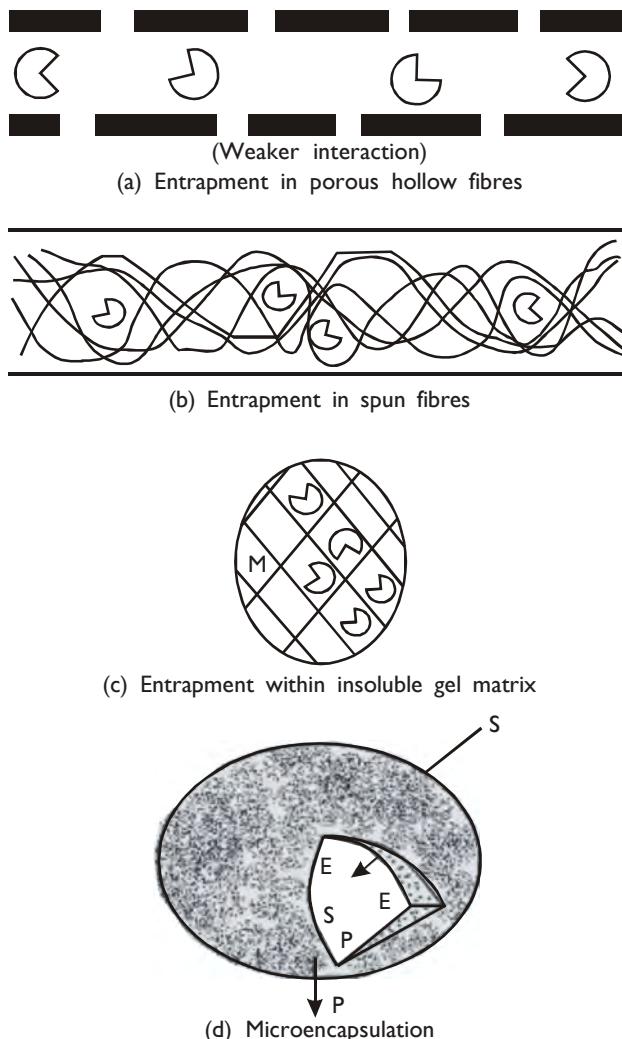


Figure 5.2 Physical methods of immobilization.

In Figure 5.2(d), the dimensions of the capsule are shown as $\phi = 300 \mu\text{m}$ capsule. Capsules surrounded by membranes have pores permitting S and P molecules to enter and leave the capsule.

A special form of membrane entrapment called microencapsulation is also used. In this technique, microscopic hollow spheres are formed. The spheres contain the enzyme solution, while the sphere is enclosed within a porous membrane. The membrane can be polymeric or an enriched interfacial phase formed around a microdrop.

Enzyme entrapment can have certain disadvantages or inherent problems:

- Enzyme leakage into the solution
- Significant diffusional limitations
- Reduced enzyme activity and stability
- Lack of control of microenvironmental conditions

These limitations can be overcome as follows. The enzyme leakage can be overcome by reducing the MW cutoff of the membrane or the pore size of solid matrices. Diffusion limitations can be eliminated by reducing the particle size of matrices and or capsules. It is seen that the reduced enzyme activity and stability are due to the unfavourable conditions relating to the microenvironment and perhaps they are difficult to control.

However, by using different matrices and chemical ingredients, by changing processing conditions, and by reducing the particle or capsule size, more favourable microenvironmental conditions can be obtained.

In general, when enzymes are immobilized for use in engineering systems, a significant decrease in overall activity is observed. The decrease in the activity may be ascribed to the following three effects:

1. *Loss due to deactivation of the catalytic activity by the immobilizing procedure itself.* This includes the destruction of the active sites of the enzyme by the reagents used and the obstruction of the active sites by the support material.
2. *Loss of overall activity by diffusional limitation external to the immobilized system.* This refers to the apparent loss in activity when the rate of reaction is controlled by transport of the substrate from the bulk of the solution to the surface of the carrier of the immobilized catalyst.
3. *Loss of overall activity due to diffusional limitation within the immobilized catalyst matrix.* It can arise when gel entrapment is being considered and can also occur when enzymes are covalently bound within pores in the inert carrier.

5.4 DIFFUSIONAL MECHANISMS AND LIMITATIONS IN THE IMMOBILIZED ENZYME SYSTEMS

It is a known fact that the enzymes which are immobilized, may be on a support porous material, non-porous material or on a matrix exhibit certain alterations in conformities in the structure and also in properties.

Enzyme immobilization is a favoured practice in industry, while enzymes undergo certain constraints so called diffusional limitations.

These diffusional limitations are well understood by knowing the diffusional resistances which are observed at different levels in the immobilized enzymes.

Resistances do vary depending upon the nature of the support materials may be porous or non-porous, hydrodynamic conditions in the vicinity of support material and the distribution of the enzyme, whether inside or on the surface of the support material.

Diffusional resistances observed have a great impact on the rate and kinetics of enzyme catalyzed reaction and overall depends upon the relative rate of reaction rate and the diffusion rate.

This relative rate of reaction rate and diffusion rate can be taken as an index of performance for immobilized enzymes over free enzymes.

The same index can be characterized by using a dimensionless variable or a number called *Damköhler number*, symbol N_{Da} , and is defined as the ratio of the maximum rate of reaction to the maximum rate of diffusion, i.e.

$$N_{Da} = \frac{\text{Maximum rate of reaction}}{\text{Maximum rate of diffusion}}$$

$$N_{Da} = \frac{v_{\max}}{k_L [S_b]} \quad (5.1)$$

where $[S_b]$ is the concentration of substrate in the bulk liquid given as g/cm³, and k_L is the liquid phase mass transfer coefficient in cm/s.

5.4.1 Characterization of Immobilized Enzymatic Reaction Rates Using Damköhler Number

The rate of enzymatic conversion may be limited by diffusion of the substrate or reaction and depends upon the value of N_{Da} i.e. Damköhler number. Here we can characterize as follows:

If $N_{Da} \gg 1.0$ the diffusion rate is said to be limiting, i.e. mass transfer resistance is large.

$$N_{Da} = \frac{\text{Maximum rate of reaction}}{\text{Maximum rate of diffusion}}$$

$$D_{Da} = \frac{v_{\max}}{k_L [S_b]} \quad \text{from (5.1)}$$

implies that diffusion rate is limiting called “diffusion limited regime”

While if $N_{Da} \ll 1.0$, the reaction rate will be a limiting, i.e. mass transfer resistance is low. This is “reaction limited regime”

Note: Any limiting factor will decide the overall rate of reaction.

Further, if $N_{D_a} = 1.0$; in this the maximum rate of reaction is equal to the maximum rate of diffusion. Therefore, the reaction resistances and diffusion resistances are comparable.

Diffusion and enzymatic reactions may be simultaneous with enzymes entrapped in a solid matrix or may be two consecutive processes for adsorbed enzymes.

5.4.2 Mechanism of Mass Transfer and Chemical Reactions in a Symmetric Slab of Immobilized Enzyme

Consider an enzyme immobilized on a support slab and the same is contacted with a substrate solution flowing over both sides of the slab.

The same is represented in Figure 5.3.

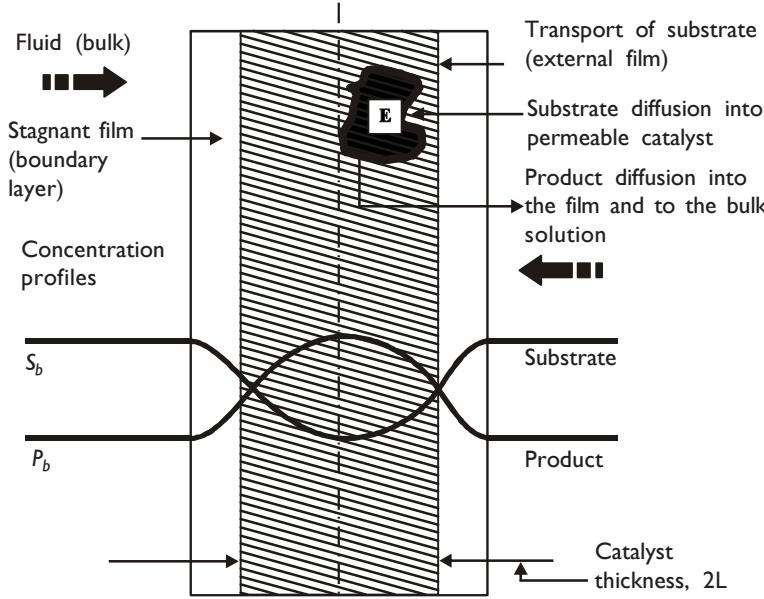


Figure 5.3 Symmetric slab of immobilized enzyme accompanying mass transfer and chemical reactions.

Far away from the catalyst, the substrate concentration and other process variables such as pH will have the values characteristic of bulk reaction mixture.

It is seen that the substrate flowing from the bulk fluid crossing the stagnant film called *boundary layer*, diffuses into the permeable catalyst, being immobilized. Here the substrate is utilized and consumed with the immobilized permeable catalyst and forms the product.

Concentration gradients develop between the bulk solution far from the catalyst and the reaction events are occurring at the active sites of immobilized enzyme molecules.

As stated earlier, substrate must be transported from the bulk solution to the outer surface to the catalyst. If the reaction mixture is stagnant, this transport takes place by molecular diffusion. But when mixing or agitation is provided, it adds to the flow of substrate solution and the mechanism developed would be convective diffusion.

If the immobilized enzyme formulation does not contain or hold enzyme in its internal volume or if substrate cannot penetrate into the internal volume, this external mass transport is the only one we have to consider. But often, the enzyme is entrapped or encapsulated or impregnated within a permeable matrix. In such cases, most of the catalyst activity is located in and distributed through the interior of the catalyst formulation.

As a result, in such systems, the substrate has to diffuse from the outer surface of the catalyst to some internal location where reaction can occur. This is also applicable for products, but in a reverse fashion compared to substrate diffusion.

This situation augments the use of intraparticle diffusion processes i.e. within the particle of an enzyme catalyst and as well external mass transport.

As per Figure 5.3, the reaction occurs within an immobilized enzyme sheet or slab at rates which are determined by the concentrations within the slab. Due to concentration gradients local reaction rates vary as a function of internal position.

The total rate of substrate consumption is the sum of the entire substrate consumption rate within the permeable enzyme catalyst. At steady state, this overall rate is also equal to the rate of substrate transport to the catalyst.

Obviously, in such situation, overall rates depend on the interaction between transport processes and catalytic reaction.

5.4.3 Effects of Diffusion in Surface-Bound Enzymes on a Non-porous Catalyst Support

Considering that enzymes are bound and are evenly distributed on the surface of a non-porous supporting material, and all enzymes show equal activity and further, substrates diffusion occurring through a thin liquid film surrounding the support material as depicted in the previous figure, we can explain the effects of diffusion in such cases.

The substrate diffuses through a thin liquid film and reach the permeable catalyst reactive surfaces, but placed on a non-porous support. This is illustrated in Figure 5.4.

In addition to the above considerations, we assume that the process of immobilization has not altered the enzyme conformity and protein structure and so also the intrinsic kinetic parameters, viz. v_{\max} and K_m also remain unaltered.

Using the steady-state situation, it is seen that the rate of reaction will be equal to the rate of mass transport.

We can equate the Michaelis-Menten equation to the diffusion or mass transfer rates taking place.

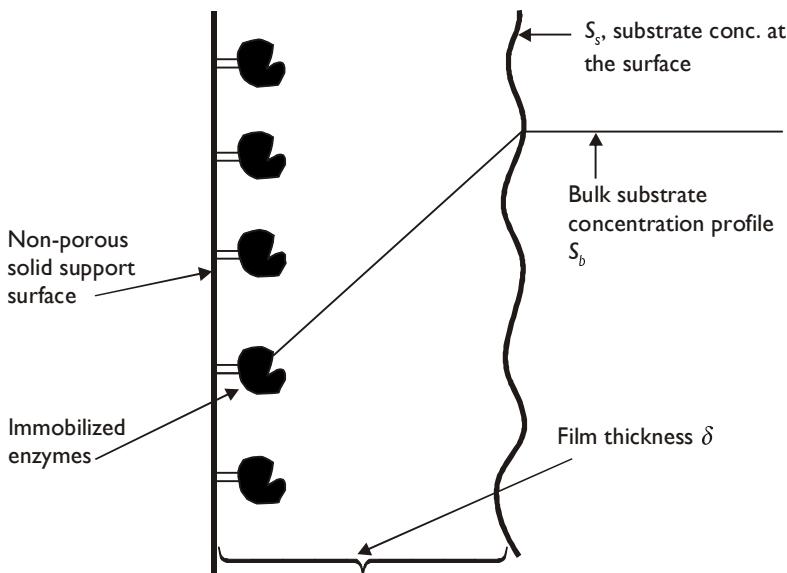


Figure 5.4 Concentration profile of substrate in a liquid film around bound enzymes on a non-porous support.

The flux, defining diffusion rates of substrates, is

$$J_s = k_L \{ [S_b] - [S_s] \} \quad (5.2)$$

and

$$\bar{r} = \frac{v_{\max} [S_s]}{K_m + [S_s]} \quad (5.3)$$

Equating the above Eqs. (5.2) and (5.3) at steady state, we have

$$J_s = k_L \{ [S_b] - [S_s] \} = \bar{r} = \frac{v_{\max} [S_s]}{K_m + [S_s]} \quad (5.4)$$

where k_L is liquid phase mass transfer coefficient and v_{\max} is the maximum reaction rate per unit of external surface area.

In the above equation, the number of parameters necessary to specify the system can be reduced from existing four (k_L , S_b , v_{\max} , and K_m) to two (N_{Da} , K) by using the dimensionless variables as defined below.

$$x = \frac{[S_s]}{[S_b]} \quad N_{Da} = \frac{v_{\max}}{k_L [S_b]} \quad \text{and} \quad \kappa = \frac{K_m}{[S_b]}$$

Using these forms, the above equation in terms of substrate mass balance is

$$k_L \left\{ \frac{[S_b] - [S_s]}{[S_b]} \right\} = \left\{ \frac{v_{\max} \frac{[S_s]}{[S_b]}}{K_m + \frac{[S_s]}{[S_b]}} \right\} \quad (5.5)$$

$$k_L \left\{ \frac{1 - [S_s]}{[S_b]} \right\} = \frac{v_{\max} \frac{[S_s]}{[S_b]}}{\frac{K_m}{[S_b]} + \frac{[S_s]}{[S_b]}} \quad (5.6)$$

On simplifying and putting the dimensionless variables defined above we can write as,

$$k_L \{1 - x\} = \frac{v_{\max} x}{\kappa + x} \quad (5.7)$$

$$\text{Therefore, } \frac{1 - x}{N_{Da}} = \frac{x}{\kappa + x} \text{ where } 0 \leq x \leq 1.0$$

The above equation is found to be quadratic for x , manipulating algebraically, we can write as,

$$x = \frac{\beta}{2} \left(\pm \sqrt{1 + \frac{4\kappa}{\beta^2}} - 1 \right) \quad (5.8)$$

$$\text{where } \beta \equiv N_{Da} + \kappa - 1$$

The + sign is used for β greater than 0 and - sign used for β less than 0, whereas when $\beta = 0$, $x = \sqrt{\kappa}$.

Using this value for $[S_s]/[S_b]$, either on the left side or right side of the equation given below

$$\frac{1 - x}{N_{Da}} = \frac{x}{\kappa + x} \text{ we can evaluate the observed dimensionless reaction rate}$$

$$\frac{\bar{r}}{v_{\max}}$$

The influence of mass transfer on the overall reaction process is very well represented using the effectiveness factor η . This effectiveness factor η is defined as the ratio of observed reaction rate to the rate without mass transfer resistance, i.e.

$$\eta = \frac{\text{Observed reaction rate}}{\text{Rate which would be obtained without mass transfer resistance}}$$

In the above definition for η , the denominator part refers to the fact that surface concentration of substrate S_s is equal to the bulk concentration S_b i.e. $S_s = S_b$.

Therefore,

$$\eta = \frac{\frac{x}{(\kappa + x)}}{\frac{1}{(\kappa + 1)}} \quad (5.9)$$

So, that $\eta \leq 1.0$ and overall, the effect of increasing the mass transfer resistance is, decrease in the observed activity of catalyst.

For N_{Da} tending to zero, $\frac{1-x}{N_{Da}} = \frac{x}{\kappa+x}$ shows that x must approach 1.0

and so for the reaction limited regime $N_{Da} \rightarrow 0$

$$\eta = 1.0 \text{ and } \bar{r} = \frac{v_{\max}[S_b]}{K_m + [S_b]}$$

The diffusion limited regimes with combination of chemical reaction and mass transfer arises when v_{\max} is greater than $k_L [S_b]$, so that $N_{Da} \gg 1.0$.

Finally by putting $N_{Da} \rightarrow \infty$ and κ being finite,

$$\eta = \frac{1+\kappa}{N_{Da}} \text{ and } \bar{r} = k_L [S_b]$$

Therefore, as long as N_{Da} is very large, the observed reaction rate \bar{r} is first order in bulk substrate concentration and totally independent of the intrinsic rate parameters v_{\max} and K_m .

All the above discussed parameters and their influences are well depicted in the following graphical representations.

Graphical representations

1. Dimensionless plot of overall reaction rate vs. bulk substrate concentration (Figure 5.5).

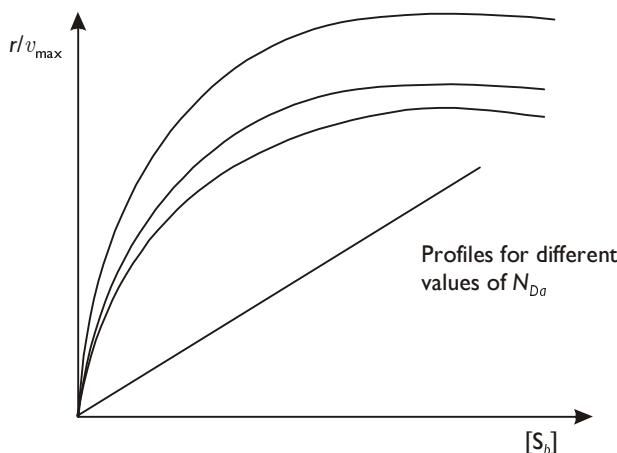


Figure 5.5 Overall reaction rates vs. bulk substrate concentration.

2. Effectiveness factor η vs. Damkohler Number N_{Da} (η vs. N_{Da}) (Figure 5.6).

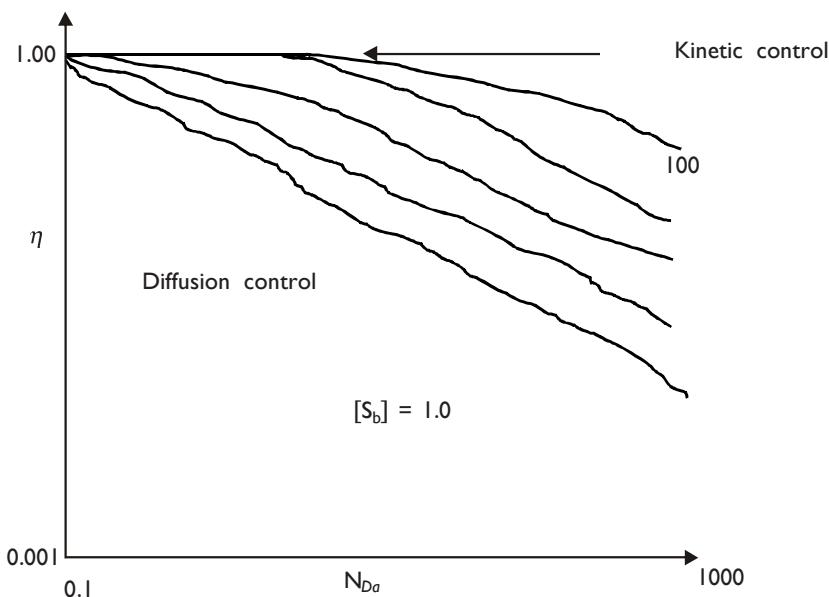


Figure 5.6 Effective factors vs. Damkohler number.

Graphical approach

The original equation:

$$J_s = k_L \{ [S_b] - [S_s] \} = \bar{r} = \frac{v_{\max} [S_s]}{K_m + [S_s]}$$

can be solved and evaluated graphically. This approach makes it easy to visualize the effects of parameter changes viz. stirring or mixing rate, bulk substrate concentration and even enzyme loading in a reactor.

The graphical solution for amount of reaction per unit surface area of immobilized enzyme on a non-porous support is depicted in Figure 5.7.

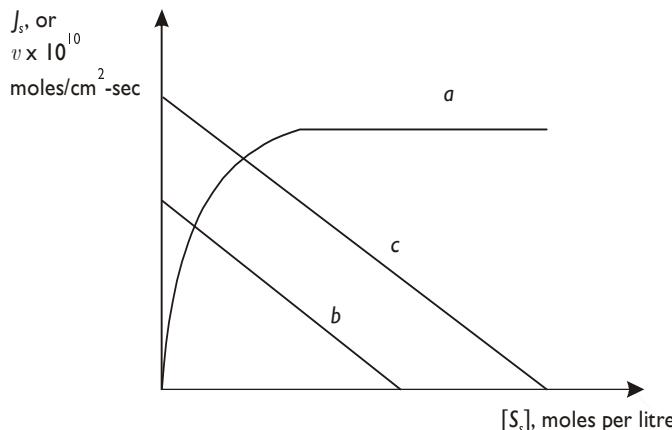


Figure 5.7 Flux J_s vs. the substrate concentration.

In Figure 5.7, curve (a) results based on the intrinsic solution-based kinetic parameters and the surface loading of enzymes, i.e. representing.

$\frac{v_{\max}[S_s]}{K_m + [S_s]}$ and line (b) is the mass transfer equation, $J_s = k_L\{[S_b] - [S_s]\}$.

The intersection of the two lines is the reaction rate \bar{r} that is sustainable. Further, the responses for two bulk concentrations are also depicted.

When the system is strongly mass transfer limited, $[S_s] \equiv 0$, since the reaction is rapid compared to mass transfer and therefore,

$$r = k_L[S_b], \text{ for } N_{Da} \gg 1.0$$

The above relation shows that the reaction system behaves as a pseudo first order.

When the system is reaction-limited $N_{Da} \ll 1.0$ the rate is expressed as

$$\bar{r} = \frac{v_{\max}[S_b]}{K_{m,app} + [S_b]} \quad (5.10)$$

where

$$K_{m,app} = K_m \left\{ 1 + \frac{v_{\max}}{k_L \{ [S_b] + K_m \}} \right\} \quad (5.11)$$

Under these conditions, the apparent MM constant will be a function of stirring speed.

Normally, the value of $K_{m,app}$ is found out based on experimentation as the value of $[S_b]$, given one-half of the maximal reaction rate.

5.4.4 Diffusion Effects in Enzymes Immobilized in a Porous Matrix Support

In this case, the enzymes are immobilized on to a support which is a porous matrix. When enzymes are immobilized on internal pores surfaces of a porous matrix, substrate diffuses through the pathway between and among pores and reacts with the immobilized enzyme.

This is typically a case of intraparticle diffusion and reaction.

The following points are earmarked in this situation:

- Some of the particle cross-section is occupied by solid and hence not available for diffusive transport.
- The pore network is complex, intrigued and entangled, so diffusion occurs only in permitted, often changing directions. Here we consider a tortuosity factor τ .
- Pores have small diameters, i.e. small sizes of pores, and much similar to substrate molecular dimensions. Therefore, it is a case of restricted diffusion process.

As mentioned above, substrate diffuses through the tortuous pathway among pores and reacts with the enzyme immobilized on pore surfaces.

This case is more or less a simultaneous mass transfer with reaction and is depicted in Figure 5.8.

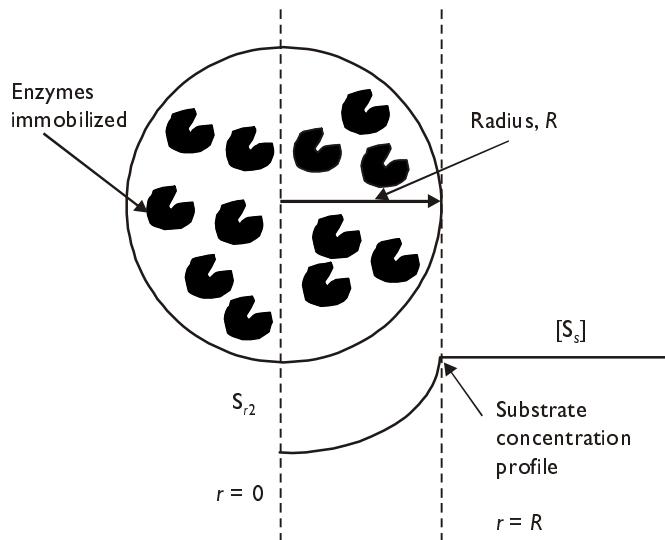


Figure 5.8 Porous support holding enzymes.

It is assumed that enzyme molecules are uniformly distributed in a support being porous and spherical one.

There is no partitioning of the substrate between the exterior and interior of the support. It is further assumed that no external substrate limitation exists so that the concentration in bulk $[S_b]$ and surface $[S_s]$ are essentially same, i.e.

$$[S_b] = [S_s]$$

At steady state writing as the rate of diffusion of substrate is equal to the rate of reaction based on the fact that no external substrate limitation exists; we have an expression for substrate diffusing across an elementary spherical shell of radius r and thickness dr as:

$$D_e \left(\frac{d^2[S]}{dr^2} + \frac{2}{r} \frac{d[S]}{dr} \right) \quad (5.12)$$

Where D_e is the effective diffusivity of the substrate within (given as cm^2/sec), a porous support and $[S]$ is the local substrate concentration, moles per litre.

The Michaelis-Menten expression is

$$r = \frac{v'_{\max}[S]}{K_m + [S]} \quad (5.13)$$

Equating (5.12) and (5.13), we get

$$D_e \left(\frac{d^2[S]}{dr^2} + \frac{2}{r} \frac{d[S]}{dr} \right) = \frac{v'_{\max}[S]}{K_m + [S]} \quad (5.14)$$

Boundary conditions applied as at $r = R$, $[S] = [S_s]$ and at $r = 0$, $\frac{d[S]}{dr} = 0$;

We can write Eq. (5.14) using dimensionless variables as $\bar{S} = \frac{[S]}{[S_s]}$;

$\bar{r} = r/R$ and $\beta = K_m / [S_s]$, so that,

$$\frac{d^2\bar{S}}{d\bar{r}^2} + \frac{2}{\bar{r}} \frac{d\bar{S}}{d\bar{r}} = \phi^2 \frac{\bar{S}}{1 + \frac{\bar{S}}{\beta}} \quad (5.15)$$

where $\phi = R \sqrt{\left(\frac{v'_{\max}}{K_m} \right) \left(\frac{K_m}{D_e} \right)}$ and is called *Thiele-modulus*. (5.16)

With boundary conditions of $\bar{S} = 1$ at $\bar{r} = 1$ and at $\frac{d\bar{S}}{d\bar{r}} = 0$ at $\bar{r} = 0$, Eq. (5.14) can be solved numerically.

Further, stating that the rate of substrate consumption is equal to the rate of substrate transfer through the external surface of the support matrix at steady state into the sphere,

We have,

$$r_s = Ns = 4 \pi R^2 D_e \left. \frac{d[S]}{dr} \right|_{r=R} \quad (5.17)$$

When there are diffusional limitations, the rate per unit volume is expressed as:

$$r_s = \eta \frac{v'_{\max}[S_s]}{K_m + [S_s]} \quad (5.18)$$

where η is effectiveness factor.

Effectiveness factor is defined as the ratio of the reaction rate with diffusion limitation (or diffusion rate) to the rate without diffusion limitation.

$$\eta = \frac{\text{Reaction rate (with diffusion limitation)}}{\text{Reaction rate (without diffusion limitation)}}$$

Its value is a measure of the extent of diffusion limitation.

For $\eta < 1.0$; conversion is diffusion-limited.

$\eta \approx 1.0$; conversion is limited by reaction-rate and diffusion limitations are ignorable.

Of course η is a function of ϕ and β as shown in Figure 5.9.

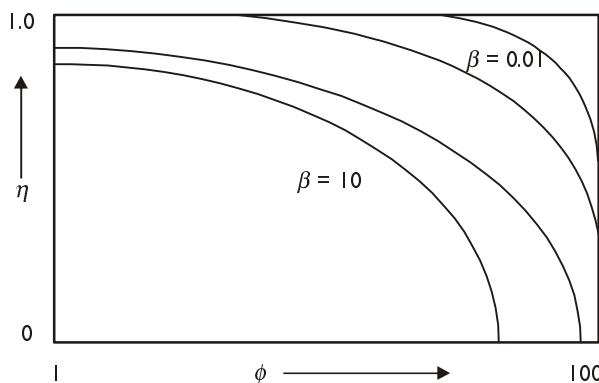


Figure 5.9 Theoretical relationships between η and ϕ for various values of β .

It is seen that, for a large range of ϕ as $1 < \phi < 100$ $\eta \approx 1.0$, the reaction is zero order rate with β tending to zero.

Also for a first order reaction rate, i.e. β tending to infinity $\eta = (\eta, \phi)$ and η approximated for high values of ϕ then

$$\eta = \frac{3}{\phi} \left(\frac{1}{\tanh \phi} - \frac{1}{\phi} \right) \quad (5.19)$$

or

$$\eta = \frac{1}{\phi} \left(\frac{1}{\tanh 3\phi} - \frac{1}{3\phi} \right) \quad (5.20)$$

Based on the above observations, we conclude here that while designing immobilized enzyme systems using a particular support, the main variables are v_{\max} and R as substrate concentration $[S]$, K_m and D_e are fixed.

The particle size R should be as small as possible but within the limitations of particle integrity, resistance to compression and nature of particle recovery systems.

The maximum reaction rate is found by enzyme activity and concentration in the support.

High concentration of enzyme will lead to high enzyme activity per unit of reaction volume, but low η . On the other hand, low concentration of enzyme will lead to lower enzyme activity per unit volume but has high η .

For maximum conversion rates, particle size should be $D_p \leq 10 \mu\text{m}$ (small), and enzyme loading to be optimized.

5.4.5 Electrostatic and Position Effects in Immobilized Enzyme Systems

Whenever enzymes are immobilized, may be on a support or a matrix, several changes are noticed in the enzyme conformities.

For instance, when enzymes are immobilized in a charged matrix as a result of a change in the microenvironment of enzyme, the apparent bulk pH optimum of the immobilized enzyme will shift from that of a soluble one.

The charged matrix will either repel or attract the substrates, products, cofactors and protons and of course depends on the type of surface charge and also on quantum of charge.

If the substrate is of a high molecular weight, the activity of an enzyme towards it is usually reduced upon immobilization.

The reason is mainly the steric hindrance by the support, e.g. starch, a carbohydrate (a polysaccharide) with a high molecular weight will not be able to penetrate to the active sites of immobilized enzymes. Also, immobilization affects the thermal stability of enzymes.

Thermal stability usually increases upon immobilization and is primarily due to the presence of thermal diffusion barriers and the constraints on protein unfolding. But in a few cases, decrease in thermal stability is also noticed.

As far the pH stability is concerned, it increases upon immobilization.

5.5 IMMOBILIZED CELL SYSTEMS

In the previous discussions, we have studied the concept of immobilization of enzymes. As a matter of fact, the meaning of immobilization, the techniques employed, the significance of immobilization of enzymes and applications have been thoroughly understood.

In this discussion we shall find the similar trends and features of immobilizing the cells as a whole.

Immobilization of cells as biological catalysts is almost as familiar as enzyme immobilization. We already are aware that immobilization refers to the restriction or localization of enzymes activity and mobility within a confined region so that finally they do not appear in the product stream.

In the same way we now shall expedite the restriction of cells and their mobilities within a confined and a well-defined space.

Undoubtedly, the performance of a biological reactor depends directly on the properties of biocatalysts employed. The developing trends in genetic engineering, so called recombinant DNA technology, have made the modification of cells at the gene level possible.

This genetic modification of cells has helped in the improvement in terms of productivities for the desired compounds.

The core feature of immobilized cells is the use of some confining or binding structure to restrict the cells in a specific region of the bioreactor.

The cells can be employed as ‘in suspension’ and ‘immobilized’ in a bioreactor. The cultures developed by adopting immobilized mechanisms have been gaining a lot of prominence in bioreactor applications.

Cell immobilization is then a process by which the cells are confined to a certain defined region of space in such a way as to exhibit hydrodynamic characteristics which differ from the surroundings. This is most usually achieved by significantly increasing the effective size or the density of the cells by aggregation or by the attachment of the cells to some support surface.

Cell immobilization can be a natural process or can be induced by chemical means or physical means similar to as what we find in the enzyme immobilizations.

It is realized that the immobilized cell cultures have the potential advantages over the suspension cultures. In the succeeding paragraphs, we find the advantages offered by these immobilized cell cultures.

1. It is because of immobilization phenomenon, the cell density or concentration in a bioreactor is always high.
2. The implications of the losses of cells are all overcome by immobilization as it provides a safe platform for the cell reuse, and indeed avoid the costly processes of cell recovery and recycle.
3. In order to achieve a high volumetric productivity, combination of high densities of cells and high flow rates with no wash out constraints is possible and this is mainly due to the immobilization.
4. During the bioreactions taking place, certain conditions that favour the cells and in turn influencing the productivity and performance viz. cell to cell contact, nutrient concentrations, nutrient to product gradients, pH differences are all met if the cells are immobilized. Consequently, leads to better product yields and rates.
5. Immobilization of cells also improvises the genetic stability, so possibility of mutations taking place is drastically reduced.
6. As immobilization leads to the formation of flocs of cells, the protection against shear damage is certain.
7. Immobilization also provides a control over the cell morphology and broth rheology. It is seen that by confining the cells or microbes to the interstices and surfaces of support particles, the rheological properties and also the mass transfer properties of the broth are well defined and do not change significantly.
8. There is a complete elimination of cell wash out problems especially at very high dilution rates.

On the contrary, the major limitation of cell immobilization is that, the product of concern should be excreted by the cells or microbes i.e. the product is usually an excreta of cells. Another complication is that immobilization often leads to the systems in which diffusional limitations are a matter of concern.

The techniques and mechanisms studied in the enzyme immobilization have a direct reflection on the whole cells. But the maintenance of a living cell in such a system with a multitude of complexities is rather difficult than maintaining the enzymatic activity.

While the main advantage of cell immobilization over enzyme immobilization is that, the former can perform multi-step, is cofactor requiring, biosynthetic reactions which are not practical using the purified enzyme preparations.

5.5.1 Formulation and Characterization of Immobilized Cell Biocatalysts

The means or the ways to immobilize the whole cells fall into two categories. These are:

Immobilization of cells—Active: This refers to entrapment or binding of cells by physical or chemical forces. Entrapment and binding are the two major strategies under active immobilization of cells.

Physical entrapment of cells within the porous matrices is the most widely used method for immobilization.

Porous polymers, viz. agar, chitosan—a polysaccharide obtained from the exoskeleton of crustaceans, polyacrylamide etc. and porous metal screens, polyurethane, silica gel, etc. can be employed as various matrices for entrapment of cells.

Use of polymer beads, alginate beads for example, is also considered for cell immobilization. These polymer beads should have certain specific properties to allow the transport of substrates and products in and out of the bead.

The methods employed to prepare porous polymer beads are:

- Polymer gelation
- Polymer precipitation
- Ion-exchange gelation
- Polycondensation
- Polymerization

Table 5.4 gives information about the polymeric networks or methods listed above used for cell immobilization.

Table 5.4 Polymer networks for cell immobilization

<i>Formation of network</i>	<i>Cross-links</i>	<i>Examples</i>
Precipitation	Non-specific	Collagen, polystyrene
Ion-exchange gelation	Ionic in nature	Alginates
Polycondensation	Covalent linkage but hetero polar	Epoxy resins
Polymerization	Covalent but homo polar	Polymethylacrylate monomer

Entrapment in polymeric networks is the most commonly applied method for cell immobilization.

Perhaps the one widely accepted and used is ionic cross links in a layer of bead of alginic, a naturally occurring polysaccharide.

Immobilization of Cells—*Passive*: It refers to the growth of biological films. Biological films are the multi-layered cells which have grown on the solid support surfaces. These support surfaces sheltering the growing cells can be inert or biologically active.

It is a common tendency of formation of biological films and usually seen both in the natural and industrial fermentation systems, for example, in wastewater treatment using biological means and in the fermentation processes using moulds respectively.

The forces of interaction between the cells and the support material are found to be very intricate and complex. Indeed it depends upon the systems incorporated.

We find that in the mixed culture systems, these biofilms are basically produced due to the presence of some polymer-producing organisms that enhance or facilitate and even stabilize the formation of biofilms.

While in the stagnant biofilms, it is seen that the nutrients diffuse into the biofilms and products diffuse out into the liquid nutrient medium.

The formation of biofilms is depicted in Figure 5.10.

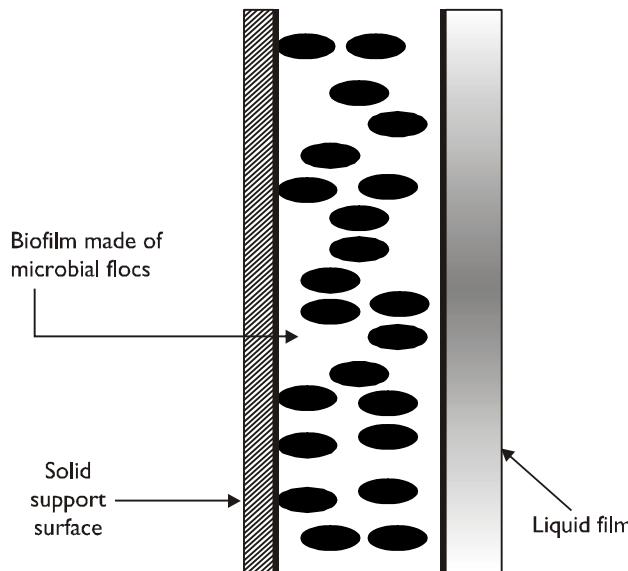


Figure 5.10 Formation of a biofilm on a support surface.

5.5.2 Techniques for Cell Immobilization

Wide spectrum of immobilization techniques is developed and many of them

taken directly from immobilized enzyme technology. It is observed that to a certain extent the choice of technique is governed by the desired physiological state of the cells and the purpose to which they are put.

Figure 5.11 represents the range of physiological states of immobilized cells.

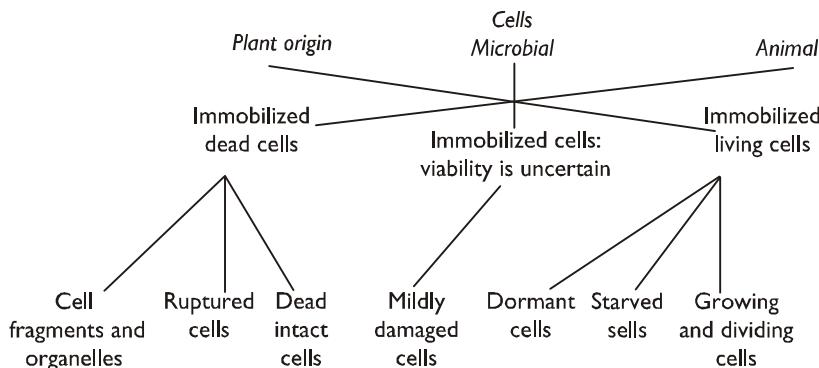


Figure 5.11 Physiological states of immobilized cells.

The numerous techniques for achieving immobilization can be divided according to the physical processes involved, viz. attachment, entrapment, containment and aggregation.

But an important distinction should be made between those techniques which allow the cells to become immobilized as a result of the natural consequence of growth by simply providing a suitable support, and those for which the cell population has to be developed or pre grown and subsequently actively treated in order to effect the process of immobilization.

In general, active techniques are more widely applicable and can be used for the cells in any physiological state.

On the other hand, the natural techniques involve no special attention and input from the operator and are therefore easily taken care of and are also cheaper compared to the induced ones.

In the succeeding paragraphs, the physical techniques for immobilization are discussed.

Attachment: In this, the cells are bound to the surface of a solid support as shown in Figure 5.12. The attachment relies on the forces of natural adhesion or be induced by certain chemical means.

The attached cells are in direct contact with the surroundings and hence subject to the relative motion of particles and fluid when placed in a reactor. It is therefore likely that some cells will become detached and enter the bulk fluid phase.

Therefore, this technique is not suitable in the situations where cell free liquor is required.

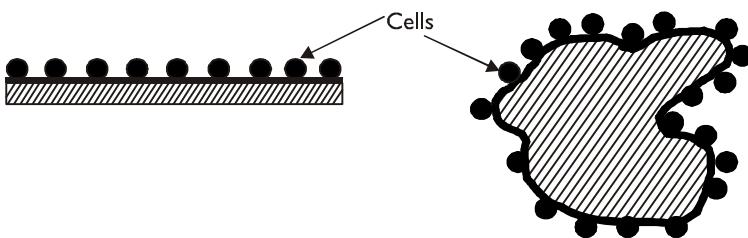


Figure 5.12 Attachment as immobilized process.

The systems developed more recently, prefer the use of particulate solid supports, often of the material such as sand, which when used in a fluidized bed for instance, provides a greatly increased surface area for attachment per unit volume of the reactor than in either of the traditional processes.

The art of cell immobilization by attachment is to provide the right surface in a suitable form for the commercial use and colonized by the desired organism or population of organisms in as high a density as is possible.

Entrapment: In a variety of porous structures the cells can be entrapped. These porous structures are either preformed or formed *in situ* around the cells. Entrapment within the preformed structures usually occurs as a natural consequence of cell growth and therefore like a natural attachment.

It is seen that the effectiveness of the immobilization varies with cell type and support type.

On the other hand the porous structures which are formed *in situ* can be used to immobilize the cell of any type, though the conditions under which the support particle is formed may be harmful to the cells in certain cases.

Figure 5.13 depicts the entrapment process.

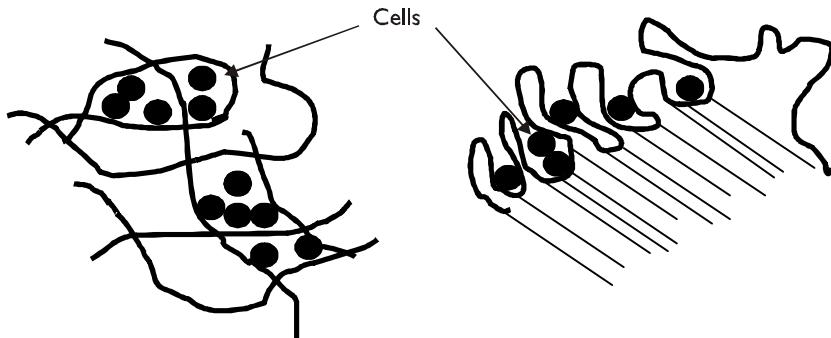


Figure 5.13 Entrapment as immobilized process.

The techniques involving the natural entrapment of cells within preformed porous structures are very similar to those used for natural attachment. In this the cells are allowed to diffuse into the pores of the structure and as they grow it becomes increasingly difficult for them to leave the pores because they get effectively entrapped as shown in Figure 5.13.

Containment and aggregation: In containment the cells are contained within the structures. This class of immobilization involves the containment of cells behind a barrier, either preformed or formed *in situ*. The barrier may be simple as an interface between the two immiscible liquids.

Preformed barriers include the semi-permeable membranes used for microfiltration and ultrafiltration processes. The nutrients are able to diffuse to the cells retained behind the membrane. These are mainly used in the area of mammalian tissue cell culture.

The process is depicted in Figure 5.14(a) and 5.14 (b).

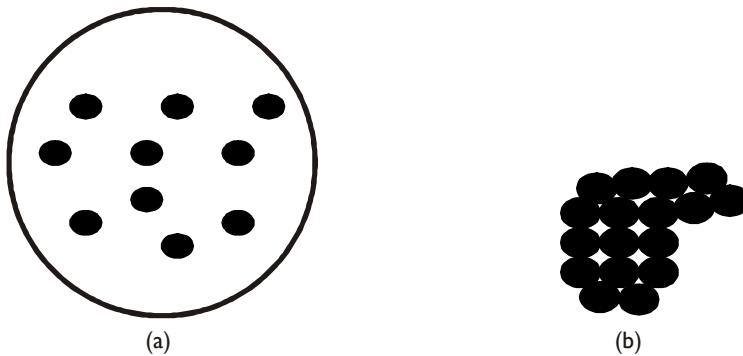


Figure 5.14 Containment and aggregation.

In aggregation of cells (b), the cells form large aggregates due to the flocculation of cells and thereby it is possible to retain them continuously in the operated bioreactors, for example, in the form of packing materials in the fluidized bed or packed bed reactors.

The artificial flocculants may also be employed to enhance the rate of aggregation. This is applied in the activated sludge process where the cells are placed in the form of aggregates.

5.5.3 Transport Across Cell Membranes

For the cell to function normally, it is quite essential that the controlled transport of ions and molecules between the cell and its surroundings occurs. We know that the cell membrane is selectively permeable in nature and provides the necessary ability of being selective to various media and cellular components, thus regulating the transport processes that serve several important purposes.

These transport processes help in maintaining the intracellular composition and pH in a narrow range constantly with required and necessary enzyme activities.

The transport across cell membrane is a significant and a vital activity for the cell survival. Membrane transport regulates the cell volume, admits and concentrates the nutrients and also aid in the secretion of toxic compounds.

There are three means of transport across the cell membranes and well identified. They are:

1. Passive diffusion
2. Facilitated diffusion
3. Active transport

It is, however, realized that the main mechanism that prevails in the transport across membranes is diffusion. Further, regardless of the mechanism, the transport characteristics of a given membrane with a given substrate are often expressed in terms of membrane permeability which is denoted as K .

Membrane permeability K can be computed by considering the concentrations in and out of the cell as shown below.

It is expressed as

$$K = \frac{V}{At} \ln \left(\frac{C_E - C_{S_0}}{C_E - C_{S(t)}} \right) \quad (5.21)$$

where V = the volume of cell

A = the cell external surface area

C_E = the external concentration

C_{S_0} = the interior substrate concentration in the initial stages

$C_{S(t)}$ = the substrate concentration after time elapse t .

K is expressed as cm/sec or m/sec, the same as the unit for velocity.

Now let us discuss on the types of transport mentioned above individually.

Passive diffusion: In this type of diffusion, the material or the constituents move across the membrane from a region of high concentration to the region of low concentration. It is seen that the diffusion rate is proportional to the overall driving force, which is concentration gradient across the membrane.

The mechanism is shown in Figure 5.15.

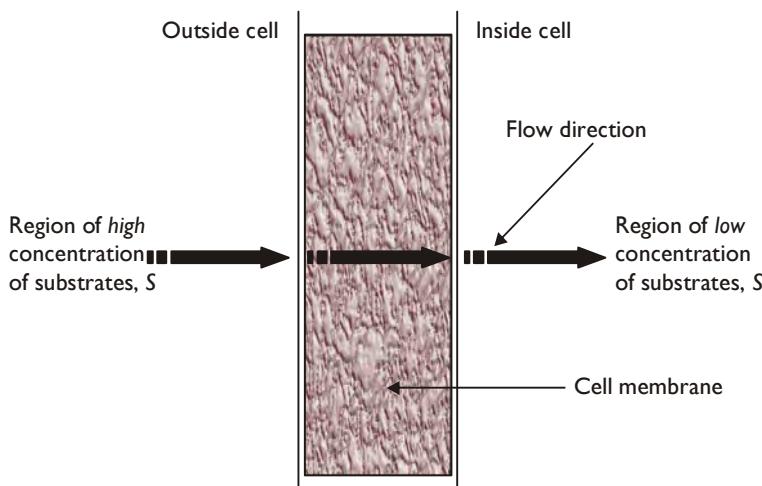


Figure 5.15 Passive diffusion across cell membrane.

Based on the thermodynamic considerations, it is found that the process of passive diffusion is spontaneous, that merely depends on the concentration differences across both the ends of the cell membrane.

By virtue of the Gibb's Free Energy changes accompanying the transport of material from a region of high concentration say C_2 to another region of low concentration say C_1 is given as:

$$\Delta G^0 = RT \ln \left(\frac{C_1}{C_2} \right) \quad (5.22)$$

It should be noted that as C_1 is smaller than C_2 in passive diffusion, ΔG^0 is negative.

Further, if the component is a charged species then we have a modified expression as given below.

$$\Delta G^0 = RT \ln \left(\frac{C_1}{C_2} \right) + Z F \Delta \psi \quad (5.23)$$

where Z = number of charges on transported molecules

F = Faraday, 23.062 kcal per $V \text{ mol}^{-1}$

$\Delta \psi$ = potential gradient across the membrane.

Facilitated diffusion: In this, it is found that the substrates combine with the carrier molecules to give a facilitated diffusion. Substrates which are on the outside of cell membrane combine with the carrier molecules present within the cell forming a complex and diffuse to the other side.

Once having diffused to the other side the complex between the carrier and substrate splits, discharging the carrier molecule inside the cell.

The same is depicted in Figure 5.16.

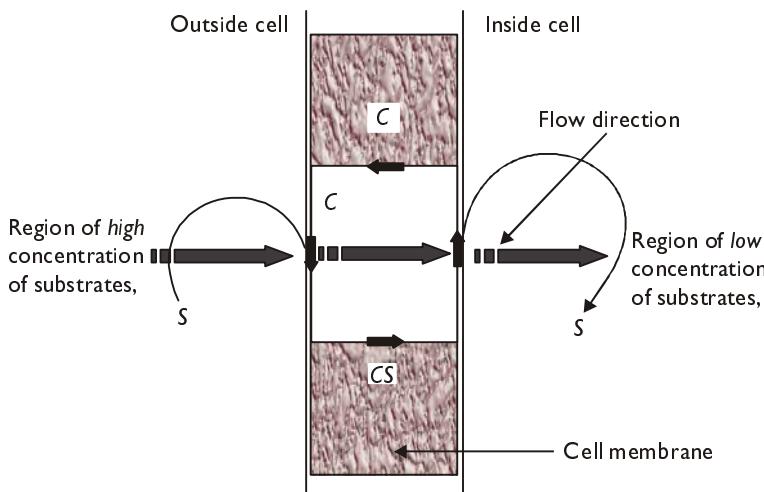


Figure 5.16 Facilitated diffusion across cell membrane.

Facilitated diffusion has several distinct features. It is seen that the diffusion instead of increasing linearly with the external solute concentration, the transport rate attains the saturation, maximum level. Further increase in the overall mass transfer gradient, i.e. concentration driving force, has no influence on the transport rate or diffusion rate.

The other features observed are: Only specific compounds are involved in the transport and specific inhibitors slow down the process. In general, the facilitated diffusion is specific in nature. Due to this behaviour, the carrier molecules are believed to be a chain of amino acids, a polypeptide, i.e. proteins.

The classical example of this mechanism is the transport of glucose in the red blood cells, i.e. RBCs of human blood.

Active transport: It is the third type of diffusion transport taking place across the cell membrane.

Active transport is said to have two distinct features. These are:

1. Movement of component(s) against the chemical gradient, i.e. from the region of low concentration to a region of high concentration.
2. It requires metabolic energy for carrying out the process.

The same is depicted in Figure 5.17.

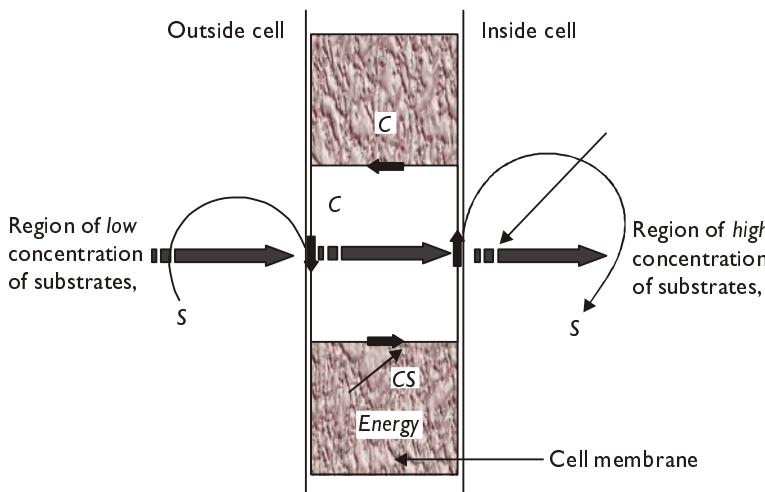


Figure 5.17 Active diffusion across cell membrane.

Active transport is important in the nerve action and seen in the transmission of nerve impulses across the dendrites and neurons accompanying the junction called *synapse*.

Almost all cells have active transport systems and are required to maintain the balance between the sodium ions and potassium ions along with water in the cell.

In specific, sodium is pumped out of the cell and potassium is pumped inside the cell. The pumping action allows the cell to offset the simultaneous passive diffusion of these ions, which is taking place continuously.

Such processes are coupled and are driven by ATP, *adenosine triphosphate* the energy currencies of the cell.

5.5.4 Reactor Configurations for Immobilized Cells

A variety of reactor configurations can be used for immobilized cell systems. It is observed that the support matrices employed in cell immobilization are usually mechanically fragile; the bioreactors with low hydrodynamic shear, for instance the packed bed reactors, the fluidized bed reactors or air lift reactors are preferred.

In certain cases, the mechanically agitated fermenters can also be used for some immobilized-cell systems, provided the support matrix is strong enough to sustain the stresses.

Multiple factors are to be considered in the selection of the reactor and the type for a particular process for immobilized-cell systems. These are: the method of immobilization, characteristics of the particles viz. size, shape, density, etc. the nature of substrate, the effects of inhibitors and as well the hydrodynamic and economic factors.

In the succeeding discussions, we find the various configurations of reactors used in immobilized-cell systems.

Stirred tank bioreactors: Although a detailed discussion on the features is dealt with in section 7.7.4, here we look for the STRs used in immobilized-cell systems.

It is seen that in the STRs, the major problem encountered with the use of immobilized cells is the harsh and vigorous treatment to which the cell particles are exposed.

A high rate of shear can have severe damaging effects, especially in the case of gel particles. Enveloping the agitator in a porous mesh is a modification to the classical STRs and provides mixing without the destruction of immobilized aggregates.

Fixed bed reactors: These are very common and are convenient to use and they usually provide the tool for the first test of the applicability of cells immobilized by certain newer techniques. These are, however, difficult to scale up and also to quantify Figure 5.18.

Plug flow packed bed reactors operated on a once-through basis may offer high rates of reaction due to high substrate concentration but are relatively poor in terms of heat and mass transfer coefficients and this is due to the low liquid velocities.

The fluidized bed fermenters with recycle show improved mass and heat transfer characteristics and also improved controllability.

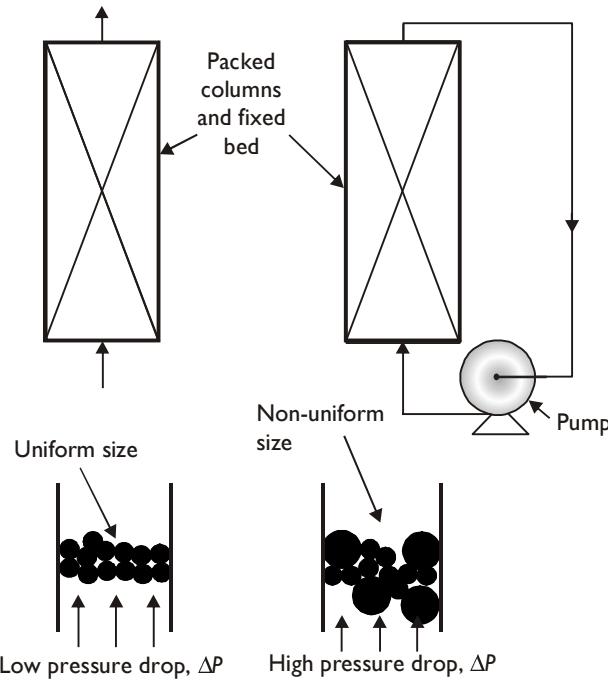


Figure 5.18 Packed bed reactors.

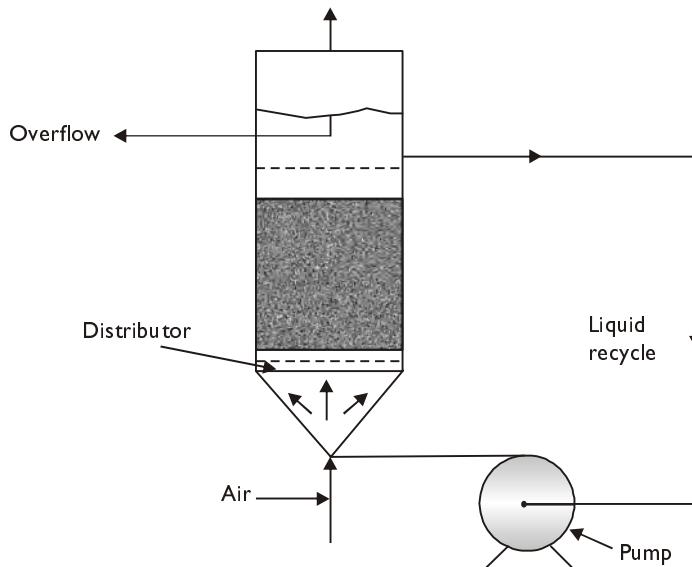


Figure 5.19 Fluidized bed reactor.

In this the particles are suspended as a result of the upflow of the fluid phase and are a focus of much attention these days (Figure 5.19).

Biological fluidized bed treatment of water and waste water is a particular application of interest in which the solid particles, e.g. sand or porous particles can be employed. The use of such reactors is done for aseptic systems.

Bubble column reactors: These are the reactors which rely on gas sparging for agitation. Bubble column reactors signify a large height to diameter ratio. Mixing or agitation is supplied entirely by forcing compressed gas through spargers into the reactor which then rises through the liquid (Figure 5.20).

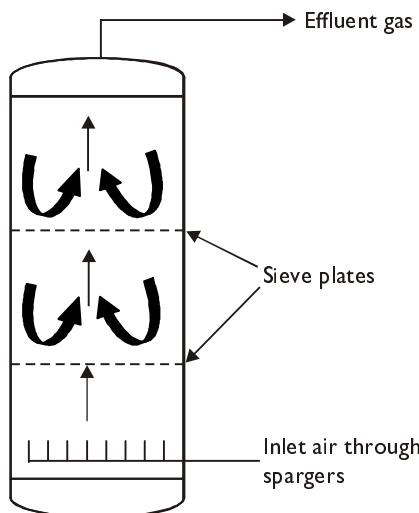


Figure 5.20 Bubble column reactor.

Bubble column reactors have certain advantages over the others. These are low capital cost, simple mechanical configuration and reduced operating costs based on the low energy requirements. These are used usually in the manufacture of beer and vinegar.

These can be used in a batch mode and also in continuous mode preferably in a counter current fashion.

Note: The other reactor types and configurations are discussed in Chapter 7.

The overall performance of immobilized cell reactors is based on the following factors:

1. The reaction properties viz. volume of bioreactor, reaction zone volume, the void volume, the volume occupied by the immobilized cells plus supports, etc.
2. The fractional particle hold-up
3. The control of biomass hold-up
4. The rate of oxygen supply, and
5. Cell physiology.

SUMMARY

In this chapter, we learnt the following:

- Confinement or localization of enzymes so that they can be reused continuously is called immobilization.
- There are physical methods and chemical methods of immobilization.
- Though immobilization is desirable, there are certain limitations.
- The physical and mechanical properties of supports are important during process consideration.
- To immobilize enzymes, both natural and artificial supports can be used.
- Enzyme immobilization is a favoured practice in industry, while enzymes undergo certain constraints so called diffusional limitations.
- Resistances vary depending upon the nature of the support materials may be porous or non-porous, hydrodynamic conditions in the vicinity of support material and the distribution of the enzyme, whether inside or on the surface of the support material.
- Relative rate of reaction rate and diffusion rate can be taken as an index of performance for immobilized enzymes over free enzymes.
- Cell immobilization can be a natural process or can be induced by chemical means or physical means similar to as what we find in the enzyme immobilizations.
- Techniques for cell immobilization are: attachment, entrapment, containment and aggregation.
- There are three means of transport across the cell membranes and well identified. They are:
 1. Passive diffusion
 2. Facilitated diffusion
 3. Active transport.
- The support matrices employed in cell immobilization are usually mechanically fragile; the bioreactors with low hydrodynamic shear, for instance the packed bed reactors, the fluidized bed reactors or air lift reactors are preferred.
- The method of immobilization, characteristics of the particles viz. size, shape, density, etc. the nature of substrate, the effects of inhibitors and as well the hydrodynamic and economic factors are the factors to be considered in selection of bioreactors for immobilized cell systems.

EXERCISES

- 5.1** What is enzyme stabilization? Discuss the various strategies for the same.
- 5.2** Why is enzyme stabilization required?
- 5.3** Immobilization of enzymes is desirable. Why?
- 5.4** Describe the process of adsorption immobilization of enzymes.
- 5.5** Enlist the chemical methods of enzyme immobilization and further with neat sketches explain the mechanism of each.
- 5.6** Explain physical methods of immobilization of enzymes.
- 5.7** Write a short note on the following:
 - (a) Interactions and carriers in adsorption method of immobilization
 - (b) Natural and synthetic supports for covalent enzyme attachment
 - (c) Microencapsulation
 - (d) Limitations of immobilization
- 5.8** Discuss the diffusional mechanisms and limitations observed in the immobilized enzyme systems.
- 5.9** Brief on the electrostatic and position effects in the immobilized enzyme systems.
- 5.10** Write on the immobilized cell systems and their reactor configurations.
- 5.11** Describe the transport mechanisms across cell membranes with their importance.

Chapter 6

Biomass Production in Cell Cultures

When a small amount of living cells is added to a liquid solution having essential nutrients at suitable temperature and pH, the cells will grow. The growth processes have two different manifestations according to the morphology of the cells involved. For unicellular organisms which divide as they grow, increase in biomass (mass of living matter) is accompanied by increase in the number of cells present. Associated with cell growth are two other processes: uptake of some material from the cell's environment and release of metabolic end products into the surroundings.

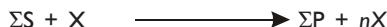
For microbes, growth is their most essential response to their physio-chemical environment. Growth is a result of both replication and also changes in cell size. Microorganisms can grow under a variety of physical, chemical and nutritional conditions.

In this chapter, we shall focus on cell growth, kinetic aspects of cell growth and situations that apply to other aspects of chemical reactions in cellular processes. Various reactor configurations with mass balance, with and without recycle, determination of kinetic parameters and quantification of growth kinetics are also treated and an insight into the introduction to the fed batch reactors is included at the end.

6.1 BIOMASS AND CELL CULTURES

Biomass is the mass of living matter in a population of particular organisms in a particular area. A culture medium is a preparation used for growing and cultivating microorganisms for experimental uses.

In a suitable nutrient medium, organisms extract nutrients from the medium and convert them into biological compounds. Part of these nutrients



is used for energy production and some other part is used for biosynthesis and product formation.

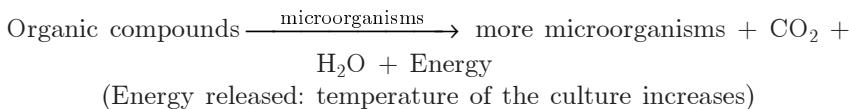
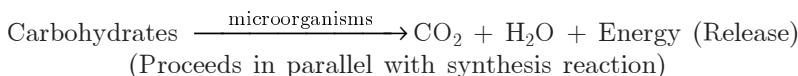
Industrial manufacturing processes that use microorganisms generally use organic compounds as a source of energy and source of carbon, for example, carbohydrates.

During the course of growth, carbohydrates are utilized by microorganisms and assimilated into the cellular materials of microorganisms because of which growth is seen. Growth is both in terms of size and numbers. We say, in general, increase in biomass (mass of living matter).

Growth features vary considerably from organism to organism. Some grow in numbers (population) and some grow in size. For example, moulds exhibit growth in terms of length, and mycelia increases as the organism grows. The growing mould thus increases in size and density and not in numbers.

Even growth requirements may differ. We know that principal elements associated with microorganisms are C, H, O, N, S and P, and the trace elements associated are Na, Ca, Fe and Co, etc. Some microorganisms require N in the form of urea and some require N in the form of ammonium ions.

If carbohydrates are organic constituents in culture as nutrients, metabolic waste products are CO_2 and H_2O with liberation of energy.



As seen, with the release of energy, the temperature of the culture increases.

The important parameters, phenomena and interactions which influence cell population are shown in Table 6.1.

The way of representation shown is very complex and it needs to be simplified to the maximum extent possible. So we have to introduce some simplifications, that it can be used with no difficulty to explain the interactions between growth medium and cell population.

The two interacting systems involved are as follows:

- Growth medium or environmental phase
- Biological phase or cell population

Cells consume nutrients and convert substrates from the environment into products. Heat is also generated; the temperature of the culture medium increases and finally sets the temperature of the cells.

Mechanical interactions occur through hydrostatic pressure and flow effects from the medium to the cells. The changes in the medium viscosity are due to accumulation of cells and of cellular metabolic products.

Table 6.1 Important parameters, phenomena and interaction which influence cell population

<i>Environmental medium or surroundings</i>		<i>Cell population or System (Biological phase)</i>
Multicomponent	Nutrients, substrates (From surroundings consumed by cell population)	Multicomponent
Reactions in solution	Products released (Enter in cell population phase)	Cell to cell heterogeneity (Each and every cell differs, based on size, age, etc.)
Acid-base equilibria (acid \rightleftharpoons base)		Multireaction
Variable pH, T, ... Changing rheology (Viscosity conditions varying)	Heat	Internal controls Adaptability, (accordingly MOs change)
Multiphase systems (G-L,L-L,G-L-L)		Stochastic; some element of probability exists
Spatial non-uniformity (Conditions at each and every point in space within the reactor are different)	Mechanical Interactions	Genetic Drift (Constitution of genes in drifting mode)

6.1.1 Environmental Medium

The environmental medium is a multicomponent system (comprises nutrients, cells, end products and more cells). So it must contain all of the required nutrients for cell growth and which will accumulate, as the cells grow, various end products of the cellular metabolism. Different parameters, phenomena and interactions depicted in Table 6.1 are detailed as follows:

Reactions in solution: Reactions occur in growth medium solution modifying the form of cell products.

Acid-base equilibria: Cells consume substrates and products are obtained. This influences the acidity of the environment medium, and further interplay of cellular consumption with acid-base equilibria determines the medium pH which in turn influences the cellular activities and transport processes.

During the course of cellular reactions, the broth temperature, pH, ionic strength and the rheological properties change with time.

Multiphase (G-L, L-L, G-L-L): It is a multiphase system having liquid with dispersed gas bubbles, of L-L systems (immiscible phases) or sometimes three-phase system with two liquid and one gas phase.

Spatial non-uniformity: Because of high viscosity and non-Newtonian behaviour of broth in some situations, it is not uncommon to find spatial non-uniformity, i.e. within the reactor, the conditions differ from point to point in space.

6.1.2 Cell Population

Now, let us discuss the parameters, phenomena and interactions depicted in Table 6.1 for cell population.

Multicomponent: Each individual cell is complex and is treated as multicomponent system within itself.

Cell to cell heterogeneity: Cell population can vary at a given point and at a given time. Some cells are old and some young (age difference), and some are newly born and some dividing.

Multireaction: Many independent chemical reactions take place even at a single-cell level. Therefore it is taken as multireaction process.

Internal controls: As many independent chemical reactions occur simultaneously in each cell, it is subjected to a complex set of internal controls.

Adaptability: The internal controls endow the cells with capability and propensity to adapt the activity. Cells adopt themselves towards the changing conditions.

Stochastic: It refers to some element of probability in structure. The situation is highly entropic, and at the same time highly probable.

Genetic drift: In long-term cultivation of a cell population, spontaneous mutations occur, which results in a slow drift in the genetic makeup of the strain.

With all the above features, it is not possible to formulate a kinetic model which can explain the cell population kinetics.

6.2 PERSPECTIVES OF CELL POPULATION KINETICS

Here we use approximations to simplify the process. Average cell and the balanced growth are the two approximations used to describe cell population kinetics.

First, with respect to environment, it is a common practice to formulate the growth medium, so that all the components but one are present at sufficient high concentrations, such that changes in their concentrations do not affect significantly the overall rates. So a single component is a rate limiting nutrient step and the concentration of only nutrient is considered while other factors can be externally controlled. Refer to Figure 6.1.

Cellular representations which are multicomponent are taken as **STRUCTURED**. Single component representations are taken as **UNSTRUCTURED**. Discrete, heterogeneous cells are **SEGREGATED**, while average cellular properties are **UNSEGREGATED**.

Actual case is structured and segregated. If cell to cell heterogeneity is not substantially influencing the kinetic process, by average cell approximation and balanced growth approximation, it is simplified to

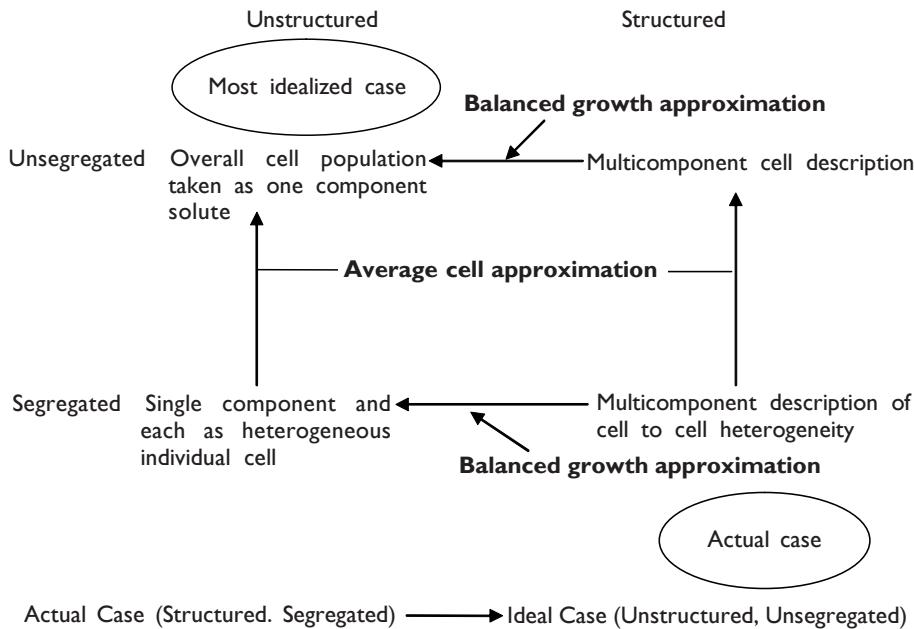


Figure 6.1 Cell population kinetics.

idealized case, i.e. unseggregated and unstructured from the actual case which was segregated and structured.

Balanced growth: Balanced growth is one of the approximations used. It is defined as that all cellular synthesis activities are coordinated in such a way that the average cellular composition is not affected by proliferation of the population.

Average cell approximation: In this single component, multicomponent description of cell-to-cell heterogeneity is considered to have a very low influence on kinetic process of interest. The average cell approximation leads to an unseggregated perspective.

6.3 IDEAL REACTORS FOR MEASUREMENT OF KINETICS

If reactors have spatially non-uniform conditions, then it is difficult to find or obtain useful kinetic information on cell population. Hence, it is desirable to study kinetics in reactors which have spatial uniformity or are well mixed. Well-mixed batch reactors and well-mixed continuous flow reactors are the ones that can provide the ideal conditions.

6.3.1 The Ideal Batch Reactor

Most of the biochemical processes involve batch growth of cell populations. Liquid medium called *culture* is seeded with an inoculum of living cells in a batch reactor.

Growth proceeds, during which nothing is added or removed from it. (some gas is possibly added). During this period, the concentration of nutrients, cells and products vary with time. Here we shall take up the material balance for the prevailing conditions.

Material balance: The material balance is made up of moles of component *i*, which shows that the rate of accumulation of the component *i* in the reactor must be equal to the net rate of formation of *i* due to chemical reactions in the vessel.

Rate of accumulation of *i* is the time derivative of the total amount of component *i* in the reactor.

$$\frac{d}{dt} [(\text{culture volume})(\text{molar concentration of } i)] = \frac{\text{Culture volume} \times \text{moles of } i \text{ formed}}{\text{Unit volume} \times \text{unit time}} = \frac{d}{dt} (V_R \times C_i) = V_R \cdot r_{fi} \quad (6.1)$$

where

$$\begin{aligned} V_R &= \text{Culture volume} \\ c_i &= \text{Moles of } i/\text{Unit culture volume} \\ r_{fi} &= \text{Rate of formation of } i \\ &= \frac{\text{Moles of } i \text{ formed}}{\text{Unit culture volume} \times \text{unit time}} \end{aligned}$$

Since no liquid is added to or removed from the reactor and gas stripping of culture liquid is low then, V_R is constant.

Therefore,

$$\frac{d}{dt} C_i = r_{fi} \quad (6.2)$$

From Eq. (6.2), rate of change in concentration of *i* is the rate of formation, i.e. measurement of rate of change of concentration of *i* allows the direct measurement of overall rate of *i* formation due to reaction. r_{fi} depends upon the state of cell population, (morphology, composition and age distribution) and also on the growth medium parameters.

6.3.2 The Ideal Continuously Stirred Tank Reactor (CSTR)

A schematic diagram of a CSTR (Chemostat) is shown in Figure 6.2.

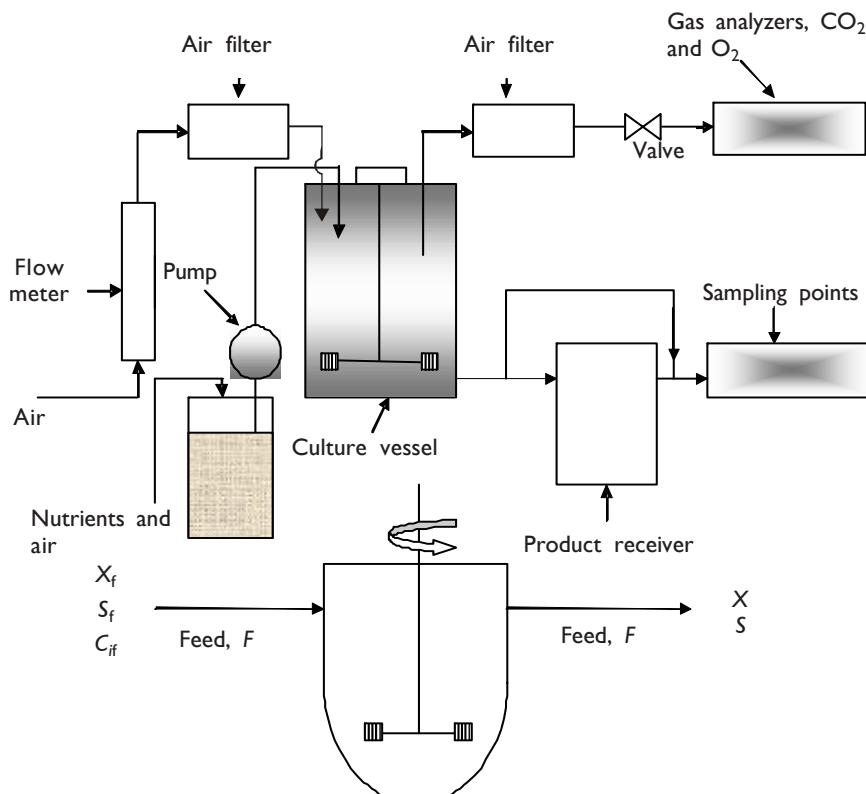


Figure 6.2 CSTR or Chemostats.

The following are the considerations in analyzing a CSTR:

F = Volumetric flow rate of feed and effluent liquid streams

C_{if} = Molar concentration of i in the feed stream

C_i = Molar concentration of i in the reactor and effluent stream

S_f = Concentration of substrates in the feed.

S = Substrate concentration in the reactor and effluent streams

C_i in the reactor and effluent are the same as complete mixing has taken place. Mixing is supplied by means of an impeller, rising gas bubbles or both. Mixing is vigorous, so that each phase of the vessel contents is of uniform composition (spatial uniformity).

Due to complete mixing, the dissolved oxygen concentration is the same throughout the bulk liquid phase.

Here we make use of two logical interpretations.

Logic 1. Decoupling of aerator: This is critically important in considering aerated CSTRs as it means that we can often decouple the aerator or agitator design from consideration of the reaction processes. So long as the aeration system maintains Dissolved Oxygen (DO) concentration in the

CSTR above the limiting concentration, we can analyze cell kinetics of the system separately. Similarly, the same logic holds good for heat transfer problems during the microbial growth.

Logic 2. Temperature controllers: During growth, temperature certainly rises. The system is maintained with adequate heat removal capacity and is equipped with a satisfactory temperature controller. We can assume that it is an isothermal operation at the desired temperature.

Material balance: Considering the steady state, where all concentrations within the reactor are independent of time, we can use material balance.

$$(\text{Rate of addition to reactor}) - (\text{Rate of removal from reactor}) + (\text{Rate of formation within the reactor}) = 0$$

Mathematically (Figure 6.2),

$$(FC_{if}) - (FC_i) + (V_R r_{fi}) = 0 \quad (6.3)$$

$$F(C_{if} - C_i) + V_R r_{fi} = 0 \quad (6.4)$$

$$F(C_{if} - C_i) = -V_R r_{fi}$$

or

$$F[C_i - C_{if}] = V_R r_{fi}$$

or

$$r_{fi} = \frac{F(C_i - C_{if})}{V_R} \quad (6.5)$$

Defining the dilution rate as

$$D = F/V_R,$$

Eq. (6.5) becomes,

$$r_{fi} = D[C_i - C_{if}] \quad (6.6)$$

The dilution rate D , characterizes the holding time or processing rate of a CSTR. It is equal to the number of tank liquid volumes which pass through the vessel per unit time. D is the reciprocal of mean holding or mean residence time used in biochemical processing. It has the units of per time.

Comparison between batch reactor and CSTR: For a batch reactor, we have

$$r_{fi} = \frac{dC_i}{dt} \quad (\text{Eq. 6.2})$$

and for a CSTR we have

$$r_{fi} = D(C_i - C_{if}) \quad (\text{Eq. 6.6})$$

In a CSTR, the kinetics determinations are straighter, since we need not measure the time dependence of concentration.

In a batch reactor, time factor is considered. In a CSTR, cell population adjusts to a steady environment and achieves almost a state of balanced growth.

Batch processes are done in a flask at a time using incubators, shakers, etc. CSTRs are more expensive and complicated. In a CSTR the steady state is achieved in hours or days, thus magnifies the problem of contamination.

6.4 KINETICS OF BALANCED GROWTH

Balanced growth is an approximation where average cellular synthesis activities are not affected by the growing cell population as the coordination is perfect.

As we know a cell can grow in size or mass and numbers. We can use here the unstructured models to characterize the bio-phase.

The net rate of cell mass growth, r_x is written as

$$r_x \propto X$$

or

$$r_x = \mu X \quad (6.7)$$

where μ is the net specific growth rate of cells given as per time and is the constant of proportionality. Using this representation for steady state CSTR, the material balance for the cell mass is as follows:

Using Feed side = Effluent side, we have

$$\frac{F}{V_R} \times X_f = \left(\frac{F}{V_R} - \mu \right) X \quad (6.8)$$

$$D \times X_f = (D - \mu) X \quad (6.9)$$

where dilution rate,

$$D = F/V_R$$

As sterile nutrient is given to the liquid feed culture, so $X_f = 0$. Now Eq. (6.9) becomes,

$$DX = \mu X$$

or

$$D = \mu \quad (6.10)$$

It means that the dilution rate is equal to specific growth rate which shows that a non-zero cell population is revealed and maintained. When Eq. (6.10) is satisfied, it seems that Eq. (6.9) does not determine X when the feed is sterile.

The experiments with continuous culture of *Bacillus linens* confirm the indeterminate nature of the population level. After a steady state, continuous operation is achieved at a 6-hour point, and two subsequent interruptions of the culture are imposed. The behaviour is shown in Figure 6.3.

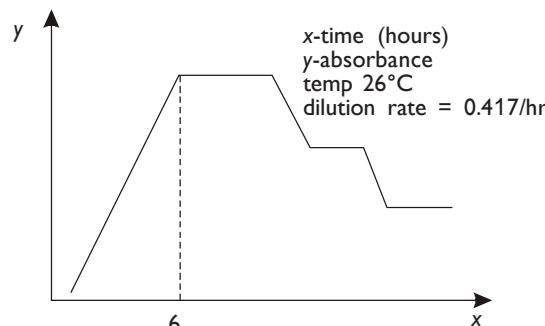


Figure 6.3 Absorbance vs time.

In each case, a portion of the reactor contents consisting of cells plus medium is removed and replaced by medium alone. Following each interruption, the system achieves a new steady population of different size.

6.5 MONOD'S GROWTH KINETICS

J. Monod in 1942 proposed the use of a saturation isotherm type of equation to relate the growth rate of a microorganism culture to the prevailing feed concentration. It is expressed as

$$\mu = \frac{\mu_{\max} S}{K_s + S} \quad (6.11)$$

where

μ = specific growth rate, hr^{-1}

S = substrate concentration, gm-mol/litre

μ_{\max} = maximum specific growth rate

K_s = Monod's constant.

The graph shown in Figure 6.4 is the general form of Monod's equation.

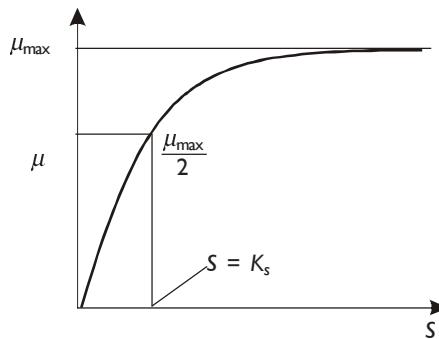


Figure 6.4 Substrate concentration vs specific growth rate.

Note: The microorganism requires several substrates for its growth but it is assumed that all but one is present in excess of requirements and the substance to which S relates is the limiting substrate component.

With reference to the above graph, the specific growth rate increases with the increase in concentration of the substrate and reaches a limiting value of μ_{\max} at high substrate levels.

In Eq. (6.11) when $S \gg K_s$, then K_s is small and ignorable.

Therefore, we get

$$\mu = \mu_{\max}$$

When $S = K_s$, Eq. (6.11) becomes

$$\mu = \frac{\mu_{\max}}{2} \quad (6.12)$$

6.6 TRANSIENT GROWTH KINETICS

During certain intervals in the batch cultivation or during the start up or disturbances to continuous flow reactors, cell population grows in a transient state.

6.6.1 Growth Patterns and Kinetics in a Batch Culture

When a liquid nutrient medium is inoculated with a seed culture, the organisms selectively take up the dissolved nutrients from the medium and convert them into biomass.

A typical batch growth curve that includes the following phases is depicted in Figure 6.5.

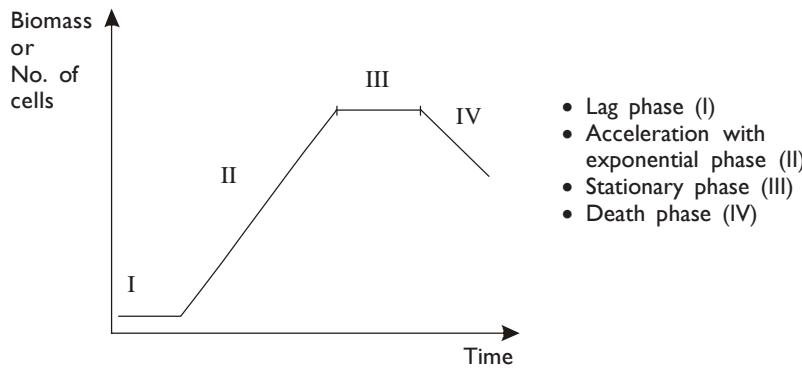


Figure 6.5 Biomass vs time.

Lag phase: In the lag phase there is no increase in microbial density with time. The length of the phase depends upon changes in the nutrient composition, age and the size of the inoculum. For instance, if the size of the inoculum is small, there will be outward diffusion of the nutrients into the bulk medium. There could be a sudden shift from the old to the new environment known as adaptability.

Acceleration with exponential phase: This phase is also called the logarithmic phase. In this phase, biomass increases exponentially with time.

Mathematical expression: We know that the growth rate is proportional to existing population. Here existing population means the microbial density X , which has been defined as mass of cells per unit culture volume.

We can write it as

$$\frac{dX}{dt} \propto X$$

or

$$\frac{dX}{dt} = \mu X \quad (6.13)$$

The above equation is called *Malthus law*.

Rearranging, we get

$$\frac{dX}{X} = \mu dt \quad (6.14)$$

Integrating Eq. (6.14) using the limits $X = X_0$ at $t = 0$ and $X = X$ at $t = t$, and simplifying, we get the resultant as

$$X = X_0 e^{\mu(t - t_{\text{lag}})} \quad (6.15)$$

Note: $t = 0 = t_{\text{lag}}$

Equation (6.15) shows that the growth is exponential.

Doubling time: At $t = t_d$, the time for doubling of biomass, $X = 2X_0$. Using the expression of the form $\ln(X/X_0) = \mu t$ and replacing the values of X and t by $2X_0$ and t_d respectively, we get

$$t_d = \frac{\ln 2}{\mu} \quad (6.16)$$

It is an expression for the doubling time of the biomass. In a CSTR, it is used to characterize the population during the growth.

Stationary phase: The stationary phase starts at the end of the exponential phase. In this phase, the net growth rate is zero as there is no cell division where we can say the growth rate is balanced by the death rate. Though the net growth rate is zero, the cells are still metabolically active and produce secondary metabolites. During the stationary phase the process called *endogenous metabolism* takes place, i.e. cell catabolizes the cellular reserves for new building blocks and for energy producing monomers.

The cell must expend some energy to maintain an energized membrane and also for the transport of nutrients for essential metabolic activities. The metabolic activities refer to the motility and repair of the damaged cells. This energy expenditure is called *maintenance energy*.

Conversion of cell mass to maintenance energy or the loss of cell mass due to the lysis during the stationary phase is given by

$$\frac{dX}{dt} \propto X$$

or

$$\frac{dX}{dt} = -K_d X$$

or

$$\frac{dX}{X} = -K_d dt$$

Further integrating, using the limits as $X = X_{s_0}$ at $t = 0$ and $X = X$ at $t = t$, we get

$$X = X_{s_0} e^{-K_d t} \quad (6.17)$$

where K_d = first-order rate constant for the endogenous metabolism

X_{s_0} = cell mass concentration at the start of the stationary phase

Death or decline phase: It follows the stationary phase. There is no clear demarcation between the stationary phase and the death phase, since some cells even die in the stationary phase because of exhaustion of essential nutrients or the accumulation of the toxic products.

The rate of death can be given as

$$\frac{dN}{dt} \propto N$$

where N is the cell concentration.

Also

$$\frac{dN}{dt} = -K'_d N$$

or

$$\frac{dN}{N} = -K'_d dt \quad (6.18)$$

Integrating the above expression using the limits as $N = N_s$ at $t = 0$ and $N = N$ at $t = t$, we get

$$N = N_s \cdot e^{-K'_d t}$$

where K'_d = first-order death rate constant (6.19)

Multiple lag phases: Multiple lag phases may sometimes be observed when the medium contains multiple carbon sources. Multiple carbon sources can be in the form of glucose, xylose, sucrose, etc.

During the growth of the cell or a microbe feeding on one particular carbon source that nears the exhaustion, the cell must divert its energy from growth to retool for the new source of carbon supply, i.e. second carbon source. This phenomenon known as *diauxic growth* which is caused by a shift in the metabolic patterns in the midst of growth. Figure 6.6 depicts multiple lag phases during the growth.

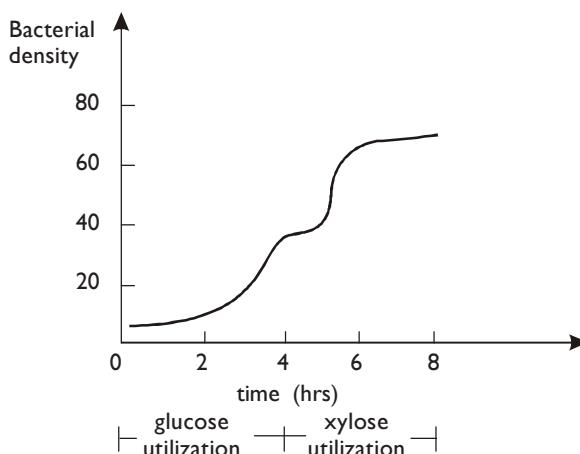


Figure 6.6 Multiple lag phase.

In a medium containing initially equal amounts of glucose and xylose, diauxic growth of *E. coli* is observed in a batch culture.

The design to minimize the culture and process times normally includes minimization of lag times associated with each new batch culture. A good design is to minimize the length of the lag phase and maximize the rate and length of the exponential phase.

To comply with, the following generalizations are drawn:

1. The inoculating culture should be as active as possible and the inoculation should be carried out in the exponential growth phase.
2. The culture medium used to grow the inoculum should be as close as possible to the final full-scale fermentation composition.
3. The use of a reasonably large inoculum of the order of 5-10 per cent of the new medium volume, is recommended to avoid the wastage by diffusion of the required intermediates or activators.

At the end of the lag phase, the population of microorganisms is well adjusted to its new environment. The cells can then multiply rapidly, and cell mass, or the number of living cells, doubles regularly with time.

6.7 CELL CULTURE MEDIUM

The cell culture medium based on the make is of two types:

- Synthetic medium
- Complex medium

Synthetic medium: In the synthetic medium, the chemical composition is well defined. The synthetic medium is a combination of the following: mineral base, carbon and nitrogen, energy sources, and vitamins.

The mineral base has buffering compounds to reduce large changes in pH values during growth. The general goal in making a medium is to support good growth and/or high rates of product synthesis. It does not mean that all nutrients should be supplied in excess of the amounts as to actually what is required. This is because, excessive concentration of a nutrient can inhibit or even poison cell growth. Moreover, if cells grow too extensively, their accumulated metabolic end products will often disrupt the normal biochemical processes of the cells. Therefore, it is a common practice to limit the total growth by limiting the amount of one nutrient in the medium. Varying the concentration of one essential medium constituent, keeping the others constant, the growth rate is as shown in Figure 6.7.

The following is the formulation of culture medium:

Common ingredients (Mineral base)		Synthetic medium
Water (1 litre)		NH_4Cl (1 g)
K_2HPO_4 (1 g)	+	Glucose (5 g*)

*Indicates that media to be sterilized by autoclaving and glucose to be added aseptically and sterilized separately.

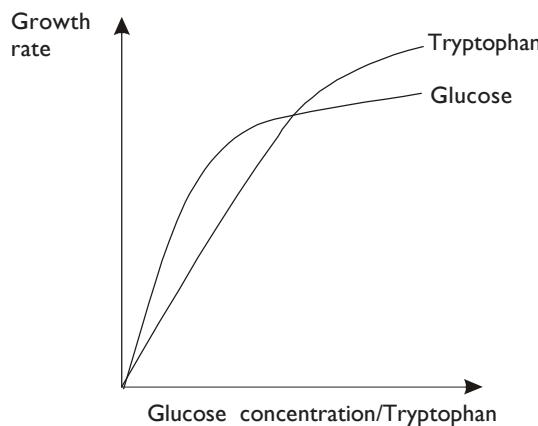


Figure 6.7 Growth rate in cell culture medium.

MgSO₄·7H₂O (10 mg)
CaCl₂ (10 mg)
Trace elements (0.02–0.05 mg)

Nicotinic acid (0.1 mg)

Complex medium

Glucose (5 g) + Common ingredients
Yeast extract (5 g)

Note: Culture medium can be synthetic or complex based on the make up.

If glucose is not sterilized separately in an autoclave, then it partially decomposes to the substances which are toxic. The exact chemical make-up of the yeast extract is unknown. The others that can be used in place of it are beef broth, blood infusion broth, corn steep liquor and sewage. Formulation or make-up is required to support the good growth and the high yield rates of product synthesis.

6.8 ENVIRONMENTAL FACTORS AFFECTING GROWTH KINETICS

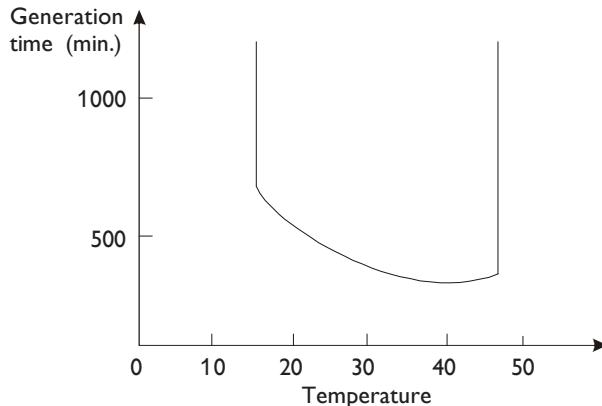
The environmental factors that affect the growth kinetics are as follows:

Temperature: It is said that the temperature range of -5 to 95°C supports life. Table 6.2 illustrates the classification of prokaryotes based on the temperature interval in which they grow.

Table 6.2 Classification of prokaryotes based on different temperature intervals

Group of Microorganisms	Minimum Temperature (°C)	Optimum Temperature (°C)	Maximum Temperature (°C)
Thermophiles; temperature loving	40–45	55–75	60–80
Mesophiles; moderate temperature conditions	10–15	30–45	35–47
Psychrophiles; humid conditions			
Obligate	–5.0–5.0	15–18	19–22
Facultative (flexible behaviour)	–5.0–5.0	25–30	30–35

Let us take an example to show the temperature dependence on *E. coli* generation (Figure 6.8).

**Figure 6.8** Generation time vs temperature.

pH: Protein configurations and the activity of cells are pH dependent. Also cellular processes, reactions and growth rates depend on pH. For example:

Yeast grows to the maximum at the pH of 4.0–5.0. Moulds grow to the maximum at the pH of 5.0–7.0. In the case of bacteria, the maximum growth is seen at the pH of 6.5–7.0. Therefore, it is seen that different microbes grow to the maximum at different pH values.

Thermodynamic activity of water and hydrostatic pressure: The thermodynamic activity of water and hydrostatic pressure influence solute activity in a medium which in turn affect the dissolved oxygen (DO) concentration.

An increase in DO content in a medium for aerobic processes favours growth rates.

6.9 CELL GROWTH AND KINETIC PATTERNS

Figure 6.9 gives us a general idea about the growth process. Here a number of substrates (ΔS) are shown entering the system which is a cell having a

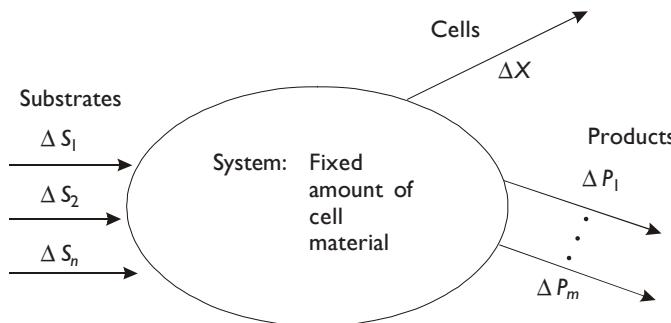


Figure 6.9 Macroscopic view of cell growth.

fixed amount of cell material. Out of the cell are shown the cells produced (ΔX) and the products liberated (ΔP) simultaneously.

It follows the basic equation, i.e.



Cell material serves as a catalyst.

Substrates react to produce more cells and metabolic products (typically an organic compound).

6.9.1 Kinetic Patterns of Growth and Product Formation in Batch Fermentation

Microbial products can be classified into three major categories:

- Growth associated products
- Non-growth associated products
- Mixed growth associated products

Growth associated products: The growth associated products are produced simultaneously with microbial growth. The rate of product formation (q_p) is proportional to the specific growth rate, i.e. μ_g . It is given as

$$q_p = \frac{1}{X} \frac{dp}{dt}$$

$$q_p = Y_{p/X} \times \mu_g \quad (6.20)$$

where X = cell mass concentration (g/L)
 μ_g = gross specific growth rate (hr^{-1})

$$Y_{p/X} = \frac{\Delta P}{\Delta X} \text{ yield coefficient of product on cell mass.}$$

Figure 6.10 shows a graphical representation of cell mass concentration or product concentration against time in the case of growth associated products.

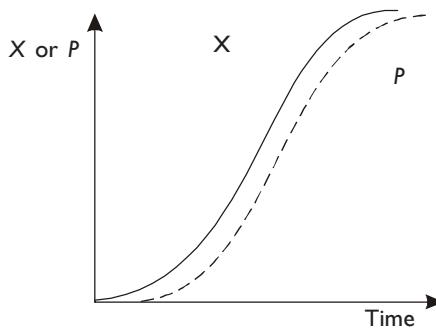


Figure 6.10 Time vs cell mass concentration/product concentration.

Non-growth associated products: This type of product formation is seen during the stationary phase when the growth rate is zero (Figure 6.11). The specific rate of production is constant. It is given as

$$q_p = \beta = \text{constant} \quad (6.21)$$

Examples include secondary metabolites as antibiotics.

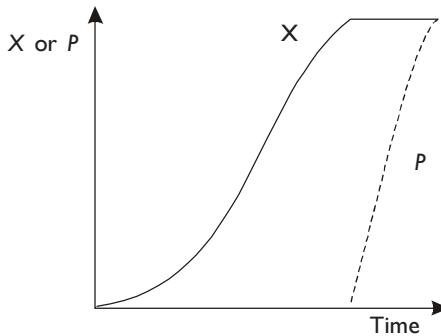


Figure 6.11 Time vs cell mass concentration/product concentration.

Mixed growth associated products: Mixed growth associated products occur during the slow growth and stationary phases. The specific rate of product formation is given as

$$q_p = \alpha \mu_g + \beta \quad (6.22)$$

where α and β are constants.

It is called the Leudeking–Piret model.

If $\alpha = 0$, the product is non-growth associated. Therefore, $q_p = \beta$ (constant).

If $\beta = 0$, the product is growth associated. Therefore,

$$q_p = \alpha \times \mu_g$$

where

$$\alpha = Y_{p/X} \quad (6.23)$$

since

$$q_p = \frac{1}{X} \frac{dp}{dt} = Y_{p/X} \times \mu_g$$

Figure 6.12 depicts a graph of time against cell mass concentration or product concentration in the case of mixed growth associated products.

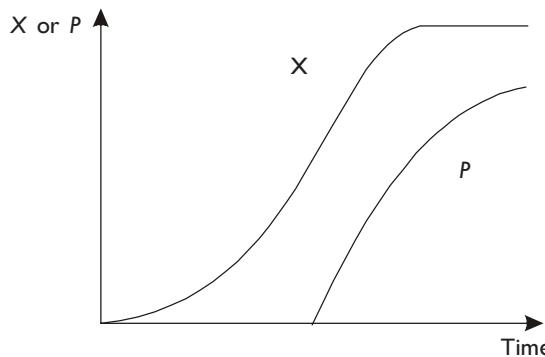


Figure 6.12 Time vs cell mass concentration/product concentration.

6.9.2 Yield Coefficient and Maintenance Coefficient

To better describe the growth kinetics, some stoichiometrically-related parameters such as yield coefficient and maintenance coefficient are defined.

Yield coefficients: The yield coefficients are based on the amount of consumption of another material. For example, in a fermentation process, growth yield is given as

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad (6.24)$$

They are also based on other substrates or product formation:

$$Y_{X/O_2} = \frac{\Delta X}{\Delta O_2}$$

and

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad (6.25)$$

where X = cellular material formed (g/mol)

S = amount of substrate consumed

P = amount of product formed

Y_{X/O_2} = yield factor relating to grams of cell formed per gram of O_2 consumed

The yield coefficients, for example, for organisms growing aerobically on glucose are as follows:

$$Y_{X/S}: 0.4 - 0.6 \text{ g/g}$$

$$Y_{X/O_2}: 0.9 - 1.4 \text{ g/g}$$

Note: Anaerobic growth is less efficient, so Y is reduced substantially.

Maintenance coefficient: The maintenance coefficient is used to describe the specific rate of substrate uptake for cellular maintenance. It is given as

$$M = -\left[\frac{dS}{dt} \right]_m / X \quad (6.26)$$

During the stationary phase in which a little external substrate is available, endogenous metabolism of biomass components is used for maintenance energy. Basically, cellular maintenance represents the energy expended to repair the damaged materials and components to derive the normal functioning of cells.

With the understanding of the concepts of growth rate, specific growth rate, yield coefficients, etc., now we shall see the steps in developing the mass balance on substrates, cell mass and products during the growth process.

The mass balance on substrates and cell mass will yield the equations that are called *Monod's chemostat models*.

6.9.3 Mass Balance on Substrates

In Figure 6.13 is shown a fermenter (reactor) into and out of which contents are added and removed. It is a continuous stirred tank reactor with volume V to keep the contents inside the reactor well mixed.

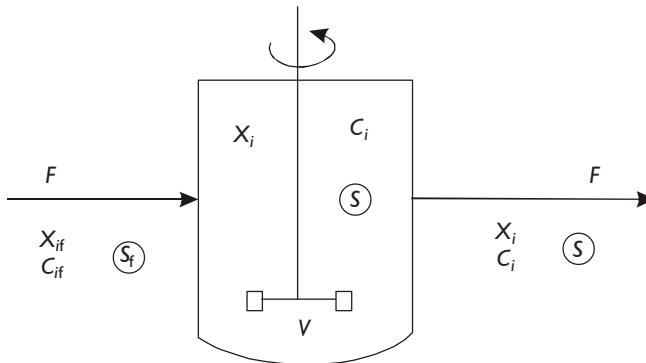


Figure 6.13 Fermenter.

At the feed side: Let F be the flow rate of the feed. C_{if} be the concentration in the feed. S_f be the substrate concentration in the feed system. In the reactor, the contents are well mixed and change to X_i and C_i with S as the concentration of substrates.

At the effluent side: Let F be the flow rate, (constant) with X_i , C_i and S , the same as in the reaction vessel, Monod's equation is given as:

$$\mu = \frac{\mu_{\max} S}{K_s + S} \quad (6.27)$$

and the yield coefficient

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad (6.28)$$

Taking the mass balance at steady state (feed side = effluent side),

$$\frac{F}{V_R} (S_f - S) = \frac{1}{Y_{X/S} \mu X}$$

$$\frac{F}{V_R} (S_f - S) - \frac{1}{Y_{X/S} \mu X} = 0 \quad (6.29)$$

$$D(S_f - S) - \frac{1}{Y_{X/S} \mu X} = 0 \quad (6.30)$$

where D = Dilution rate = F/V_R

Using Eq. (6.27) in Eq. (6.30), we get

$$\begin{aligned} D(S_f - S) - \frac{1}{Y_{X/S} \left(\frac{\mu_{\max} S}{K_s + S} \right) X} &= 0 \\ \therefore D(S_f - S) - \frac{\mu_{\max} \times S \times X}{Y_{X/S} (K_s + S)} &= 0 \end{aligned} \quad (6.31)$$

The above equation is called *substrate balance* equation.

Correspondingly, cell mass balance is:

$$D \cdot X_f = (D - \mu) \cdot X$$

or

$$DX_f + (\mu - D)X = 0 \quad (6.32)$$

$$\frac{F}{V_R} \times X_f = \left(\frac{F}{V_R} - \mu \right) X$$

$$\frac{F}{V_R} \times X_f + \left(\mu - \frac{F}{V_R} \right) X = 0$$

Substituting Eq. (6.27) in Eq. (6.32), we get

$$D \cdot X_f + \left[\frac{\mu_{\max} S}{K_s + S} - D \right] X = 0 \quad (6.33)$$

The above equation is called *cell mass balance equation*. Equations (6.31) and (6.33) are known as *Monod's chemostat models*.

6.9.4 Products Balance

We can define

$$Y_{P/X} = \frac{\text{mass of product formed}}{\text{increase in cell mass}}$$

Writing in terms of dilution rate and yield coefficients for the products,

$$D(P_f - P) + Y_{p/X} \times \mu \times X = 0$$

or

$$P = P_f + \frac{Y_{p/X} \times \mu \times X}{D} \quad (6.34)$$

From Eq. (6.34), the concentration of product in the effluent is calculated.

6.9.5 Other Forms of Growth Kinetics

Besides Monod's chemostat models, different forms have been suggested to understand the rate of growth in a vessel. They are as follows:

1. Tessier $\mu = \mu_{\max} (1 - e^{-S/K_s})$
2. Moser $\mu = \mu_{\max} (1 + K_s \times S^{\lambda})^{-1}$
3. Contois $\mu = \mu_{\max} \left(\frac{S}{BX + S} \right)$ where $BX = K_{sx}$

6.9.6 Estimation of Lag Time

In the lag phase during transient growth kinetics, there is hardly any increase in the microbial density with time. Biomass concentration versus the time values are plotted to get a curve as shown in Figure 6.14 to estimate lag

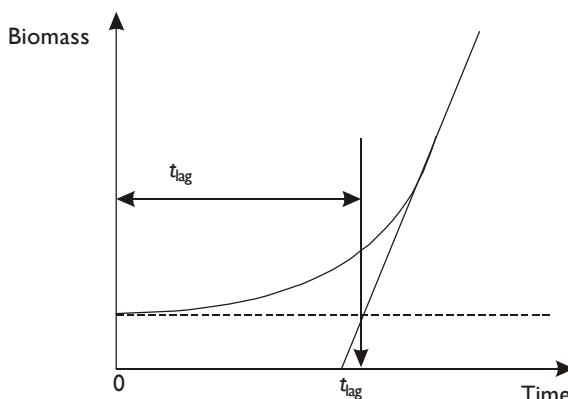


Figure 6.14 Biomass concentration vs time.

time. The tangents to the curve are drawn and the point of intersection will produce the lag time t_{lag} , and shows that virtually there is no increase in the microbial density or the biomass concentration with time. Lag phase should always be shorter for a good design.

6.9.7 Relation between Initial Nutrient Concentration and Maximum Cell Population in Batch Culture

The expected dependence of maximum population on the initial level of a given nutrient is shown in Figure 6.15. The diminution of nutrient concentration finally brings the culture to a maximum size and is

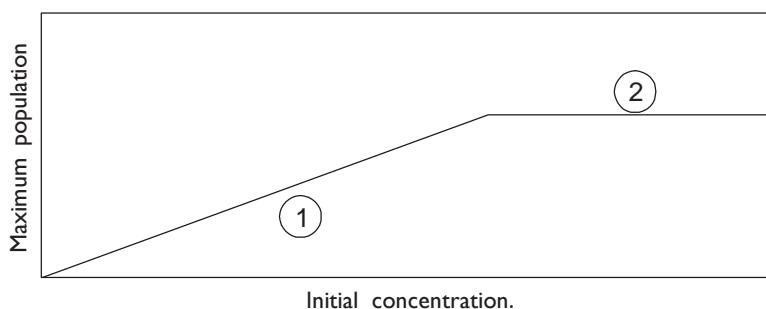


Figure 6.15 Maximum population vs initial concentration.

proportional (linear) in the initial nutrient concentration. The figure shows two regions:

1. Substrate (nutrient) exhaustion
2. Toxic products liberated during the process

Both can act as limiting factors during the growth.

Nutrient depletion cause the cessation of exponential growth. Conversely, a rise in initial nutrient supply may lead to excess amounts and eventually suggests the accumulation of toxic products which could prove to be rate limiting factors. When the nutrient concentration is large and has no influence on the maximum population, accumulation of toxic products may be the factor which limits the population size.

6.9.8 Pearl and Reed Model (Unstructured)

Pearl and Reed in 1920, contributed to a theory which included an inhibiting factor to population growth. It is unstructured model and depicts microbial density X versus time t , with the profile as shown in Figure 6.16.

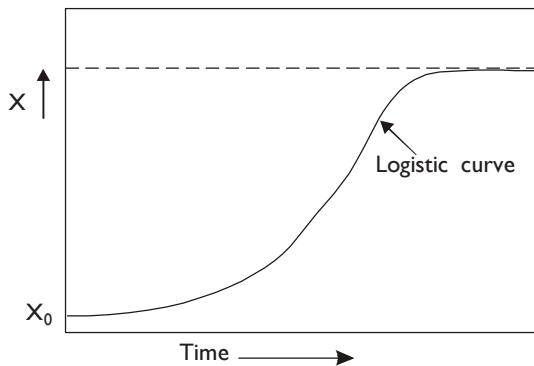


Figure 6.16 Microbial density vs time.

In this case, the relation $\frac{dX}{dt} = \mu X$ called the *Malthus law* is modified as:

$$\frac{dX}{dt} = KX - K\gamma X^2 \text{ (assuming that inhibition is proportional to } X^2)$$

$$\frac{dX}{dt} = KX(1 - \gamma X); X(0) = X_0$$

The above equation is called the *Riccati equation*.

Here is an inclusion of γ on account of inhibition at high biomass concentration. Inhibition is proportional to the square of biomass concentration.

On integration, we get

$$X = \frac{X_0 \cdot e^{Kt}}{1 - \gamma X_0(1 - e^{Kt})}$$

This is called *Logistic equation* and the profile shown in Figure 6.16 is called the *Logistic curve*.

6.10 REACTORS AND THEIR CONFIGURATIONS

We know that enzymes are quite often used as catalysts to promote specific reactions in free solution. Indeed these are typically needed in small amounts. They provide the nutritional support which would be required for microorganisms to perform the same conversion, and the subsequent removal of those microbes.

It is not necessary that the enzyme is of a microbial origin, so there could be a wider choice of operating conditions and plenty of characteristics may be made available.

We have seen that in a batch reactor, where a substrate is converted into

a product by enzymatic means, i.e. enzymatic conversion, a mass balance in terms of substrate can be given as,

$$-\{\text{Rate of formation of substrate}\} = \{\text{Rate of enzyme activity/reaction}\}$$

Further, substrate concentration being S , volume of reactor V , and t is the time; then we have

$$-\frac{Vd[S]}{dt} = Vr \quad (6.35)$$

$$r = -\frac{d[S]}{dt} = +\frac{d[P]}{dt} \quad (6.36)$$

It has been discussed in section 3.3.4, the integral MME for batch reaction kinetics, incorporating material balance and use of MME, we would finally have

$$\frac{(S_0 - S)}{\ln\left(\frac{S_0}{S}\right)} = \frac{v_{\max} \cdot t}{\ln\left(\frac{S_0}{S}\right)} - K_m \quad (\text{according to 3.19})$$

Or also can be written as,

$$K_m \cdot \ln\left(\frac{S_0}{S}\right) + (S_0 - S) = v_{\max} \cdot t \quad (6.37)$$

6.10.1 Growth of Microorganisms (MOs) in a Batch Reactor

This is also referred to as batch growth of microorganisms. Invariably, batch growth of MOs involves the addition of a small amount of MOs or their spores called the *seed culture* or *inoculum*, to a quantity of nutrient material in a suitable vessel. The process can be taken up by either aerobic means or anaerobic. In the case of aerobic fermentation growth process requires the presence of molecular oxygen, the contents of the vessel are well stirred and aerated and later growth is allowed to proceed. The conditions inside the vessel being complex and very intricate, let us support the discussion with certain assumptions.

For convenience, the feed material is present in aqueous solution and the feed has carbon content, which is an energy source and later acts as a limiting substrate during the growth of culture. Further, during growth; it is also assumed that there is no lag phase.

Note: Although in aerobic culture, aeration is very essential, the fact that air enters the vessel and exits enriched in CO_2 . This shall be ignored and emphasis laid on the changes occurring in the liquid phase.

The general balance equation for fermenter is:

$$\{\text{Rate of flow of material in}\} + \{\text{Rate of formation}\} - \{\text{Rate of flow of material out}\} = \text{Accumulation.} \quad (6.38)$$

But in a batch process, the flow in and out is both zero. Therefore, Eq. (6.38) reduces to

{Rate of formation} = Accumulation.

Mathematically, we can write as

$$r_f \cdot V = \frac{dX}{dt} \cdot V \quad (6.39)$$

or

for biomass,

$$r_f \cdot V = \mu \cdot V \cdot X = \frac{dX}{dt} \cdot V \quad (6.40)$$

In (6.40) as usual, μ is the specific growth rate, V is the volume of the reactor and X is the concentration of biomass prevailing.

If S is considered to be a concentration of limiting substrate, then an equivalent expression for substrate is:

$$r_s \cdot V = \frac{d[S]}{dt} V \quad (6.41)$$

Where r_s is the rate of conversion of substrate per unit volume of the reactor. Using the yield coefficient $Y_{X/S} = \frac{\Delta X}{\Delta S}$ as defined in section 6.9.2.

We can write in terms of Y as

$$Y \frac{dS}{dt} = - \frac{dX}{dt} \quad (6.42)$$

$Y \cdot r_s = -\mu \cdot X$ (as per Malthus law, section 6.6.1)

$$\begin{aligned} Y \cdot \frac{dS}{dt} &= -\mu \cdot X \\ \frac{-1}{Y} \mu \cdot X &= \frac{d[S]}{dt} \end{aligned} \quad (6.43)$$

The yield coefficient can also be expressed in its integral form as

$$Y = Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{X - X_0}{S_0 - S} \quad (6.44)$$

On rearranging, we get

$$S = S_0 - \frac{(X - X_0)}{Y_{X/S}} \quad (6.45)$$

Let us consider that the growth pattern follows Monod's kinetic model,

then we have $\mu = \frac{\mu_{\max}[S]}{K_s + [S]}$. Using (6.40) and substituting for μ as shown below.

$$\mu \cdot V \cdot X = \frac{dX}{dt} \cdot V$$

Substitute for μ

$$\frac{\mu_{\max}[S]}{K_s + [S]} V \cdot X = \frac{dX}{dt} \cdot V$$

or

$$\frac{\mu_{\max}[S]}{K_s + [S]} X = \frac{dX}{dt}$$

or

$$\frac{dX}{dt} = \frac{\mu_{\max}[S]}{K_s + [S]} X \quad (6.46)$$

The condition of fermentation of culture after any time t , is understood by integrating (6.46) using boundary conditions as, at $t = 0$, $X = X_0$ and $t = t$, $X = X$.

$$\int_{X_0}^X \frac{K_s + [S]}{\mu_{\max}[S]} \frac{dX}{X} = \int_0^t dt \quad (6.47)$$

Substituting for S from (6.45) in (6.47) and later integrating to get,

$$\frac{(K_s \cdot Y + S_0 Y + X_0)}{\mu_{\max}(YS_0 + X_0)} \cdot \ln\left(\frac{X}{X_0}\right) + \frac{K_s \cdot Y}{\mu_{\max}(YS_0 + X_0)} \cdot \ln\left(\frac{YS_0}{YS_0 + X_0 - X}\right) = t \quad (6.48)$$

A similar expression for substrate concentration is written as

$$\frac{(K_s \cdot Y + S_0 Y + X_0)}{\mu_{\max}(YS_0 + X_0)} \cdot \ln\left(1 + \frac{Y(S_0 - S)}{X_0}\right) - \frac{K_s \cdot Y}{\mu_{\max}(YS_0 + X_0)} \cdot \ln\left(\frac{S}{S_0}\right) = t \quad (6.49)$$

Equations (6.48) and (6.49) can be used to estimate the time needed during batch growth of MOs.

The main disadvantage is that the changes in biomass and substrate concentration during batch fermentation are not clearly understood as they are not explicit in X or S .

Therefore, a trial and error procedure has to be used to find the values at particular value of time t .

6.10.2 Continuous Culture of Microorganisms (MOs)

In this, perhaps the growth of MOs is continuous and can be achieved as continuous chemical reactions that are carried out either in tubular

fermenters (plug flow) or in a well-mixed vessel called the *back mix fermenters*.

Let us now consider the features observed in continuous stirred tank fermenter (CSTF) analogous to a continuous stirred tank reactor (CSTR).

The CSTR or a CSTF is characterized by containing a liquid phase which is homogeneous and into which the nutrients for growth are continuously added and also the suspension of MOs and depleted feed are removed continuously. Both the input to the reaction vessel (fermenter) and the output from the vessel are carried out continuously.

It is also seen that the contents in the fermenter are well-mixed, so that the samples withdrawn from any location in the fermenter shall be identical and in particular, the composition of the exit stream will be identical to that of the liquid agitated in the fermenter. In particular, the CSTF can be operated as *turbidostat*, which refers to the feed metered in such a way that a constant biomass concentration is maintained.

This is indicated in the form of turbidity and, of course, it is more commonly used as a chemostat as discussed in section 6.3.2 and features of CSTR mentioned in Appendix A.4.2.

The following schematic representation depicts a continuous stirred tank fermenter (Figure 6.17).

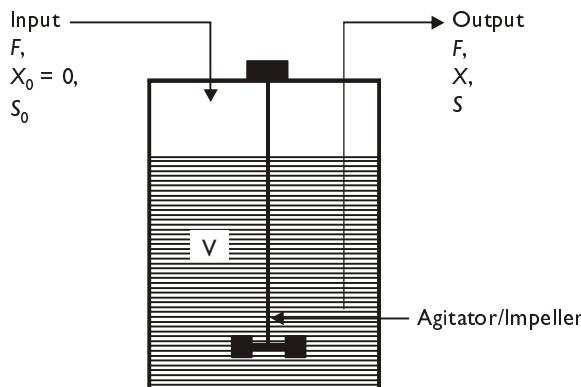


Figure 6.17 Continuous stirred tank fermenter.

Let us carry out the material balance for a CSTF. Consider a volumetric flow rate of feed as F , influent substrate as S_0 , biomass concentration as X_0 ($= 0$) during initial stages, altogether in a volume V of a fermenter called the working volume.

Due to reaction taking place, conversion is seen and the conditions change to S , the substrate concentration, i.e. $(S_0 \rightarrow S)$ X , the biomass concentration, i.e. $(X_0 \rightarrow X)$ while the quantity F remains unchanged.

The overall material balance can be written as

$$\{ \text{Material flow in} \} + \{ \text{Rate of formation by biochemical reaction} \} - \{ \text{Material flow out} \} = \text{Accumulation.}$$

Here we can develop the mass balance for both substrate and biomass. One should note that substrate is consumed and biomass is formed during the reaction.

For biomass, referring to the figure,

$$FX_f + r_{fx} V - FX = \frac{dX}{dt} \cdot V \quad (6.50)$$

Where r_{fx} is the rate of formation of biomass per unit volume of fermenter.

For substrate, referring to the figure,

$$FS_0 + r_s \cdot V - F \cdot S = \frac{dS}{dt} \cdot V \quad (6.51)$$

(Here r_s is *-ve*, as the substrate is consumed).

In the section 6.3.2 we have defined quantity called dilution rate, $D = \frac{F}{V_R}$. Here, writing $V_R = V$ the working volume or reactor volume and developing further Eq. (6.50) as

$$FX_f + r_{fx} V_R - FX = \frac{dX}{dt} \cdot V_R$$

Divide throughout by V_R

$$\frac{F}{V_R} X_f + r_{fx} \frac{V_R}{V_R} - \frac{F}{V_R} X = \frac{dX}{dt} \cdot \frac{V_R}{V_R}$$

$$D X_f + r_{fx} - D X = \frac{dX}{dt}$$

$$D (X_f - X) = - r_{fx}$$

And further at steady state we can write $\frac{dX}{dt} = 0$.

But $r_{fx} = \mu \cdot X$ (Malthus law)

$$D (X_f - X) = - \mu \cdot X \quad (6.52)$$

$$D = - \frac{\mu \cdot X}{X_f - X}$$

$$D = - \mu \cdot \frac{X}{X_f - X} \quad (6.53)$$

Further, as we know if the feed is sterile we can write $X_f = 0$.

$D = \mu$. (Same as Eq. (6.10) of section 6.4)

Further, using $D = \mu$ and introducing Monod's equation for μ as

$$\mu = \frac{\mu_{\max} [S]}{K_s + [S]}$$

We get,

$$D = \frac{\mu_{\max}[S]}{K_s + [S]} \quad (6.54)$$

Rearranging the above equation we get,

$S = \frac{D(K_s + S)}{\mu_{\max}}$ and at steady state, the substrate concentration is

$$S = \frac{DK_s}{\mu_{\max} - D} \quad (6.55)$$

Equation (6.55) shows that steady state substrate balance is independent of feed substrate concentration S_f whereas biomass concentration depends on the value of S_f .

Further, the yield coefficient

$$Y = Y_{X/S} = \frac{\Delta X}{\Delta S}$$

$$Y_{X/S} = \frac{X_f - X}{S_f - S}$$

Rearranging as

$$X_f - X = Y_{X/S} (S_f - S)$$

or

$$X = X_f + Y_{X/S} (S_f - S) \quad (6.56)$$

Substitute for S from (6.55) in (6.56)

$$X = X_f + Y_{X/S} \left(S_f - \left\{ \frac{DK_s}{\mu_{\max} - D} \right\} \right) \quad (6.57)$$

For a sterile feed $X_f = 0$,

$$X = Y_{X/S} \left\{ S_f - \left(\frac{D \cdot K_s}{\mu_{\max} - D} \right) \right\} \quad (6.58)$$

Cell productivity: Cell productivity is defined as the specific rate of production of biomass by CSTF which is the product of biomass concentration and the volumetric flow rate offered divided by the volume of

the fermenter, i.e. $\frac{FX}{V_R}$ = specific rate of production or cell productivity.

But

$$\frac{F}{V_R} = D$$

$$DX = \frac{FX}{V_R} \quad (6.59)$$

Washout and critical dilution rate D_{critical} : From Eqs. (6.55) and (6.58), we find that substrate and biomass concentration vary inversely with each other. At low dilution rates, such that $D \ll K_s$, then S is small and X is large.

At high dilution rates, it is seen that substrate concentration at steady state will increase and biomass concentration decreases.

As D nears the value of the μ_{max} , ($D \approx \mu_{\text{max}}$), X becomes infinitesimally small. This condition is called washout and the value of the D will be critical called, D_{critical} .

The behaviour is depicted in Figure 6.18.

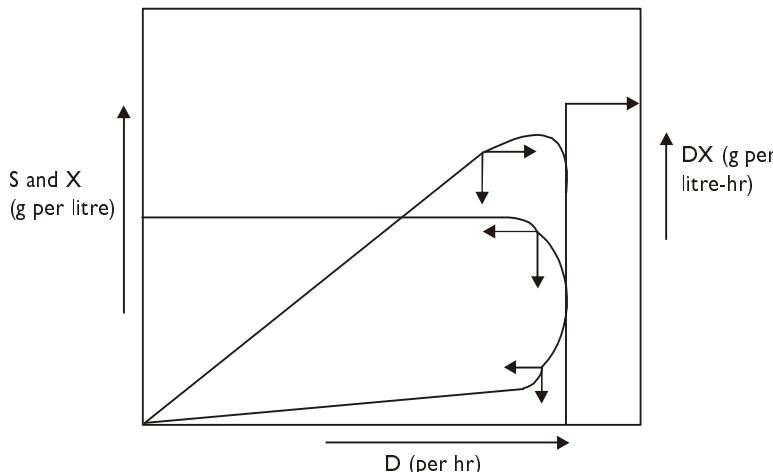


Figure 6.18 Performance curves for CSTF at steady state.

Variation of DX with varying D and at different K_s values: It is seen that DX becomes zero when $D = 0$ and also when $D = D_{\text{critical}}$. It passes through a maximum when plotted as DX vs. D as shown in Figure 6.19.

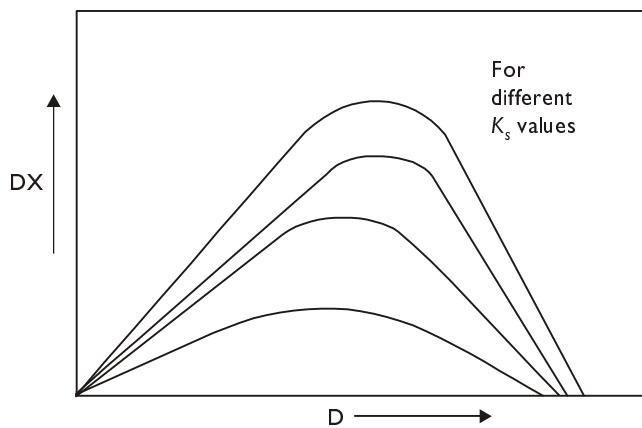


Figure 6.19 DX vs. D for different K_s values.

Location of this maximum can be obtained as follows:
Using Eq. (6.58),

$$X = Y_{X/S} \left\{ S_f - \left(\frac{D \cdot K_s}{\mu_{\max} - D} \right) \right\}$$

Multiplying the above equation by D

$$D \cdot X = D \cdot Y_{X/S} \left\{ S_f - \left(\frac{D \cdot K_s}{\mu_{\max} - D} \right) \right\} \quad (6.60)$$

The above is an expression for cell productivity.

Now when DX is maximum,

$$\frac{d}{dD} \left[D \cdot Y_{X/S} \left\{ S_f - \left(\frac{D \cdot K_s}{\mu_{\max} - D} \right) \right\} \right] = 0$$

On differentiating, we get

$$S_f - \frac{(\mu_{\max} - D_{\text{opt}}) 2D_{\text{opt}} \cdot K_s + D_{\text{opt}}^2 \cdot K_s}{(\mu_{\max} - D_{\text{opt}})^2} = 0$$

where D_{opt} is the value of dilution when DX is maximum.

Eliminating D_{opt} , we get

$$D_{\text{opt}} = \mu_{\max} \left[1 - \sqrt{\frac{K_s}{K_s + S_f}} \right] \quad (6.61)$$

The above Eq. (6.61) is a criterion for design of a CSTF.

Example 6.1 A continuous fermenter is said to have an operating volume of 20 litres. It is given a feed which is sterile or free of contaminants and containing a limiting substrate at 1000 mg per litre and a feed rate of 5 litre per hour to the vessel. Assuming, Monod's growth kinetics is applicable, calculate the steady state substrate and biomass concentration taking the values of K_s as 10 mg/litre and μ_{\max} as 0.4 per hour and the yield coefficient as 0.45.

Solution

Data: Operating volume of a continuous fermenter is 20 litres. Feed is sterile, $X_f = 0$; limiting substrate concentration, $S_f = 1000$ mg/litre, feed rate F is 5 litre/hr; $K_s = 10$ mg/litre and $\mu_{\max} = 0.4$ and $Y_{X/S}$ or $Y = 0.45$.

To calculate: steady state substrate concentration and steady state biomass concentration. First calculate the dilution rate for the fermenter i.e.

$$D = F/V_R \text{ or } F/V$$

$$D = 5.0/20 = 0.25 \text{ hr}^{-1}$$

Using the equation for the steady state substrate concentration, i.e.

$$S = \frac{DK_s}{\mu_{\max} - D} \quad \text{Eq. (6.55), we get after substitution:}$$

$$S = 0.25 (10) / 0.4 - 0.25 = 16.67 \text{ mg/litre.}$$

Further substituting in Eq. (6.56) for calculating the biomass concentration X taking $X_f = 0$ as the feed is sterile, we get

$$X = 0 + 0.45 (1000 - 16.67) = 442.5 \text{ mg/litre.}$$

Example 6.2 Using the same information as in Example 6.1, further calculate the cell productivity and the optimum dilution rate for the CSTF.

Solution

Cell productivity is given as $\frac{FX}{V_R}$ = specific rate of production.

Substituting the values in the above equation, we get

$$5 (442.5) / 20 = 110.62 \text{ mg/litre-hr.}$$

$$\text{Optimum dilution rate is } D_{\text{opt}} = \mu_{\text{max}} \left[1 - \sqrt{\frac{K_s}{K_s + S_f}} \right] \text{ [Eq.] (6.61)}$$

Substituting the corresponding values in the above equation we get, $D_{\text{opt}} = 0.360 \text{ hr}^{-1}$.

Example 6.3 It is observed that in continuous culture experimentation, when a culture is fed with substrate concentration of 1.0 g/litre, the critical dilution rate for wash out is 0.2857 hr^{-1} . The dilution rate is found to change to 0.0983 hr^{-1} , if the same microorganism is used but the feed concentration is 3.02 g/litre. Further, when the fermenter is operated at its maximum productivity, i.e. when it has attained optimum dilution rate; calculate the substrate concentration seen in each case in the effluent stream.

Solution (Hint)

Data: Substrate concentration given to a culture is 1.0 g/litre; D_{critical} is 0.2857 hr^{-1} ; Change in the dilution rate to 0.0983 hr^{-1} same organism used but feed concentration is found to be 3.0 g/litre.

To calculate: The substrate concentration in the effluent stream when the fermenter is operating at its maximum productivity.

Using

$$D = \frac{\mu_{\text{max}}[S]}{K_s + [S]} \quad \text{[Eq. (6.54)]}$$

Rewriting as

$$\frac{D}{\mu_{\text{max}}} = \frac{S}{K_s + S}$$

Inserting the condition $S \rightarrow S_f$ as $D \rightarrow D_{\text{critical}}$, we get

$$\frac{D_{\text{critical}}}{\mu_{\text{max}}} = \frac{S_f}{K_s + S_f}$$

Rearranging the above equation and using it to put in terms of μ_{\max}

$$\begin{aligned}\mu_{\max} &= D_{\text{critical}} (K_s + S_f) / S_f \\ \mu_{\max} &= 0.2857 (K_s + 1.0) / 1.0 = (0.2857 K_s + 0.2857) / 1.0 \\ \mu_{\max} &= 0.2857 K_s + 0.2857\end{aligned}\quad (\text{i})$$

When the feed rate is increased, we have

$$\begin{aligned}\mu_{\max} &= 0.0983 (K_s + 3.0) / 3.0 = 0.0983 K_s + 0.2949 / 3.0 \\ \mu_{\max} &= 0.03276 K_s + 0.0983\end{aligned}\quad (\text{ii})$$

Solving Eqs. (i) and (ii) get the values for K_s and μ_{\max} .

Maximum cell productivity takes place at D_{opt} and is given as

$D_{\text{opt}} = \mu_{\max} \left[1 - \sqrt{\frac{K_s}{K_s + S_f}} \right]$ and the substrate concentration for any dilution rate below D_{critical} is

$$S = \frac{DK_s}{(\mu_{\max} - D)}$$

Using the above equations for the initial conditions and for the increased conditions solve for D_{opt} and S respectively.

6.10.3 Stirred Tank Reactor with Recycle of Biomass

This is yet another specialized feature of CSTF's where recycling of the biomass is taken up. The recycling can be partial or complete and is desirable particularly in the field of wastewater treatment using biological methods. It also serves to operate a continuous fermenter at high dilution rates. Use of CSTFs with recycling encompasses two effects. One is that the substrate concentration in the effluent will increase and the other is that such a system in practice tends to be unstable. At the same time, a solution to overcome these ill-effects is to utilize a fermenter with the larger working volume. While the other alternative is to device a strategy to retain the biomass in the fermenter while permitting the spent feed to exit out. Although there are several other methods, the net effect is the same in all, whereas the analysis may vary based on the configuration. Below shown are certain strategies.

- (a) Use of a centrifuge
- (b) Use of internal filter
- (c) Use of an external cross flow filter.

Figure 6.20 is indicating the adoption of a centrifuge (a), an internal filter (b) and an external cross flow filter (c) respectively.

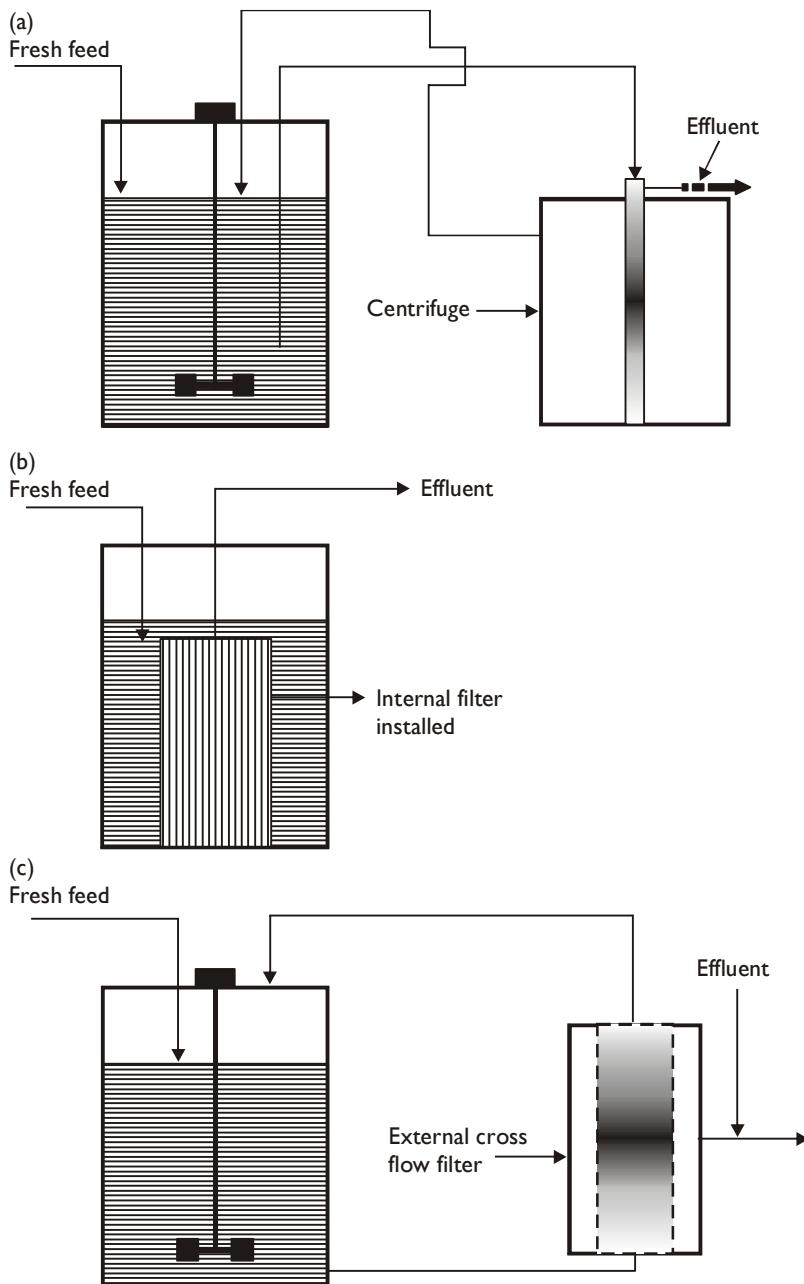


Figure 6.20 Strategies in CSTFs with recycling.

The most important strategy is passing the effluent to the settler-thickener and the concentrated biomass or a portion of it is returned to the fermenter. It is depicted in Figure 6.21.

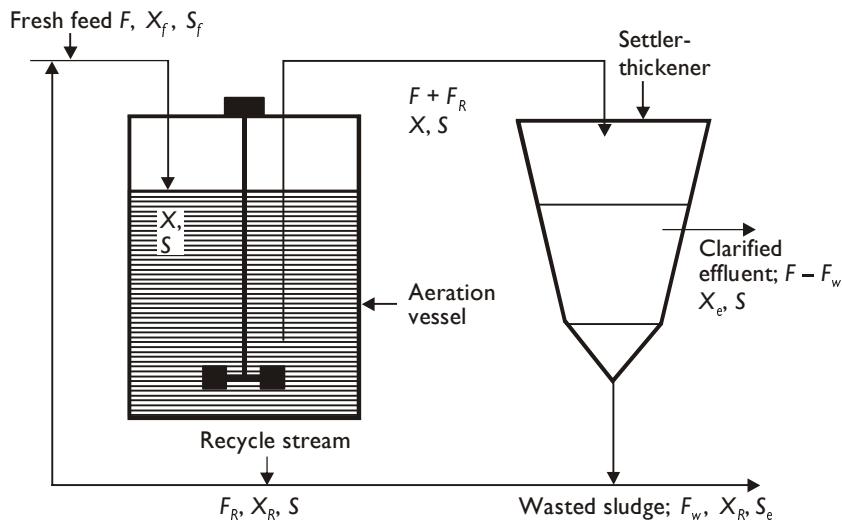


Figure 6.21 CSTF with recycle of biomass using a settler-thickener.

For Figure 6.21, carrying out material balance, we assume that no biochemical reaction or growth occurs in the separator so that the substrate concentration in the entering stream is the same as the clarified liquid effluent stream, in the recycle stream and also in the exit biomass rich stream.

Therefore,

$$\{\text{Material in with fresh feed}\} + \{\text{Material in with recycle}\} + \{\text{Rate of formation by bioreaction}\} - \{\text{Output to separator}\} = \text{Accumulation.}$$

We can use the above general equation of material balance individually as discussed below.

Substrate balance

$$FS_f + F_R S + r_S V_R - (F + F_R)S = V \cdot \frac{dS}{dt} \quad (6.62)$$

For biomass

$$FX_f + F_R X_R + r_x V_R - (F + F_R)X = V \cdot \frac{dX}{dt} \quad (6.63)$$

We know that r_x , the rate of formation of biomass is given as $r_x = \mu X$ (as per Malthus law).

and assuming steady state condition gives $\frac{dX}{dt} = 0$

and further saying that feed is sterile, $X_f = 0$. In the above Eq. (6.63)

$$\begin{aligned} F(0) + F_R X_R + \mu X V_R - (F + F_R)X &= 0 \\ F_R X_R + \mu X V_R - (F + F_R)X &= 0 \end{aligned} \quad (6.64)$$

Divide Eq. (6.64) by F throughout, we get

$$\begin{aligned} \frac{F_R}{F} X_R + \mu X \frac{V_R}{F} - (1 + \frac{F_R}{F}) X &= 0 \\ \frac{F_R}{F} X_R + \mu X \frac{V_R}{F} - X - \frac{F_R}{F} X &= 0 \end{aligned} \quad (6.65)$$

Putting $\frac{F}{V_R} = D$, dilution rate and $\frac{F_R}{F} = \text{recycle ratio } R$, we can write

$$R X_R + \frac{\mu X}{D} - X - R X = 0$$

or $\frac{\mu X}{D} = X(1 + R) - X_R \quad (6.66)$

Defining the ability of a separator to thicken or concentrate as ξ which is equal to the ratio of $\frac{X_R}{X}$, i.e.

$$\xi = \frac{X_R}{X} \quad (6.67)$$

which in fact depicts the performance of a separator.

Dividing Eq. (6.66) by X and using Eq. (6.67), we get

$$\mu/D = 1 + R - \xi R \quad (6.68)$$

Rearranging Eq. (6.68), we get

$$D = \frac{\mu}{1 + R - \xi R}$$

or

$$D = \frac{\mu}{1 - R(\xi - 1)} \quad (6.69)$$

In the above Eq. (6.69) if $R = 0$ or $\xi = 1.0$ then $D = \mu$, i.e. as good as simple CSTF with no recycle.

Therefore, as per Eq. (6.69), the net effect of recycle is to allow the fermenter to be operated at higher dilution rates, which seems to be beneficial.

To find the concentration of substrate at steady state: Using Eq. (6.69) $D = \frac{\mu}{1 - R(\xi - 1)}$ and substituting into Monod's equation $\mu = \frac{\mu_{\max}[S]}{K_s + [S]}$ we have

$$D [1 - R(\xi - 1)] = \mu = \frac{\mu_{\max}[S]}{K_s + [S]}$$

$$(K_s + S) \cdot D [1 - R(\xi - 1)] = \mu_{\max} [S]$$

$$K_s \cdot D [1 - R(\xi - 1)] + S \cdot D [1 - R(\xi - 1)] = \mu_{\max} [S]$$

or

$$K_s \cdot D [1 - R(\xi - 1)] = \mu_{\max} [S] - S \cdot D [1 - R(\xi - 1)]$$

$$K_s \cdot D [1 - R(\xi - 1)] = \{\mu_{\max} - D(1 - R(\xi - 1))\}S$$

Finally, we have the following equation for substrate concentration:

$$S = \frac{K_s \cdot D[1 - R(\xi - 1)]}{\mu_{\max} - D[1 - R(\xi - 1)]} \quad (6.70)$$

To find the biomass concentration at steady state: Using Eq. (6.62)

$$FS_f + F_R S + r_S V_R - (F + F_R)S = V_R \frac{dS}{dt}$$

Dividing throughout by V_R and putting $\frac{dS}{dt} = 0$ (since steady state),

$$\frac{F}{V_R} S_f + \frac{F_R}{V_R} S + r_S - \left(\frac{F}{V_R} + \frac{F_R}{V_R} \right) S = 0$$

$$D \cdot S_f + r_S - \frac{F}{V_R} S = 0$$

$$D \cdot S_f + r_S - D \cdot S = 0$$

$$D[S_f - S] = -r_S \quad (6.71)$$

Introducing Monod's Kinetics with constant yield coefficient, $Y_{X/S} = \frac{\Delta X}{\Delta S}$ into Eq. (6.71), we have

$$D[S_f - S] = \frac{\mu_{\max} S \cdot X}{Y_{X/S} (K_s + S)} \quad (6.72)$$

On rearrangement, at steady state the biomass concentration is as given below.

$$X = \frac{D \cdot Y_{X/S} (S_f - S)(K_s + S)}{\mu_{\max} S} \quad (6.73)$$

If we put $S \rightarrow S_f$ as $D \rightarrow D_{\text{critical}}$, we can impose the conditions for wash out with recycle stream of biomass.

Further incorporating Monod's equation into Eq. (6.69)

$$D = \frac{\mu}{1 - R(\xi - 1)}, \text{ we have}$$

$$D = \frac{\mu_{\max} \cdot S}{\{1 - R(\xi - 1)\}(K_s + S)} \quad (6.74)$$

Therefore, the ratio of

$$\frac{D_{\text{critical}}}{\mu_{\max}} = \frac{S_f}{\{1 - R(\xi - 1)\}(K_s + S_f)} \quad (6.75)$$

will be arrived at. The above equation confirms that the dilution rate for wash out will be greater than that for simple CSTF.

6.10.4 Continuous Stirred Tank Fermenters (CSTFs) in Series

Certain situations demand the use of the CSTF's in series or in a cascade form. It will be evident that the effluent of the preceding vessel will be the feed for the succeeding vessel, and so on. Use of two, three and even more CSTF's in series is seen in the treatment of industry effluents.

The schematic representation for the two CSTFs in series is shown in Figure 6.22.

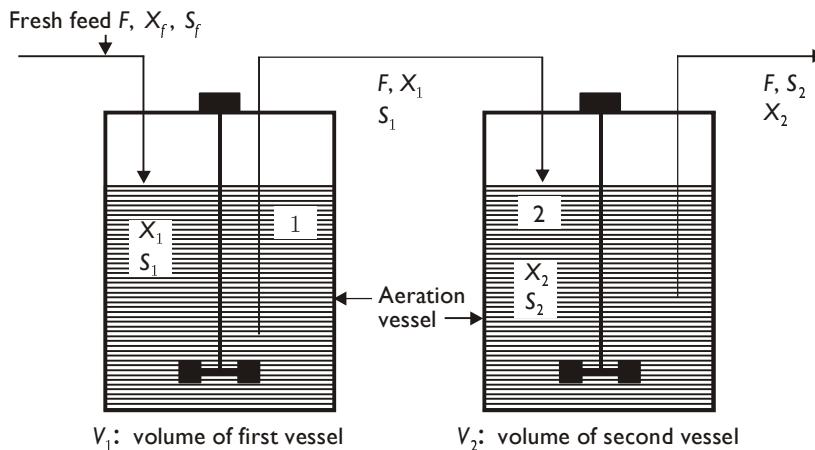


Figure 6.22 Two CSTFs in series.

Note: In Figure 6.22, the first vessel will behave as a simple chemostat and the performance equation of the same will be valid, while the second vessel will not receive a sterile feed.

As per Figure 6.22, the feed to the second fermenter will have biomass concentration X_1 and substrate concentration S_1 , while the first tank (vessel) will receive a feed with $X_f = 0$.

To get substrate concentration: Equation (6.70) will reduce to

$$S = \frac{K_s \cdot D[1 - 0]}{\{\mu_{\max} - D[1 - 0]\}}$$

We can write as

$$S_1 = \frac{K_s \cdot D_1}{\{\mu_{\max} - D_1\}} \quad (6.76)$$

Using yield coefficient $Y_{X/S} = \frac{\Delta X}{\Delta S}$, we can write for this case as shown below.

$$Y_{X/S} = \frac{X_1 - X_f}{S_f - S_1} \quad (6.77)$$

But according to Eq. (6.76), we have

$$S_1 = \frac{K_s D_1}{\{\mu_{\max} - D_1\}}$$

Substitute for S_1 in (6.77), we get

$$Y_{X/S} = \frac{X_1 - X_f}{S_f - \left(\frac{K_s \cdot D_1}{\{\mu_{\max} - D_1\}} \right)}$$

$$(X_1 - X_f) = Y_{X/S} \left\{ S_f - \left[\frac{K_s \cdot D_1}{(\mu_{\max} - D_1)} \right] \right\}$$

Since $X_f = 0$, (during initial stages)

$$X_1 = Y_{X/S} \left\{ S_f - \left[\frac{K_s \cdot D_1}{(\mu_{\max} - D_1)} \right] \right\} \quad (6.78)$$

where D_1 is the dilution rate for the first vessel.

Carrying out mass balance for second tank, we get

$$\{ \text{Flow of material in} \} + \{ \text{Rate of formation of biomass} \} - \{ \text{Flow of material out} \} = \text{Accumulation.}$$

$$FX_1 + r_2 V_2 - FX_2 = \frac{dX_2}{dt} \cdot V_2 \quad (6.79)$$

$$FX_1 + \mu_2 X_2 \cdot V_2 - FX_2 = \frac{dX_2}{dt} \cdot V_2 \quad (6.80)$$

At steady state,

$$\frac{dX_2}{dt} = 0$$

$$FX_1 + \mu_2 X_2 \cdot V_2 - FX_2 = 0 \quad (6.81)$$

$$F(X_1 - X_2) + \mu_2 X_2 \cdot V_2 = 0$$

Dividing by V_2 throughout, we get

$$\begin{aligned} \frac{F}{V_2}(X_1 - X_2) + \mu_2 X_2 &= 0 \\ D_2(X_1 - X_2) + \mu_2 X_2 &= 0 \\ D_2 = \frac{\mu_2 X_2}{(X_2 - X_1)} \end{aligned} \quad (6.82)$$

where D_2 is the dilution rate and μ_2 is specific growth rate for second tank.

Using $\mu = \frac{\mu_{\max}[S]}{K_s + [S]}$ in Eq. (6.82), we get

$$D_2 = \left\{ \frac{\mu_{\max} S_2}{K_s + S_2} \frac{X_2}{X_2 - X_1} \right\} \quad (6.83)$$

To find the biomass concentration in the second vessel, material balance over both the fermenters is

$$X_2 = Y[S_f - S_2] \quad (6.84)$$

Substituting for X_2 from Eq. (6.84) in Eq. (6.83), we get

$$\begin{aligned} D_2 &= \left\{ \frac{\mu_{\max} S_2}{K_s + S_2} \frac{Y[S_f - S_2]}{X_2 - X_1} \right\} \\ D_2 &= \frac{\mu_{\max} S_2}{K_s + S_2} \frac{S_f - S_2}{\left(\frac{D_1 K_s}{\mu_{\max} - D_1} - S_2 \right)} \end{aligned} \quad (6.85)$$

Solving Eq. (6.85) for S_2 results in

$$(\mu_{\max} - D_2) S_2^2 + \left\{ \frac{D_1 D_2 K_s}{(\mu_{\max} - D_1)} - D_2 K_s - \mu_{\max} S_f \right\} S_2 + \frac{D_1 D_2 K_s^2}{(\mu_{\max} - D_1)} = 0 \quad (6.86)$$

Example 6.4 An arrangement consists of two stirred tanks fermenters connected in series. The first fermenter has an operational volume of 75 litres and the second fermenter having an operational volume of 25 litres. A sterile feed is given to the first fermenter containing 3.5 g/litre of substrate and fed at the rate of 15 litres/hr⁻¹.

Using Monod's kinetic model to describe the growth kinetics, it is found that the value of K_s is 0.11 g/litre and maximum specific growth rate is 0.22 hr⁻¹.

Determine the steady state concentration of substrate in the second fermenter. What is the outcome if the flow were reversed from 25 litres to 75 litres fermenter?

Solution (Hint)

Data: Two CSTFs in series arrangement. Operational volume of the first fermenter is 75 liters and second fermenter is 25 litres.

Sterile feed is given to the first fermenter i.e. $X_f = 0$; $S_f = 3.5$ g/litres; $F = 15$ litres/hr.

Using the flow diagram (Figure 6.22) we first find out the dilution rates in both the fermenters.

Dilution rate for the first fermenter, $D_1 = F/V_R = 15/75 = 0.2$ hr⁻¹.

Dilution rate for the second fermenter, $D_2 = F/V_R = 15/25 = 0.6$ hr⁻¹.

Now using the Eq. 6.86 and substituting the values and from which we can find out the value of S_2 .

$$(\mu_{\max} - D_2) S_2^2 + \left\{ \frac{D_1 D_2 K_s}{(\mu_{\max} - D_1)} - D_2 K_s - \mu_{\max} S_f \right\} S_2 + \frac{D_1 D_2 K_s^2}{(\mu_{\max} - D_1)} = 0$$

Substituting the values in the above equation, we get a quadratic form of equation by which S_2 can be determined.

Alternatively adopting Monod's equation and using $D_1 = \mu_1$ with usual notations, for the first fermenter as it behaves like a simple chemostat.

Writing as $\mu_1 = D_1 = \mu_{\max} S_1 / (K_s + S_1)$ or $S_1 = D_1 K_s / (\mu_{\max} - D_1)$, by which we can find out the steady state concentration S_1 of substrate in the first fermenter.

But first find out the dilution rate, $D_1 = 0.2$ hr⁻¹ then finding out S_1 using the above equation.

$$S_1 = 0.2 (0.11) / (0.22 - 0.2) = 1.1 \text{ g/litre.}$$

$$\text{Now using } Y_{X/S} = \frac{X_1 + X_f}{S_f - S_1} \text{ (Eq. 6.77)}$$

Taking the yield coefficient as $Y = Y_{X/S} = 0.4$ and $X_f = 0$ (sterile feed) we can find out the value for X_1 the biomass concentration at steady state.

$$X_1 = Y(S_f - S_1)$$

$$X_1 = 0.4 (3.5 - 1.1) = 0.96 \text{ g/litre. (For the first fermenter)}$$

Similarly, mass balance over the second fermenter gives:

$$D_2 = \mu_2 X_2 / (X_2 - X_1) \text{ (Eq. 6.82) with usual notations.}$$

The yield coefficient $Y = (X_2 - X_1) / (S_1 - S_2)$, where S_2 is the steady state substrate concentration in the second fermenter.

Rearranging in terms of $X_2 = X_1 + Y(S_1 - S_2)$, we get:

$$X_2 = 0.96 + 0.4 (1.1 - S_2)$$

$$= 1.4 + 0.4 S_2$$

Now substituting the value of X_2 in $D_2 = \mu_2 X_2 / (X_2 - X_1)$ along with the values for D_2 , μ_2 and X_1 lead to a quadratic equation in S_2 from which S_2 can be determined and later finding the value of X_2 .

If the flow is reversed from 25 litres to 75 litres fermenter, then the dilution rate for the first vessel would be above the D_{critical} and wash out can occur in that fermenter and consequently the feed would enter the second fermenter unchanged and having no microorganisms.

To find D_{critical} :

$$\frac{D_{\text{critical}}}{\mu_{\text{max}}} = \frac{S_f}{K_s + S_f}$$

$$\text{i.e. } D_{\text{critical}} = \frac{\mu_{\text{max}} S_f}{K_s + S_f}$$

$$D_{\text{critical}} = 0.22(3.5)/0.11 + 3.5 = 0.213 \text{ hr}^{-1}.$$

6.10.5 Stirred Tank Fermenters in Series (Considering N no. of Reactors)

It is a more generalized case of CSTF in series. The analysis is carried out considering N such fermenters with V as a volume of each and F being the fresh feed volumetric flow rate introduced to the first tank. A due consideration is taken up and is that since no stream enters or leaves intermittently the flow rates between the stages and the final product leaving the last fermenter remain constant, i.e. F .

The same is shown in Figure 6.23.

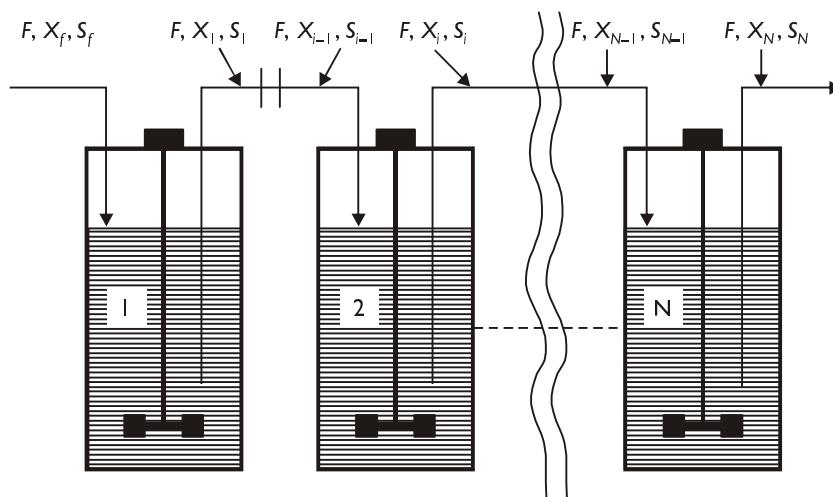


Figure 6.23 CSTFs in series (N no. of fermenters).

Carrying out the material balance over the i th fermenter,

$$FX_{i-1} - FX_i + V\mu_i X_i = V \cdot \frac{dX_i}{dt} \quad (6.87)$$

where μ_i is the specific growth rate in the fermenter.

At steady state $\frac{dX_i}{dt} = 0$

$$FX_{i-1} - FX_i + V\mu_i X_i = 0$$

Dividing throughout by V , we get

$$\frac{F}{V} [X_{i-1} - X_i] + \frac{V}{V} \mu_i X_i = 0$$

or $\frac{F}{V} X_{i-1} - \frac{F}{V} X_i + \frac{V}{V} \mu_i X_i = 0$

$$DX_{i-1} - DX_i + \mu_i X_i = 0$$

$$DX_{i-1} - X_i (D - \mu_i) = 0$$

$$X_i = \frac{DX_{i-1}}{D - \mu_i} \quad (6.88)$$

Similarly, for $i = N$, the biomass concentration is

$$X_N = \frac{DX_{N-1}}{D - \mu_N} \quad (6.89)$$

For the $(N - 1)$ tank, Eq. (6.88) can be written as

$$X_{N-1} = \frac{DX_{N-2}}{D - \mu_{N-1}} \quad (6.90)$$

Substituting Eq. (6.90) in Eq. (6.89) X_{N-1} may be eliminated as:

$$X_N = \frac{D \cdot DX_{N-2}}{(D - \mu_{N-1})(D - \mu_N)} = \frac{D^2 \cdot X_{N-2}}{(D - \mu_{N-1})(D - \mu_N)} \quad (6.91)$$

Finally, the effluent concentration for N growth tanks or fermenters may be expressed as

$$X_{N-1} = \frac{D^{N-1} X_1}{\sum_{i=2}^N (D - \mu_i)} \quad (6.92)$$

Note: The feed to the first fermenter has $X_f = 0$, as it is sterile. If the tanks are of the same volume, the wash out condition is similar to the case of a single stirred tank fermenter and therefore

$$\frac{D_{\text{critical}}}{\mu} = \frac{S_f}{(K_s + S_f)} \quad (6.93)$$

6.10.6 Stirred Tank Fermenter in Series with Biomass Recycle

Undoubtedly, the performance of a set of fermenters in series can be improvised with inclusion of a recycle stream. A series of fermenters with recycle stream is shown in Figure 6.24.

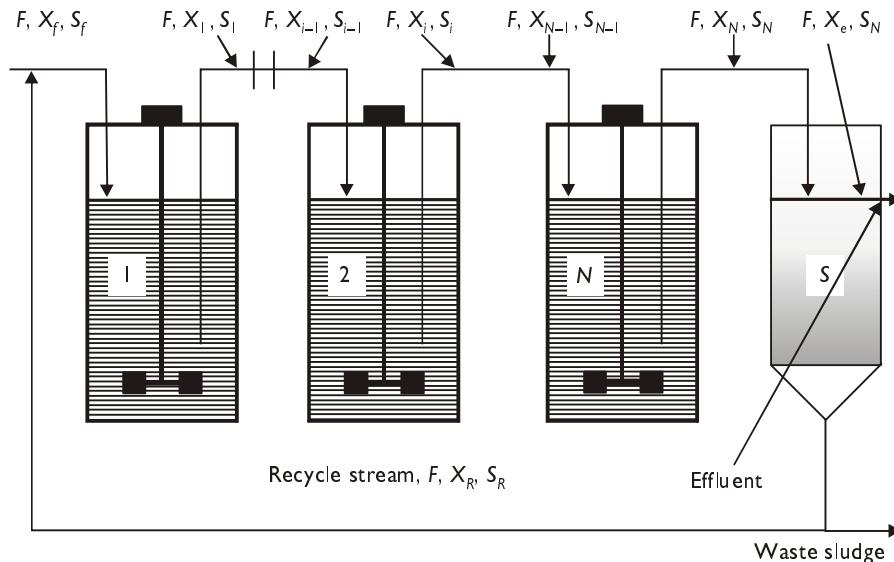


Figure 6.24 CSTFs in series with a recycle of biomass.

Material balance over i th fermenter:

$$F_0(1 + R)X_{i-1} - F_0(1 + R)X_i + \mu_i X_i V = \frac{dX_i}{dt} \cdot V \quad (6.94)$$

At steady state

$$\frac{dX_i}{dt} = 0$$

On simplification, we have

$$D(1 + R) \frac{X_i - X_{i-1}}{X_i} = \mu_i \quad (6.95)$$

Eliminating X_i by rearrangement, we get

$$X_i = \frac{DX_{i-1}}{D(R+1) - \mu_i} \quad (6.96)$$

Similarly, biomass concentration in the N^{th} tank is:

$$X_N = \frac{D^{N-1} X_1}{\sum_{i=2}^N \{D[R+1] - \mu_i\}} \quad (6.97)$$

where $R = \text{recycle ratio} = \frac{F_R}{F}$ (recycle stream)

We know that the biomass concentration entering the first tank is not zero. Then,

$$X_f^1 = X_R \frac{F_R}{F_R + F_0} \quad (6.98)$$

$$X_f^1 = X_R \frac{R}{R+1}, \text{ since } R = \frac{F_R}{F}$$

Therefore

$$X_N = \frac{D^N X_R \frac{R}{R+1}}{\sum_{i=1}^N \{D[R+1] - \mu_i\}} \quad (6.99)$$

Writing $\mu_i \rightarrow \mu_{\text{critical}}$ since $D \rightarrow D_{\text{critical}}$

$$X_N = \frac{D_{\text{critical}}^N X_R \frac{R}{R+1}}{\sum_{i=1}^N \{D_{\text{critical}}[R+1] - \mu_{\text{critical}}\}^N} \quad (6.100)$$

or using $\xi = \frac{X_R}{X}$ and rearranging to give D , we get

$$D_{\text{critical}} = \frac{\mu_{\text{critical}}}{(1+R) \left[1 - \left\{ \frac{\xi R}{1+R} \right\}^{1/N} \right]} \quad (6.101)$$

$$\text{where } \mu_{\text{critical}} = \frac{\mu_{\text{max}} \cdot S_f}{K_s + S_f}$$

Substituting for μ_{critical} in Eq. (6.101), we get

$$D_{\text{critical}} = \frac{\frac{\mu_{\text{max}} S_f}{K_s + S_f}}{(1+R) \left[1 - \left\{ \frac{\xi R}{1+R} \right\}^{1/N} \right]} \quad (6.102)$$

6.10.7 Plug Flow Fermenters (PFR/PFF)

These have some explicit features over the usual CSTF's. In its ideal form, the PFR/PFFs are characterized by the fact that the liquid phases pass through the fermenter without any back mixing.

The fresh feed and inoculum enter the fermenter at one end, the mixture of feed and growing cells move in combination towards the exit.

Figure 6.25 shows a representation of a plug flow fermenter.

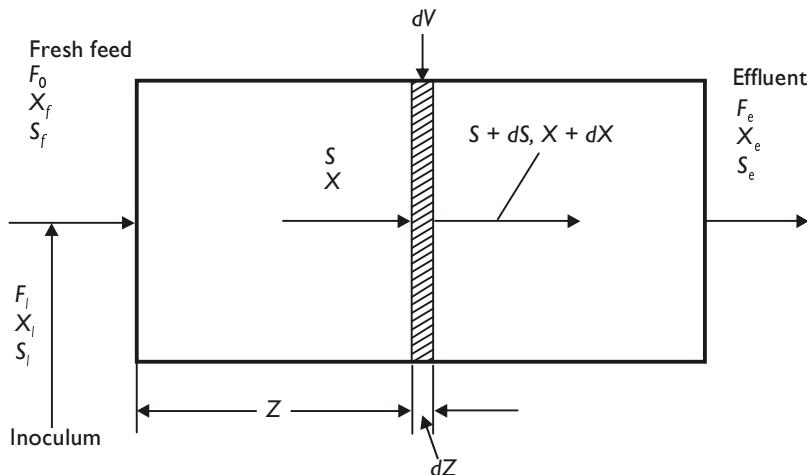


Figure 6.25 Plug flow fermenter.

The analysis in a PFF is carried out as follows:

Respective stream represent their traits as shown in Figure 6.25. Inoculum is added to initiate the growth. It has F_I the volumetric flow rate, X_I the biomass concentration and S_I the substrate concentration.

It is further mixed with feed stream having F_0 , X_f and S_f respectively, to produce the final product.

Considering an elemental volume dV and carrying out material balance.

$$\{ \text{Material entering by bulk flow} \} + \{ \text{Formation by biochemical reaction} \} - \{ \text{Material exit by bulk flow} \} = \text{Accumulation.}$$

Referring to Figure 6.25, we get

$$(F_0 + F_I)X + r_f \cdot dV - (F_0 + F_I)(X + dX) = \frac{dX}{dt} \cdot V \quad (6.103)$$

If A_c is the cross-sectional area of fermenter, then we can write

$$dV = A_c \cdot dZ$$

and further at steady state writing $\frac{dX}{dt} \cdot V = 0$, we get

$$(F_0 + F_I)X + r_f \cdot A_c \cdot dZ - (F_0 + F_I)(X + dX) = 0$$

Simplifying further

$$\begin{aligned} F_0 \cdot X + F_I \cdot X + r_f \cdot A_c \cdot dZ - (F_0 \cdot X + F_0 \cdot dX + F_I \cdot X + F_I \cdot dX) &= 0 \\ r_f \cdot A_c \cdot dZ - F_0 \cdot dX - F_I \cdot dX &= 0 \\ r_f \cdot A_c \cdot dZ - F_A \cdot dX &= 0 \end{aligned} \quad (6.104)$$

Since after mixing the inoculum with feed, let us say an entry stream of flow rate F_A , with X_A and S_A is produced.

Rearranging and later integrating

$$\int_{x_A}^{x_e} \frac{dX}{r_f} = \frac{A_c}{F_A} \int_0^z dZ \quad (6.105)$$

$$\int_{x_A}^{x_e} \frac{dX}{r_f} = \frac{A_c}{F_A} \cdot Z = \gamma \quad (6.106)$$

where γ is the residence time of liquid in the fermenter.

Here $X_A = \frac{F_0 X_f + F_1 X_1}{F_0 + F_1}$ and $X_e = X$, the boundary conditions which are altered.

Using Monod's kinetics for growth rate, $\mu = \frac{\mu_{\max}[\text{S}]}{K_s + [\text{S}]}$, we have

$$\frac{(K_s Y + S_A Y + X_A)}{\mu_m (Y S_A + X_A)} \cdot \ln \left[\frac{X_e}{X_A} \right] + \frac{K_s \cdot Y}{\mu_m (Y S_A + X_A)} \cdot \ln \left[\frac{Y S_A}{Y S_A + X_A - X_e} \right] = \gamma \quad (6.107)$$

Expression for substrate concentration: Material balance for substrate is

$$r_S \cdot A_c \cdot dZ - F_A \cdot dS = 0$$

Integrating,

$$\int_{S_A}^{S_e} \frac{dX}{r_s} = \gamma \quad (6.108)$$

where

$$S_A = \frac{F_0 S_f + F_I S_I}{F_0 + F_I} \quad (6.109)$$

Considering a constant yield coefficient Y , the final equation is

$$\frac{(K_s Y + S_A Y + X_A)}{\mu_{\max}(YS_A + X_A)} \cdot \ln \left[\frac{1 + Y(S_A - S_e)}{X_A} \right] - \frac{K_s \cdot Y}{\mu_{\max}(X_A - YS_A)} \cdot \ln \left[\frac{S_e}{S_A} \right] = \gamma \quad (6.110)$$

6.10.8 Plug Flow Fermenter (PFF) with Biomass Recycle

Figure 6.26 shows recycling of biomass using plug flow fermenter.

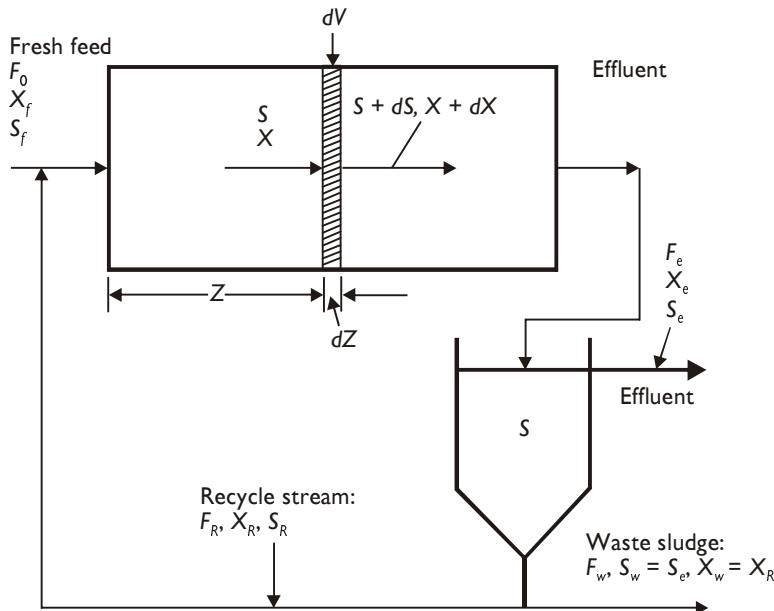


Figure 6.26 Plug flow fermenter with biomass recycling.

Considering recycle of biomass using the plug flow fermenter the equation based on the material balance is:

$$(F_0 + F_R)X + r_f \cdot dV - (F_0 + F_R)(X + dX) = \frac{dX}{dt} \cdot V \quad (6.111)$$

Writing again, $dV = A_c dZ$ and at steady state, $\frac{dX}{dt} \cdot V = 0$

$$r_f \cdot A_c \cdot dZ - (F_0 + F_R)dX = 0 \quad (6.112)$$

or

$$r_f \cdot A_c \cdot dZ - F_0(1 + R)dX = 0 \quad (6.113)$$

where

$$R = \frac{F_R}{F_0} = \text{recycle ratio}$$

The performance of a plug flow fermenter is

$$\int_{X_A}^{x_e} \frac{dX}{r_f} = \int_0^z \frac{A_c}{F_0(1+R)} dZ \quad (6.114)$$

For substrate, the form is

$$\int_{S_A}^{S_e} \left(\frac{dS}{r_s} \right) = \int_0^z \left(\frac{A_c \cdot dZ}{F_0(1+R)} \right) \quad (6.115)$$

The material balance for substrate about the mixing point of fresh feed and the recycle stream is

$$S_f \cdot F_0 + S_R \cdot F_R = S_A(F_0 + F_R) \quad (6.116)$$

$$\text{where } S_A = \frac{S_0 + RS_e}{(1+R)} \quad (6.117)$$

Similarly, for biomass, the material balance

$$X_f \cdot F_0 + X_R \cdot F_R = X_A(F_0 + F_R) \quad (6.118)$$

Using $\xi = \frac{X_R}{X_e}$; so that $X_R = \xi X_e$ and $R = \frac{F_R}{F_0}$, the recycle ratio; above equation can be rearranged as:

$$X_A = \frac{X_e + \xi R}{(1+R)} \quad (6.119)$$

now material balance over element of dV is

$$(F_0 + F_R)S + r_s \cdot dV - (F_0 + F_R)(S + dS) = \frac{dS}{dt} \cdot V \quad (6.120)$$

using $R = \frac{F_R}{F_0}$ and $\frac{dS}{dt}$ at steady state, it is written as

$$-F_0(1 + R) dS + rS \cdot dV = 0 \quad (6.121)$$

Putting $dV = A_c \cdot dZ$ in the above equation,

$$-F_0(1 + R) dS + rS \cdot A_c \cdot dZ = 0 \quad (6.122)$$

using Monod's kinetics and constant yield, we can write

$$r_s = -\frac{\mu_{\max} [S] X_A}{Y(K_s + [S])} \quad (6.123)$$

then

$$F_0(1 + R) dS = - \frac{\mu_{\max} [S] X_A A_C}{Y(K_s + [S])} \cdot dZ \quad (6.124)$$

Since $S = S_I$ when $z = 0$ and $S = S_e$ at exit, then for reactor of length Z ,

$$\int_{s_A}^s \frac{(K_s + S)dS}{S} = - \frac{\mu_{\max} X_A A_C}{YF_0(1 + R)} \int_0^Z dZ \quad (6.125)$$

yields the following:

$$(S_I - S_e) + K_s \ln \left(\frac{S_I}{S_e} \right) = - \frac{\mu_{\max} X_A A_C}{YF_0(1 + R)} dZ \quad (6.126)$$

Using the yield coefficient

$$Y = \frac{\Delta X}{\Delta S} = \frac{X_e - X_I}{S_I - S_e}$$

$$(X_e - X_I) = \frac{\mu_{\max} X_A A_C}{F_0(1 + R)} dZ - YK_s \cdot \ln \left(\frac{S_I}{S_e} \right) \quad (6.127)$$

6.10.9 Evaluation of Kinetic Parameters

It is a well-known fact that, the Monod's growth kinetics equation is predominant in estimating the performance of microbial growth phenomena. Of course this has led to the development of certain techniques to determine the constants of the equation, such as K_s and μ_{\max} .

The maximum specific growth rate is dependent upon several factors, viz. temperature and pH. For a good understanding and design of fermenter, in addition to the above parameters also is required the yield coefficient, to have a better link of the calculations of microbial growth to the concentration of substrates.

In order to carry out the measurement of these constants, we can use either a batch cultivation method or the continuous cultivation method. Indeed, different results can be obtained by different methods and this is due to the fact that the growth conditions in each case are typically different.

In other words, the substrate concentration in the batch fermenter is much higher than in the continuous fermenter.

In the succeeding paragraphs are discussed these two methods in brief.

Batch culture experiments or batch cultivation method: This is perhaps a simple and a rapid approach. It involves a batch experiment, while we ensure that the initial substrate concentration S_0 is much higher than the probable value of K_s , the Monod's constant. The approach in this case is already mentioned in the sections 6.5 and 6.6 of Chapter 6.

Continuous culture experiments: In this the analysis is more straight forward and logical. Use of chemostat (section 6.3.2) is made for the determination of kinetic constants.

The experimental method involves organizing a CSTF to grow the microorganisms using the substrates in the feed which is sterile. The feed conditions like flow rate, is adjusted to the desired value and should produce the dilution rate below the critical value for wash out and later the system is allowed to reach the steady state.

As and when a steady state is reached, careful measurements are carried out in terms of microbial density or cell population X , the substrate concentration S and the flow rate F . As a matter of fact further, the operation is repeated at a series of suitable dilution rates.

A simple configuration for the same is depicted in Figure 6.27.

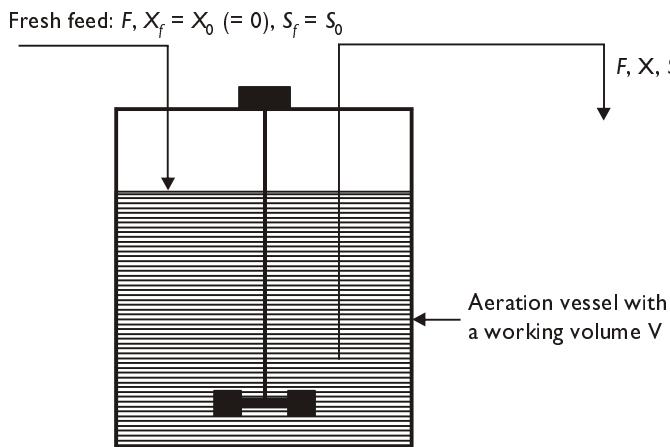


Figure 6.27 The chemostat (simple configuration) for estimation of kinetic parameters.

Using the above chemostat, and employing the conditions as mentioned above, we can determine the kinetic parameters as follows. We have earlier carried out the material balance for the CSTF and yielded the following equation.

$$FS_0 + r_s \cdot V - F \cdot S = \frac{dS}{dt} \cdot V$$

(Here r_s is $-ve$ as the substrate is consumed). which at steady state where $dS/dt = 0$, reduces to

$$D(S_0 - S) = -r_s \quad (6.128)$$

(After simplifying and using $D = F/V_R$, the dilution rate and dividing throughout).

Further, assuming that the yield coefficient Y for the conversion of substrate into the microbial cells is constant, and further applying Monod's equation incorporating yield coefficient, we get

$$D(S_0 - S) = \frac{\mu_{\max} S X}{Y(K_s + S)} \quad (6.129)$$

Writing in the reciprocals form for the above equation, we get a linear form of expression as

$$\frac{X}{D(S_0 - S)} = \frac{K_s Y}{\mu_{\max} S} + \frac{Y}{\mu_{\max}} \quad (6.130)$$

Using Eq. (6.131) to plot $X/D (S_0 - S)$ versus $1/S$ it is seen that a straight line is obtained as shown in Figure 6.28.

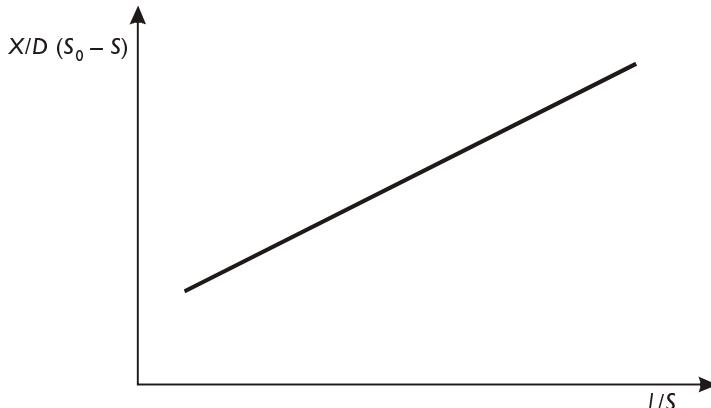


Figure 6.28 Graph showing $X/D (S_0 - S)$ vs. $1/S$ for a chemostat culture.

In order to find the value of K_s evaluate the slope of the straight line and calculate the intercept.

To find the value of μ_{\max} , at first we should calculate the value for Y . Ignoring the maintenance requirements of the culture, we find that the yield coefficient for growth is equal to the overall observed yield coefficient and is given as

$$Y = \frac{X - X_0}{S_0 - S} \quad (6.131)$$

But since the feed is sterile, we can write $X_0 = 0$

$$Y = \frac{X}{S_0 - S} \quad (6.132)$$

Note: The mean value of Y calculated at each dilution rate used in the experiments will allow μ_{\max} to be determined from the graph.

In a more realistic way it is better we calculate the specific growth rate considering endogenous respiration K_d as defined in section 6.6.1 and the equation is given below.

$$\mu = \frac{\mu_{\max} S}{(K_s + S)} - K_d \quad (6.133)$$

When dilution rate is equal to specific growth rate, i.e. $D = \mu$, we can write

$$D = \frac{\mu_{\max} S}{(K_s + S)} - K_d \quad (6.134)$$

Therefore, Eq. (6.129) can be rearranged to give:

$$\frac{\mu_{\max} S}{(K_s + S)} = YD \frac{(S_0 - S)}{X} \quad (6.135)$$

Substituting the above Eq. (6.135) in Eq. (6.134), we get:

$$\frac{(S_0 - S)}{X} = \frac{K_d}{YD} + \frac{1}{Y} \quad (6.136)$$

Using the above equation to plot $(S_0 - S)/X$ versus $1/D$, we find that a straight line is produced which has a slope of K_d/Y and an intercept $1/Y$ (Figure 6.29).

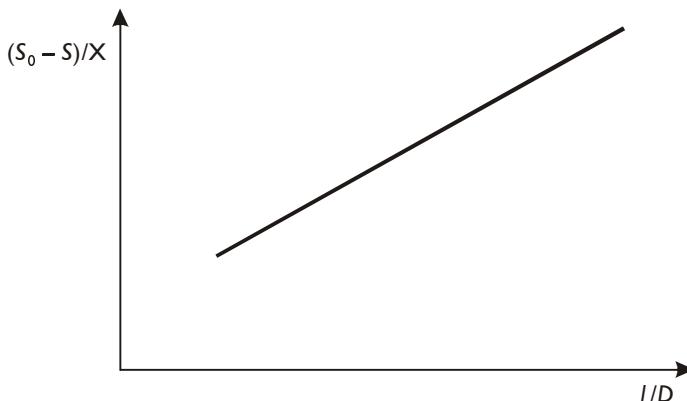


Figure 6.29 Graph showing $(S_0 - S)/X$ vs. $1/D$ for a chemostat culture.

All the above discussions permit the determination of both endogenous respiration K_d and the yield coefficient Y and subsequently all the rate or kinetic parameters for the microbial growth.

Example 6.5 A continuous stirred tank fermenter for continuous microbial cultures is operated at various dilution rates. The fresh feed concentration is 500 mg/litre. After having reached a steady state, the concentrations for biomass and the substrates at various dilution rates were measured and the values are mentioned in Table 6.3.

Table 6.3 Example 6.5

Dilution rate $D = F/V_R$, (per hour)	Substrate concentration S , (mg per litre)	Biomass concentration X , (mg per litre)
0.30	45	325
0.25	40	328
0.20	15	341
0.15	8.0	343
0.06	3.5	345

Using this information estimate the following:

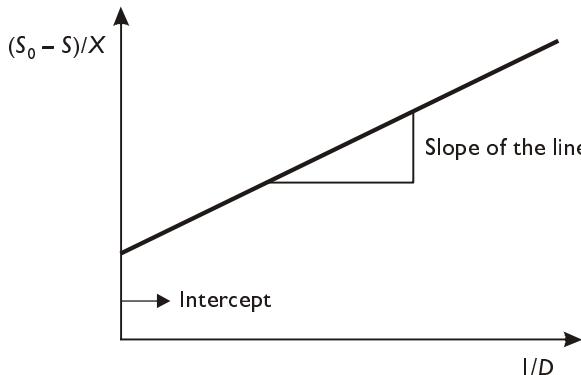
1. Monod's constants
2. Yield coefficient, Y
3. Endogenous respiration coefficient, K_d

Solution: Hint: First, using the information given in the table above calculate the values for $(S_0 - S)/X$; $1/D$; $1/S$; $X/D(S_0 - S)$ using $S_0 = 500$ mg/litre, the fresh fed concentration.

Then represent in the form of a table respectively as indicated.

$(S_0 - S)/X$	$1/D$ (hr.)	$1/S$ (ltr./mg)	$X/D(S_0 - S)$

Next using the values $(S_0 - S)/X$ versus $1/D$, plot the graph as shown below in Figure 6.30.

**Figure 6.30** Example 6.1: $(S_0 - S)/X$ vs. $1/D$.

From the graph find the intercept and slope of the line.

From the intercept, using $\text{intercept} = 1/Y$ we can find out the value for Y , the yield coefficient.

Using the slope of the line = K_d/Y , find out the value for K_d , the endogenous respiration coefficient (per hour).

Next plot the graph of $X/D(S_0 - S)$ versus $1/S$ as shown in Figure 6.31.

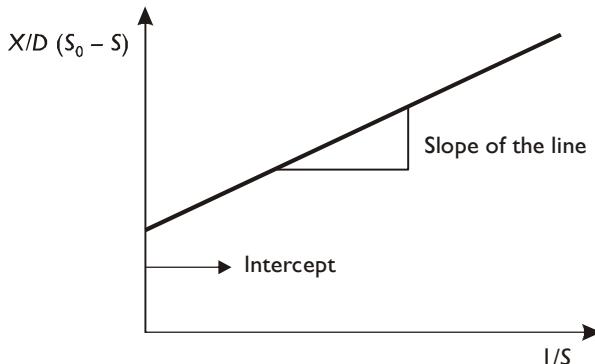


Figure 6.31 $X/D(S_0 - S)$ vs. $1/S$.

From the above figure, find the intercept = Y/μ , in terms of hours. Therefore maximum specific growth rate μ_{\max} (per hour) = $Y/\text{intercept}$.

Using the slope = $\frac{K_s Y}{\mu_{\max}}$ (mg per litre per hour)

Find the value for K_s , the Monod's constant in terms of mg/litre.

Quantification of growth kinetics: In the preceding discussions, we have studied certain important concepts pertaining to the growth of cultures. The biomass and the cell cultures, cell population kinetics and its perspectives, balanced growth, transient growth kinetics, etc. were given a qualitative treatment to understand the growth of cultures.

In addition to this, we can further contemplate the discussions on the growth dynamics in terms of kinetic descriptions.

We have seen that the cell composition and the biosynthetic capabilities do change in response to the new conditions imposed during the growth process, so called the unbalanced growth. This is indeed contrary to the concept of balanced growth (section 6.2) wherein the cellular synthesis activities are coordinated in such a way that the average cellular composition is not affected by proliferation of cell population.

Balanced growth certainly predominates in the exponential phase. Further, if the deceleration phase is due to the depletion of substrates rather than the inhibition by metabolites and toxins, the rate of growth decreases as the substrate concentration decreases, whereas in the stationary phase and the death phase, the distribution of properties among the individual cells is important. (Section 6.6.1)

Although these ideas are evident in batch cultures, but they are equally important and significant in the other modes of growth of cultures for instance, continuous cultures.

The clear explanation of growth kinetics of a cell culture will involve the identification and recognition of the structured nature of each cell and the segregation of cultures into the individual cells or units, (refer section 6.2 for structured, segregated and unstructured and unsegregated models) that may differ from each other.

We have discussed in the preceding sections about the models being structured, segregated and unstructured and unsegregated in describing the cell population kinetics. Also we know that the ones which are structured and segregated pertain to the actual case and are more realistic, while unstructured and unsegregated are considered to be the ideal ones.

Structured and segregated models are complex for computations, although being realistic in nature. The degree of realism and complexity needed in a model depends upon what is being described.

An unstructured model assumes fixed cell composition which is as good as assuming the state of balanced growth. The concept of balanced growth as understood is valid primarily in a single stage and steady state conditions of a continuous culture and also in the exponential phase of growth of batch cultures. However, it fails to validate during the transient conditions.

Overall, the use of either the structured or unstructured and segregated or unsegregated depends upon the conditions or the situations prevailing, but all have some significance.

In the succeeding paragraphs, we find the use of certain models to predict the specific growth rate of cultures.

6.10.10 Use of Unstructured, Unsegregated Models to Predict Specific Growth Rate

1. Substrate-limited Growth: It tells us about the substrate being a limiting factor during the growth. The relationship between the specific growth rate and the substrate concentration often assumes the form of saturation kinetics as depicted in Figure 6.32 which resembles Figure 6.4.

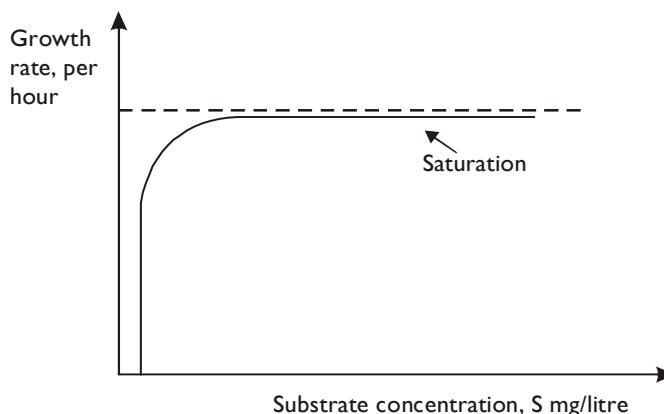


Figure 6.32 Saturation kinetics profile for culture growth.

Substrate being growth rate-limiting (i.e. increase in S influences the growth rate while changes seen in the other nutrient concentrations have no effect), is assumed to be a single component species. The nature observed is similar to the Michaelis-Menten kinetics in enzymatic reactions.

For cellular systems, we can apply Monod's equation as:

$$\mu = \frac{\mu_{\max}S}{K_s + S} \text{ (according to Eq. (6.11) with usual significance of terms)}$$

Note: The same is explained in section 6.5 for different versions of S with respect to K_s .

We see that the Monod's equation explains the substrate-limited growth only when the growth is slow and cell population density is low. This is of course a limitation observed in the Monod's equation. In reality, if growth is fast and the cell population density is high, Monod's equation cannot be used.

The release of metabolites, waste products and other toxins is likely during the process, and is due to the carbon-energy substrates. Due to this, the situation is different and therefore at high population levels, as the build-up of toxic products dominates, similar but modified Monod's equation can be used as given below.

$$\mu = \frac{\mu_{\max}S}{K_{s0}S_0 + S}$$

or

$$\mu = \frac{\mu_{\max}S}{K_{s1} + K_{s0}S_0 + S}$$

where S_0 is the initial substrate concentration and K_s is a dimensionless quantity.

The others proposed as alternative to Monod's are mentioned in section 6.9.5.

In order to calculate the specific growth rate on net basis as μ_{net} , we have an equation,

$$\mu_{\text{net}} = \frac{\mu_{\max}S}{K_s + S} - K_d$$

where K_d is endogenous respiration coefficient as already discussed in the section 6.6.1.

2. Models with growth inhibitors: The process of growth is bound to get inhibited due to the high concentrations of substrates or products released and also in the presence of inhibitory substances that could be present in the growth medium.

Therefore, the growth rate depends upon the concentration of inhibitors.

It is seen that the pattern of inhibition of microbial growth is analogous and similar to the one observed in the enzymatic inhibitions.

We here shall consider the growth inhibiting components individually.

Substrate Inhibition: Verily it is observed that the microbial growth rate gets inhibited due to the high concentration of substrates. The substrate inhibition phenomenon can be competitive or non-competitive. Importantly, if a single-substrate enzyme catalyzed reaction is the rate limiting step in the microbial growth, then the inhibition of enzyme activity results in the inhibition of microbial growth by the same pattern.

In the succeeding lines, we find the major substrate inhibition patterns and their relevant expressions.

For non-competitive substrate inhibition:

$$\mu_g = \frac{\mu_{\max}}{\left(1 + \frac{K_s}{S}\right)\left(1 - \frac{S}{K_I}\right)}$$

For competitive substrate inhibition:

$$\mu_g = \frac{\mu_{\max}S}{K_s\left(1 + \frac{S}{K_I}\right) + S}$$

Product inhibition: It is also found that the very high concentration of products during the process is inhibitory for the microbial growth. This is quite evident as the products concentration goes on elevating due to accumulation within the vessel. Here also we find that the inhibition process is either competitive or non-competitive in nature.

The expressions for the above two types are mentioned below.

For competitive product inhibition:

$$\mu_g = \frac{\mu_{\max}S}{K_s\left(1 + \frac{P}{K_{PI}}\right) + S}$$

For non-competitive product inhibition:

$$\mu_g = \frac{\mu_{\max}}{\left(1 + \frac{K_s}{S}\right)\left(1 + \frac{P}{K_{PI}}\right)}$$

where K_{PI} is the product inhibition constant.

Inhibition by toxic compounds: The toxic compounds are likely to enter the reaction vessel or are generated during the release of metabolites and finally inhibiting the growth rate of the cultures and microbes.

The expressions suggested below are all analogous to the enzymatic inhibition mechanisms.

For competitive (toxic) inhibition:

$$\mu_g = \frac{\mu_{\max} S}{K_s \left(1 + \frac{I}{K_I} \right) + S}$$

For non-competitive (toxic) inhibition:

$$\mu_g = \frac{\mu_{\max}}{\left(1 + \frac{K_s}{S} \right) \left(1 + \frac{I}{K_I} \right)}$$

For uncompetitive (toxic) inhibition:

$$\mu_g = \frac{\mu_{\max} S}{\left(\frac{K_s}{1 + \frac{I}{K_I}} + S \right) \left(1 + \frac{I}{K_I} \right)}$$

It is also observed that in some instances the presence of toxic compounds viz. metal ions, in the culture medium will lead to the deactivation of cells and the cellular material and eventually the death. In such cases where death is observed, the specific growth rate, i.e. the net specific growth rate is estimated by the following expression.

$$\mu_{\text{net}} = \frac{\mu_{\max} S}{K_s + S} - K'_d$$

where K'_d is the death rate constant, given as per hour.

Note: The logistic model or approach that refers to the inhibition due to high biomass concentrations is discussed in section 6.9.8.

6.10.11 Models for the Growth of Filamentous Organisms

Filamentous organisms are usually those that exhibit the growth in terms of size and not in terms of numbers. For example, moulds. It is discussed in section 1.6.4 that moulds which are a higher class of fungi have a vegetative structure called *mycelium*.

The growth in such cases is referred in terms of mycelia growth, i.e. hyphae. In general, the growth rate is measured in terms of elongation of hyphae and the overall size of the organism in the cultures.

It is seen that these filamentous organisms frequently form microbial pellets at high cell populations or densities in the suspension cultures. Inside these pellets the cells which are growing are quite likely to be subjected to the diffusion limitations.

The growth models for such cases should include simultaneous diffusion and nutrient consumption within the microbial pellet at large pellet sizes.

Interestingly, it is also observed that these filamentous organisms also grow on the moist solid surfaces, so called *attached growth*. Indeed such growth is a complicated process that involves not only the growth kinetics but also the diffusion of nutrients and toxic metabolites.

To elaborate the description of filamentous organisms we consider that a microbial pellet is formed in a submerged culture of a mould colony growing on the surface of an agar. This is showing growth that varies linearly with time.

A microbial pellet of radius r is growing as a function of time t . It is to be assumed here that there are no mass transfer limitations during the process.

We can write the above statement as:

$$\frac{dr}{dt} = k_{\text{pellet}} = \text{constant}$$

The growth rate of a mould colony can be further expressed as:

$$\frac{dM}{dt} = \rho 4\pi r^2 \frac{dr}{dt}$$

$$\frac{dM}{dt} = \rho 4\pi r^2 k_{\text{pellet}}$$

or

$$\frac{dM}{dt} = v M^{2/3}$$

where $v = k_{\text{pellet}} (36 \pi \rho)^{1/3}$

Integrating the above equation, we get

$$M = \left(M_0^{1/3} + \frac{vt}{3} \right)^3$$

where M_0 is the initial biomass.

This is congruent to $(vt/3)^3$.

The initial biomass M_0 is very small compared to M and therefore M varies with cube of time factor, i.e. t^3 .

6.10.12 Models for Transient Behaviour

In this we discuss the models or the approaches that predict the transient behaviour of the growing cultures, i.e. the ones which are time dependent.

We have seen that the conditions or the situations pertaining to the environment and surroundings of a microbial culture do shift and change continually, although slowly that affects cellular compositions and biosynthetic capabilities. This makes the growing culture of microbes to behave in a transient mode.

These cellular changes are not instantaneous but take place at an observable time period.

This is understood in two ways. These are:

1. The models with time delay and
2. Chemically structured models.

Models with time delay: Models with time delay basically refer to those which are unstructured models. These unstructured models are limited to balance or pseudo-balanced growth conditions. Such cases can be improvised with the inclusion of time delays so as to use in dynamic conditions.

The basic premise is that the dynamic response of a cell is dominated by an internal process with a time delay on the order of the response time under observation while the other internal processes are either too fast to be called at pseudo-equilibrium state or too slow to affect the observed response.

These models are limited to cultures with similar growth histories and subjected to perturbations.

Note: A detailed discussion of the above two models is beyond the scope of this book.

Introduction to fed batch reactors: In batch type reactors, it is seen that the input of the raw materials is given and the products formed are removed, forming a batch. It is always felt necessary that the liquid streams be added to a batch fermenter as the reaction is progressing.

In compliance with this, the nutrients are added continuously or semi-continuously, while the effluent is removed discontinuously. This is also called a repeated *fed batch culture*.

Addition or feeding during the process aids in precursors for the desired products i.e. the desired product(s) can be obtained as these are carried forward for withdrawal.

In addition to this, regulating compounds as inducers and intermittent substrates are also fed at a desired point in the batch operation so as to maintain low nutrient levels to minimize catabolite repression and substrate inhibition.

If the substrate is inhibitory in nature, intermittent addition of substrate improves the productivity of the process by keeping the substrate concentration very low. When a liquid feed stream is added and enters the reactor, the culture volume also gets altered.

Therefore, fed batch reactors are also referred to as the *semicontinuous or variable volume continuous culture reactors*.

The process of fed batch culture is shown in Figure 6.33 schematically.

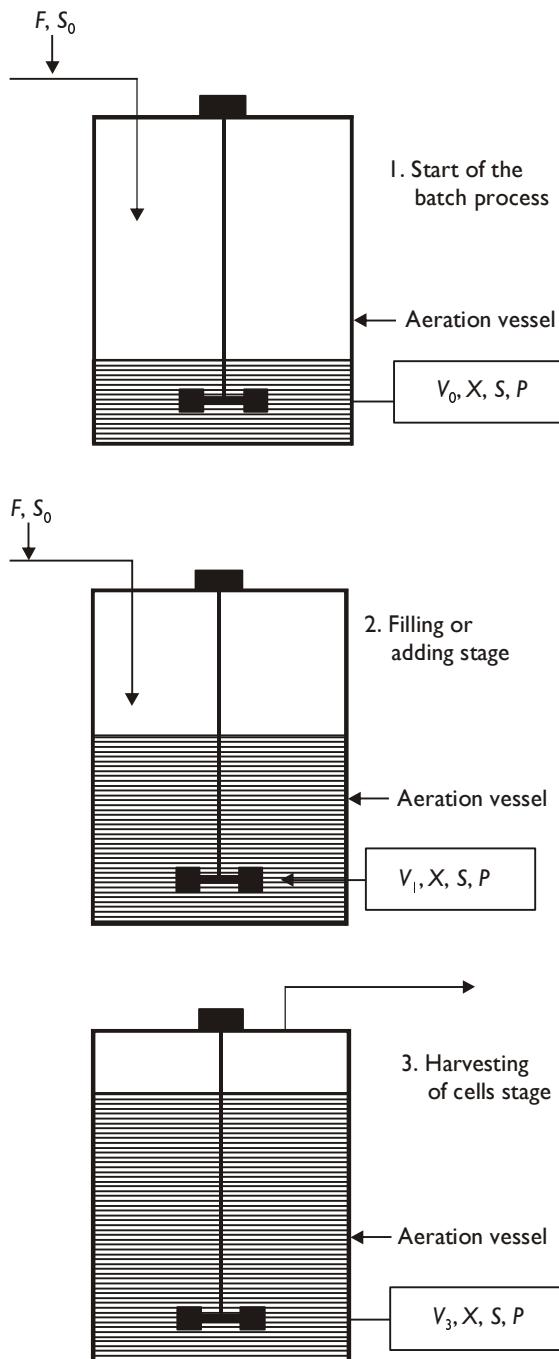


Figure 6.33 Stages of operation of fed batch reactor.

From Eq. (6.1), we have

$$\frac{d}{dt}(V_R C_i) = V_R \times r_{fi}$$

If $F(t)$ is the volumetric flow rate of the feed entering at time t and $C_{if}(t)$ is the concentration of this component in the feed stream, then from above equation we can further deduce as

$$\frac{d}{dt}(V_R C_i) = V_R \times r_{fi} + F(t) \cdot C_{if} \quad (6.137)$$

We assume here that the densities (feed stream and culture) are equal and say it is ρ . Then we get the total mass balance on the reactor contents as

$$\frac{d}{dt}(V_R \times \rho) = \rho \times F(t) \quad (6.138)$$

Further assuming that the density does not change appreciably with time during the batch process then, we get

$$\frac{d}{dt}(V_R) = F(t) \quad (6.139)$$

Differentiating Eq. (6.137) and then substitute for dV_R/dt using (6.139), we get on final rearrangement as

$$\frac{dC_i}{dt} = \frac{F(t)}{V_R} (C_{if} - C_i) + r_{fi} \quad (6.140)$$

The above equations are used to assess the fed batch reactor performance.

SUMMARY

In this chapter, we learnt the following:

- Cell growth kinetics can be modelled. They can be structured or unstructured. Unstructured models quantify cell mass as a single component and cannot describe the transient behaviour.
- In batch cultivation, a population of cells typically exhibits several different growth phases.
- Lag phase, exponential phase, stationary phase and death phase are four growth phases.
- Products formed by cells can be related to the batch-culture growth cycles. Primary products are growth associated, secondary products are non-growth associated and are made in the stationary phase.
- Some products are both growth and non-growth associated components.

- There are also segregated and unsegregated models seen during growth. These models can also be applied to the continuous culture besides batch culture.
- A graph of biomass concentration vs time helps to determine lag time.
- The contents in the fermenter are well mixed so that the samples withdrawn from any location in the fermenter shall be identical and in particular the composition of the exit stream will be identical to that of the liquid agitated in the fermenter.
- In its ideal form, the PFR/PFFs are characterized by the fact that the liquid phases pass through the fermenter without any back mixing.
- Structured and segregated models are complex for computations, although being realistic in nature. The degree of realism and complexity needed in a model depends upon what is being described.
- Filamentous organisms frequently form microbial pellets at high cell populations or densities in the suspension cultures. Inside these pellets the cells which are growing are quite likely to be subjected to the diffusion limitations.
- Transient behaviour can be understood by these two:
 1. The models with time delay and
 2. Chemically structured models.

EXERCISES

- 6.1** Define the following terms:
 - (a) Biomass
 - (b) Cell culture
 - (c) Synthetic and complex mediums
 - (d) Cell population
- 6.2** Explain the important parameters, phenomena and interactions between the growth medium and the cell population which determine cell population kinetics.
- 6.3** Elucidate different perspectives for cell population kinetic representations.
- 6.4** In an ideal batch reactor, for kinetic measurements, show that the rate of formation of a component is the change in its concentration with time.
- 6.5** Define dilution rate in a CFSTR and show that $D = F/V_R$.
- 6.6** Describe Monod's growth kinetics and define specific growth rate.
- 6.7** Describe the formulation of synthetic and complex mediums for cell cultures.

- 6.8** What are the environmental factors that affect growth kinetics? Explain.
- 6.9** Describe transient growth kinetics. With a neat sketch, explain the phases of growth.
- 6.10** Write a note on the following:
- Multiple lag phases
 - Doubling time
 - Yield and maintenance coefficients
 - Chemostat
 - Growth, non-growth and mixed growth associated products
- 6.11** During the batch growth of microorganisms, derive an expression for time needed in terms of substrate concentration.
- 6.12** In the continuous culture systems, explain the following:
- Cell productivity and
 - Wash out and critical dilution rate.
- 6.13** What are the strategies adopted in the recycling of biomass using a CSTF? Explain.
- 6.14** Using a settler-thickener arrangement in a CSTF with recycle, arrive at the following expressions:
- Substrate concentration at the steady state.
 - Biomass concentration at the steady state.
- Further prove that the dilution rate for wash out in a CSTF with recycle is greater than that in a simple CSTF with no recycle.
- 6.15** Derive the expressions for CSTF arrangement in series.
- 6.16** The steady state substrate and biomass concentrations for a continuous stirred tank fermenter operated at various dilution rates are shown in the following data. Given that the fresh feed concentration is 695 mg per litre, calculate the values for Monod's constants, the yield coefficient and the endogenous respiration coefficient.

Dilution rate hr ⁻¹	Concentration of substrate, mg/litre	Concentration of biomass, mg/litre
0.30	45	325
0.23	40	326
0.20	16	345
0.14	8.2	348
0.05	3.5	350

- 6.17** Calculate the steady state substrate and biomass concentrations in a continuous stirred fermenter which has an operating volume of 25 litres when the sterile feed stream contains limiting substrate at 2100 mg/litre and entering the fermenter at 7.9 litres/hr. The values for K_s and μ_{\max} are 10.5 mg/litre and 0.428 hr⁻¹ respectively. The yield coefficient may be taken as 0.45.

- 6.18** The growth rate of a microorganism can be expressed as a relation given below.

$v = \mu_{\max} (1 - e^{-S/K_s} X)$ where $\mu_{\max} = 0.36 \text{ hr}^{-1}$ and $K_s = 6.8 \text{ g/litre}$ and the cell yield is 0.45. If the microorganism is cultivated in a 10 litre CSTF with a flow rate of 2.8 litre/hr, what is the steady state biomass concentration of the outlet stream? The substrate in the inlet stream is 13 g/litre.

- 6.19** For a specific culture the specific growth rate is 1.4 hr^{-1} . Determine the doubling time for the cells.

- 6.20** A chemostat shows the following results with variation in the inlet flow rate and concentration of substrates.

Flow rate, ml/hr	31	50	70	90	200
Cell concentration	5.9	5.6	5.5	5.42	0.00
Substrate concentration, mg/litre	0.5	1.0	2.0	4.0	100

The inlet concentration of substrate is 100 g/litre and the operational volume of the fermenter is 500 ml. The inlet feed is sterile.

(i) Find the rate equation for the cell growth.

(ii) What should be the range of flow rate for the prevention of wash out?

- 6.21** Deduce the expressions for the CSTF in series with a recycle of biomass.

- 6.22** Explain the features of a plug flow fermenter.

- 6.23** Deduce expressions for a PPF with biomass recycle.

- 6.24** Discuss the evaluation of kinetic parameters of continuous growth in a culture vessel.

- 6.25** With models explain the quantification of growth kinetics.

Chapter 7

Biological Reactors

A handful of basic bioreactor designs are used to produce a wide range of products, from antibiotics to food products to fuels. Here is how to pick the best options for our application. Traditionally, microbiologists have played a dominant role in the development of bioreactions with the assistance from those in multiple disciplines, including biochemists, geneticists and chemical engineers. Fermentation process that is in use since antiquity is a precursor to modern bioreactions. However, major advancements of the last half century have much to do with the technology as with biology.

In this chapter, emphasis is given on the illustration of the relevance of established chemical engineering practices and processes as they apply to today's bioreaction engineering, as chemical engineers make further inroads into a field once thought to be the sole domain of biology-based scientists. Further, it discusses key engineering issues in bioreactor design and operation, focusing on similarities between traditional chemical reactor engineering and bioreaction engineering and even key differences that must be taken into consideration for successful bioreactions are also stated. Various types of reactors used in the biochemical industry with their significance and utilities are also met with. Alternate reactor configurations are briefly touched upon with the concept of medium formulation and aseptic conditions.

7.1 BIOREACTORS vs CHEMICAL REACTORS

A bioreactor is a system in which a biological conversion is effected. This biological conversion involves use of enzymes, microorganisms and animal or plant cells. A bioreactor includes mechanical vessels in which (a) organisms are cultivated in a controlled manner, and (b) specific reactions are involved in converting or transforming materials.

Some bioreactors are specifically designed to influence the metabolic pathways, among these are the CSTRs, CFSTRs, PFRs (single or in series), Ebullized Bed (Bubbling or Boiling) and Fluidized Bed Reactors.

Bioreactors support and control biological entities. These bioreactors should be designed in such a way so as to provide a higher degree of control over process upsets and contaminations.

Some causes of process upsets and contaminations are as follows:

1. Microorganisms which are more sensitive and less stable than chemicals are likely to cause process upsets and hinder the process.
2. The mutations taking place in microorganisms alter the biochemistry of the reaction or physical properties of the reaction.
3. Microbes are relatively shear tolerant compared to animal or plant cells (shear sensitivity, which is morphology dependent).

Bioreactors differ from conventional chemical reactors—they support and control biological entities. Although a majority of fundamental bioreactor engineering and design issues are similar, maintaining desired biological activities and eliminating or minimizing undesired activities often presents a greater challenge than traditional chemical reactors typically require.

The viscosities of reaction mixture inside a reactor change and usually are non-Newtonian. Mixing forms an integral part of design for efficient heat and mass transfer, so the design should include the mixer or an agitator design that is really complicated.

Selectivity and rate of course is the key difference between bioreactors and chemical reactors. Activity and selectivity complies with a smaller range of conditions. Deactivation of biomass leads to severe consequences and can affect the process as a whole. Rates are secondary in this case. Incubation period is considered as it is needed to prepare a culture of microorganisms.

A bioreactor is not an isolated unit, but is a part of an integrated unit operation with both upstream (preparation) and downstream (recovery) unit operations. In a bioreactor, bioreaction can take about 10–20 days for completion of a batch.

7.2 PRODUCTS OF BIOREACTION

The products of the bioreactions are formed by three basic processes:

Process 1: Product is produced by cells either extracellular, e.g. alcohols or citric acid, or intracellular, e.g. a metabolite or an enzyme.

Production of cellular products is of two types based on when they are produced within a biological cycle. They are as follows:

Primary metabolites: Primary metabolites are produced during the growth phase and are needed for growth. Examples include amino acids, nucleotides and proteins.

Primary products for industrial use are ethanol, citric acid, acetone, butanol, lysine, vitamins, etc.

Secondary metabolites: Secondary metabolites are produced from intermediates and are products of primary metabolism. These may be toxic and possess the antibiotic properties.

Note: Not all microorganisms produce secondary metabolites, only a few, filamentous microorganisms like fungi, plant cells, etc.

Process 2: During this type of process a cell mass is produced. Baker's yeast that is used in baking industry, is an example of a produced cell mass. The others include single-cell proteins for food sources.

Process 3: This type of process modifies a compound that is added to the fermentation process and is referred to as *biotransformation*. The process can be by way of dehydration, oxidation, hydroxylation, amination, etc. By way of biotransformations, the products produced are steroids, antibiotics, hormones, etc.

7.3 KEY ISSUES IN BIOREACTOR DESIGN AND OPERATION

The goals of an effective bioreactor are to control, contain and positively influence the biological reaction.

A bioreactor should comply with:

1. GMP-Good Manufacturing Practice, regulations (cGMP—Current GMP)
2. Three Qs:
 - Design Qualifications (DQ)
 - Installation Qualifications (IQ)
 - Operation Qualifications (OQ)

The three Qs lead to Factory Acceptance Test (FAT).

3. Validation—Established Documented Evidence (purpose to be served).
4. cGMP—Employed strictly in drug industry, where stringent requirements are met.

To accomplish this, a chemical engineer must consider the following two areas:

1. Suitable reactor parameters (for desired biological, chemical, physical systems)
2. Bioreaction parameters:
 - Controlled temperature
 - Optimum pH
 - Sufficient substrate (carbon source)
 - H_2O availability

- Salts for nutrition
- Vitamins
- Oxygen
- Gas evolution
- Product or by-product removal

In addition to this, a bioreactor must be designed to both promote formation of the optimal morphology and eliminate or reduce contamination by unwanted microorganisms or mutation. Microbiology, sterilization, rheology, mixing, agitator design, fluidization, heat transfer/mass transfer, surface phenomenon, transport enhancements, kinetics, hydrodynamics, scale-up, modelling, instrumentation and process control are the other parameters to be considered.

Further designed for sterility, cleanability (spray devices), drainability (steam condensate), avoidance of dead zones and dead legs, avoidance of low velocity flow zones, ensure accessibility in cleaning and inspection.

The guidelines generally followed in bioreactor design and operations are as follows:

- ASME-BPE-2000 (American Standards for Bioprocess Pressure Vessels)
- GAMP for automation
- ISO for qualification
- EN-46001 (for medical devices)
- Electrical guidelines.

Note: Avoidance of cross contamination is considered and for which once-through processes are preferred.

The material of construction generally used is stainless steel that has a surface finish of $0.03 \mu\text{m}$ roughness average.

7.4 COMPONENTS OF A TYPICAL FERMENTATION PROCESS

Figure 7.1 shows the components and operations of a typical fermentation process.

Medium preparation (formulation): Nutrients in proportion to minerals are added. These are provided in enough quantities and proper proportions for a specified amount of biomass or products to be synthesized. Sugars (sources of carbon) are used. Instead of purified sugars, crude sources as beet, cane, corn, molasses, etc. are used that are 50–70% fermentable sugars. In some cases, process wastes like whey and cannery wastes also provide carbon.

Medium sterilization: Steam or chemical addition accomplishes the medium sterilization. It is an important component of a fermentation process. The purpose served here is to avoid process upsets and contaminations.

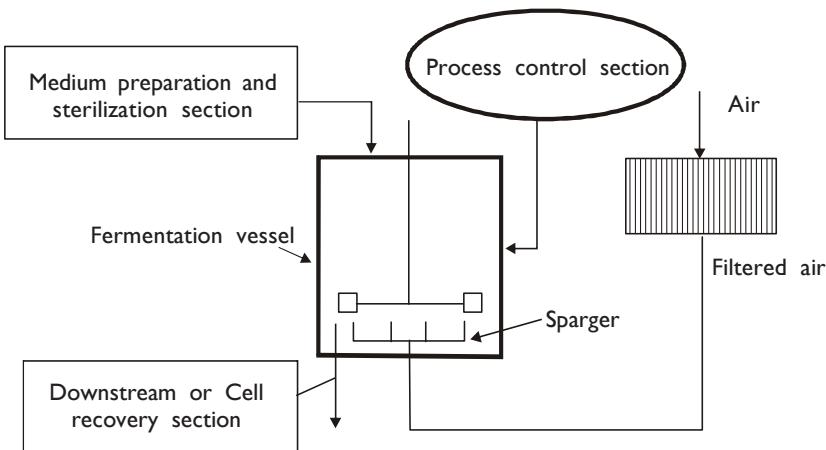


Figure 7.1 Components and operations of a typical fermentation process.

Inoculum preparation: It comprises 5–10% of new medium volume. The process of inoculum preparation is discussed in section 7.5.

Air filtration: Air filtration is done to remove the traces of various components that are present in the gaseous form and also to remove moisture. Filtered air is inducted through the spargers.

Process control: The overall monitoring of the process is spearheaded by this department.

Cell recovery: Cell recovery is a downstream processing part and recovers all those valuables present in the downward section of a bioreactor.

7.5 DESIGN AND OPERATION OF A TYPICAL ASEPTIC AND AEROBIC FERMENTATION PROCESS

Why is the fermentation process to be aseptic? It has to be aseptic so as to avoid any kind of intrusions, process upsets or contaminations.

To see that the aseptic conditions are achieved, the following sequence of steps are used:

Preparation of inoculum: Preparation of an inoculum needs a careful development of relatively a few cells to a dense suspension of from 1–20% of the volume of the fermenter.

The starting point is the stock culture which is a carefully maintained collection of a particular microbial strain. In order to achieve maximum genetic stability in stock, minimize its metabolic activities during storage by freeze drying or lyophilization process to achieve a dormant state. Besides lyophilization or freeze drying to maintain the microorganisms in a dormant state, a thorough drying dispersions of spores on sterile soil or sand can be used. Then the cells are suspended in a sterile liquid. From this, a drop of

suspension is transferred to the surface of an agar slope or agar slant. Later it is placed in an incubator. During incubation, where sufficient growth is seen, cells are again suspended in liquid and added to a larger agar surface in a Roux bottle or a shaking flask. Further, it is agitated to promote growth and for adequate transport of gases to and from the microorganisms. Finally, proliferation is achieved in one or more seed vessels (tanks).

Figure 7.2 followed by explanation will give us an idea about the valve and the piping configurations for aseptic inoculation of a large-scale fermenter.

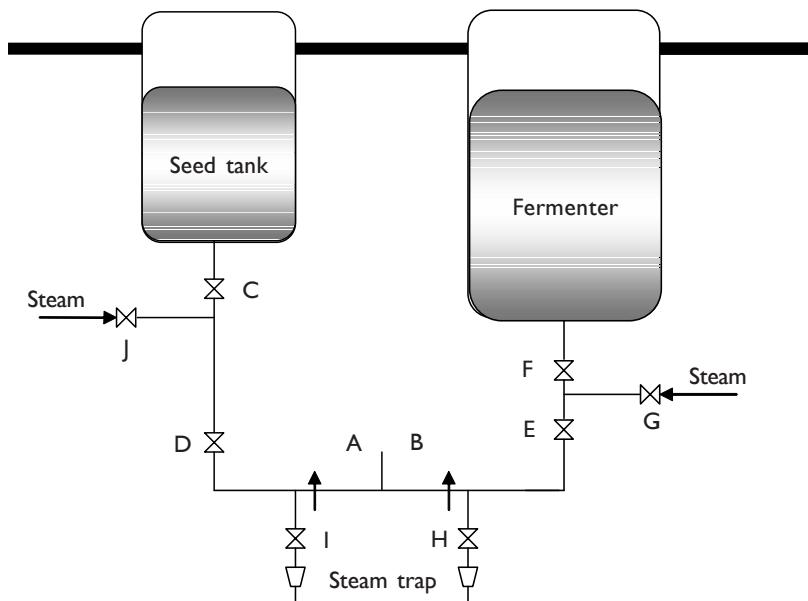


Figure 7.2 Valve and piping configurations.

Operation sequence: Referring to Figure 7.2, follow the steps mentioned below that govern the operation and maintenance.

1. Install pipe section AB.
2. Sterilize connection with 15 lb/inch^2 gauge stream for 20 minutes. Valves D and J are open and valve C is closed.
3. Cool the fermenter under sterile air pressure with valves C, G, H, I and J closed and valves D, E and F open. Sterile medium fills the connection.
4. Increase pressure in the seed tank to 10 lb/inch^2 gauge. Lower the fermenter pressure to 2 lb/inch^2 gauge.
5. Transfer the inoculum by opening valve C.
6. Steam seal the fermenter-seed tank connections by closing C and F and opening G and J. Steam and condensate are bled from partially open D and E.

With a proper and close check on the steps mentioned above, aseptic inoculations can be achieved for long-scale fermenters.

7.6 ALTERNATE BIOREACTOR CONFIGURATIONS

Figure 7.3 depicts the alternate bioreactor configurations. A number of alternate bioreactor configurations have been proposed and examined in various scales ranging from laboratory to pilot to full-scale processes. Bioreactors have been classified into three main classes of energy input. They are as follows:

1. Bioreactors using mechanically moved internals for energy input which is shown in Figure 7.3.
 2. Bioreactors in which energy input is provided by liquid circulation using an external pump.
 3. Bioreactors in which energy input is provided in the form of compressed gas.

Note: Classes 2 and 3 are not shown.

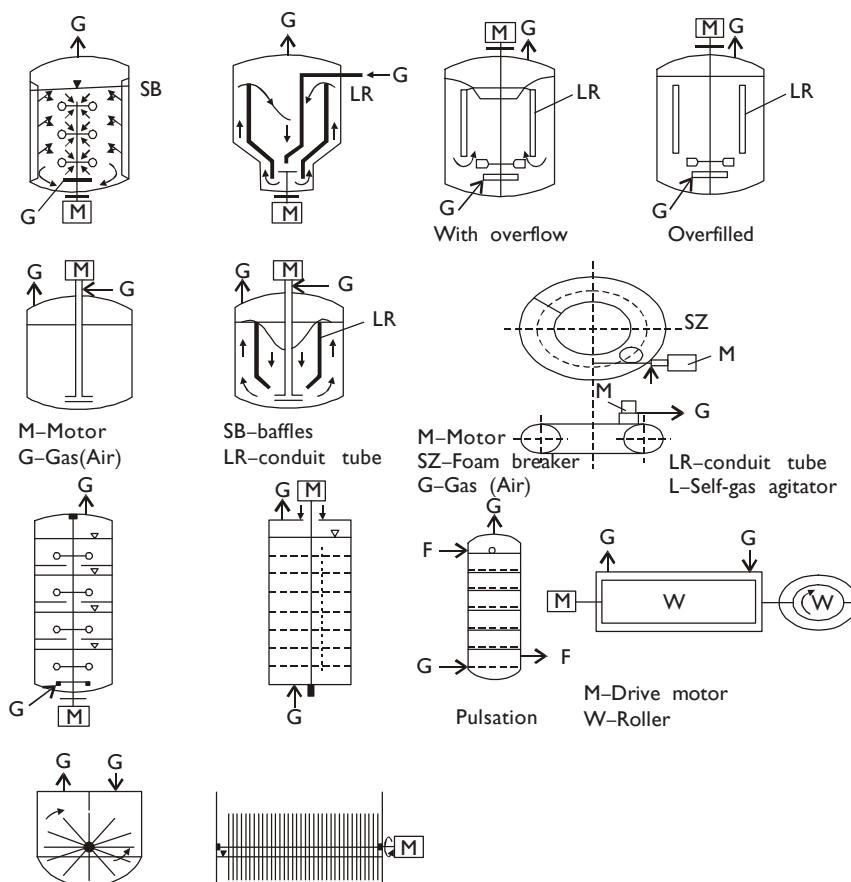


Figure 7.3 Alternate bioreactor configurations showing the use of mechanically moved internals for energy input. (Source: Bailey and Ollis, *Biochemical Engineering Fundamentals*, 2nd ed., McGraw-Hill, 1986.)

7.7 TYPES OF BIOREACTORS/BIOREACTIONS

In this section, we will discuss different types of bioreactors which are invariably used in a bioprocess industry. We will also discuss applications of a variety of bioreaction/fermentation technologies, including the challenges of each and the advantages of the respective technologies. The various types of bioreaction systems include batch, continuous, semicontinuous, surface/tray, submerged, airlift loop and trickle-bed units. There can be overlapping characteristics in several of the technologies.

7.7.1 Batch Bioreactions

Figure 7.4 shows a tray type bioreactor which is loaded with the culture medium and organisms, and then air flow is started to initiate the reaction. As the reaction progresses, the exhaust gas produced is discharged. When the bioreaction is complete, end product is removed from the trays.

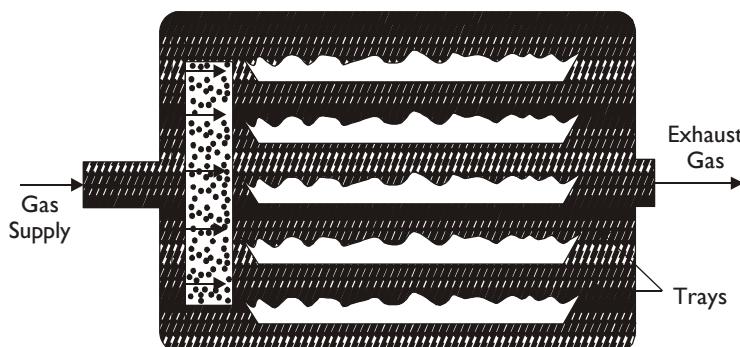


Figure 7.4 Tray bioreactor.

We know that a majority of bioreactions are batch-wise. A batch bioreaction serves the following advantages:

- Reduced risk of contamination and cell mutation and can have a brief growth period
- Lower capital investment when compared to continuous processes for the same bioreactor volume
- More flexibility with varying product/biological systems
- Higher raw material conversion levels due to the controlled growth period

At the same time it has some disadvantages which are as follows:

- Lower productivity levels as time is required for filling, heating, sterilization, cooling, emptying and cleaning the reactor
- Requires increased focus on instrumentation due to frequent sterilization

- Greater expenses incurred in preparing several sub-cultures for inoculation
- Higher labour costs and process control
- Larger industrial hygiene risks as there can be a potential contact with pathogenic microorganisms

Batch bioreactions are used in several situations:

- Products that are to be produced with minimal risk of contamination or mutation
- Operations in which only small amounts of product are produced
- Processes that use one reactor to make various products
- Processes in which batch or semicontinuous product separation is adequate

7.7.2 Continuous Bioreactions

The defining characteristic of continuous bioreactions is a perpetual feeding process. A culture medium that is either sterile or comprised of microorganisms is continuously fed into the bioreactor to maintain a steady state. Of course, product is also drawn continuously from the reactor. The reaction variables and the control parameters remain consistent, establishing a time-constant state within the reactor. The result is continuous productivity and output.

The following are the advantages that are provided by such systems:

- Increased potential for automating the process
- Reduced labour expense, due to automation
- Less non-productive time expended in emptying, filling and sterilizing the reactor
- Consistent product quality due to invariable operating parameters
- Decreased toxicity risks to the staff due to automation
- Reduced stress on instruments due to sterilization

The disadvantages include:

- Minimal flexibility, since only slight variations in the process are possible
- Mandatory uniformity of raw material quality is necessary to ensure that the process remains continuous
- Higher investment costs in control and automation equipment, and increased expenses for continuous sterilization of the medium
- Greater processing costs with continuous replenishment of non-soluble, solid substrates such as straw
- Higher risk of contamination and cell mutation due to the relatively brief cultivation period

Continuous bioreaction is frequently used for the processes with high-volume production; for processes using gas, liquid or soluble solid substrates; and for processes involving microorganisms with high mutation stability.

7.7.3 Semicontinuous Bioreactions

A semicontinuous bioreaction is a hybrid of batch and continuous operations, which is found in many types of processes. This system provides a number of advantages such as:

- Higher yield, resulting from a well-defined cultivation period during which no cells are added or removed
- Increased opportunity for optimizing the environmental conditions of the microorganisms in regard to the phase of growth or production and age of the culture.
- Nearly stationary operation with slightly mutation of microorganisms.

The disadvantages include the following:

- Lower productivity levels due to time-consuming procedures for filling, heating, sterilizing, cooling, emptying and cleaning the reactor.
- Greater expenses in the labour and/or dynamic process control for the process.

Semicontinuous bioreactions are used when continuous methods are not feasible, for example, those in which slight mutation or contamination of microorganisms occurs. Such bioreactors are also used when batch methods do not offer desired productivity levels.

7.7.4 Submerged Bioreactors—Stirred Tank Bioreactor

The most common type of aerobic bioreactor in use today is the stirred tank bioreactor. It features a specific internal configuration designed to provide a specific circulation pattern. Ideal for the industrial applications, this unit offers manufacturers both low capital and operating costs. A schematic diagram of a stirred tank bioreactor is shown in Figure 7.5.

The figure depicts a stirred tank bioreactor that uses baffles and an agitator for optimal mixing and recycling biomass. The operating principle of this type of bioreactor is relatively simple. The sterile medium and the inoculum are introduced into a sterilized tank, and air supply typically enters the bottom. For optimal mixing, the tank features not only an agitator system but also the baffles that help prevent a whirlpool effect that could impede proper mixing. The number of baffles may range from four to eight.

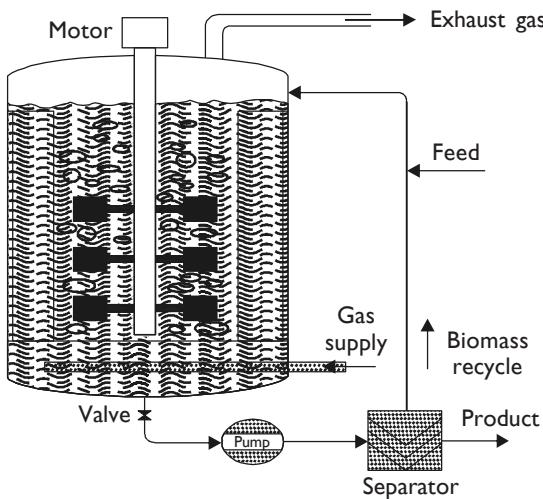


Figure 7.5 Stirred tank bioreactor.

As the reaction progresses, the bubbles produced by the air supply are broken up by the agitator as they travel upwards. Many types of agitators are used, with the most common being a four-bladed disk turbine. The turbine is a cylindrical structure with the baffles that serve the purpose as usual. Concentric to it is an agitator driven by a motor at the top that helps in vigorous mixing. At the bottom is given gas supply so as to aerate reactor contents. After the reaction is complete, the contents are drained from the bottom through the drain valve and fed to the pump. This is given to the separator that segregates into the product and the biomass to be recycled. From the top, exhaust gases emanate.

Stirred tank bioreactors have been modified to reduce shear rates on the cells in suspension. Sail-type and axial-flow hydrofoil agitators have been developed and used for cell suspension cultures. The agitation rate in these reactors is of the order of 10 to 40 rpm, providing a low shear rate.

7.7.5 Airlift Bioreactor Systems

Also known as a tower reactor, an airlift bioreactor can be described as a bubble column containing a draught tube. Many types of airlift bioreactors are currently in use today. The simple design of a concentric draught tube bioreactor with annular liquid downflow is shown in Figure 7.6.

The gas supply is given by means of a sparger at the bottom into the inner cylinder and moves into the annular space creating a swarm of bubbles that induce significant aeration.

Air is fed through the sparger ring into the bottom of a central draught tube that controls the circulation of air and the medium. Air flows up the tube, forming bubbles, and exhaust gas disengages at the top of the column. The degassed liquid then flows downwards and the product is drained from

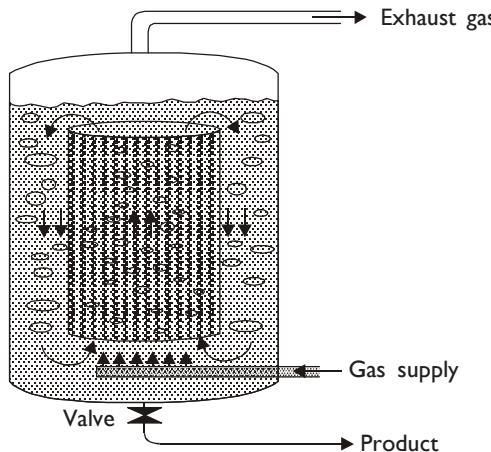


Figure 7.6 Concentric draught tube reactor.

the tank. The tube can be designed to serve as an internal heat exchanger, or a heat exchanger can be added to an internal circulation loop.

Airlift systems provide some advantages against more conventional bioreactors as fermentors:

- Simple design with no moving parts or agitator shaft seals for less maintenance, less risk of defects and easier sterilization
- Lower shear rate, for greater flexibility—the system can be used for growing both plant and animal cells
- Efficient gas phase disengagement
- Large, specific interfacial contact area with low energy input
- Well-controlled flow and efficient mixing
- Well-defined residence time for all phases
- Increased mass transfer due to enhanced oxygen solubility achieved in large tanks with greater pressure
- Large volume tanks possible, increasing the output
- Greater heat removal against conventional stirred tanks.

The main disadvantages are as follows:

- Higher initial capital investment due to large-scale processes
- Greater air throughput and higher pressures needed, particularly for large-scale operations
- Low friction with an optimal hydraulic diameter for the riser and the downcomer
- Lower efficiency of gas compression
- Inherently impossible to maintain consistent levels of substrate, nutrients in oxygen with organisms circulating through the bioreactor and conditions changing
- Inefficient gas/liquid separation and foaming occurs

7.7.6 Airlift External Loop Reactors

Another type of airlift system is the airlift external loop reactor (AELR) system that is used primarily for batch operation. Figure 7.7 shows a schematic diagram of an airlift external loop reactor.

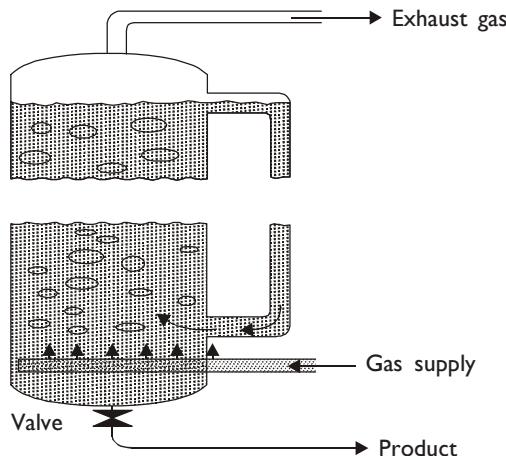


Figure 7.7 Airlift external loop reactor.

The AELR has some advantages over standard airlift reactor and they are as follows:

- Effective heat transfer and efficient temperature control
- Low friction with an optimal hydraulic diameter for the riser and the downcomer
- Well-defined residence time in the individual section of the AELR
- Increased opportunity for measurement and control in the riser and the downcomer
- Independent control of the gas input-rate and liquid velocity by a throttling device between the riser and the downcomer

It has induced circulation that directs air or liquid throughout the vessel. Loop reactors have intermediate characteristics between the bubble columns and stirred tanks. The motion of gas carries fluid and cells up a draft tube. At the top, the gas disengages from the liquid, and the degassed liquid descends in the reactor. Such type of reactors can handle somewhat more viscous fluids than the bubble columns, and coalescence is not so much of a problem. The largest agitated fermentor ever built by Imperial Chemical Industries (ICI) is an airlift design for the production of single-cell protein.

7.7.7 Trickle Bed Reactor

A trickle bed reactor employs adhered, immobilized enzymes to accomplish bioreaction. It is a three-phase system containing a packed bed of heterogeneous catalyst and flowing gas and liquid phases.

One (or more) reactant is provided in each feed liquid and gas phase, so that biochemical reaction depends on contacting of liquid, containing a sparingly soluble reactant from the gas phase, with the catalyst surface.

The performance of such reactors is substantially influenced by the physical state of gas-liquid flow through the fixed bed and by the associated mass transfer processes.

The important characteristics of such a reactor are the surface area of the packing, the efficiency of wetting the catalyst by the flowing liquid phase, the gas-liquid flow pattern, mass transfer of sparingly soluble reactants from the gas to liquid phase, mass transfer of both reactants to the catalyst surface and, in case of a porous or permeable catalyst, diffusion of reactants to the intra particle catalyst sites.

In Figure 7.8, is shown a schematic diagram of a trickle bed reactor.

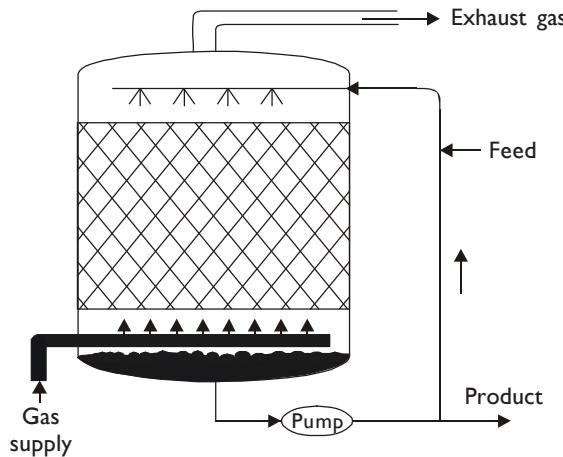


Figure 7.8 Trickle bed reactors.

The trickling biological filter is a trickle bed reactor and is used for wastewater treatment. Trickle bed reactors are used in the production of vinegar and also in petroleum and petrochemical industry for hydrocracking, hydrotreating and other multiphase reaction processes.

The bioreactors will be integral to the development of many high-value products and the replacement of existing chemical-based commodity processes. The proper selection and design of a bioreactor will determine an optimal commercial bioprocess and corresponding capital investment.

7.7.8 Membrane BioReactors

We know that many bioproducts, viz. food products, pharmaceuticals, hormones, steroids, vitamins, etc. are manufactured using conventional bioreactors. These can be operated both in batch and continuous modes. In the operation of batch bioreactors, we know that enzymes or microbial cultures or cells are employed in their soluble form or free form, whereas in

the continuous bioreactors these are immobilized. Perhaps, immobilizing enzymes in continuous reactors will overcome the deficiencies found in the batch reactors like less efficiency, low yield, substrate depletion, product inhibition, etc. At the same time even the continuous bioreactors too have certain limitations. For instance, losses in the activity of enzymes are noticed and are due to its repeated use.

An alternative approach is the use of membrane bioreactors. In this we find that use of synthetic semi-permeable membranes of exact chemical nature and physical configuration meant to immobilize enzymes within the vessel of a reactor and also to isolate from the reaction mixture.

Use of membrane bioreactor serves the following advantage. It shows increase in the efficiency of cellular reactions. This is by way of influencing the concentration of the primary substrates in the vicinity of the biomass, control over favourable and unwanted substrate species and by removing the inhibitory metabolites.

Figure 7.9 depicts the working of a membrane recycle bioreactor.

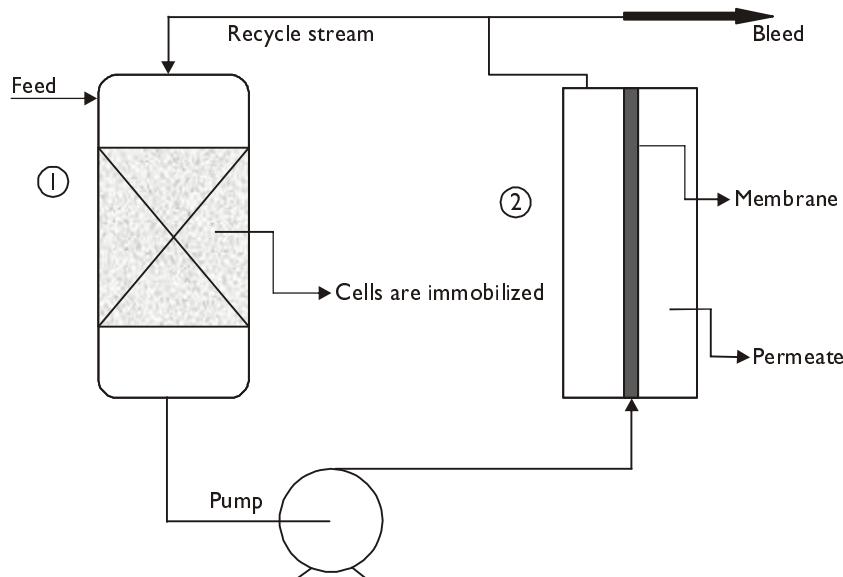


Figure 7.9 Membrane recycle bioreactor.

Typically, a reactor ① is coupled with a membrane module ② via a suitable pump. This membrane module has a semi-permeable membrane. The enzymes or cells are suspended in the main reactor. Feed is pumped into it at the same rate at which permeate flows. Complete reaction broth is pumped through the membrane module where the products of reaction or any other which are small to permeate are removed. The membrane used should retain the enzyme and at the same time minimize the retention of the product.

The membrane recycle bioreactor is operated usually under mixed conditions depending upon the relative volume of the reaction vessel and the membrane module and also on the ratio of recycling to the flux. These are found to be more suitable for the substrate inhibited reactions than the product inhibitions when the conversions are high.

Membrane bioreactors are being applied for many processes and some are commercialized. These are applied for cell culture and fermentation processes, light hydrocarbon gas phase catalytic reactions, chiral drug separations, etc. In applying membrane reactors, three important configurational features of membranes are used.

1. The membrane as a *contactor*, in which membrane separates the reaction broth medium in one chamber from a second chamber that would contain an enzyme or a catalyst or a cell culture. The configuration of such reactors is shown in Figure 7.10. Indeed, such application of membranes as contactors has a long history that dates back to early fermentation processes.

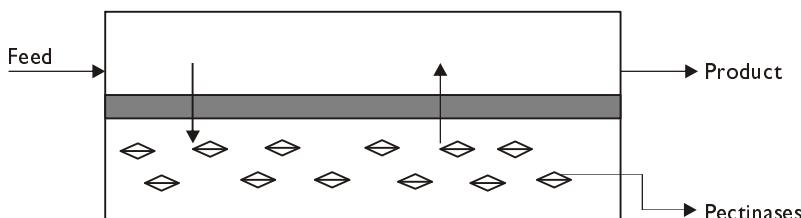


Figure 7.10 Membrane as a contactor.

Feed: juice + pectin

Product: juice + degraded pectin

Pectin degrades to galacturonic acid, i.e. pectin \longrightarrow Galacturonic acid

In Figure 7.10, the membranes as contactors are used to separate pectin from citrus fruit juice. Generally, citrus fruit juice has pectin, a high molecular weight polysaccharide that causes undesirable haze in the juice. In order to reduce the pectin content the juice is passed across the membrane on one side of which the *pectinases*, enzymes that degrades the pectin are available. Pectinase degrades pectin to produce galacturonic acid thereby eliminating the haze.

2. In the second, membranes are used as *separation barriers*. For example, in the dehydrogenation of *n*-butane to form butadiene and hydrogen. It finds more application in a petrochemical industry than in a biochemical industry.
3. In the third type, membrane reactor combines the features of the previous two, i.e. as a *contactor and as a separator*. The configuration is shown in Figure 7.11.

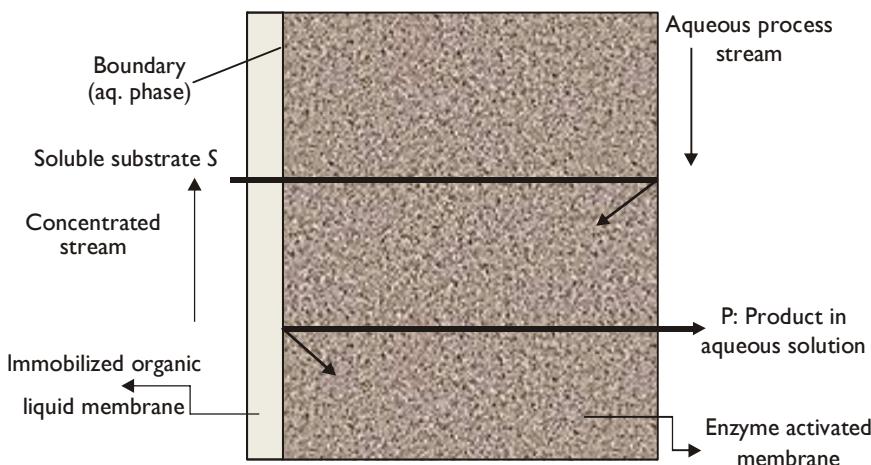
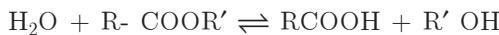


Figure 7.11 Membrane as a contactor and separator.

Figure 7.11 shows the process of de-esterification reaction using an enzyme. The reaction taking place is as follows:



Membrane used is multilayered and composite. The layer towards the organic feed side is an immobilized organic liquid membrane; the layer towards the aqueous product solution side contains an enzyme as a catalyst to bring about de-esterification.

The organic soluble ester is brought to the reactor with the organic feed side and is seen freely permeating into the immobilized organic liquid membrane thereby reaching the enzyme catalyst zone or layer. Here the ester undergoes hydrolysis. Due to hydrolysis the products as alcohol and acids are found to be more polar than the ester so that these can solubilize in water easily than in an organic. Later these products diffuse to the aqueous permeate solution.

As it can be noticed, the membrane provides both an active site for the reaction and separates the products from the feed. Therefore, it serves both as a contactor and a separator.

SUMMARY

In this chapter, we learnt the following:

- A bioreactor is a system in which a biological conversion is effected.
- A bioreactor should not be regarded as an isolated unit, but as part of an integrated unit operation with both upstream (preparation) and downstream (separations) unit operations.
- There are different types of bioreactors/bioreacting—batch, continuous semicontinuous, surface/tray submerged, airlift loop and trickle bed units.

- Bioreactors will be integral to the development of many new high-value products and replacement of existing chemical-based commodity processes.
- The proper selection and design of a bioreactor will determine an optimal commercial bioprocess and corresponding capital investment.
- Immobilizing enzymes in continuous reactors will overcome the deficiencies found in the batch reactors like less efficiency, low yield, substrate depletion, product inhibition, etc.
- The membrane recycle bioreactor is operated usually under mixed conditions depending upon the relative volume of the reaction vessel and the membrane module and also on the ratio of recycling to the flux.
- In applying membrane reactors, three important configurational features of membranes are used. Membrane as contactor, as a barrier or separator and as a contactor and separator combined.

EXERCISES

- 7.1 Discuss the features of bioreactors and explain how these are different from the chemical reactors?
- 7.2 Explain the products of bioreactions.
- 7.3 Highlight key issues to be considered in the design and operation of bioreactors.
- 7.4 With the help of a neat flow sheet, explain the components of a typical fermentation process.
- 7.5 Discuss with the help of a neat sketch the operation of a typical aseptic aerobic fermentation process.
- 7.6 Enlist the steps of the preparation of inoculum.
- 7.7 Write on the alternate bioreactor configurations.
- 7.8 With a neat sketch, describe the working and maintenance of a tray type bioreactor.
- 7.9 Write a note on the following:
 - (a) Trickle bed reactors
 - (b) Concentric draught tube reactors
 - (c) Airlift reactors
 - (d) Stirred tank bioreactors
- 7.10 With advantages and disadvantages, explain the different configurations of membrane bioreactors.

Chapter 8

Fermentation Technology

Traditional Processes and Products

With an ever increasing knowledge about microorganisms, we have now developed skills and technology to utilize microorganisms for industrial purposes. From baking bread to the synthesis of steroids, microorganisms have a wide range of industrial uses. The major areas of thrust in the field of industrial microbiology are food processing, manufacture of alcohol and alcoholic beverages, pharmaceuticals and drugs. Of course, the phenomenon common to all these major areas of thrust is fermentation. As seen, enzymes and microbial cells are used for the production of fine chemicals, steroids, vitamins, alkaloids, flavours and fragrances. In addition to the above-mentioned areas microorganisms are also exploited in wastewater treatment. Each of these bioprocesses is unique in terms of characteristics, processing and separation technologies involved.

This chapter is an addition to the earlier chapter and emphasizes the concepts and processes employed to manufacture traditional products of biotechnology.

8.1 FERMENTATION

Our knowledge of the use of fermentation is as old as the civilization itself. Right from the leavening of bread, the rising of wet ground, preparation of *idli* and *dosa*, to the transformation of milk to cheese and the making of wine and vinegar are all well-understood current practices wherein we have continually used microorganisms even without being aware of it. Today, this knowledge is intentionally used to devise and design innovative and practical applications of microbial activities. In the succeeding discussions, emphasis is placed on the processes and technologies used in the production of various fermentable products.

8.1.1 Anaerobic Fermentation and Products

Bread: In the making of bread dough, wheat or rye flour is mixed with selected strains of the yeast, *Saccharomyces cerevisiae*. The yeast utilizes the sugar present in the dough to produce ethanol and carbon dioxide. Fermentation proceeds only for a few hours and only small quantity of ethanol is produced. During baking the volatile ethanol is lost and the carbon dioxide left bubbles out and is trapped in the bread. This carbon dioxide escapes from the bread leaving behind the slots or holes in that making it lighter in weight. This process is called the *leavening of bread*. Of course, the leavening helps in the slicing of bread.

Curd and buttermilk: Industrially, cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are grown separately or in mixed cultures in large containers of milk. The acids produced during the fermentation coagulate milk; separate it into a semi-solid portion, curd and a watery portion called whey. By changing the cultures of microorganisms and by adjustment of the temperature of fermentation, curd with different flavours and tastes can be obtained. Buttermilk is prepared by using the cultures of *Streptococcus lactis* and *Leuconostoc citrovorum* or *L. dextranum*.

Cheese: The production of cheese involves the following steps:

1. Milk is curdled to form curd and whey by adding lactic acid bacteria along with enzyme rennet, which is obtained from the calf's stomach.
2. Curd is now processed to remove moisture. At this stage cheese is called *cottage cheese*.
3. Curd after removal of moisture is salted. This is done either by rubbing salt on the surface or dipping it in the brine. Salt serves as a hygroscopic agent to help further removal of moisture and also prevents the growth of unwanted microorganisms as it serves as a preservative.
4. Ripening of curd to cheese is carried on in a room where temperature and humidity are maintained under controlled conditions. Different microorganisms are used to ferment cheese, and the choice depends upon the variety of cheese to be produced.

Ripening period varies from 1–16 months. The composition of cheese is 20–30% fat, 20–35% proteins and a small amount of minerals and hence it is nutritious.

Vinegar: Vinegar literally means *sour wine*. It is produced by making wine to undergo sour under controlled conditions. The sources that can produce cheap quality vinegar are apple, cider, industrial alcohol, grape or grains. Basically, the production of vinegar accomplishes two types of biochemical changes. They are as follows:

- Carbohydrates undergoing alcoholic fermentation to produce alcohol
- Alcohol oxidized to acetic acid

In the first step, which is fermentation of carbohydrates to produce alcohol, yeast fermentation is done. Later, alcohol concentration is done up to 10 to 13% and then exposed to the action of acetic acid bacteria.

In the second step, that is oxidation of alcohol to produce acetic acid, acetic acid bacteria are employed. One of the industrial processes adopted is the Frings method to manufacture vinegar. Refer to Figure 8.1.

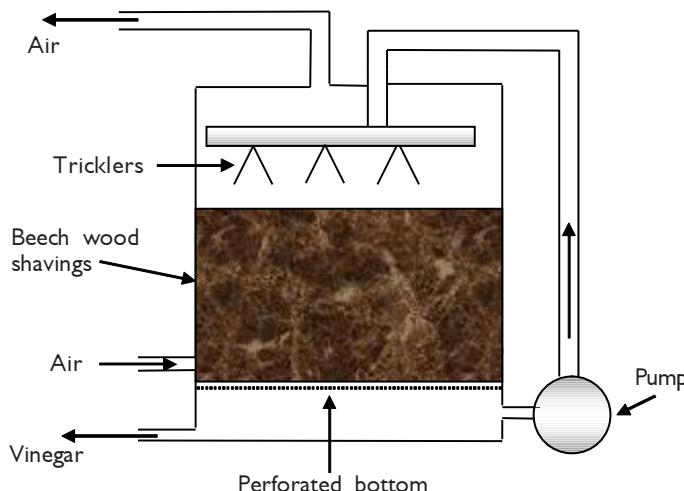
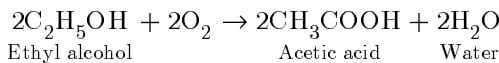


Figure 8.1 Frings vinegar generator.

An adjusted solution of alcohol acidified with acetic acid and special nutrients for growth of acetic acid bacteria are all mixed and fed to the chamber. Inside the chamber, beech wood shavings are used. *Acetobacter*, species of acetic acid bacteria, are inoculated onto the beech wood shavings inside the chamber.

The mix of alcohol and nutrients is applied through the feed line and the mix trickles down into the trough or chamber containing inoculated beech wood shavings. During this process, acetobacter oxidize some of the alcohol to acetic acid. The reaction is as follows:



Later, the mix is collected at the bottom and can be recirculated over the shavings that result in further oxidation of alcohol to acetic acid, until vinegar of desired strength is obtained.

The liquid is then stored anaerobically to prevent further degradation. Ageing takes place during the storage. Esters are formed and the harsh flavour disappears. Further, vinegar is clarified by filtration, pasteurized and bottled.

An adequate supply of air must be available throughout the chamber. It is also necessary to keep the temperature between 15 and 35°C.

Lactic acid: Lactic acid was first isolated from sour milk in the year 1780. It has two optically forms called D- and L-lactic acids. Most of the lactic acid is produced by fermentation process. For the production of lactic acid, several carbohydrate substances like corn, starch from potatoes, molasses and whey are used.

Let us emphasize the production of lactic acid using whey. Large quantities of whey constitute a waste during the manufacture of certain dairy products. Whey is a satisfactory medium for the growth of certain bacteria as it contains growth favouring constituents like carbohydrates (lactose), nitrogenous substances including vitamins and salts. *Lactobacilli* are the microorganisms that are suitable for this process, more specifically, *Lactobacillus bulgaricus*.

Figure 8.2 depicts the production of lactic acid from whey.

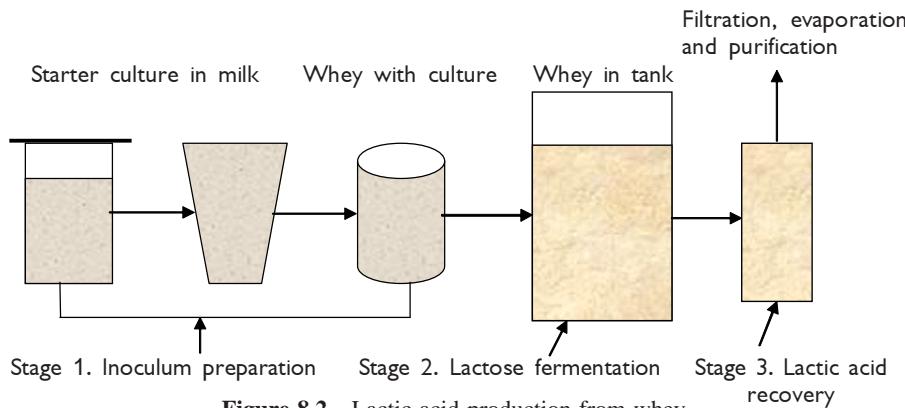


Figure 8.2 Lactic acid production from whey.

The starter culture in milk or stock cultures of the microorganism used are maintained in a skim-milk medium. In order to get a sufficient amount of inoculum to be used for the addition to the main fermentation vessel, the culture is transferred and incubated in increasing amounts of sterile milk, pasteurized-skim milk and finally whey. Later, 5 to 10% of the fermenting volume of the inoculum obtained from the incubation tank is added to the fermentation vessel.

Inside the fermentation tank, the whole process is governed by the following reaction:



Lactose

Glucose + Galactose

Lactic acid

Note: \bullet Lactase $\bullet\bullet$ System of enzymes

During fermentation, temperature of nearly 45°C is maintained and a period of two days required for fermentation to complete. It is seen that protein in whey will coagulate. Later, it is filtered and processed for use as animal-feed supplement.

Calcium hydroxide is added during fermentation to neutralize the effect of acid, wherein calcium lactate is produced. The neutralization of acid by using $\text{Ca}(\text{OH})_2$ is recommended as the presence of acid would retard the fermentation process.

The filtrate containing calcium lactate is concentrated by evaporation followed by the purification of lactic acid, the final product.

A flow sheet for the production of lactic acid is shown in Figure 8.3.

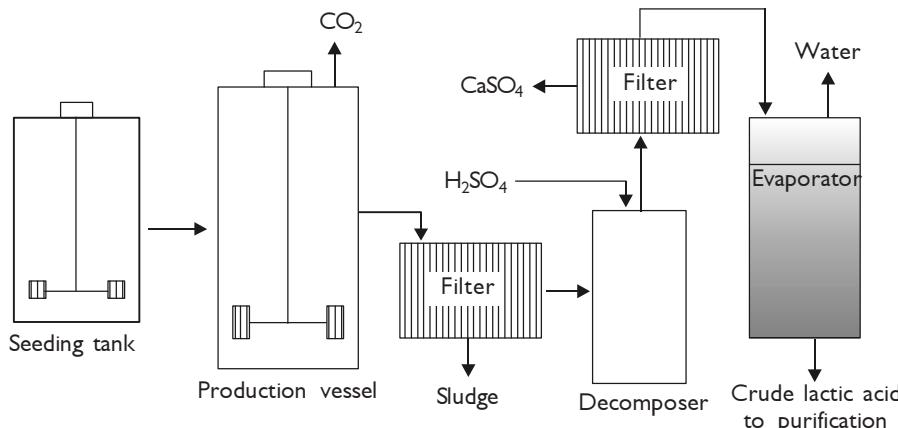


Figure 8.3 Flow sheet for the production of lactic acid.

The first step is to increase the temperature to about 80–100°C and the pH to 10–11. Due to this, the organism is killed and coagulates proteins, solubilizes calcium lactate and degrades some of residual sugar. The liquid is then filtered to remove the biomass, and sulphuric acid is added to obtain lactic acid. Calcium sulphate is removed by filtration and lactic acid is then concentrated by way of evaporation. Further, crude lactic acid is taken for purification.

Recovery of lactic acid from the fermentation broth constitutes a significant part of the production cost.

The major use of lactic acid is in food as an acidulant and preservative. It is also used as a chemical intermediate to produce other chemicals and in pharmaceutical industry.

In the production of lactic acid, industrial processes are operated batchwise. The features of the fermentors and process conditions for the various organisms employed are discussed as follows:

Fermentors are made of stainless steel and are equipped with heat transfer coils. Prior to the addition of a pasteurized medium to the vessel, it is steam sterilized to see that there is no unwanted microbial growth. In order to prevent the settling of calcium carbonate, slow agitation is provided with top mounted mechanical stirrers. For each industrial producer, the fermentation conditions are different:

- $T = 45\text{--}60^\circ\text{C}$ and $\text{pH} = 5\text{--}6.5$ for *L. delbrückii*

- $T = 43^{\circ}\text{C}$ and $\text{pH} = 6\text{--}7$ for *L. bulgaricus*
- $T = 30\text{--}50^{\circ}\text{C}$ and $\text{pH} < 6$ for *Rhizopus*

The fermentation time periods are also different for different sources—1–2 days for a 5% sugar source like whey and 2–6 days for a 15% glucose or sucrose source. If the conditions during fermentation are optimum, the processing time can be reduced to 1–2 days.

The rate of lactic acid formation depends on the factors such as pH, sugar, nitrogen and lactic acid concentrations. In order to improve the rate of lactic acid formation, temperature and pH values should be at the optimal levels. Finally, the produced lactic acid must be neutralized by the addition of calcium carbonate or calcium hydroxide. CO_2 is continuously released during fermentation and it thus creates the anaerobic conditions in the fermentor.

The productivities of lactic acid formation fall in the range of 1–3 kg/ $\text{m}^3\text{.h}$. 90–95% of initial sugar concentration is the final yield of lactic acid at the end of the process, while the cell mass yield is less than 15% of initial sugar concentration, but it can be increased up to 30% depending upon the organism and the culture conditions.

Alcohol and alcohol beverages: Alcohol is one of the major industrial products from biological material utilizing the physiological activities of microorganisms. Ethyl alcohol is one such obtained from starch sources. Starch is hydrolyzed into sugar which is then fermented to alcohol.

The common raw materials are potato, molasses, waste sulphite liquor and wood sugar. Figure 8.4 is a flow sheet for the production of ethyl alcohol.

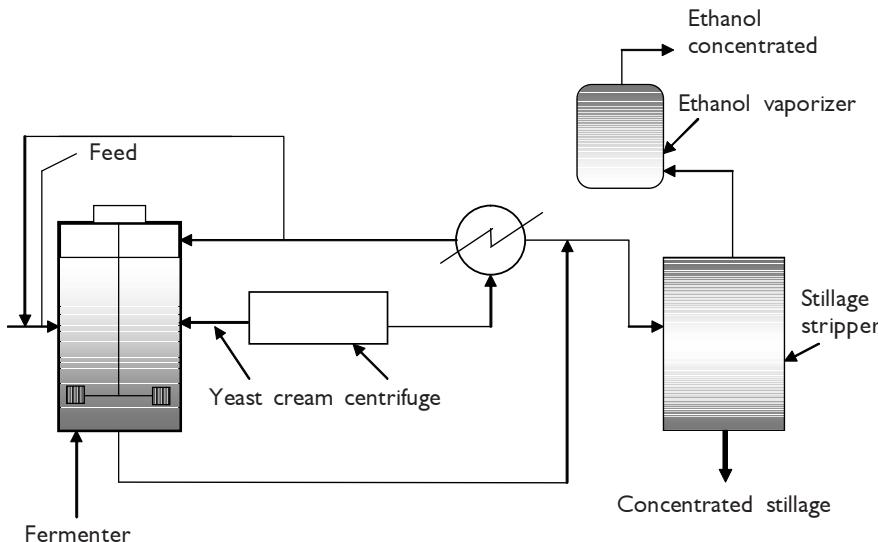


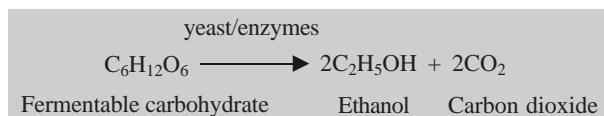
Figure 8.4 Ethanol production.

The above process flow sheet depicts a bio-still process for the production of ethanol. Continuous operations with cell recycle and a distillation column for ethanol separation are employed. A stainless steel fermenter with mechanical agitation is operated in the continuous mode, and the effluent is centrifuged for yeast separation. Yeast is used in the manufacture of ethanol. Part of the separated yeast is recycled to the fermentor as it has two facets of importance: the yeast recycle provides high conversion rates and liquid recycle decreases the amount of wastewater generated and also dilutes the feed sugar concentration down to non-inhibitory levels.

Yeast immobilization within porous or polymeric matrices will result in high cell concentration inside the reactor as a result of which loss of yeast is reduced. This increases the rates of ethanol production. The immobilized cell reactors are in the form of packed columns or fluidized beds. Certain flocculating yeast strains that can settle rapidly are also used in the fermentors in order to obtain high cell concentrations.

Conventional ethanol fermentations operate in the batch mode under the aseptic conditions. But, the use of continuous mode is preferred as the risk of contamination by microbes is minimized with the use of continuous sterilization reactors.

The reaction accomplished by the yeast is as follows:



At the end, to get the ethanol of very high purity, azeotropic distillation is employed. As we know that ethanol forms an azeotrope with water at 95.7 wt% or 89 mol% ethanol. The recovery by distillation is done in two steps. First, a three-column conventional distillation train produces a high alcohol product stream. For anhydrous alcohol production as needed for motor fuel usage, this binary solution is mixed with benzene that acts as an entrainer to break the binary azeotrope and allowing 100% recovery of ethanol.

Figure 8.5 depicts the process of distillation to get ethanol.

Alcoholic beverages and their manufacture represent one of the largest industrial applications of microbial activity. In brewing both carbon dioxide and ethanol formed as a result of fermentation become desirable products. Further, in brewing, the growth of yeast in a sugary medium should take place under anaerobic conditions or else the process of fermentation gets inhibited.

Wine and beer are produced in large quantities as alcoholic beverages. Of course, the raw materials and the processes employed in both are different.

Wine: Wine is made mostly from the juice of grapes and to a lesser extent from the juices of other fruits. The juices containing a large concentration of sugar cane, on fermentation by yeast, be converted to ethanol and carbon dioxide.

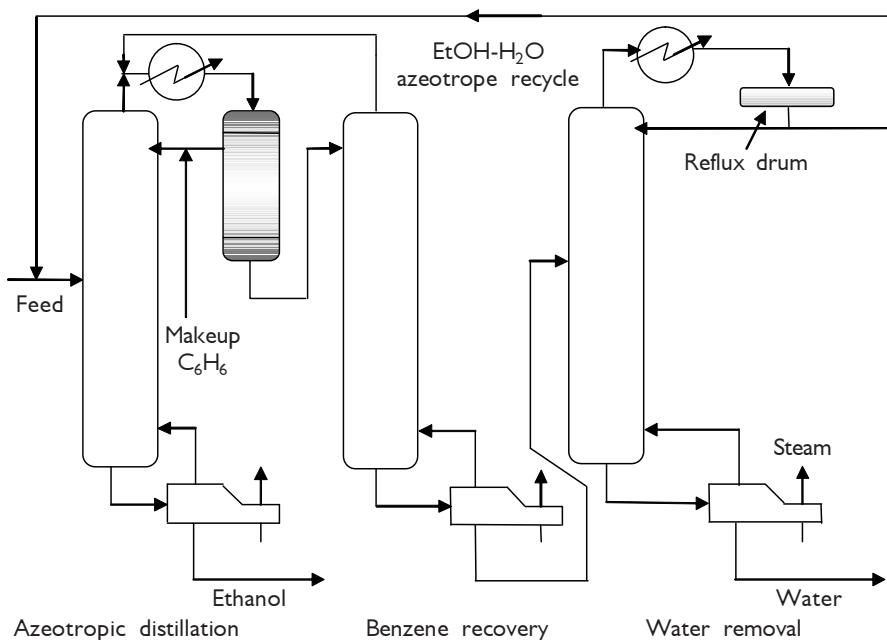


Figure 8.5 Azeotropic distillation of ethanol.

Grapes are crushed to extract juice. The juice itself is a strong solution as it contains many moulds, yeasts and bacteria from the surface of grapes. This solution is treated with sulphur dioxide to remove microbial population and inoculated with starter cultures of the yeast *Saccharomyces ellipsoideus*.

The duration of fermentation ranges from a few days to about 15 days. Temperatures in the range of about 10–15°C, are considered to be the best for fermentation.

The product is then cured to allow the wine to age. This is a slow complex process and a moderate supply of oxygen is required.

Only a properly aged wine will have a desirable aroma and flavour and such wine alone is bottled. Dry wine has a little or no unfermented sugar. Sweet wine has unfermented sugar giving it a sweet taste.

Rum: Rum is prepared by fermentation of black strap molasses containing 12–14% sugar with *S. cerevisiae*, to which ammonium sulphate and phosphate are added. After fermentation, the alcohol is distilled and bottled.

Beer: Beer is prepared from grains like barley, rice and maize as principal carbohydrate, starch is present in them. Yeasts cannot ferment starch but only sugar. Beer is manufactured by the breaking down of starch of sprouted barley into suitable sugar by amylase obtained from other sources.

Fermentation is allowed to start thereafter and is allowed to proceed slowly. The characteristic bitter flavour of beer is produced by the addition of flower buds of the hop plant.

Commercial preparation of beer takes about eight days to ferment and six weeks to mature.

Acetone/butanol: Acetone is used mainly as a solvent for fats, oils, waxes, resins, lacquers and rubber plastics. Butanol is used in the production of lacquers, rayon, detergents and brake fluids and as a solvent for fats, waxes and resins. Acetone and butanol are produced from petrochemical industry intermediates.

A schematic of the process for the manufacture of acetone and butanol is shown in Figure 8.6.

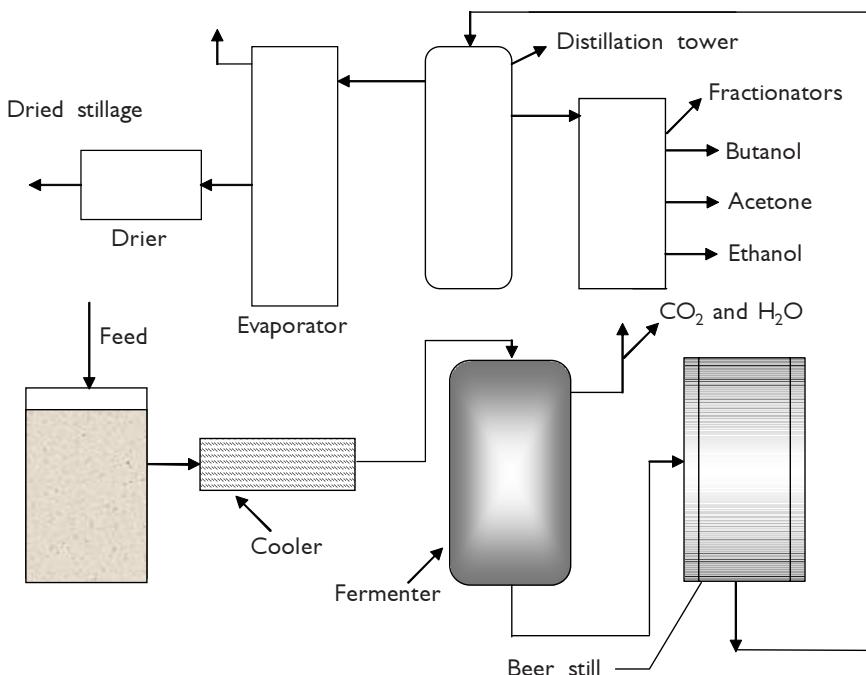


Figure 8.6 Production of acetone/butanol.

In this the raw material used is starch. Corn starch (gelatinized) is hydrolyzed to glucose and maltose with amylolytic enzymes using *C. acetobutylicum*. The process is as follows:

Grains which are mashed are gelatinized at 65°C for 20 minutes and later sterilized at 105°C for a period of 60 minutes. Now the cooked mash is cooled to 35°C using heat exchangers and is pumped to pre-sterilized fermenters of a volume of 250–2000 m³.

The fermenter is inoculated with a 5% inoculum from a 24-hour culture. With the increase in the age of inoculum, the final butanol to acetone ratio increases. For batch fermentations, the period is usually 2–3 days. During the initial stages, there is a rapid growth and the production of acetic/butyric acids and carbon dioxide and hydrogen as well. During this phase, the pH

drops from 6.5 to 4.5. The second phase exhibits cessation of growth and organisms convert acetic and butyric acids to neutral acetone and butanol. At the end of fermentation the pH is around 5.0. In order to improve the solvent yield in acetone/butanol fermentations, and butanol production following steps are taken up: addition of acetic and butyric acids and moderate to high agitation (300 rpm) during the acid phase followed by low agitation (25 rpm) during the solvent phase respectively. Besides this, the removal of inhibitory products (butanol/acetone) by adsorption employing activated carbon is done. This improvises the fermentation productivity.

At the end of solvent phase to recover acetone and butanol, broth is transferred to a beer still that concentrates the solvents. Further, solvents are separated by distillation and fractionation and stillage is dried. Finally, three streams, butanol, acetone and ethanol are recovered.

8.1.2 Aerobic Fermentation Processes

Aerobic bioprocesses are widely used for the production of organic acids (citric, acetic, gluconic), vitamins, antibiotics, enzymes, flavours-fragrances, amino acids, etc.

Citric acid: Figure 8.7 depicts the process of citric acid production.

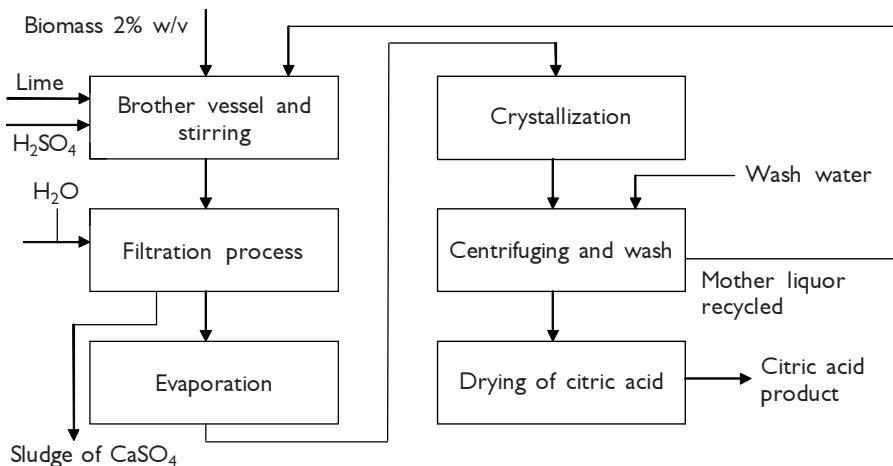


Figure 8.7 Production of citric acid.

Citric acid is commercially produced by microbes and is commonly used as a food preservative, flavouring agent of fruit juices, candy, ice cream and marmalades. It is also used as a preservative of stored blood, in ointments and cosmetics.

Citric is produced from sugar beet and sugar cane molasses by *A. niger* that converts 42% of raw sucrose into citric acid. This citric acid is excreted by the organisms into the culture which is then precipitated. Precipitation is usually accomplished by addition of calcium hydroxide to the heated fermentation broth to obtain calcium citrate tetrahydrate.

The precipitate is then washed and treated with dilute sulphuric acid, yielding an aqueous solution of citric acid calcium sulphate precipitate.

After bleaching and crystallization, either anhydrous or monohydrate citric acid is obtained. Later, centrifugal recovery is done followed by drying crystals. Solvent extraction is another option for the recovery of citric acid, although it is not used commercially.

Baker's yeast: The use of yeast as a leavening agent in the baking process dates back to the very early history of the Jews. In modern baking practice, pure cultures of selected strains of *S. cerevisiae* are mixed with the bread dough to bring about the desired changes in the texture and the flavour.

The desirable characteristics of strains selected for the commercial production of baker's yeast include the ability to ferment sugar in dough and to grow rapidly and should be relatively stable.

Figure 8.8 depicts the steps in the production of baker's yeast.

In the production of baker's yeast, the medium that contains molasses and corn-steep liquor is inoculated with a stock strain, while the pH of the medium is brought to acidic value (4–5). This aids in the retardation of bacterial growth, the unwanted ones. The total medium is incubated during which aeration is provided. At the end of incubation period, the yeast cells are harvested by adopting centrifugation and washing of the cells by suspending in water and again centrifuging the cells out.

The cells are finally recovered by filtration process using a filter press. Over the press, cells are present and these are treated with small amounts of edible oil which serves as a plasticizer in giving the form to the cells as they are molded into blocks.

Fine chemicals: Fine chemicals include the bioproduction of high value molecules like antibiotics, vitamins, hormones, enzymes, monoclonal antibiotics, etc.

Antibiotics: Penicillin was the first antibiotic to be produced industrially. The major steps in the commercial production of penicillin are as follows:

- Preparation of inoculum
- Preparation and sterilization of the medium
- Inoculation of the medium in the fermenter
- Forced aeration with sterile air during fermentation
- Removal of the mold mycelium after fermentation
- Extraction and purification of penicillin

Antibiotic production yields either a bulk salt form, e.g. sodium penicillin or a more purified precipitate, procaine penicillin for clinical use. Figure 8.9 shows the method to manufacture and purify crude penicillin, to get procaine penicillin.

Penicillin is a secondary metabolite with a non-growth associated production. The process to produce penicillin involves an initial batch phase in which cell growth occurs.

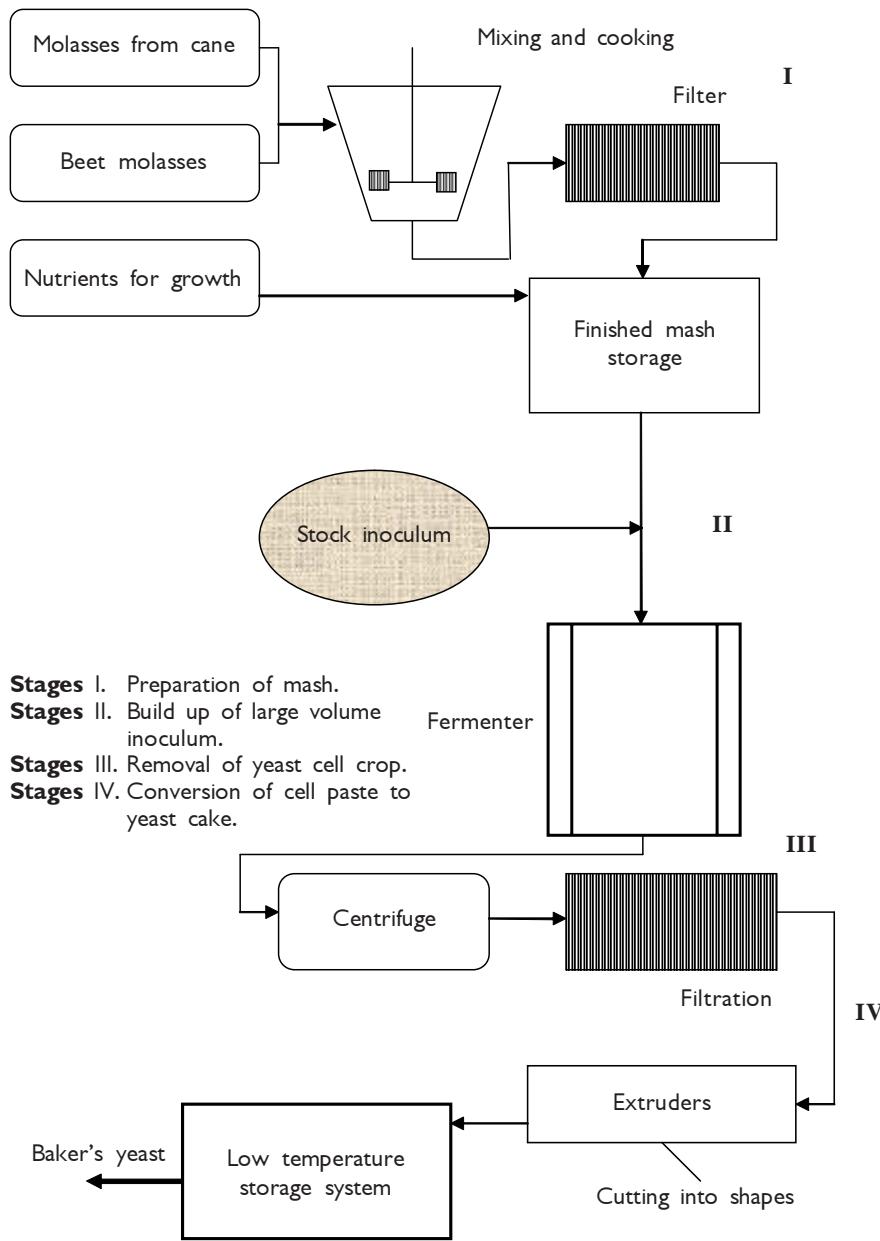


Figure 8.8 Steps in the production of baker's yeast.

Culture preparation starts with freeze drying of spores and agar slant cultures. Vegetative cells are cultivated in the shake flasks and then transferred to the seed fermenters.

These are agitated tanks of 200–500 m³ in volume made of stainless steel. Mechanical agitation is provided at a rate of 100–300 rpm. Temperature is controlled around 25–28°C by using cooling coils.

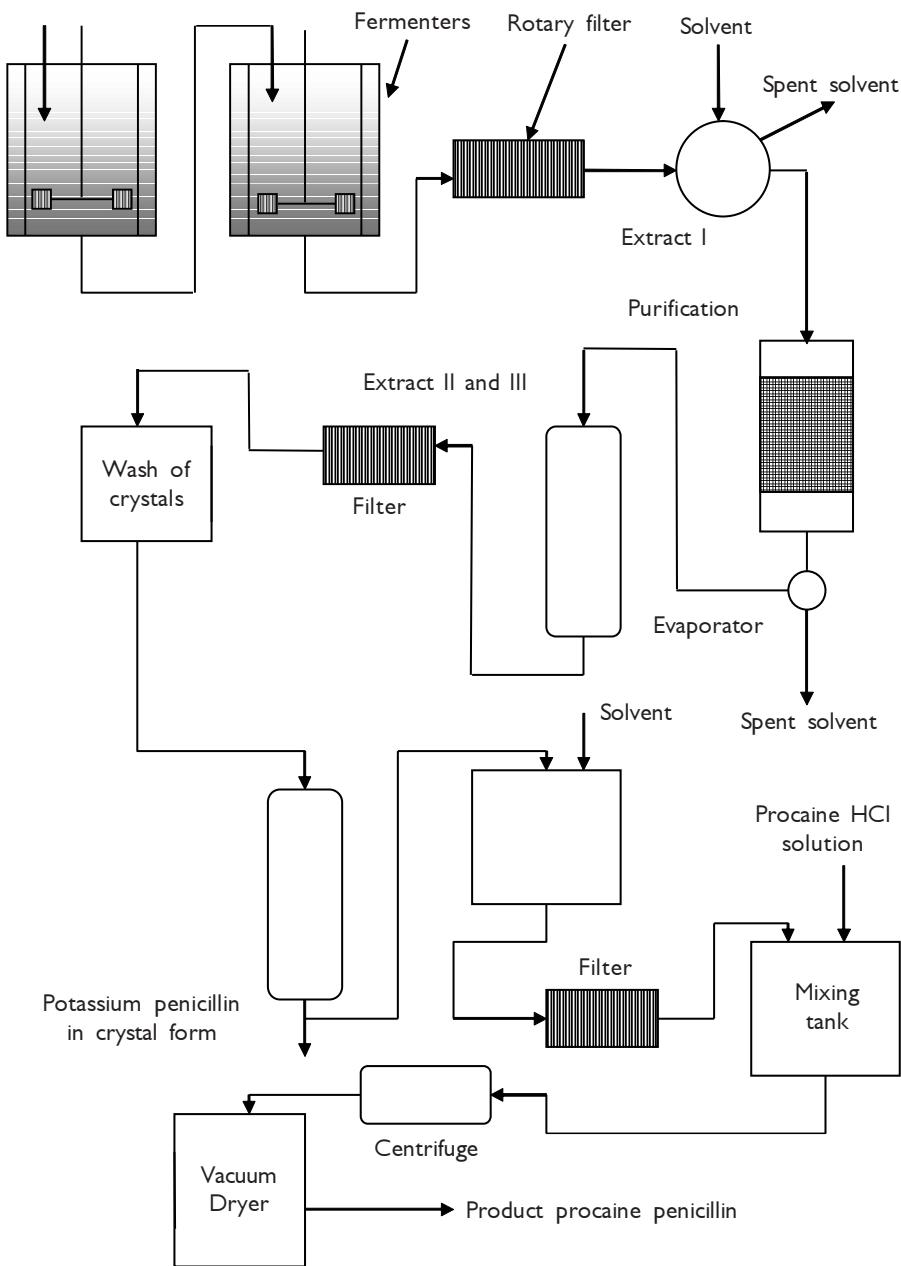


Figure 8.9 Production of penicillin.

Antifoam is added to reduce foam formation. DO (Dissolved Oxygen) content is controlled at 2 ppm and pH at 6.5. Vigorous aeration is provided at the bottom of the fermenters.

The fermentation is stopped when the oxygen uptake rate of the culture exceeds the oxygen transfer rate of the reactor, or when 80% of the fermenter is full.

The recovery process includes filtration, extraction, adsorption, crystallization and drying. Filtration is usually done by using a high capacity, rotary vacuum drum filters for separation of the mycelia. The mycelia are washed on the filter and disposed. The penicillin rich filtrate is cooled to a temperature of 2–4°C to avoid the chemical or enzymatic degradation of penicillin.

Solvent extraction is accomplished at low pH values, i.e. at 2.5–3.0, using amyl acetate or butyl acetate as a solvent. Two extractors are used in series and the efficiency of extraction is 99%. Carbon adsorption is used to remove the impurities from penicillin-rich solvent after extraction.

Crystallization is performed from the solvent or the aqueous phase. Na, K and penicillin concentrations need to be adjusted along with the pH, temperature, etc.

Excess amounts of Na or K are added to the penicillin-rich solvent before crystallization in an agitated vessel.

The crystals are separated by a rotary vacuum filter and are washed and predried using anhydrous butyl alcohol to remove impurities. Large horizontal belt filters are used for collection and drying of crystals for which usually warm air or radiant heat is used.

Final spray drying of the procaine antibiotic precipitate is accomplished in a low temperature freeze dryer. Biomass may be fully recovered and sold as an animal food supplement.

Crystalline penicillins G or V are sold as an intermediate or converted to 6-aminopenicillanic acid, used in the production of semi-synthetic penicillin.

Penicillin for medical applications and feed has an annual demand of \$4,400 million. More than 80% of penicillin produced is utilized for the synthesis of 6-APA and other intermediates.

Vitamins (Vitamin B₁₂): Vitamin B₁₂, is cyanocobalamin, the deficiency of which leads to pernicious anaemia. The requirement of the human body for this vitamin is met from diet. Vitamin B₁₂ is produced as a supplement for animal feeds, for use in pharmaceutical industry, as a food additive and as a vitamin supplement.

Vitamin B₁₂ is a primary product, commercially produced by *Propionibacterium freudenreichii*. The vitamin is extracted from the cells by a heat treatment.

Most commercially produced riboflavin, vitamin B₂ is made chemically and sold as a vitamin supplement or added to vitamin-enriched bread.

Riboflavin can be produced microbiologically by the yeast *Ashbya gossypii* that produces up to 7 g of riboflavin per litre of the growth medium.

Steroids: Cortisone is a well-known steroid and in the production of this steroid, a strain of bread mould *Rhizopus arrhizus* is used that is able to hydroxylate progesterone, an early intermediate in the synthesis of cortisone by means of microbial hydroxylation.

The commercially important steroids like corticosteroids, cortisone, hydrocortisone, prednisone and dexamethasone, androgen testosterone and estrogen estradiol (used in contraceptives) have been synthesized using microorganisms. The raw material for all these are complex alcohols called *sterols*.

Polysaccharide (Dextran) and biogum (Xanthan Gum): Polysaccharides and their closely-related derivatives are precipitable by alcohol addition, forming the basis for recovery.

Dextrans provide a high value polysaccharide product with uses which may include food or medicinal applications. Hence, their primary uses are in purification of alcohol precipitation and use of washes with pyrogen-free water. Figure 8.10 illustrates the process of dextran manufacture.

The sequence of steps employed are as follows:

Partial hydrolysis of initial precipitate is done and is followed by the removal of solids, further repeated fractional crystallizations and final impurities are removed by ion exchange.

The food processing industry uses polysaccharides to alter or control physical properties of foods. Many are incorporated into foods as thickeners, gelling agents and agents to control ice crystal formation in frozen foods. They are used in instant foods, salad dressings, sauces, whips, toppings, processed cheeses and dairy products.

Dextran is commercially produced using an organism called *Leuconostoc mesenteroides* and xanthan gum in the presence of *Xanthomonas campestris*.

Xanthan gum is one such that is produced on a large scale to meet the demands of both the food and the pharmaceutical industries.

High fructose corn syrup: High fructose corn syrup (HFCS) is a low-calorie sweetner commonly used in beverages, desserts, and other sweet foods. The first HFCS was produced in 1967 and contained 15% fructose. Further process improvements yielded 42% and 55% fructose-containing HFCS.

There are three major HFCS products that differ by their fructose content: 42%, 55%, and 90%. HFCS containing 42% fructose is mainly used in most of the food products as a liquid sweetner. HFCS with higher level of fructose (55%) is used as a low-calorie sweetner.

Figure 8.11 illustrates the process of the commercial production of HFCS.

Production of HFCS from corn starch is an enzymatic process. The overall process scheme has 18 steps as shown in Fig. 8.11 with five major operations.

Five major operations are explained as follows:

1. Dextrose production by enzymatic hydrolysis of corn starch: In this operation corn starch is gelatinized by cooking at high temperatures, 65°C in a two-stage continuous reactor, due to which it gets converted to dextrose. The product is a dextrose-rich syrup having 10 to 15 DE (Dextrose Equivalent).

2. Primary physical and chemical treatment of dextrose syrup: Dextrose syrup which is liquefied is said to have some proteins both total

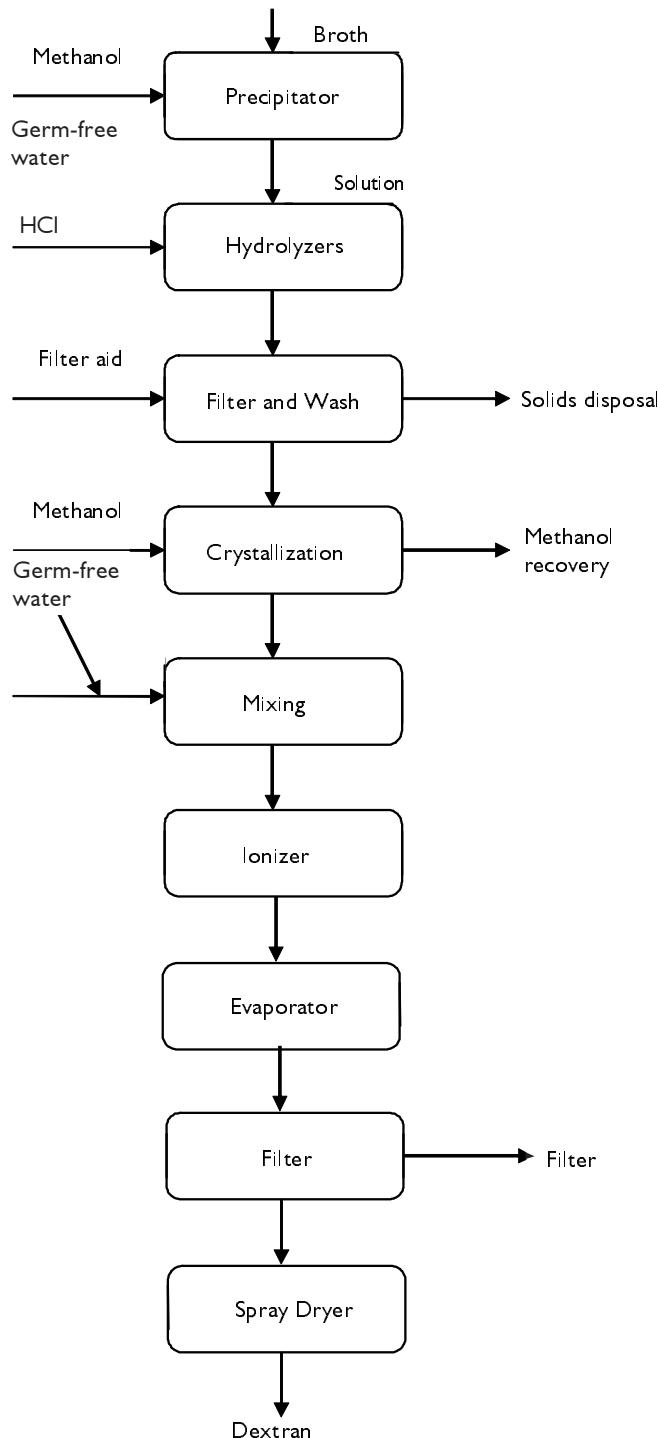


Figure 8.10 Dextran manufacture.

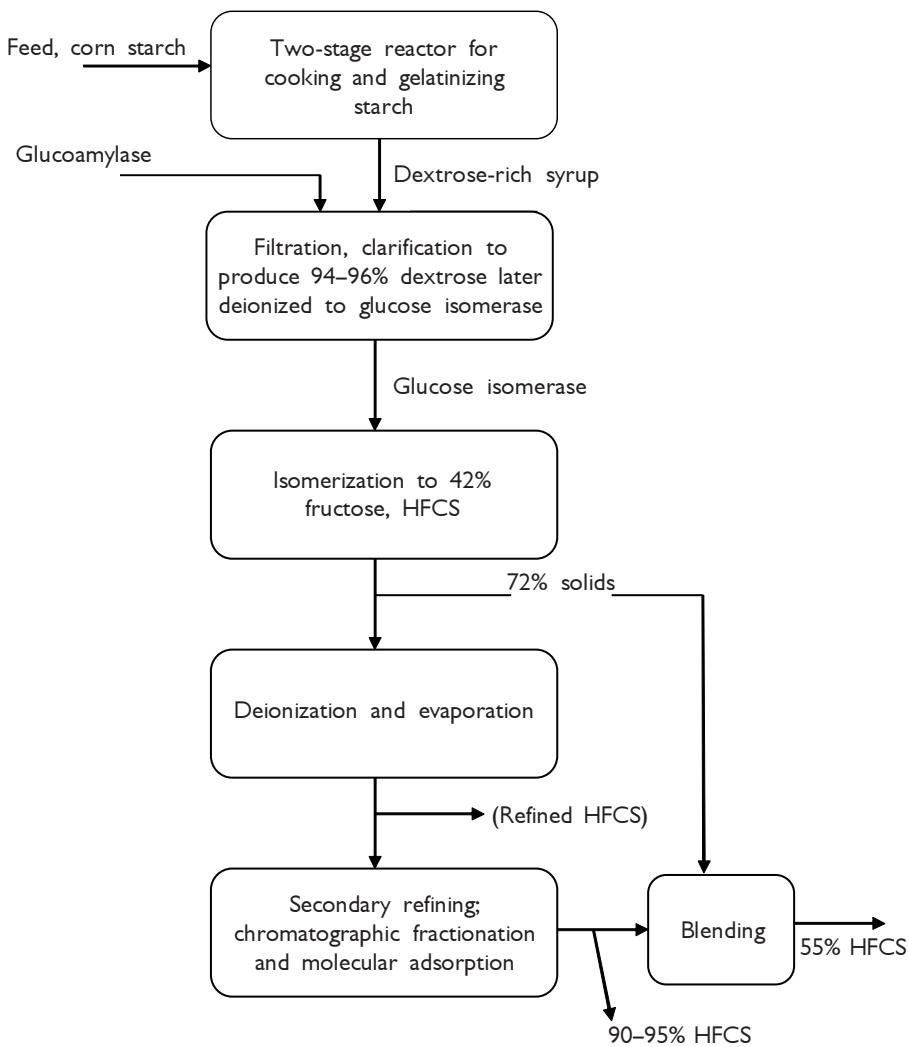


Figure 8.11 Production of HFCS.

and soluble. The total and soluble protein contents should be lower than 0.3% and 0.03% respectively. This is to avoid colour formation which is a consequence of the Maillard reaction between amino acids and sugars at high temperatures.

This is done by saccharifying the liquefied starch slurry using the enzyme glucoamylase that produces more dextrose from the branched chains of starch. Glucoamylase is added to the 10–15 DE liquefied starch after temperature and pH adjustment and fed to the reactors. The conditions for this step are 60°C, and pH of 4.3 with a holding time in reactors of 65 to 75 hours. Finally, the product is said to have 94–96% dextrose.

Further, it is refined to remove ash, metal ions and proteins. This step is essential as proteins and metal ions can interfere in the subsequent

isomerization reactions. These are removed by using filters like rotary vacuum filters into which dextrose syrup is fed. This step removes solids, proteins and oil if present.

Later, the filtered liquor is sent for the removal of traces of particles for which polish filters are used. Decolorization is done by adsorption using granular activated carbon.

3. Isomerization of dextrose to 42% fructose: Deionized and decolorized dextrose syrup is subjected to isomerization wherein the enzyme glucose isomerase is used. The enzymatic isomerization of glucose to fructose is reversible. Also, the dextrose syrup is concentrated in an evaporator, while the Mg ions are added to activate the isomerase. It yields HFCS containing 42% fructose and 72% solids.

4. Secondary refining of fructose corn syrup: In this enrichment of HFCS which is 42% is done by using either an inorganic resin for selective molecular adsorption of fructose or by chromatographic fractionation employing organic resins during the course of which fructose gets selectively held and dextrose does not.

5. Conversion of 42% fructose to 55% fructose containing HFCS: The enriched syrup contains nearly 90% fructose and is called *very enriched fructose corn syrup* (VEFCS). It is blended with 42% fructose to obtain the desired fructose content, i.e. 55% HFCS.

HFCS has replaced sucrose and glucose as a low-calorie sweetner, which has been used in soft drinks, canned fruits, desserts, ice creams and bakery products over the last 20 years.

SUMMARY

In this chapter, we learnt the following:

- Fermentation processes are carried out to manufacture a variety of industrial alcohols, food products, beverages and fine chemicals.
- Hormones, steroids, alkaloids and vitamins are also manufactured by fermentation processes.
- Fermentation processes can be aerobic or anaerobic depending upon the requirements of the product to be manufactured.
- Enzymes and microbial cells are used for the production of all the fermentation products.
- Yeasts are the preferred organisms for the production of industrial-scale ethanol.
- Antibiotics like penicillin are produced by using moulds.
- Dextran and xanthan gum are also the products obtained from the fermentation industry.
- HFCS is a low-calorie sweetner used in beverages, desserts and other food products.

EXERCISES

- 8.1 Describe the manufacture of penicillin in detail. Further, how is it recovered to a purer form?
- 8.2 With the help of neat flow sheet, describe the production of HFCS and explain its uses.
- 8.3 Write a note about the manufacture of the following:
 - (a) Vinegar
 - (b) Beer
 - (c) Rum
 - (d) Cheese
- 8.4 Describe the production of citric acid with a neat flow sheet and indicate its uses.
- 8.5 Differentiate anaerobic and aerobic fermentation processes with the help of examples.
- 8.6 Enlist the microorganisms that are used in a bioprocess industry.
- 8.7 With a neat flow sheet, describe the production of the following:
 - (a) Baker's yeast
 - (b) Acetone
 - (c) Butanol

Downstream Processing

Downstream processing refers to the recovery and purification of products of various biochemical processes. It is bound to be essential to any commercial process and an important activity in biotechnological industries by means of which a wide variety of products such as pharmaceuticals, drug intermediates, food chemicals, beverages, organic fine chemicals and solvents, industrial enzymes, dairy products, etc. are manufactured. On a wide basis, these are also called *bioseparations*.

In downstream processing also called *product recovery operations*, a spectrum of techniques, viz. isolation, purification, concentration, etc. are adopted to bring the desired product from a complex mixture comprising starting materials, products of reaction, by-products, etc. to a final form prior to which formulation is done. This envisages the product for long-term storage, transportation and marketing before it reaches the end users, i.e. customers. In order to achieve the same, the techniques range from the conventional chemical engineering unit operations to the sophisticated high resolution techniques.

Prominently saying, biotechnology without downstream processing is incomplete.

This chapter emphasizes the use of several conventional and advanced techniques to isolate the product in a purer form.

9.1 NEED FOR DOWNSTREAM PROCESSING

Downstream processing is an integral part of biotechnology, which is needed for product isolation, purification, and formulation for different end uses. The products can be biomass itself, an extracellular product, an intracellular product, unconverted substrates and unconvertible compounds that overall forms fermentation broth. As the chemical nature of fermentation broth is very complex, extremely high purity is needed for some products especially

pharmaceuticals. Products are manufactured using a variety of equipment. For instance, not only the bioreactors, other special reactors such as airlift, membrane and immobilized cell reactors are also used. All these operate under sterile conditions. Different kinds of microbes including genetically modified species are used for the production of desired products.

In view of the above, the products finally formed are usually low in concentrations necessitating the handling of large volumes and in some cases, the broths are viscous creating additional problems of fouling of equipment.

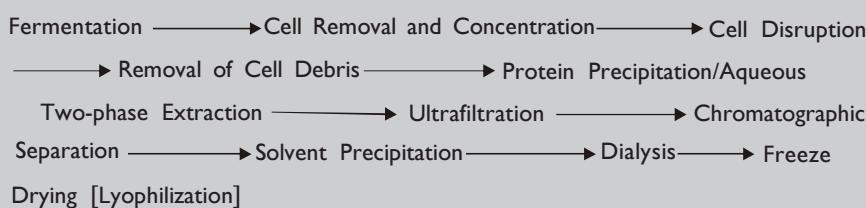
The recovery and the purification of products incur major expenses in a fermentation industry, i.e. 50% of the total cost of manufacturing. However, the ultimate challenge is to select the best combination of substrate, enzyme/organism, bioreactor and separation for a given product.

Each separation needed depends on initial broth characteristics like viscosity, product concentration, impurities, undesired particulates and final product concentration and also the form required. The forms can be a crystallized product, concentrated liquid, crude solution or a dried powder. In general, the separation methods vary with the size and nature of product.

The operational sequence through which a bioreactor broth must pass for a highly purified product is as follows:

- **Removal of particulates or insolubles:** The removal of particulates or insolubles is achievable by adopting filtration, centrifugation, settling, sedimentation and decantation processes.
- **Primary isolation:** Primary isolation refers to the concentration of product that is achievable by removing water content. Water is removed through solvent extraction, sorption, precipitation, ultra filtration, etc.
- **Purification:** Purification complies with the removal of contaminating chemicals and it is achievable by fractional precipitation, chromatography, adsorption, etc.
- **Final product isolation:** The final product isolation refers to the product preparation by drying through drum driers or spray driers, and by freeze drying also called lyophilization.

The steps explained above are depicted as follows:



Now, let us discuss the steps one by one.

9.2 REMOVAL OF PARTICULATES (INSOLUBLES)

The removal of particulates or insolubles is the first and foremost step in the sequence of product recovery operations.

The fermentation broth is pretreated to facilitate solid separation. This is done by heating the fermentation broth, by adjusting pH/ionic strength, by adding coagulants and flocculants, etc.

The pretreatment is desirable so as to increase the size of these insolubles that can later facilitate the removal by using either of the physical separation methods.

Heating the fermentation broth, which is quite a least expensive method, is to be used when the volume of the broth is small. By heating, the handling characteristics of broth improvise and also pasteurize it. But, addition of chemicals such as acids, bases, simple electrolytes and poly electrolytes promotes the process of coagulation and flocculation. Ferric chloride and aluminium sulphate act as flocculants whereas poly acrylics and polyamines act as poly electrolytes. Flocculants, however, have the disadvantage of fouling the membrane filter presses.

9.2.1 Filtration

In the case of filtration (refer to Figure 9.1), rotary vacuum filters, micro-filters, or ultra filters can be employed depending upon the requirements and can prove to be cost effective. Figure 9.1 shows the mechanism of filtration. Fermentation broth is passed through a filter medium over which cake is obtained on the surface. This medium can act as a septum and hold cake on its surface. In the case of a rotary drum filter (Figure 9.2), the drum is covered with a pre-coat layer, i.e. diatomaceous earth. Also filter aids are used that can enhance the rate of filtration.

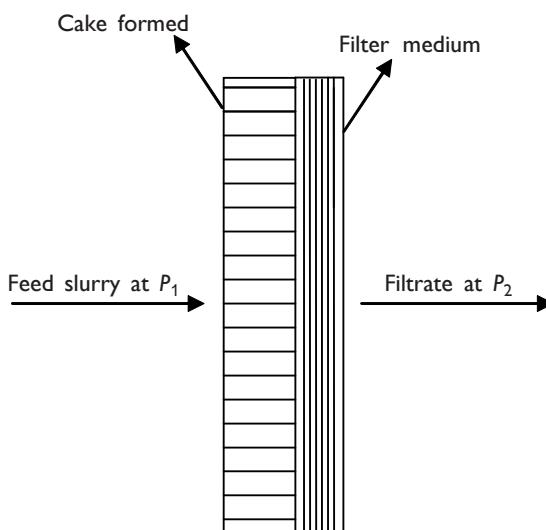


Figure 9.1 Schematic for filtration.

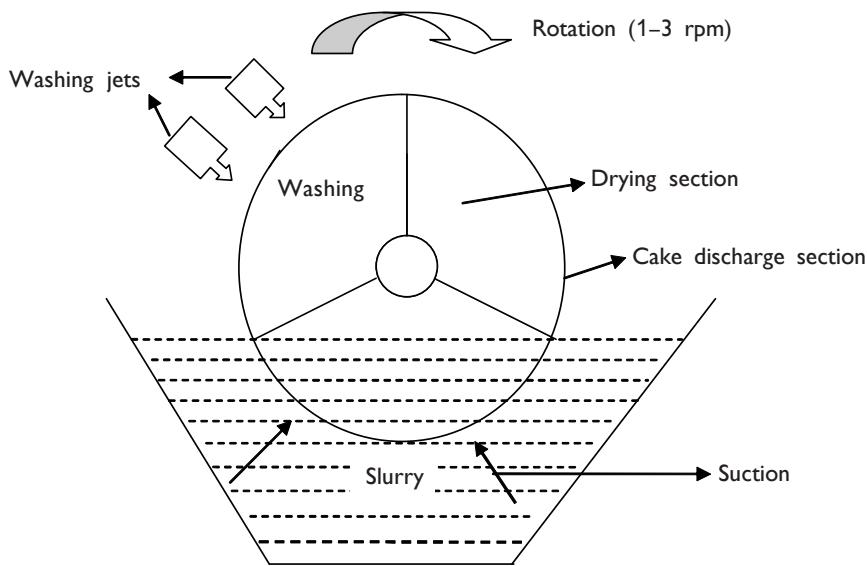


Figure 9.2 Rotary drum vacuum filter.

The filtration process is commonly used to separate the mycelium from the fermentation broth in the manufacture of antibiotics. It is reported that the filtration process produces cell sludges or cell solids to an extent of 25–30% weight by volume.

Filtration is defined as the separation of solid in a slurry consisting of solid and fluid. The separation of solid is done by passing the slurry through the septum called the filter medium. The slurry is pumped usually at a pressure in perpendicular direction to the filter medium as shown in Figure 9.1. The filter medium allows the fluid (filtrate) to pass through it and retains the solid.

The cake gets deposited on the surface of the filter medium. The thickness of the cake increases from an initial value of zero to a final thickness at the end of the filtration process.

The rotary drum vacuum filter is a continuous filtration device used in the industries for large volumes of feed. It consists of a rotating hollow segmented perforated drum covered with a fabric or metal filter mesh.

Filtration through a rotary drum vacuum filter has three steps:

- Cake formation
- Cake washing
- Cake discharge

The drum is partially submerged in a trough containing the feed slurry to be filtered. The interior of the drum has a series of compartments. Vacuum is applied that helps in sucking in the liquid through the filter medium forming a cake at the exterior surface of the filter medium.

Washing is done so as to facilitate the process and this is taken up by using water jets. Using the doctor's blade the cake is scraped.

Filtration theory: In addition to the use of filtration for separation of mycelium from the fermentation broths during antibiotic manufacture, it is also used in the treatment of wastewater.

Generally, we have two mechanisms adopted in filtration. They are as follows:

(a) Constant pressure filtration or vacuum filtration, and (b) Constant rate filtration.

It is always seen that during filtration, the cake forming develops the resistance called *cake resistance* denoted as R_c and also the filter medium develops the resistance called *filter medium resistance* denoted as R_m . The rate of filtration, i.e. flow of filtrate for a constant pressure filtration is determined mainly by the resistance offered by the cake and the medium combined together.

As the flow of filtrate is time dependent, we can define as the volume flow of filtrate as dV/dt and is given as,

$$\frac{dV}{dt} = \frac{\Delta P \cdot A \cdot g_c}{(R_m + R_c)\mu} \quad (9.1)$$

where dV/dt is the volume flow rate of filtrate and is time dependent.

μ is the viscosity of the filtrate, kg/m-sec.

A is the surface area of filter, m^2 .

ΔP is the pressure drop through the cake and filter medium and R_m and R_c with their usual meanings and significance.

Filter medium resistance R_m and its value is usually a characteristic of filter medium, whereas the cake resistance R_c is seen to increase as filtration proceeds and later after a start up period R_c supercedes R_m , i.e. the value of R_c will be greater than the value of R_m .

The value of R_c is expressed as

$$R_c = \alpha(W/A) \quad (9.2)$$

where α is the average specific cake resistance, m/kg

W is the total weight of cake formed on the filter, kgs

A is the surface area, m^2 .

Equation (9.2) can be further expressed as

$$R_c = \alpha(CV/A) \quad (9.3)$$

where $W = CV$ and C is the concentration of the cake, i.e. the weight of the cake formed per unit volume of filtrate, kgs/m^3 , and V is the total volume of filtrate.

Substituting Eq. (9.3) in (9.1), we get

$$\frac{dV}{dt} = \frac{\Delta P \cdot A \cdot g_c}{[R_m + \alpha(C \cdot V/A)]\mu} \quad (9.4)$$

Considering area of filtration, A (m^2) to be constant, it yields the following equation.

$$\frac{d(V/A)}{dt} = \frac{\Delta P \cdot g_c}{[(R_m + \alpha(C \cdot V/A))\mu]} \quad (9.5)$$

Integrating the above equation using the boundary conditions as at $t = 0$,

$$V = 0 \text{ and at } t = t, V = V,$$

$$\int_0^V d(V/A) = \frac{\Delta P \cdot g_c}{[(R_m + \alpha(C \cdot V/A))\mu]} \int_0^t dt$$

Integration and further simplification yields

$$V^2 + 2VV_0 = Kt \quad (9.6)$$

where $V_0 = \frac{R_m A}{\alpha \cdot C}$ and $K = \left(\frac{2A^2}{\alpha \cdot C \mu} \right) \Delta P \cdot g_c$

i.e.

$$V^2 + \frac{2VR_m}{\alpha \cdot C} A = \left(\frac{2A^2}{\alpha \cdot C \mu} \right) \Delta P \cdot g_c t \quad (9.7)$$

Equations (9.6) and (9.7) are called Ruth equations for constant pressure filtration and can be further rearranged as

$$\frac{t}{V} = \frac{1}{K} (V + 2V_0) \quad (9.8)$$

Using the above Eq. (9.8) and plotting t/V vs. V by way of which a straight line is obtained with a slope $1/K$ and an intercept $2V_0/K$ as shown in Figure 9.3(a).

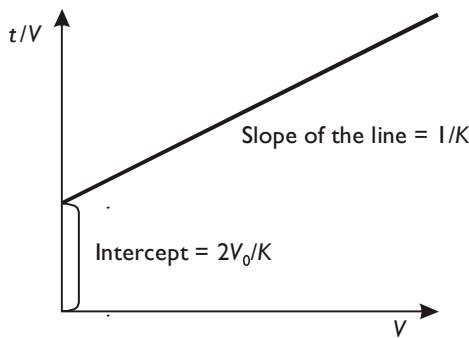


Figure 9.3(a) Plot of t/V vs. V .

This is used in the filtration practice to calculate specific cake resistance α and filter medium resistance R_m from experimentally determined values of K and V_0 .

Referring to Figure 9.2, i.e. rotary drum vacuum filter, it is observed that the drum rotates at a constant speed of n revolutions per second and only

a fraction of its surface area will be immersed in the slurry reservoir. This fraction is represented as f .

The time period during which the filtration is carried out is f/n per revolution of the drum. Therefore, Eq. (9.6) can be written as

$$\left(\frac{V'}{n}\right)^2 + \frac{2V'}{n}V_0 = K \cdot \frac{f}{n} \quad (9.9)$$

where $V = V'/n$ and V' is the filtrate volume per unit time and V'/n is the volume of filtrate for one revolution of the drum.

During filtration, certain complexities in situation are faced. To simplify the same, certain assumptions are employed for the analysis of filtration. The first and foremost is that the cake formed is assumed to be incompressible although cakes formed after filtration are compressible.

Holding the assumption valid, results in constant specific cake resistance to be constant, while in reality it is not so and varies with the pressure drops.

It is also found that the use of filter aids, say around 1% to 5%, to enhance the rate of filtration, also exhibits a significant effect on specific cake resistance. This effect is illustrated in Figure 9.3(b).

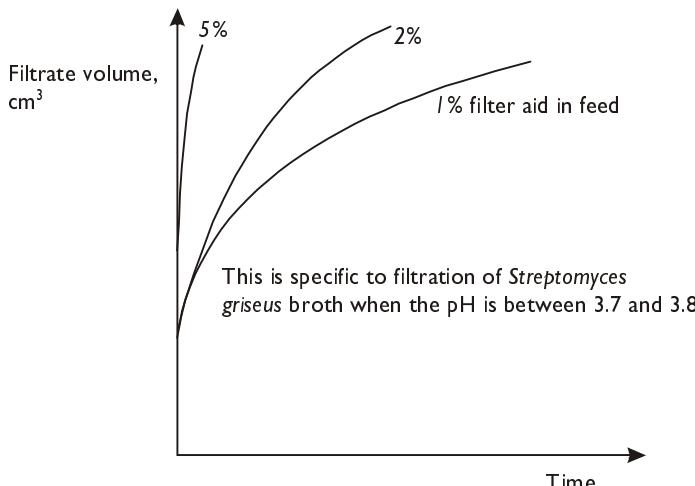


Figure 9.3(b) Effect of filter aids concentration in the feed on specific cake resistance.

According to Figure 9.3(b) it is found that, specific cake resistance decreases as the concentration of filter aids increases. Further, it is also seen that the specific cake resistance is also influenced by conditions like pH, viscosity and composition of broth or medium during fermentation.

In general, the filterability of fermentation broth is affected by the conditions during fermentation. In Figure 9.3(c) shown below is the effect of pH on cake resistance. It is found that specific cake resistance decreases with decreasing pH.

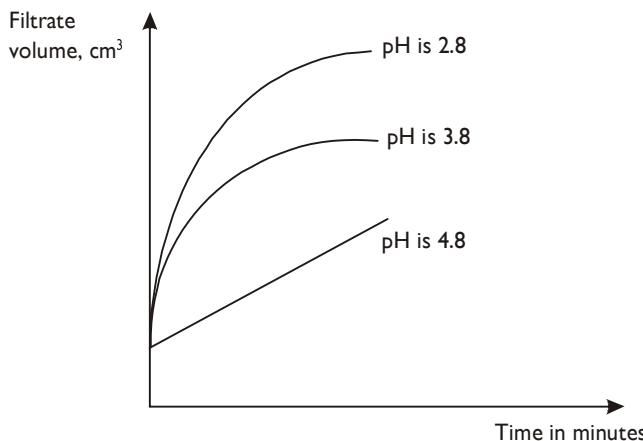


Figure 9.3(c) pH effects on filtration rate and specific cake resistance (specific to *Streptomyces griseus*).

Therefore, in order to avoid or at least minimize the above effects, it is essential that in a typical filtration process, the conditions are maintained favourably: please note pH is 3.6, filter aids content is 2–3% and followed by heat treatment of 30–60 minutes at temperatures of 80 to 90°C specific for streptomycin fermentation.

Example 9.1 For the filtration of a cell broth, the constant pressure filtration was employed. The characteristics of filter employed are:

- (i) Area $A = 0.3 \text{ m}^2$
- (ii) Weight of the cake deposited per unit volume of filtrate $C = 1950 \text{ kg/m}^3$
- (iii) Viscosity of the medium $\mu = 3.0 \times 10^{-3} \text{ kg/m-s}$ and
- (iv) Average specific cake resistance $\alpha = 3.0 \text{ m/kg}$.

During the process the following observations were made and recorded.

Time, t (min)	3.0	15	30	65	110
Volume of filtrate, V (litre filtrate)	90	300	650	820	1150

Estimate the following: (a) Pressure drop across the filter and
 (b) Filter medium resistance.

Solution

With the data provided first find out the values for t/V and prepare the table for t/V and V .

V , litre	90	300	650	820	1150
t/V , (min/litre)	0.034	0.05	0.46	0.08	0.0956

Then plot the graph of t/V vs. V as shown below in Figure 9.3(d).

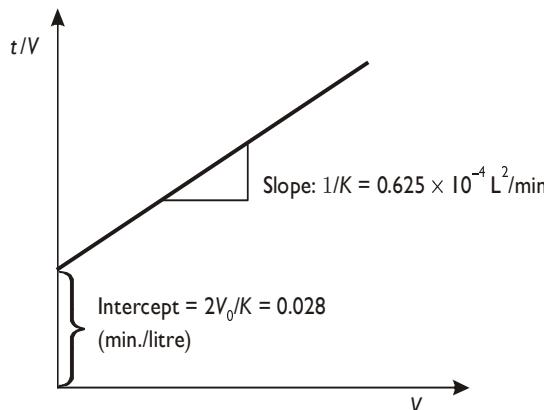


Figure 9.3(d) t/V vs. V values.

(a) To calculate the pressure drop across the filter:

Using the equation, $\Delta P = K C \alpha \mu/2 A^2 g_c$ (Take $g_c = 1.0$)

Substituting the values in the above equation, we get

$$\Delta P = 1.56 \times 10^4 \text{ N/m}^2.$$

(b) To calculate filter medium resistance R_m :

Since intercept is $2V_0/K = 0.028$ from graph.

Therefore,

$$V_0 = 224$$

Using the equation $R_m = \alpha V_0 C/A$ we get $R_m = 4368000 \text{ m}^{-1}$

Example 9.2 In a constant pressure filtration process of a biomass suspension, the specific cake resistance and the filter medium resistance is to be calculated on the basis of the following experimental observations.

The characteristics of filter used are as follows:

Area of filter (A): 1m^2 ; Density of suspension (C): 500 kg/m^3 ; Viscosity of filtrate (μ) is $1.1 \times 10^{-3} \text{ kg/m-sec}$. and the pressure drop $\Delta P = 2 \text{ bar}$ and the observations recorded are given below.

Observations:

Vol. of filtrate collected, $V \times 10^{-3} \text{ m}^3$	2.0	4.0	6.0	8.0	10.0
Time, t secs.	25	60	113	185	268

Solution (Hint)

Data: Constant pressure filtration, Area of filter (A) = 1m^2 ; Density of suspension (ρ) = 500 kg/m^3 ; Viscosity of filtrate (μ) = $1.1 \times 10^{-3} \text{ kg/m-sec}$. and the pressure drop $\Delta P = 2 \text{ bar}$.

To determine: Filter medium resistance R_m and specific cake resistance α .

Using the tabulated readings calculate t/V and plot the graph of V vs. t/V as shown in Figure 9.3(d).

Slope of the line $= 1/K$ from which get the value of K and the Intercept $= 2V_0/K$. Using this find the value for V_0 .

$$V_0 = \frac{R_m A}{\alpha C} \text{ and } K = \frac{2A^2}{\alpha C \mu} \Delta P \cdot g_c$$

In the above equations substitute the values and get the R_m and α .

9.2.2 Centrifugation

Centrifugation is taken up whenever the size of the particles is in the range of 100–0.1 μm . It produces a cell-concentrated stream (cream), since the solids content in a slurry is upto 15% weight by volume. This process is employed for yeast fermentation recovery. It is used to separate the materials of different densities when the gravitational force is insufficient for separation.

In industry, centrifugal force is used (i) to separate fine solids from liquids by centrifugal sedimentation, (ii) to separate immiscible liquids whose density difference is small by centrifugal decantation, and (iii) in the filtration of solids from liquids by centrifugal filtration.

The common types of centrifuges that find application in bioseparations include the following:

- Tubular bowl centrifuge (Figure 9.4)
- Multi-chamber bowl centrifuge (Figure 9.5)
- Disc bowl centrifuge with/without nozzle (Figure 9.6)
- Decanter centrifuge
- Basket centrifuge (Figure 9.7)

Tubular bowl centrifuge: The tubular bowl centrifuge (Figure 9.4) is the simplest type and provides high centrifugal force. It has the provision for cooling and so can be used for protein separation and other products that are thermally sensitive.

It consists of a long narrow cylindrical bowl suspended from the top rotating at a high speed, i.e. 10,000 rpm, in an outer casing that is stationary. The bowl measures from 8 to 15 cm in diameter and upto 150 cm in height.

Multi-chamber bowl centrifuge: A multi-chamber bowl centrifuge (Figure 9.5) consists of a number of concentric tubes connected in such a way that a zigzag flow of the suspension of feed through the chamber is obtained. Solids get deposited on the outermost chamber wall and solid discharge is done manually.

Disc bowl centrifuge: The disc bowl centrifuge (Figure 9.6) has a shallow wide cylindrical bottom driven bowl rotating at a speed of about 6000 rpm in a stationary casing. The bowl is 30 to 100 cm in diameter and contains

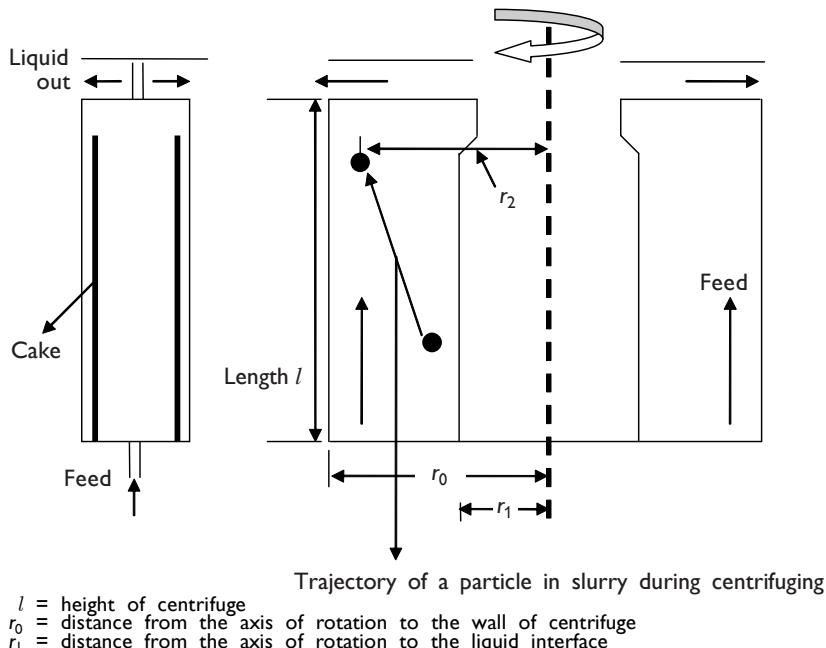


Figure 9.4 Tubular bowl centrifuge with flow patterns shown.

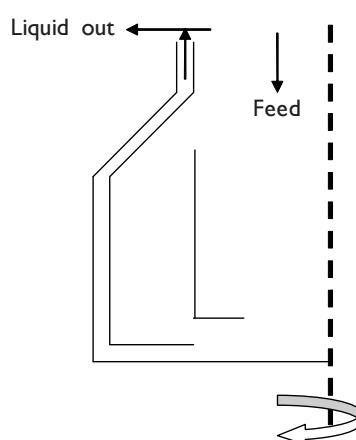
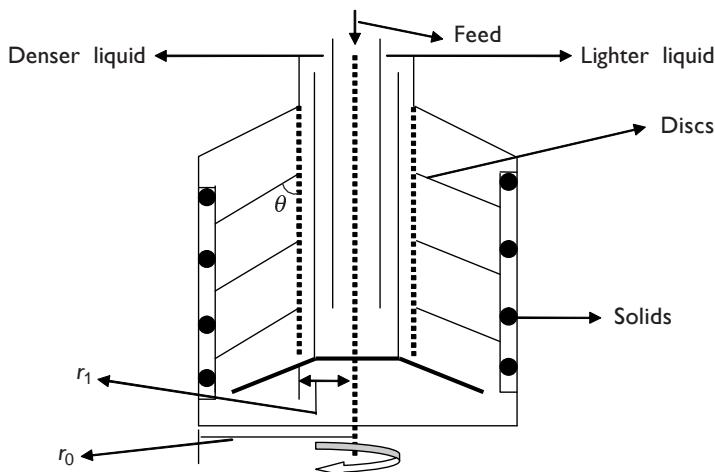


Figure 9.5 Multi-chamber bowl type centrifuge.

closely spaced metal discs that are located one above the other. The feed is introduced at the bottom through a centrally located feed pipe from above and the clear liquid flows out through an annular slit near the neck of the bowl.

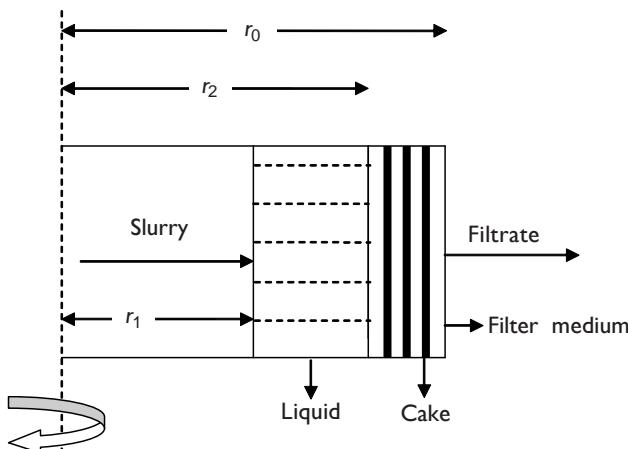


r_0 = distance of the outer edge from the feed axis
 r_1 = distance of the inner edge from the feed axis
 θ = angle at which the discs are fitted.

Figure 9.6 Disc bowl centrifuge.

Basket centrifuge: A basket centrifuge (Figure 9.7) involves a combination of a centrifuge and a filter, and consists of a perforated cylindrical basket which rotates rapidly. Suspension is fed along the axis of the bowl and solids accumulate on the wall of the basket. The liquid flows out under centrifugal forces through the cake and the perforations in the basket wall. It is used for washing the accumulated solids.

Settling and sedimentation are carried out by using the settling tanks wherein the coagulants and the flocculants are added to create the flocs by



r_0 = distance of the medium from the feed axis
 r_1 = distance of the liquid suspension from the feed axis
 r_2 = distance of the cake formed from the feed axis

Figure 9.7 Basket centrifuge.

agglomeration aided with the addition of poly electrolytes or salts as calcium chloride.

If the products are intracellular, the process of cell disruption is used.

Decanter centrifuge: A decanter centrifuge is also called *scroll type centrifuge*. It consists of a rotating horizontal bowl, with a length to diameter ratio of 1:4, fitted with a screw type conveyor and an adjustable feed pipe which is positioned in the middle of the decanter.

The speed of rotation of the bowl is slower than the screw. Solids that get deposited on the wall are scraped off while the liquid leaves the decanter through an overflow weir. It is used to thicken the slurries with high dry solid concentrations.

Centrifugation theory: It is a solid-liquid separation process. Prior to the application of centrifugal and gravity based separations as sedimentation, it is advisable to understand the concept of the theory of solid-liquid separations in a gravitational field.

In gravitational settling or separation, are encountered the free settling and hindered settling. The particle settling in a high particle density suspension is called *hindered settling* and resembles solid-liquid separations in a centrifugal field.

It is seen that the major component of forces acting on a solid particle while settling in a liquid by gravitation are gravitational force (F_G) itself, drag force (F_D) and a buoyant force (F_B).

We can apply a condition as the gravitational force (F_G) is a combination of (F_D) and (F_B), i.e. drag forces and buoyant forces respectively, when the particles reach a terminal settling velocity.

During terminal settling velocity, forces acting on the particle balance each other resulting in the net force to be zero, i.e.

$$F_G = F_D + F_B \quad (9.10)$$

The respective forces are defined by the equations given below.

$$F_G = \frac{\pi}{6} D_p^3 \rho_p \cdot \frac{g}{g_c} \quad (9.11)$$

$$F_D = \frac{C_D}{2g_c} \rho_f \cdot U_0^2 A \quad (9.12)$$

and

$$F_B = \frac{\pi}{6} D_p^3 \rho_f \cdot \frac{g}{g_c} \quad (9.13)$$

where C_D is drag coefficient, ρ_f is the fluid density, U_0 is the relative velocity between the fluid and particle also called terminal settling velocity, A is the cross-sectional area of the particles perpendicular to the direction of fluid flow; and for a sphere is given as:

$$A = \left(\frac{\pi}{4}\right) D p^2$$

It is a well established fact in fluid mechanics that, for spherical particles when the particle Reynolds number, $N_{Re,p} < 0.3$, the drag force is then given by Stokes equation as

$$F_D = 3\pi\mu D_p U_0 \frac{1}{g_c} \quad (9.14)$$

Equating Eqs. (9.12) and (9.14), we get

$$\frac{C_D}{2g_c} \rho_f \cdot U_0^2 \cdot A = 3\pi\mu D_p U_0 \frac{1}{g_c} \quad (9.15)$$

After simplifying, we have

$$C_D = \frac{24\mu}{\rho_f U_0 D_p}$$

or

$$C_D = \frac{24}{N_{Re,p}} \quad (9.16)$$

where particle Reynolds number $N_{Re,p} = \frac{\rho_f U_0 D_p}{\mu}$

Further, when $N_{Re,p}$ is between 1.0 and 10,000, C_D for a sphere is approximated by

$$C_D = \frac{24}{N_{Re,p}} + \frac{3}{\sqrt{N_{Re,p}}} + 0.34 \quad (9.17)$$

Now writing for $F_G = F_D + F_B$ using the pertinent equations and solving for U_0 , we have

$$U_0 = \frac{g D p^2 (\rho_p - \rho)}{18\mu} \quad (9.18)$$

Eq. 9.18 is an expression for calculating terminal settling velocity.

In a centrifugal field, the terminal separation velocity of particles is given as

$$U_{0,CT} = \frac{r\omega^2 D p^2 (\rho_p - \rho)}{18\mu} \quad (9.19)$$

where $g = r\omega^2$ i.e. gravitational acceleration is replaced by centrifugal acceleration.

or

$$U_{0,CT} = \frac{gZDp^2(\rho_p - \rho)}{18\mu} \quad (9.20)$$

and

$$U_{0,CT} = Z U_0 \quad (9.21)$$

i.e. $Z = \frac{r\omega^2}{g}$ called *centrifugal factor* while r is the radial distance from the central axis of rotation and ω is the angular speed or angular velocity of rotation and is given as $\omega = 2\pi Nr$.

All the above expressions are for dilute suspensions where particle interactions are ignorable.

For hindered settling that is found in the concentrated suspensions, and where the particle concentrations are high, the phenomenon is somewhat complex. The particles interact to form a swarm and consequently their average velocity decreases from U_0 to U .

It is interesting to note that the separation of cells or particles in a centrifugal field is similar to hindered settling under gravity, since particle concentration is high under conditions of centrifugation.

In hindered settling, the drag force on the particles is given as

$$F'_D = \frac{1}{g_c} 3\pi\mu D_p U \left(1 + \beta_0 \frac{D_p}{L} \right) \quad (9.22)$$

where U is the terminal settling velocity of particles under hindered settling conditions, L is the average distance between the adjacent particles and β_0 is the hindered settling coefficient and is given a value of 1.6 for a rectangular arrangement of particles.

In dilute solutions, since D_p/L is far lesser than 1.0; we have $F'_D \approx F_D$.

Therefore, hindered settling becomes more prominent when D_p and L are comparable.

9.3 CELL DISRUPTION

The process of cell disruption is a next step after the removal of particulates. The examples of intracellular products formed by natural producers include glucose isomerase, phosphatase, β -galactosidase, ethanol dehydrogenase, Dnase and Rnase. The others are interferon, immunoglobulin, plasminogen, somatotropic hormones, etc. In such cases the process of cell disruption becomes necessary.

Any process of cell disruption must ensure that the labile materials are not denatured by the process or hydrolyzed by the enzymes present in the cell.

The methods of cell disruption can be classified broadly as:

- Physical methods
- Chemical and enzymatic methods
- Mechanical methods.

9.3.1 Physical Methods

Physical methods include:

- Thermal shock or Thermolysis
- Osmotic shock
- Ultrasonication.

Thermolysis: Thermolysis refers to splitting or rupturing of cells by using heat. It is relatively an easy and economical method, although to be used only for the materials that are thermally stable. By way of this process, an organism gets inactivated where the cell wall is ruptured without affecting the products.

Heat shocks or thermal shocks provided rely on certain parameters, viz. pH, ionic strength and the presence of chelating agents also known as sequestering agents like EDTA.

Osmotic shock: The osmotic shock refers to a process in which the cells are dumped into pure water of about twice the volume of cells. Due to dumping of cells in pure water, the cells swell. The swelling is mainly because of osmotic flow of water into cells. Consequently, cells burst thereby releasing the products into the surrounding medium. The susceptibility of cells to undergo disruption by osmotic shock depends on the type of cell incorporated, for example, the RBCs are found to be easily disrupted. Animal cells do require mincing or homogenizing the tissues before being disrupted, whereas the plant cells that do have the cell wall which is cellulose based does not undergo the cell disruption so easily. In the case of bacterial cell, the effect of osmotic shock is low. But this method is more useful in releasing the hydrolytic enzymes and membrane binding proteins from Gram negative bacteria, viz. E.coli and salmonella typhimurium.

Ultrasonication: In this technique, the ultrasound waves of frequencies greater than 20 kHz are employed on the cells, thereby rupturing the cells by a process called *cavitation*. When ultrasonic waves are applied into a liquid medium, they create areas of compression and rarefaction alternatively that change rapidly. Due to these compressions the bubbles are produced in the cavities and are subjected to several thousand atmospheres pressure. This leads to the collapse of bubbles creating the shock waves that disrupt the cell wall in the regions adjacent to it.

It is an expensive technique and the efficiency of which depends on several factors like the biological conditions of the cells (age and maturity), pH, temperature, ionic strength and of course the time of exposure.

9.3.2 Chemical and Enzymatic Methods

Chemical and enzymatic methods are as follows:

- Alkali treatment
- Detergent solubilization

- Lipid solubilization
- Enzymatic method.

Alkali method: The alkali method is considered to be a cheap and effective method. Alkali acts on the cell wall including saponification of lipids. Alkali treatment is carried out at pH of 11–12 for about 20–30 minutes due to which proteases, protein splitting enzymes, are inactivated. This method is found to be more useful in the preparation of pyrogen free therapeutic enzymes. Pyrogens are the fragments of cell wall of Gram negative bacteria and when ingest in the body cause increase in the temperature of the body. By this method enzyme L-asparaginase is successfully isolated.

Detergent solubilization: The solubilization of detergents involves the addition of a concentrated solution of detergent to about half the solution's volume of cells to disrupt the cell wall. The process depends on pH and temperature.

Detergents like sodium dodecyl sulphate (SDS), sodium sulphonate and sodium taurocholate which are anionic and detergents such as cetyltrimethyl ammonium bromide (CTAB) which are cationic, and detergents that are non-ionic, Triton X-100, are employed.

Lipid solubilization or cell wall permeabilization: The solubilization of lipids or the permeabilization of the cell wall is achieved by the addition of organic solvents that bring about the cell wall disruption. The solvent is absorbed by the cell wall resulting in swelling and ultimate rupture. Toluene is one such organic solvent. The amount of solvent added is 10% of cell volume. Figure 9.8 depicts the cell wall permeabilization process.

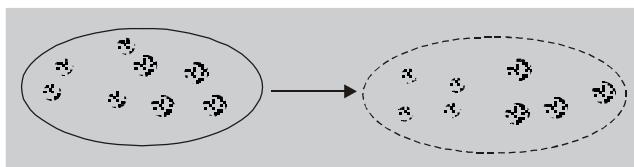


Figure 9.8 Cell wall permeabilization.

Enzyme digestion: In the case of enzyme digestion, lytic enzymes are added to the cell suspension. Lytic enzymes being more effective, selective in nature, gentle and of course very expensive, are employed in some special cases.

With the addition of these enzymes, the digestion of cell wall is achieved. For example, hydrolyses of α -1, 4-glycosidic bonds in the mucopeptide moiety of bacterial cell wall is achieved by the enzyme lysozyme. Yeast cells require a mixture of different enzymes such as glucanase, protease, mannanase or chitinase.

9.3.3 Mechanical Methods

Mechanical methods include the use of ultrasonic vibrators or sonicators,

bead mills and high-pressure homogenizers. These methods find the use in the laboratory as well as in the industrial scale operations.

A waring blender is particularly effective with animal cells and tissues as well as with mycelial organisms.

9.3.4 Sequential Disruption of Cells

The following procedure suggests the sequential disruption of cells (Figure 9.9). It involves the use of lytic enzymes that depend upon the parameters like process conditions, type of cells, the products to be harvested, etc. It is carried out sequentially as shown below. The fermentation broth when subjected to filtration and centrifugation releases extracellular products. Removal of extracellular products is necessary as it can affect the concentration of the product desired. The whole cells in suspension form but in a favourable osmotic medium are subjected to the action of lytic enzymes that disrupt the cell wall followed again by centrifugation. The cell wall proteins are released leaving behind the protoplast intact.

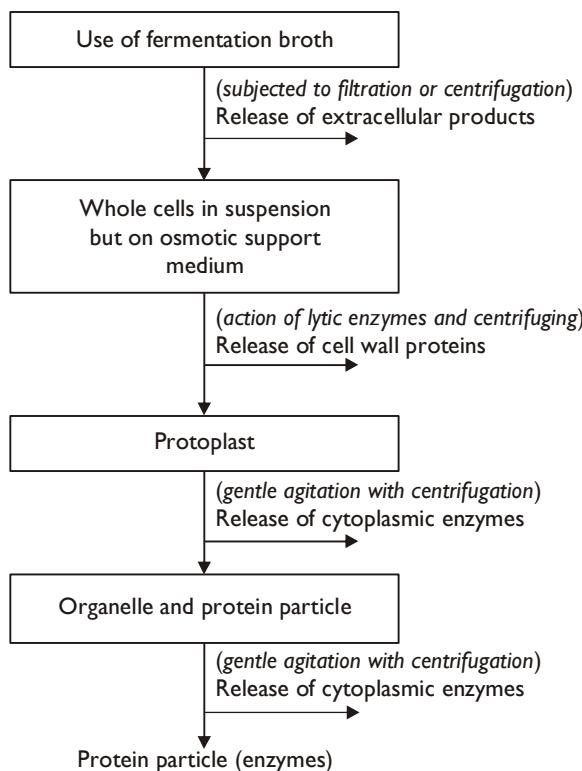


Figure 9.9 Sequential disruption of cells.

In the subsequent step, the protoplasm is disrupted by gentle mechanical agitation resulting in the release of cytoplasmic enzymes. Later these are separated from recombinant proteins and mitochondria. In the final step use of appropriate reagent or enzyme is done to release the organelle products and as a result of which the protein particle called enzyme is liberated.

9.4 PRIMARY ISOLATION

Primary isolation involves the removal of water and concentration of the product. This is achievable by the use of the processes such as solvent extraction, sorption, precipitation, etc.

9.4.1 Solvent Extraction

Liquid-liquid extraction or commonly called solvent extraction is a classical and versatile method for the recovery as well as concentration of a variety of products. The advantages of solvent extraction are as follows:

- Selectivity
- Reduction in the product loss due to hydrolytic or metabolic or microbial degradation as the product is transferred to a second phase with different physical and chemical properties
- Suitability over a wide range of scales of operation

This method separates the inhibitors used in the recovery of antibiotics, using amyl acetate as a solvent. In selecting a solvent, the characteristics are to be considered. These are surface tension, viscosity, miscibility, flammability, selectivity and recoverability to list a few.

Besides the characteristics of solvents for extraction listed above, they should also be non-toxic, selective in nature, inexpensive and most importantly high partition coefficient for the product.

The principle of extraction shows that it is based on the differences in selectivity values of the compound in one phase relative to the other. We can define the partition coefficient or distribution coefficient as follows.

When a compound is distributed between the two phases of liquid being immiscible, the ratio of concentrations in the two phases is called *partition coefficient* and is given as

$$K = Y/X \quad (9.23)$$

where Y is the concentration of solute in the lighter phase and X is the concentration of same solute in the heavier phase. In majority of the cases it is seen that the organic solvent is a lighter phase and the aqueous fermentation broth is a heavier phase.

Solvent extraction is carried out in a single stage or multistage processes, depending upon the quantity and the purity of the product.

A single stage process is depicted in Figure 9.10(a).

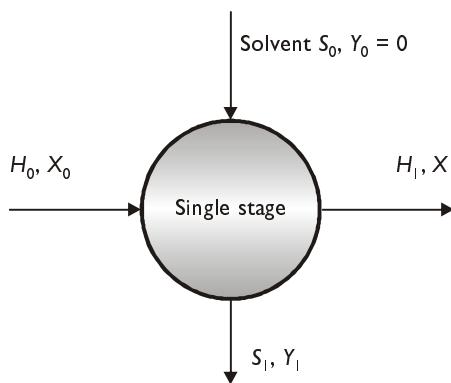


Figure 9.10(a) Single stage extraction.

In Figure 9.10(a), it is considered that the solvents are totally immiscible, such that the mass flow rates of the light and heavy phases are non-varying. Therefore, we can write $S_0 = S_1 = S$ kg/hr and $H_0 = H_1 = H$ kg/hr.

Carrying out the material balance on the solute extracted component, we have

$$HX_0 - HX_1 = SY_1 - SY_0 \quad (9.24)$$

$$H(X_0 - X_1) = S(Y_1 - Y_0) \quad (9.25)$$

Since $Y_0 = 0$, solvent being fresh in the initial stages, we further can write as

$$H(X_0 - X_1) = S(Y_1) \quad (9.26)$$

On rearranging,

$$X_1 = X_0 - \frac{SY_1}{H} \quad (9.27)$$

Further, using the equation for partition coefficient, $K = Y/X$ and assuming that K is constant, we can write,

$$K_1 = Y_1/X_1 \quad (9.28)$$

Therefore, substituting for Y_1 from (9.28) in (9.27), we get

$$X_1 = X_0 - \frac{S}{H} X_1 K_1 \quad (9.29)$$

$$\frac{X_1}{X_0} = 1 - \frac{SK_1}{H} \frac{X_1}{X_0}$$

Calling $SK_1/H = E$, the extraction factor, then

$$\frac{X_1}{X_0} = 1 - \frac{EX_1}{X_0}$$

On further simplification, we have

$$\frac{X_1}{X_0} = \frac{1}{1+E} \quad (9.30)$$

Using Eq. (9.30) we can estimate the extraction factor E in a single stage extraction process.

Multistage extraction processes can be carried out in various modes. These are continuous cross current, counter current and co current. Of these, the counter current is the most preferred one as it can provide better contact between the extracting solvent and the feed.

In a multistage countercurrent extraction processes, we use a number of stages to increase the extraction efficiency. The same is depicted in Figure 9.10(b).

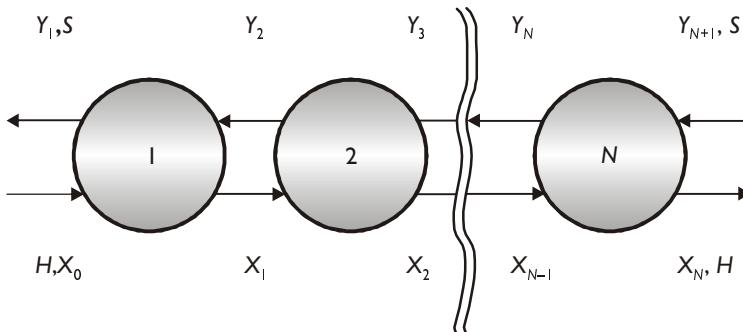


Figure 9.10(b) Multistage countercurrent extraction.

The material balance on the solute extracted is

$$R = E \frac{(E^n - 1)}{E - 1} \quad (9.31)$$

Where R is rejection and defined as the weight ratio of the solute leaving in the light phase to that leaving in the heavy phase and n is the number of equilibrium stages.

The fraction of the solute extracted is estimated as

$$\text{Percentage solute extraction} = 1 - \frac{1}{R+1} \quad (9.32)$$

Further, when the solute enters the system in the light phase, the rejection ratio R is given as

$$R = \frac{E^n(E - 1)}{E^n - 1} \quad (9.33)$$

The relation between E , X_n/X_0 and n is depicted in Figure 9.10(c).

The ratio X_n/X_0 is given as

$$\frac{X_n}{X_0} = \frac{E - 1}{E^{n+1} - 1} \quad (9.34)$$

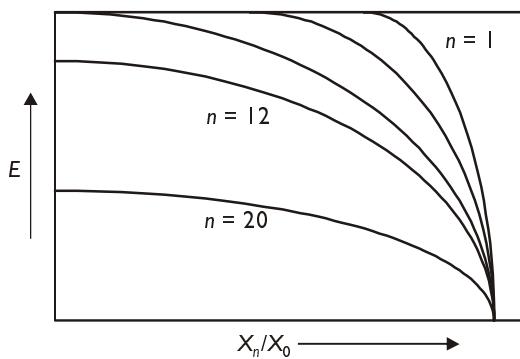


Figure 9.10(c) Graph showing the relation between the unextracted solute, extraction factor and no. of stages in continuous countercurrent extraction.

Also extraction processes can be carried out in two ways: batch and continuous. The various processes that are envisaged are physical, dissociative and selective extraction.

For the extraction of biologically active polymers such as proteins, enzymes and nucleic acids, it is necessary to have a two-phase extraction system that will not denature the biopolymers. Of course, the two-phase extraction process is of immense importance in the process of biotechnology.

Aqueous two-phase extraction process: Figure 9.11 depicts the entire process. The process of extraction involves two steps:

- Mixing the aqueous feed with the two-phase solvent for extraction
- The separation of phases

The very first step in the separation of a desired biopolymer from a fermentation broth is the removal of cell debris into the heavier phase. The salt phase in PEG (poly ethylene glycol-potassium phosphate) is a two-phase system by contacting the aqueous feed with the solvent system.

Biopolymers like proteins enzymes, etc. are extracted into the PEG-rich lighter phase.

Later, centrifugal separation of two phases is done thereby the lighter phase is contacted with a solution of the salt at a fixed pH value. The second stage of extraction isolates other biopolymers, viz nucleic acids, polysaccharides, etc. leaving purer enzyme or protein in the PEG-rich phase which is lighter. These two phases are separated by centrifugation.

Next is the purification of the desired protein or enzyme, that is achieved by a third stage extraction of protein/enzyme into the salt phase by adding salt solution and changing the pH conditions. It proceeds to give a heavier salt phase that contains the desired protein at one end and the PEG phase at the top is a residue that can be recycled. Ultra-filtration is taken up for the final recovery of the product desired.

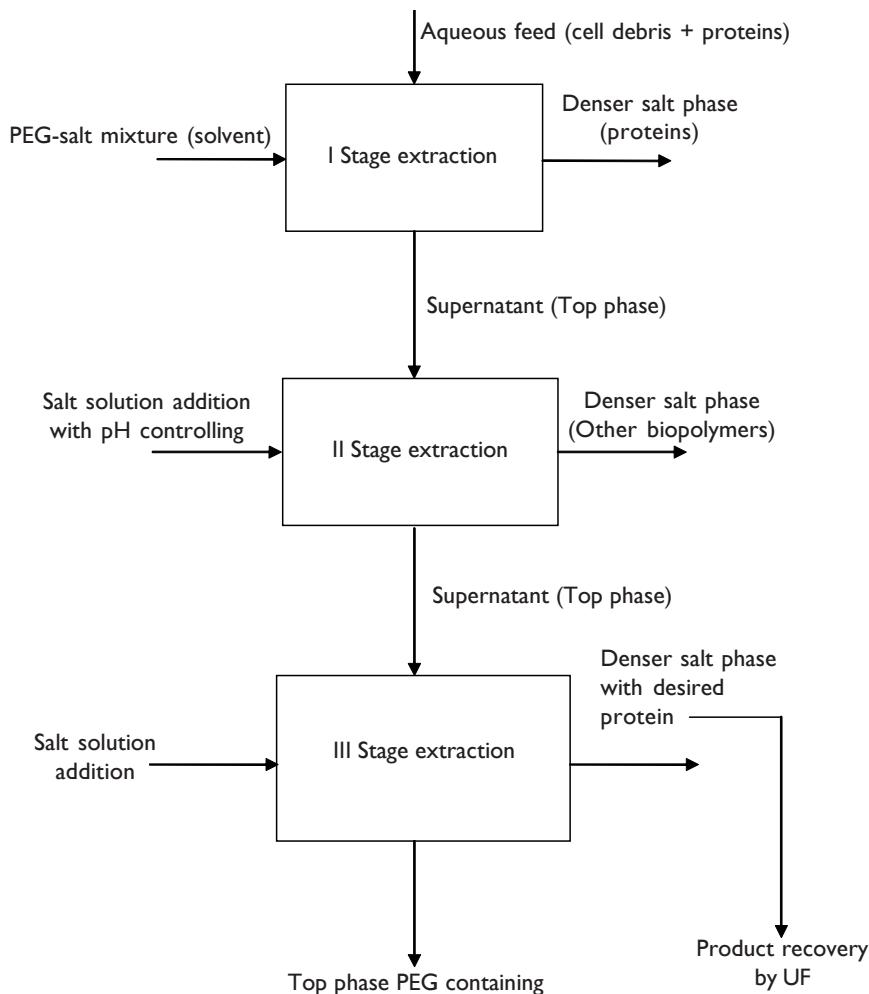


Figure 9.11 Aqueous two-phase extraction for the recovery of biological polymers.

9.4.2 Supercritical Fluid Extraction Process

The extracts from agro-products like spices, herbs, aromatic plants and medicinal plants are used for various end applications such as cosmetics, flavours, medicines, perfumes, etc. To get these end products we can apply the conventional methods of extraction, i.e. solvent extraction; but it has been realized that these conventional methods have a number of demerits such as lower efficiency, lower purity of the product, requires long processing times and of course the overall efficacy of the process is bound to decrease. In order to overcome these limitations, the process called supercritical fluid extraction is developed.

The technology of supercritical fluid extraction has gained a lot of prominence these days and is considered to be one of the advanced separation processes applied in the chemical engineering and biochemical engineering practice.

Supercritical fluid extraction (SCFE), applications are finding their ways today not only in laboratories, i.e. these are not only confined to the laboratories but are also applied on a commercial scale in the industry.

As stated earlier that SCFE is applied to overcome the limitations of the conventional methods of extraction and as realized that in these conventional methods of solvent extraction, the residual contents of the solvent is found in the final products. For example, in the process of decaffeination of coffee beans the traces of the solvent methylene chloride are found and these traces are proven to be toxic. In order to eliminate these residual contents in the products, the motivation for the SCFE was enforced. Also the solvent residues were found in the pharma and food products and were becoming increasingly the focus of regulatory attention in the 1970s.

Not only the elimination of solvent residues, but also the use of SCFE enhances the flavour, aroma characteristics, etc. in the final product which cannot be obtained by the traditional or conventional organic solvent extraction processes.

We know that the near-critical fluids and SCFs, in contrast to gases in general, that have poor solubility, can be employed as solvents for various substances at the moderate values of temperatures. Therefore, on both the technical and economical basis is made the unique combination of the properties and attributes of SCFs that are harmoniously applied in proliferation of the improved versions of the processes and products.

Where then are these processes employed? The following situations indicate the compliance of the SCFE processes. They are:

1. Where the environmental issues and compliance procedures will immediately require a change or alteration in the process.
2. Where the regulatory pressures will immediately require a change in the product purity.
3. Where the increased product performance is needed.
4. Where the improved product version can create a new market position and demand.

This section emphasizes the supercritical fluids, their properties, characteristics, usefulness in the succeeding processes, the SCF extraction principles, applications to the various situations with advantages and disadvantages and finally their applications.

Supercritical fluids The SCFE process is one that uses a dense gas like CO_2 as a solvent for extraction well above its critical parameters, i.e. critical pressure and critical temperature. In this case the critical temperature is 31°C and the critical pressure is 74 bar or 73 atm. ($1 \text{ bar} = 0.9869 \text{ atm} = 10^5 \text{ N/m}^2$). SCFs are the gases with properties that lie between that of a

normal gas and a normal liquid and this is advantageous and is exploited in the process of extraction. Of course, the changes made in the pressure values will influence on the properties and alter their behaviour that is from more gas like to a more liquid like.

In this context, at first we will try to envisage as to what are the critical parameters, viz. critical temperature and critical pressure?

What is a critical temperature and critical pressure? The critical temperature of a substance is defined as the temperature above which a distinct liquid phase cannot exist regardless of the pressure. And the vapour pressure of the substance at its critical temperature is called the *critical pressure*. The other way of defining is, the pressure used to liquefy the gas at its critical temperature. In general, the substance above and close to its critical temperatures and pressures will exist as a supercritical fluid. It is an interesting fact to note that there exist a number of potential extracting critical solvents that include CO_2 , N_2O , SO_2 , NH_3 , C_2H_6 , C_3H_8 , C_4H_{10} and ethylene. Of these, CO_2 is widely used because of its advantages over the others in terms of being non-toxic and non-hazardous in nature.

The transport properties of a substance at first are to be understood to decide as to which of these solvents are to be employed. We know that the transport properties of a solvent as density, viscosity and diffusivity as well as thermo physical properties as heat capacity and the latent heat of vaporization are of significance in the extraction processes. Interestingly, SCFs combine the high solvating power, a characteristic feature of liquids with low viscosity and high penetrating power or ability, a characteristic feature of gases.

The physical properties of these SCFs such as viscosity, density and diffusivity and other similar properties fall intermediate between the substances in the gases state and in the liquid state. Table 9.1 illustrates the properties of liquids gases and the SCFs.

Table 9.1 Physical properties of gases, liquids and SCFs

Property	Gas (STP)	SCF	Liquid
Density ρ (g/cc)	$(0.6\text{--}2.0) \times 10^{-3}$	0.2–0.5	0.6–2.0
Viscosity μ (g/cm-s)	$(1.0\text{--}3.0) \times 10^{-4}$	$(1.0\text{--}3.0) \times 10^{-4}$	$(0.2\text{--}3.0) \times 10^{-2}$
Diffusivity (cm ² /s)	$(1.0\text{--}4.0) \times 10^{-1}$	$10^{-3}\text{--}10^{-4}$	$(0.2\text{--}2.0) \times 10^{-5}$

From the above table it is clear that the SCFs have low viscosity and high diffusivity. It is because of this reason the SCFs are used in the extraction process. It is simply evident that the SCFs due to their low viscosity and greater diffusivity compared to the liquids diffuse more rapidly into the substances and even penetrate into the solids. The solvent strength or the solvating power of SCF can be controlled easily just by increasing the pressure on the fluid. We know that the increase in the pressure on the fluid

increases the density of the fluid and further the solvent strength. Near the critical point, small changes in the pressure values will create appreciable changes in the density of the SCFs.

Also the latent heat of vaporization is found to decrease in the SCF region and is zero at the critical point and it is due to the fact that there is absolutely no phase change involved. With all these properties of SCF, shall enhance the heat capacity as well. It is found that the heat capacity of SCFs will increase several folds than that of the normal liquid.

Phase diagram: The phase diagram shown in Figure 9.12 is actually a pressure-temperature diagram also called a P-T diagram of a pure substance. This is a thermodynamic phase diagram referring to one of the volumetric properties of a pure substance.

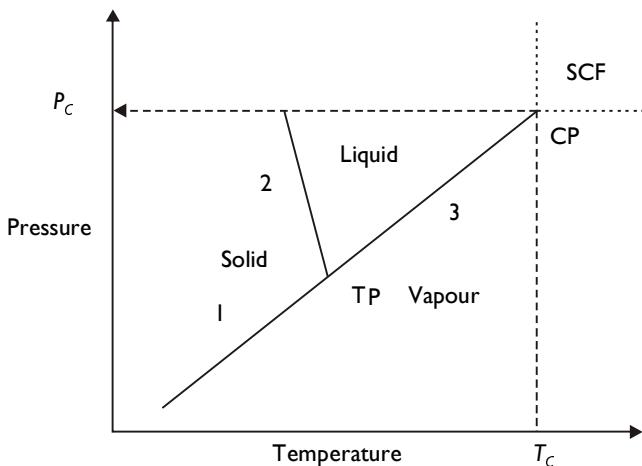


Figure 9.12 Pressure-temperature diagram of pure substance (CO₂).

In the above figure, the importance of physical properties, particularly density of a SCF as a function of temperature can be properly understood. We find that there are three points mentioned that segregate the curves into 3 regions. They are as follows: Point 1 refers to the partitioning between solid and the vapour regions by a curve called the *sublimation curve*, point 2 refers to *fusion curve* that partitions the solid and the liquid regions and point 3 is the one that refers to the *vaporization curve* that partitions between the liquid and the vapour regions respectively. The point where all the three curves meet is called the *triple point* (TP). At the triple point, all the three phases coexist with each other. The vapour pressure curves for each substance is unique but each exhibits generally similar characteristics as shown in the figure. Liquid and vapour represented by points on the vapour pressure curve are called *saturated liquid* and *saturated vapour* respectively. The vapour or gas at a temperature above the saturation is called *superheated*.

A unique feature is also noticed at CP, called the *critical point* and beyond which is a region for the SCFs. At and above the critical point there

is hardly any distinction between the three states of matter. We cannot easily identify the phases that exist at and above the critical point. At this point the distinction between the liquid and the vapour phases disappears and all the properties of the liquid such as density, viscosity, refractive index, etc. are identical with those of vapour. The substance above the critical point is the gas and will then not be liquefied regardless of how high the pressure is applied. Therefore, these are called *super critical fluids*.

In particular to CO₂, is shown the values for both the critical temperature and the critical pressure. From the graph it is found that the values for T_c and P_c are 31°C and 74 bar respectively. As stated above, with the use of the phase diagram, the significance of physical properties, particularly density of a SCF as a function of temperature and pressure can be best understood. For instance, in CO₂ as an SCF, the phase diagram can be further extrapolated as shown below.

Types of super critical fluids: Table 9.2 illustrates the variety of supercritical fluids that can be employed in the SCFE process.

Table 9.2 Critical fluids and properties

Solvent	T_c °C	P_c MPa	Critical Density g/cm ³
Ethylene	9.3	5.04	0.22
Xenon	16.6	5.84	0.12
Carbon dioxide	31.1	7.38	0.47
Ethane	32.22	4.88	0.2
Nitrous oxide	36.5	7.17	0.45
Propane	96.7	4.25	0.22
Ammonia	132.5	11.28	0.24
<i>n</i> -Butane	152.1	2.8	0.23
<i>n</i> -Pentane	196.5	3.37	0.24
Isopropanol	235.2	4.76	0.27
Methanol	239.5	8.1	0.27
Toluene	318.6	4.11	0.29
Water	374.2	22.05	0.3

SCF extraction uses total extraction region (TER) where the density is between 0.6 and 0.9 and fractionation region (FR) where the density is between 0.2 and 0.6 grams per cc.

Properties of supercritical fluids: The following are the properties of supercritical fluids in general.

1. There is a drastic change in some of the important properties of a pure liquid as its temperature and pressure are increased and approaches the critical point.
2. The other properties of a liquid change near the critical point as thermal conductivity, surface tension, viscosity, etc.

3. The solvent strength of a given supercritical fluid is related directly to the fluid density. This solvent strength may be manipulated over a wide range by making small changes in temperature and pressure values.

Figure 9.13 presents the density variations with pressure and temperature for an SCF.

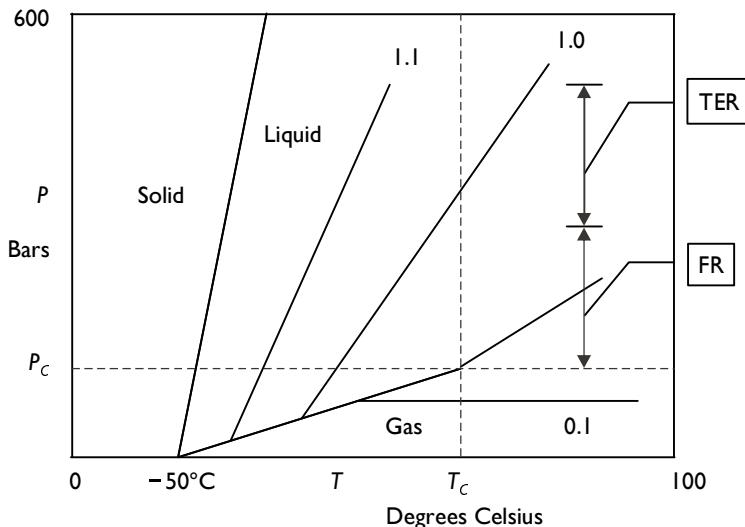


Figure 9.13 Density variations with pressure and temperature for a SCF.

Supercritical fluid extraction principles: The principle employed relies on the solvent power, i.e. the ability of the solvent to extract a particular component in a mixture of a supercritical fluid. The solvent power is highly dependent on the density of the supercritical fluid which in turn depends on the pressure and the temperature. We know that the close proximity of molecules in a liquid phase, i.e. larger density imparts the solvent power as the intermolecular forces allow the solvent molecules to surround the solute molecule and hold it in the solution. Therefore, there is a direct relationship between the density and the solvent power.

We can find out from the relationship in the Figure 9.13 as shown earlier that the solvent power of a SCF increases with increasing density at constant temperature and at constant density, it increases with increasing temperature. In case of CO_2 , the solvent power also depends upon the similarity of physical and chemical properties of the solute to be extracted and the extracting solvent. Of course, the close proximity results in the high solubility of solute in the extracting solvent. With the solutes of comparable polarity, volatility decreases with increasing molecular weight of the solute and the solubility in CO_2 also exhibits a similar behaviour. With increase in the solvent power of CO_2 , the percentage solubility of a solute increases, the range of extractable solutes also increases and hence at high solvent power selectivity is low.

On the other hand, at low solvent power, CO_2 exhibits high selectivity in dissolving the solutes. Thus, supercritical CO_2 offers potential scope of tailoring the extracting parameters in optimizing the recovery and purification of the desired solute components from a mixture. The solubility of a solute in super critical CO_2 may be enhanced by the addition of a co-solvent or entrainer. The addition of a co-solvent modifies the thermodynamic affinity between the solute and the extracting solvent favourably.

Co-solvents and Surfactants: Many non-volatile polar substances cannot be dissolved at moderate temperature in nonpolar fluids such as CO_2 . For this reason co-solvent also called *entrainers*, modifiers, moderators; such as alcohols and acetone are added to the fluids to increase the solvent strength. Of late, surfactants are also employed to form the reverse micelles, micro emulsions, and polymeric latexes in the SCFs including CO_2 . These organized molecular assemblies can dissolve hydrophilic solutes and ionic species such as amino acids, and also proteins. The surfactants that interact with CO_2 are fluoroethers, fluoroacrylates, fluoroalkanes, propylene oxides and siloxanes.

Schematic diagram of a typical SCFE process unit: Figure 9.14 illustrates the single stage extraction process that utilizes the feature of adjustability of the solvent strength with pressure as a variable.

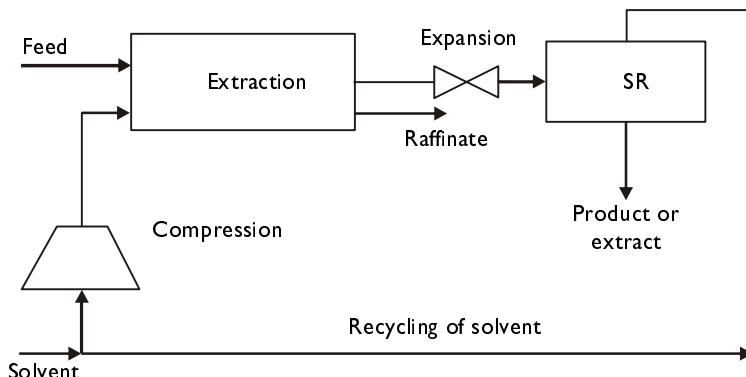


Figure 9.14 Typical SCFE unit.

The feed is given to the extraction chamber to which the solvent is also fed at relatively high pressure by using a compressor. Within the extraction chamber the solute/solutes of interest are extracted from the feed. The products are recovered in the separator by releasing the pressure using an expansion valve where heating is taking place. The raffinate from the extraction chamber is collected from the downstream end. From the expansion valve the solvent is recovered in a solvent recovery system and the extract also withdrawn simultaneously. The solvent is recompressed and recycled. The products can also be precipitated from the extract phase by raising the temperature after the extraction to lower or decrease the solvent

density. Using the profile of increasing pressures, the conditions are set in such a way that only the lightest components in the feed are extracted in the first phase. The recovery vessel is then replaced and the pressure elevated to collect the next heavier fraction.

Using the profile of decreasing pressures with multistage isothermal conditions, all but the heaviest fraction is extracted in the first vessel. Then on the extract passes through a series of recovery vessels which are held at successively lower pressures and each of which precipitates the next lower molecular weight fraction in the raffinate.

Schematic diagram of SCFE unit using carbon dioxide as a solvent in both liquid-liquid and liquid-solid extraction: Figure 9.15 can be used both for the liquid-liquid and solid-liquid extractions. If the feed is L-L, then this aqueous feed is pumped into the extraction column and the same is made to contact with the supercritical solvent CO_2 at higher pressures (HP). The solvent is compressed and is recycled into the lower end of the extraction column. Due to the contact of the feed with the solvent in the first column the solutes of interest are extracted. The extract phase is then brought to a lower pressure in a separator still (LP) in order to recover the extract, i.e. the product from the solvent used.

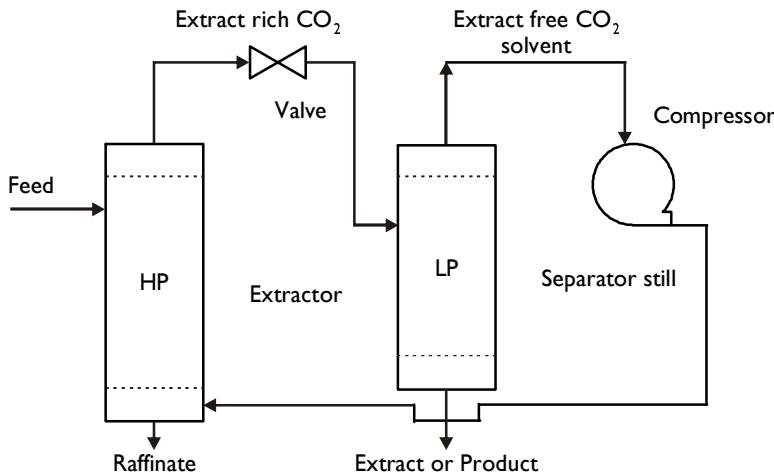


Figure 9.15 SCFE unit using CO_2 solvent.

If the feed is S-L, then in that case the solid bed is prepared in the first column into which the solvent is pumped. This encompasses the leaching of the solutes from the solid phase and into the solvent phase. Now the extract is said to be solvent rich, further requires the separation of solvent from it which is called recovery step. In order to achieve this, extract phase is pumped to a separator which is held at lower pressures that facilitate the precipitation of the solid product. Consequently, the solute-lean fluid is then compressed and recycled.

Note: The pressures used fall in the range of 100–480 bar.

Advantages of supercritical fluid extraction: In comparison to the conventional extraction, the process of SCFE has several advantages as follows:

1. *Solvent strength* employed in these processes is adjustable in terms of its strength or solvating power so as to tailor selectivities and the yields of the desired product/products.
2. *Transport properties* of SCF facilitate better extraction and phase separation. We have seen that these SCFs have low viscosities, surface tension compared to liquids and also higher diffusion coefficients, which are in fact the characteristic feature of SCFs, results in better mass transfer rates during the extraction process and also improved phase separation.
3. *Selectivity during separation*, offers a high degree of control over the selective extraction of the desired component from a complex mixture.
4. *Economy and Safe mode of separation* is proven to be very economical and safe as large requirements of organic solvents are ruled out and it eliminates the possibility or presence of traces that could appear in the final product for example, use of solvent CO_2 does not contaminate the final product as any residual content of the solvent volatilizes off. The solvent is completely non-toxic to almost all the bioproducts.
5. *Efficiency of separation* is proved and realized that the recovery rates are higher than the conventional methods of extraction. Therefore, the overall efficiency of extraction is found to be much better than the conventional means. For example, 98% of piperine extracted from black pepper and 97% of capsicum in chillies.
6. *The use of CO_2* , as a solvent has proven to be very attractive as it is environmentally acceptable solvent for waste minimization, non-toxic, nonflammable, inexpensive, usable at mild temperatures, etc. As can be seen in this case, low extraction temperature permits heat labile compounds to remain intact without getting damaged.
†Any thermal means, for instance, use of steam distillation step is not incorporated; therefore there is no hydrolytic degradation of the natural flavour compounds in the spices.
7. *Water as a solvent*, favours the efficiency as it is non-toxic, non-flammable, substitute for organic solvents and has extreme wide spectrum of solvent strength with temperature and pressure.
8. *Thermal properties*, as the solvent have low latent heat of vaporization, it permits its separation from the extracted product with low energy usage. While the heat capacity of the SCF is found to be several times that of a normal liquid, the heat transfer efficiency is found to be greater during the product recovery.

9. *Prevention of collapse of structure in case of solids*, during the solvent removal.
10. *Samples as solids, semisolids and liquids*, of different chemical nature can be handled safely by SCFE.

Disadvantages of SCFE: There are several disadvantages as well. They are as follows:

1. It requires a high capital cost.
2. Requirement of high level of automation and operator skills.
3. After a semi continuous process, the extractor has to be depressurized, the solid residue to be removed and fresh raw material charged before it can be brought on-line again. This is applicable for the solid-liquid extraction process.

Applications of SCFE: This process has an exhaustive list of applications that are commercialized. These processes are used in the following industries.

1. Food Industries: Carbon dioxide, a non-toxic material can be used to extract food components at near ambient temperatures. Thus, the food product is not contaminated with residual solvent as liquid solvents such as methylene chloride or ethane. Therefore in the food industry, the solvent CO_2 is not recorded as a foreign substance or an additive.

For instance, it is used for the following purposes.

- Coffee and tea decaffeination that will be discussed later and also in the extraction of flavours from hops in the manufacture of beer.

The hops are actually dried flowers of hop plant and are used to impart bitter taste to beer. The non-volatile soft resins of the hops contain the major active ingredients of α -acids which are desirable and as well as harsh bitter taste imparting β -acids.

- Extraction of flavours from fruits as apples, oranges, etc.
- Oils from black pepper, almond, lemon peel, lilac, nutmeg, basil, ginger, paprika, rosemary, chamomile and ground chilies for use as flavours and perfumes.
- β -carotene from plant materials.
- Monoglycerides from vegetable oils for use as emulsifiers.
- Oils from the seeds of sunflower, soybean, and rape.
- Cholesterol and fat from eggs.
- Nicotine from tobacco.

2. Pharmaceutical Industries: This is an another area where supercritical fluid using CO_2 is attractive, especially where solvent toxicity and the presence of a toxic residual solvent are concerns, e.g. extraction of vitamin E from soybean oil.

Other applications are separation of acetone from antibiotics.

3. Petroleum Industries: The petroleum industry includes the following:

- Fractionation of residuum oil and petroleum deasphalting.
- Polymer fractionation.
- Extraction of butanol from isobutene.
- Using ROSE process for separation of heavy components from crude oil.

4. Environmental applications: The most environmental and eco-friendly solvents such as carbon dioxide and water are least expensive. Carbon dioxide at a pressure of 4 MPa is a leading replacement for organic solvents to reduce the organic waste and volatile organic carbon emissions.

It is also used for extraction of organics like alcohols, ketones, carboxylic acids, esters, etc. from the aqueous media. Recently it has been applied for hydrothermal oxidation of organic wastes in water which is a reactive separation.

Decaffeination of coffee beans: By using SCFE, caffeine ($C_8H_{10}N_4O_2$) removal from coffee beans also called *decaffeination* is achieved. In this process use of water saturated with carbon dioxide as a solvent, the caffeine content of coffee beans can be reduced from 3.0% to 0.2%. There are several processes to decaffeinate coffee beans, but the most widely accepted is the *Kraft process* which employs high pressure absorption with water. Figure 9.16 illustrates the process and the mechanism behind the extraction of caffeine from coffee beans.

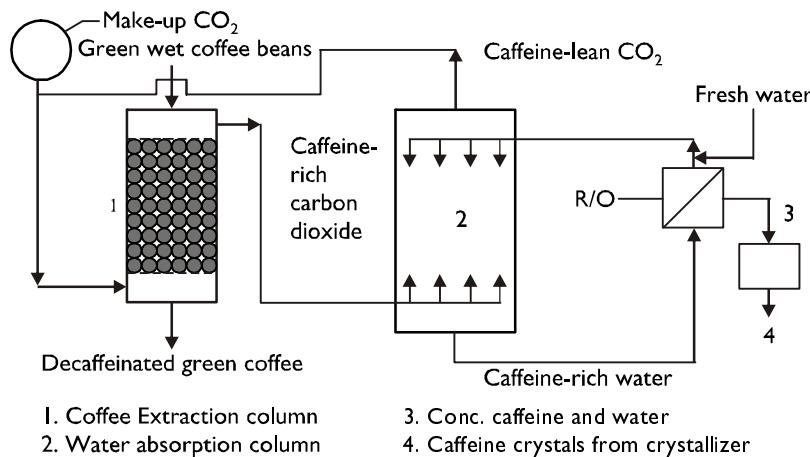


Figure 9.16 Schematic diagram of the Kraft process for decaffeination of coffee beans.

In this process the feed comprising moist and mashed coffee beans enter from the top of the coffee extractor and then carbon dioxide is passed through the bottom of the extractor. The process of extraction is achieved within the extractor. The countercurrent flow is preferred over other configuration as it provides a better contact and the equilibrium attainment

is guaranteed. The decaffeinated green coffee beans are removed from the bottom of the extractor and are later sent to the roasting tower to produce coffee while caffeine-rich carbon dioxide is withdrawn as a product from the first column.

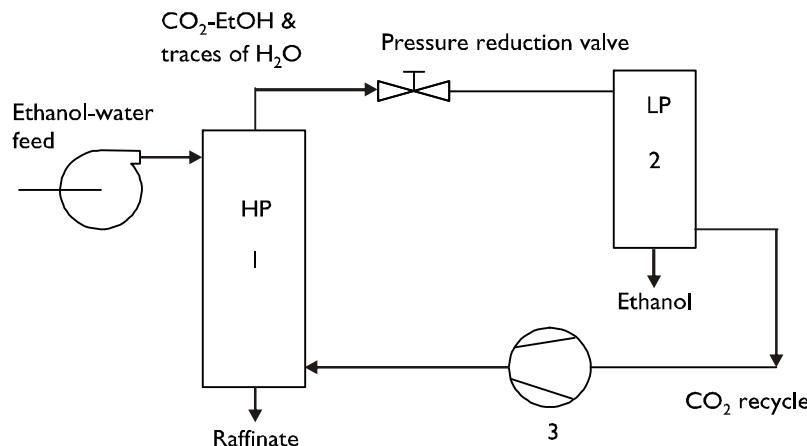
Caffeine-rich carbon dioxide stream now requires the treatment with water as the separation of CO_2 from the caffeine is accomplished. We know that CO_2 is soluble in water to form carbonated water or carbonic acid. Therefore fresh water is added from the top of the tower through spray device. Due to this, from the top of the water column the caffeine-lean CO_2 is recycled to the first column where it is supplied with make-up CO_2 . The downstream end of the second column which is caffeine-rich water is fed to the reverse osmosis (RO) plant in order to purify the used water so that it can be reused for the absorption of CO_2 and simultaneously concentrate the amount of caffeine in the product stream by way of membrane separations.

The product stream from the RO plant is concentrated caffeine and to some extent water. This is later sent to a crystallizer in order to produce the purer crystals of caffeine.

Overall it is seen that all the 3 separation steps are taking place at high pressures by using pumps (not shown in the figure).

Note: In commercial practice the dimensions of an extraction vessel are: diameter is approximately 7 ft. and the height is usually 70 ft.

As indicated earlier, there are several methods proposed for the recovery of caffeine including washing with water and absorption, but the Kraft process is widely accepted one. It is apparent that the recovery of a particular component of an extract is the key challenge in the SCF extraction process. This is the reason as to why the SCFE is combined with another process such as distillation, absorption and even adsorption.



I. Extraction column. 2. Separator. 3. Compressor.

Figure 9.17 Separation of Ethanol-water mixture using CO_2 .

Separation of Ethanol-water by pressure reduction: This basically describes the separation of EtOH-H₂O mixtures by using super critical CO₂. Figure 9.17 describes the same.

Ethanol-water mixture that is a feed is pumped as a liquid to the pressure of the extraction column (1) which is at a high pressure (HP). Inside the column it is contacted with SCF CO₂. This supercritical CO₂ has the tendency to dissolve in water as well in ethanol. The raffinate which is enriched with water comes at the bottom whereas the extract containing most of the CO₂, some ethanol and traces of water is collected from the top of the extraction column. Later it is sent through the pressure reduction valve (PRV) in order to get the reduced pressure.

Within the PRV, expansion takes place at a lower pressure. The downstream end of the PRV is a flash drum held at the reduced or low pressure (LP) into which is flashed the mixture of EtOH-CO₂ to get the ethanol separated and later CO₂ recycled through the compressor. This is because, by this time the solvent has lost sufficient pressure and in order to regain the pressure, the same as the column pressures it is sent through the compressor.

It is to be noted that unless the pressure is greatly reduced across the valve, resulting in large compression costs, little of EtOH is condensed.

Note: Ethanol-water can also be separated by using azeotropic distillation but due to the energy crisis somewhere in the 1970s the energy efficient processes were tried and later were complied and as well commercialized. One among them is SCF CO₂ use in the separation of the same. Of course due the unlimited capacity of CO₂ to dissolve ethanol and water, today pervaporation processes are also employed.

Separation of ethanol-water by high pressure distillation: This concept was first enunciated by Fillipi and Vivian. In this process, the flash drum that was used in the pressure reduction process is replaced by a high pressure distillation column (HPDC).

The process is illustrated in Figure 9.18. As stated, the flash drum is replaced by a high pressure distillation column that operates at a pressure just below the pressure of the extraction column.

Ethanol-water feed mixture is given to an extraction column to which supercritical CO₂ is added from the bottom. Introduction of the feed and CO₂ is at higher pressure as pumps are used. Ethanol-water is in a liquid state and CO₂ gets absorbed which is a gas absorption phenomenon. Depending on the distribution coefficient, and the unlimited capacity to dissolve both EtOH and water, CO₂ takes along with both the components in varying and different proportions. Thus, extraction prevails in the first column.

The extract phase, rich in ethanol content along with CO₂ is withdrawn overhead and the raffinate rich in water with CO₂ to some extent is taken from the bottoms. The raffinate is sent to the separator where water gets separated and CO₂ is sent to recovery.

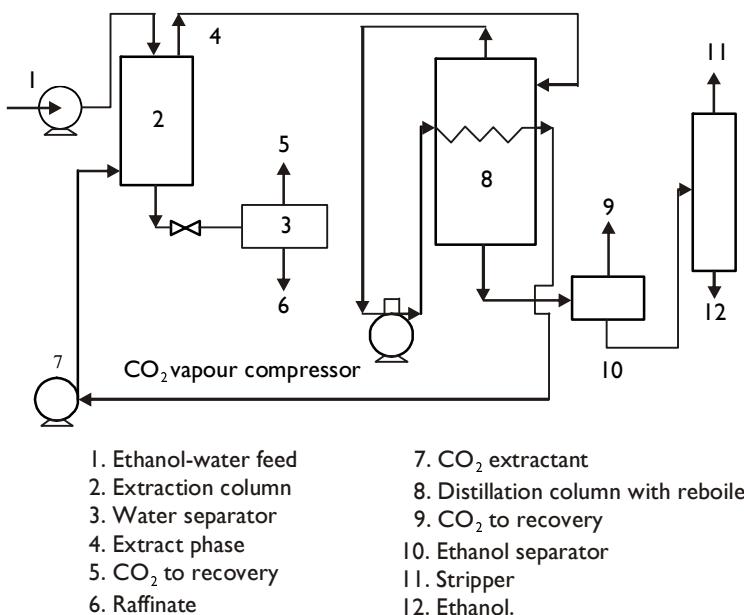


Figure 9.18 High pressure distillation for separation of EtOH-water mixture using CO_2 .

While the extract phase is sent to an HPDC from the top to distill CO_2 vapours from the overheads and in turn given to the compressor and further recycled to the extraction column. Ethanol obtained in the bottoms in an HPDC with traces of CO_2 is sent to the recovery unit where even the traces of CO_2 are recovered. Consequently, ethanol is stripped and obtained as a separate product.

This process is no doubt more complex but said to be versatile in practice.

By way of SCFE processes it is understood that we can extract up to 95% to 97% by use of a common solvent which is a supercritical fluid carbon dioxide. With all the advantages understood it is evident that most of the conventional methods are now replaced by these novel or advanced SCFE processes.

9.4.3 Sorption

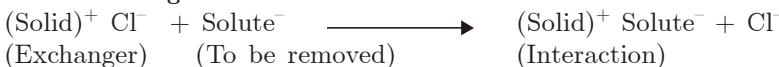
Sorption involves the partitioning of a solute between a bulk solution phase and a typically porous or high surface area solid. For example, the sorption of antibiotic streptomycin is done by ion exchange materials.

The ion exchange materials are merely dissociable ion pairs having one type of charge and being immovable.

For example,

Cation exchanger:



Anion exchanger:

Antibiotic streptomycin is likely to adsorb onto a carboxylic acid cation exchange resin as shown below.



The above mechanism shows the recovery of antibiotics from the fermentation broth. On the right-hand side is shown that the antibiotic loaded resin which requires some treatment for the separation of resin from the recovered antibiotic. At the same time, the recovery of resin and regeneration are equally important.

This is done by elution process using acid water, further reversing the original adsorption step and simultaneously getting the resin regenerated.

9.4.4 Precipitation

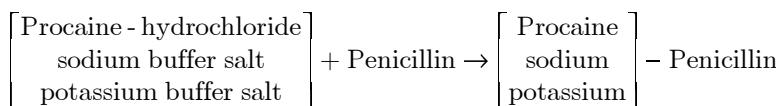
Precipitation refers to the transformation of soluble solutes into insoluble solids also called *infusible mass* which is a precipitate. Here the transformation means conversion and this conversion is possible because of the decrease in solubility of the original solute in solvent. This solubility reduction is due to the addition of a third component. The infusible mass of solid is subsequently separated from liquid by filtration or centrifugation. It serves as a means of isolation or recovery and concentration of desired products.

Organic solutes have solubility that depends upon factors like pH of solution, temperature, composition, ionic strength, etc.

The process of precipitation is brought about in the following ways:

Addition of a precipitant: A precipitant serves to react with a solute and produces an insoluble product called *precipitate*, usually a salt. For example, the recovery of antibiotics is done by adding a precipitant.

Let us see how precipitation is done to isolate penicillin. Penicillin in a combined form is available as procaine penicillin, sodium penicillin or potassium penicillin. To isolate pure penicillin, precipitation is done that separates procaine, sodium or potassium from penicillin.



Streptomycin with sulphuric acid is added with a third solvent which is organic and this addition reduces the solubility of the original solute in a solvent and reacts to give a precipitate called di-hydro-streptomycin sulphate.

Organic solvent + Streptomycin + Sulphuric acid \rightarrow Di-hydro-streptomycin sulphate.

The process of precipitation is also used in the recovery of biopolymers.

Solvent-driven precipitation: The process of solvent-driven precipitation is used in the production of microbial biopolysaccharides like dextran, xanthan gum, etc.

Protein precipitation techniques: The protein precipitation technique results in a phase change forming a precipitate that reduces the solubility of the solute and ultimately forms the product desired.

For example, *salting* is done by the addition of a polyelectrolyte that acts as a flocculating agent resulting in flocs. This is also achieved with the addition of polyvalent metal ions, addition of non-ionic polymers, organic solvents, etc. In general, with the addition of any of the above, the solubility of the original solute decreases and results in a precipitate.

In addition to the techniques discussed above, we also have several others to bring about precipitation.

They are as follows:

1. pH adjustment to a protein pH of neutral charge, so called the isoelectric pH or isoelectric point, at which generally the protein exhibits least solubility and comes out as a precipitate.
2. Reduction of medium dielectric constant and this is to enhance electrostatic interactions and further carried out by adding the miscible organic solvents.
3. Addition of non-ionic polymers that reduce the amount of water available for the protein solvation. It is as good as concentration process.

9.5 PURIFICATION

The purification process refers to the removal of contaminating chemicals from the precipitate formed. This is rather done by taking up the processes of fractional precipitation, adsorption and chromatography.

9.5.1 Fractional Precipitation

Fractional precipitation is used for the separation of proteins with different isoelectric pH values. As protein molecules are amphoteric, they have no net charge at the isoelectric pH value, i.e. pI and some charge at different pH. The electrostatic repulsive forces between the solute molecules are reduced at this pI . At a given pH, the proteins with the nearest pI will tend to precipitate. By varying the pH value, different fractions of proteins are separated.

9.5.2 Adsorption Process

The adsorption process is a reversible phenomenon occurring at the surface of a solid. The forces of adsorption are purely physical and are not strong.

So the desorption of an adsorbate in this case is feasible and thus it is called physical adsorption. On the contrary, in the case of chemical adsorption, the forces are strong and so the bonding between the adsorbent and the adsorbate is strong enough. Therefore, the separation is very difficult and hence it is an irreversible process.

The advantages of this process are several in cases of bioseparation. It is used for the primary isolation as well as for the concentration of the desired product. This process does not denature sensitive biomolecules and hence preferred for isolation of proteins. The adsorbents conventionally used in the bioseparations are activated carbon of vegetable origin, clay minerals, natural and synthetic zeolites and molecular sieves, alumina, silica gel and ion exchange resins based on the synthetic polymers made from styrene and cross-linked with divinyl benzene. The various modes of operations are used in these cases. They are batch, continuous, etc. Also used is the fixed bed and moving bed adsorption process.

In the fixed bed adsorption processes, we find that it is a vertical cylindrical column like structure filled with the adsorbent beads or any other adsorbent packed inside. The fluid containing solutes flows through the column of packed bed from one end to another at a constant flow rate.

In such cases the situation is complex than for a simple stirred tank vessel or a batch process which reaches equilibrium. Mass transfer resistances are important in the fixed bed processes and the overall process is in an unsteady state. The overall dynamics of a system shall determine the efficiency of the system rather than just the equilibrium considerations.

It is always seen that the concentrations of the solute in the fluid phase and on the solid adsorbent change with time and also with positions in the fixed bed as adsorption progresses.

Usually the measurements are done at the end of the column and are used to infer the variations of the solute concentration with time factor.

At the inlet of the adsorbent bed the adsorbent is fresh and contains no solute at the start of the process. As the fluid first contacts the inlet end of the bed, most of the mass transfer and adsorption takes place here. Further, as the fluid passes through the bed, the concentration of the solute in the fluid decreases very rapidly with distance in the bed to zero even before the outlet end of the adsorbent bed is reached.

After a short interval of time, the adsorbent bed at the inlet end is almost saturated and most of the mass transfer and adsorption now takes place in a relatively narrow zone called *mass transfer zone*.

Further as the fluid continues to flow, this profile moves downward indicating the progress of adsorption. It is realized that the concentration differences is the driving force for mass transfer.

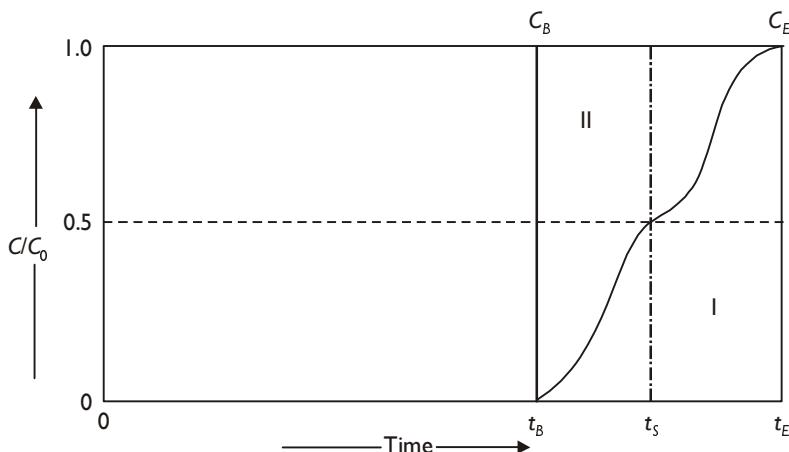
The exit concentration of the fluid remains near zero until the mass transfer zone starts moving down and reaching the outlet. The exit concentration starts to rise and the saturation of the adsorbent is indicated.

This abrupt rise in the solute concentration in the effluent is called *break point* at which the concentration is C_b .

The concentration profile in the fluid phase or the variation of solute concentration in the effluent as a function of time is obtained by plotting the solution concentration ratio C/C_0 , versus adsorbent bed length and is called *break through curve*.

C is the solute concentration in the liquid at a given time and C_0 is the feed concentration.

The following schematic represents the breakthrough curve for fixed bed adsorption (Figure 9.19).



I—No adsorption zone; below the curve, and
II—The mass transfer zone; above the curve

Figure 9.19 Breakthrough curve for fixed adsorption.

After the break point time t_B , the concentration abruptly rises to C_D or C_E which is the end of the breakthrough curve, where the bed is said to be saturated.

The break point concentration represents the maximum that can be discarded in the effluent and is often between 0.01–0.05 for the ratio C_B/C_0 .

The value of C_B/C_0 is taken as the point where C_D or C_E is approximately equal to C_0 .

For a narrow mass transfer zone, the breakthrough curve is very steep and most of the bed capacity is used at the breakpoint. This makes efficient use of the adsorbent and hence lower costs for regeneration.

Once the breakpoint is reached, the feed is stopped and the adsorbed material is eluted using different solvents or pH or ionic strength conditions.

Capacity of the column and scale-up design method: The equilibrium bed capacity is given as a combination of usable capacity and mass transfer zone.

When the entire bed comes to saturation with the feed, the total capacity of the bed may be taken to be proportional to the area under the curve and the line $\frac{C}{C_0} = 1.0$.

The total capacity or the time equivalent to the total capacity or the stoichiometric capacity of the bed is given as

$$t_{\text{total}} = \int \left(1 - \frac{C}{C_0}\right) dt \quad (9.35)$$

The usable capacity of the bed is given by the breakpoint time t_B . The time t_U is the time equivalent to the usable capacity or the time at which the effluent concentration reaches its maximum permissible limit and usually is very close to the t_B . It is given as

$$t_U = \int \left(1 - \frac{C}{C_0}\right) dt \quad (9.36)$$

Now the ratio t_U/t_{total} , the fraction of the total bed capacity or the length of bed utilized up to the breakpoint, H_B is given as

$$H_B = \left(\frac{t_U}{t_{\text{total}}} \right) H_T \quad (9.37)$$

where H_T is the total length of the bed.

The length of unused bed H_{UNB} is then the unused fraction of the total length and is given as

$$H_{\text{UNB}} = \left(1 - \frac{t_U}{t_{\text{total}}}\right) H_T \quad (9.38)$$

Whenever vapour is made to adsorb adiabatically from a gas mixture in a similar manner, there is always the evolution of heat of adsorption and causes a temperature wave to flow through the bed in a manner similar to adsorption wave. The rise in temperature of the bed at the fluid outlet is merely an indication that the breakpoint is achieved.

For liquids the temperature rise is very small and can be ignored. The shape of the breakthrough curve and the time of appearance greatly influence the method of operating the packed bed adsorber. Generally, S shaped curve or sigmoidal curve is exhibited, but also can be steep or relatively flat and in some cases is distorted.

If the adsorption process were to be infinitely rapid, the breakthrough curve would be a straight vertical line. There are several factors that affect the shape of the breakthrough curve.

The actual mechanism of adsorption, the rate of adsorption, the nature of adsorption equilibrium, the fluid velocity, the concentration of solute in the influent or the feed and the length of the bed, particularly if the solute

concentration in the feed is high, all contribute to the shape of the curve produced.

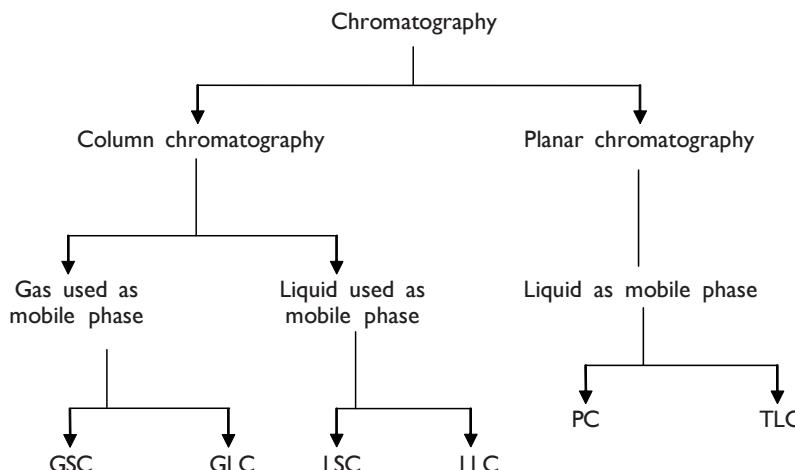
The breakpoint is sharply defined in some case and in others poor. Generally, the breakpoint time t_B , decreases with decrease in the bed height, increase in the particle size of an adsorbent, increased flow rate of the fluid through the bed and increased initial solute content of the feed.

9.5.3 Chromatography

Chromatography, a separation technique, refers to a group of closely-related techniques that are quite useful in the analysis of chemical substances. These techniques find use in the separation, purification and identification of compounds before quantitative analysis is taken up.

The basis of separation in any of the chromatographic techniques is the selective distribution of the components of a mixture between two immiscible phases in intimate contact with each other. One of these phases, called *stationary phase*, is a solid or an immobilized liquid (i.e. a liquid coated on a finely-divided inert solid). The other phase is called *mobile phase* (eluent or carrier gas depending on whether it is a liquid or gas) and percolates through the stationary phase. The sample to be analyzed is usually dissolved in the mobile phase.

Figure 9.20 shows the classification of chromatographic techniques.



GLC: Gas-Liquid Chromatography; GSC: Gas-Solid Chromatography; LSC: Liquid-Solid Chromatography; LLC: Liquid-Liquid Chromatography; PC: Paper Chromatography; TLC: Thin Layer Chromatography

Figure 9.20 Classification of chromatographic separations.

Column chromatograph: Figure 9.21 represents the Column chromatographic separation of a two-component mixture say A and B.

Sample mixture is introduced along with a compatible solvent phase at the head of column which is packed with a stationary phase. The components

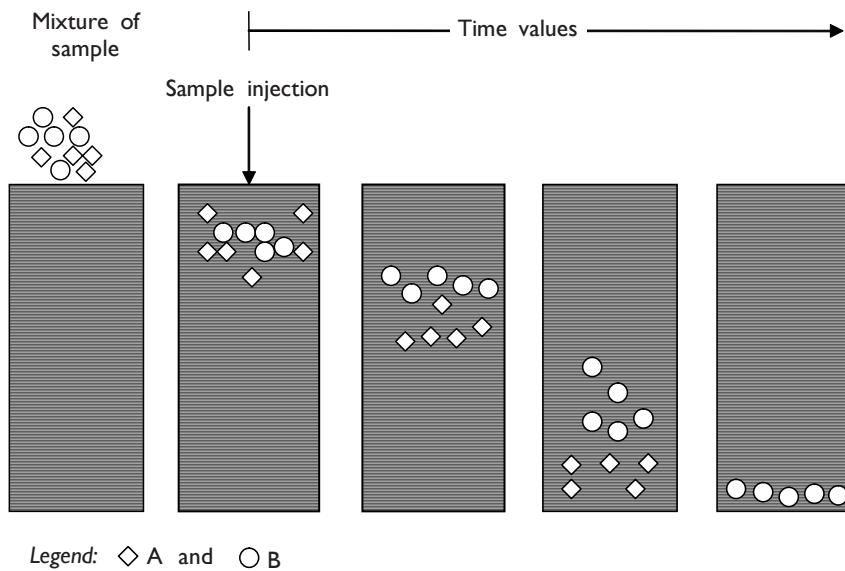


Figure 9.21 Chromatographic separation of a two-component sample mixture.

start distributing themselves between the two phases. Once the adsorption is complete, the mobile phase is added continuously, with a constant flow rate at the head of the column. With this, there is a redistribution of the solute components with a simultaneous migration down the column along with the mobile phase. Series of such distributions occur between the mobile and stationary phases leading to different rates of migration of solutes A and B. It leads to separate bands which elute from the column at different times.

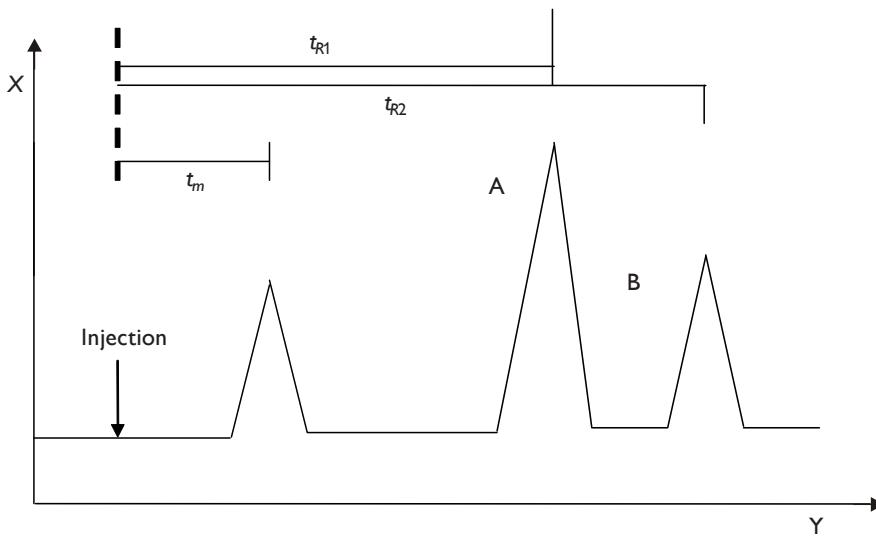
Online chromatogram: Figure 9.22 represents an online chromatogram, which shows different resolutions of peaks detected with respect to different times.

Retention time (t_R): Retention time is defined as the time taken by the solute to reach the detector from the moment it is injected into the column. It is determined by measuring the distance between the sample injection point to the apex of the peak on an on-line *chromatogram*.

Planar chromatography: Planar chromatography (Figure 9.23) uses a strip of filter paper instead of a column with an adsorbent in it.

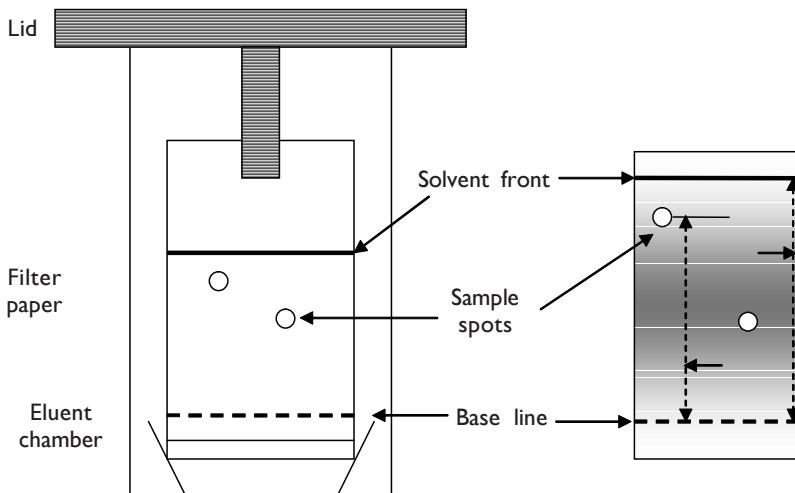
The stationary phase is immobilized water held by cellulose molecules of the filter paper and the mobile phase is a homogenous liquid. It consists of a glass jar with a lid within which is placed a Petri dish called *eluent reservoir*. A small amount of sample is spotted at one end of the filter strip using a capillary or a hypodermic syringe. It is placed inside the jar, thereafter a chromatogram develops.

The solute components of the sample move up the paper based on their distribution coefficients between the stationary and the mobile phases. Different solutes of the mixture will move to different extents and get



X-axis = detector signal
 Y-axis = time for elution of components
 t_m = the time for the mobile phase to flow through the column and retain in the column
 t_{R1} = retention time of the solute eluting in peak 1.
 t_{R2} = retention time of the solute eluting in peak 2.
 A and B = components in a sample mixture to be analyzed.

Figure 9.22 Development of on-line chromatogram.



d_m and d_s = the linear distances of mobile and solute phases respectively from the starting point.

Figure 9.23 Planar chromatography and paper strip after development.

separated. Later the paper strip is taken out for development of a chromatogram.

Affinity chromatography: This technique has been gaining a widespread importance in the recent times for purification of various components which are sensitive to the conditions during the separation.

Affinity chromatography is one in which one or more components in the sample mixture to be analyzed show an affinity or attraction towards their counterparts. This makes the process overall highly specific and efficient compared to the others.

There are certain techniques which are closely related to this affinity chromatography. They are bioaffinity chromatography, dye ligand chromatography and immobilized metal ion affinity chromatography.

It is seen that in all the above techniques, the stationary phase matrix is specially prepared to isolate or purify the biological molecules based on their specific interactions exhibited between the matrix immobilized functional groups and the functional groups available on the surface of the biological molecules.

A less commonly used technique called *covalent chromatography* is also available. But all these are relatively mild and are capable of high resolution of the desired component species from a complex sample mixture.

Specific and non-specific interactions: It is always observed that the interactions can be specific or non-specific. These decide the degree of separation or isolation and purification of the desired component from the complex multicomponent mixtures.

Some kind of interaction being simple or complex does prevail between the components in the sample that requires purification, separation or concentration.

It is even realized that in most of the chromatographic separations the interactions are non-specific. Non-specific means, the similar components in a given sample mixture interact in a similar manner with a solid phase, while the separation is solely based on the minor differences in their extent or degree of interaction.

For example, in ion-exchange chromatography, though involving a simple and well-defined interaction and depending upon the affinity of a charged species for its counter-ion is non-specific. It is because all the ions of like or similar charges will either adsorb or do not adsorb. The same drawback or the limitation holds good for all the types of chromatographic separations.

This lack of specificity should be viewed seriously as it is likely to affect not only the separation efficiency, but also the recovery of the desirable product.

Affinity chromatography is one where the problem of non-specificity is overcome, as this has been proved to be highly specific and thus aiding in the precision, specificity and better separations. This is immensely used in the biological separations as is evident with the increasing applications.

It is therefore also referred to as *bioaffinity chromatography*.

Bioaffinity chromatography: This term is used to include different categories of chromatographic separations involving the biological functional pairs. The biological functional pairs which exhibit high specificity are enzyme-inhibitor, antigen-antibody, hormone-receptor, etc.

The others such as dye-ligand chromatography, involves biomimetic ligands particularly dyes, which binds the active sites of the functional enzymes and immobilized metal ion affinity chromatography are the ones treated as pseudo-affinity chromatography processes as in true sense they do not exhibit specificity during the interactions.

Principle of bioaffinity chromatography: It is always understood that there exists a natural affinity displayed between the biological macromolecule and its complementary ligand. Ligands are basically the coordination compounds that possess an atom or a group of atoms attached to a central atom. While a coordination compound is one in which a molecule contains a central atom surrounded by the atoms called ligands, e.g. ferricyanide is a coordination compound.

An enzyme has always an affinity for its substrate, cofactors and inhibitors, etc. Similarly, an antibody for the antigens which stimulates the immune response, a lectin for some sugar residue, a hormone for the receptor or a carrier, a polynucleotide for its complimentary strand, and so on.

Bioaffinity exploits this mutual biological affinity to effect the separation or purification of the component from complex mixtures. It exploits the formation of specific and reversible complexes between a pair of biological molecules.

One of the pairs is called *ligand* and is usually immobilized onto a stationary phase while the other is called *counter ligand* which is adsorbed from the extract that is passing through the column containing an immobilized ligand.

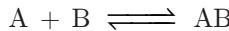
Table 9.3 illustrates ligands and counter ligands in affinity chromatography.

Table 9.3 Ligands and counter ligands

<i>Ligand</i>	<i>Counter ligand</i>
Enzyme	Substrate, substrate analogue, cofactor and inhibitor.
Sugar	Lectin, sugar or enzyme binding proteins
Antibody	Antigen, virus or cells.
Nucleic acid	Nucleic acid binding proteins, enzyme or histones
Hormones and vitamins	Receptor or carrier proteins
Lectin	Glycoproteins, polysaccharides, membrane proteins, cell surface receptors or cells.

It is mainly because of the van der Waal's weaker forces of attraction, hydrogen bonding and hydrophobic interactions that generate the specificity or affinity of a ligand towards the counter ligand.

As usual, the interaction between the two biomolecules can be represented by equilibrium as shown below.



Where K is equilibrium constant, i.e. the ratio of two velocity constants k_1 (forward) and k_{-1} (backward) and further depends upon the nature of interaction and the interacting species or biomolecules.

Certain examples are mentioned below to signify the values of K in order to understand the magnitude of interactions.

- (i) $E + S \rightleftharpoons ES$; here $K = 10^4$ to 10^6 (lower)
- (ii) Avidin + Biotin \rightleftharpoons Avidin – Biotin; $K = 10^{15}$ (higher)
- (iii) Antigen – Antibody; here $K = 10^6$ to 10^{12} (moderate)

In general, it is observed that, if the value of K is too low, affinity adsorption of the counter ligand does not occur, while if the value of K is too high, there exists a strong affinity during the interaction and subsequently leading to almost irreversible mechanism of adsorption.

It should be noted here that in either case of K being low or high, the separation or purification of counter ligand from ligand is very difficult. In such cases, the changes in pH values, addition of salts or surfactants as detergents is complied with, that affect the stability of the ligand-counter ligand complex without affecting or destroying the active conformation of the biomolecules.

Affinity binding may be molecular size specific and also shape specific.

Affinity chromatography practice: The practice of affinity chromatography totally depends upon the several important issues pertaining to the following; the choice of a support matrix, choice of a ligand, availability of spacer arms and finally coupling of ligands.

In actual practice, the ligand is normally attached to a water insoluble polymeric stationary phase matrix or gel in a covalent fashion and immobilized. The polymeric stationary phases which are water insoluble are agarose, cepharnose, dextran, polyacrylamide gel derivatives, cellulose, etc. that form tailor-made chromatographic adsorbent material suitable for adsorbing, specifically the desired component species from a sample mixture.

The choice of a support matrix should meet the following requirements.

1. Should have a surface area.
2. Should be physically rigid to withstand the operational stresses.
3. Should be permeable and macroporous.
4. Should contain the reactive functional groups as amino, carboxylic, hydroxyl, alcohol, etc. in order to derivatize and bind the ligand molecules covalently.
5. Should be stable towards the harsh conditions found during derivatization and regeneration.
6. Should have minimal non-specific adsorption characteristics, reasonable useful life and preferably economical.

The choice of a ligand mainly depends on two factors mainly:

1. Availability of chemically modifiable groups on the ligand so as to facilitate the attachment to the matrix. This cannot be at the expense of binding capability towards the counter ligand.
2. Most importantly the value of K , which defines the affinity for binding of counter ligand and preferably, should be in the range of 10^4 to 10^8 .

It is to be noted that in the preceding paragraphs, the significance of K is explained.

The functional groups for immobilization of ligands to the matrix as mentioned in the earlier discussions should be available and facilitate the multipoint attachment of ligand to the matrix rather than the single point attachment.

This is basically to prevent the leakage of ligands from the matrix over a period of time or whenever exposed to the harsh conditions of elution or washing.

Spacer arms and their choice carry a lot of significance during binding of ligands to counter ligands. It is as good as a junction or a bridge that narrows the gap between the matrix and the ligand.

Spacer arms between the matrix and ligand are usually the aliphatic hydrocarbons as 6-aminohexanoic acid, $H_2N(CH_2)_6COOH$ and hexamethylene diamine, the molecular formula being $H_2N(CH_2)_6NH_2$, an intermediate used in the manufacture of nylon 6.

It is always found that the adsorbents are prepared by coupling small ligands to the matrix directly, and often exhibit low binding capacity which is mainly due to the steric or position or configuration constraints.

This affects the binding capacity by hindering the interactions between the immobilized ligand and the active site of counter ligand. On the contrary, the spacer arms eliminate this problem as shown.

This mechanism is depicted in Figure 9.24.

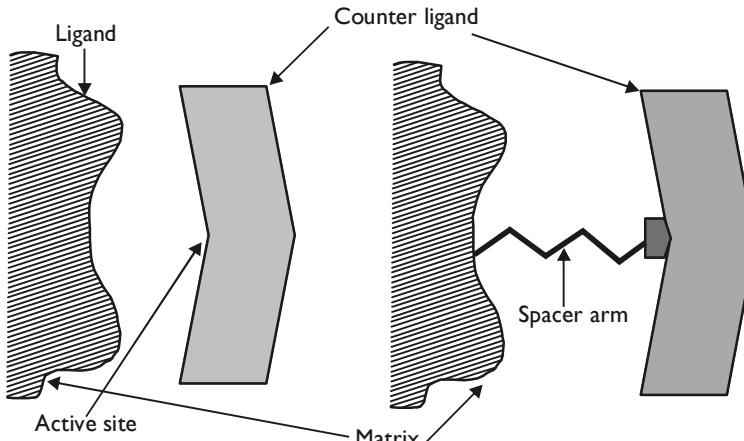


Figure 9.24 Ligand-counter ligand interactions.

The coupling of a ligand also has the influence over the interactions between. The steps involved in the coupling or pairing of chosen ligand to the selected matrix are as follows.

1. Chemical activation of the matrix, rather than going for thermal activation as this is thermally labile.
2. Immobilization of ligand via the chosen functional groups as mentioned above.
3. Blocking or deactivating the residual active groups that do not take part in the interactions.

In general, the proper selection of support matrix, ligands and selection of spacer arms will lead to an efficient binding and interactions that later provide the values of K , an equilibrium constant in the moderate range as mentioned in the above paragraphs.

This value of K (10^4 to 10^8) shall provide a reversible and at the same time a proper interaction between the ligand and counter ligand so that finally the separations and purifications are efficient.

In the succeeding paragraphs, is described the actual mechanism by showing the conceptual representation of affinity chromatography and the generation of on-line chromatogram (Figure 9.25).

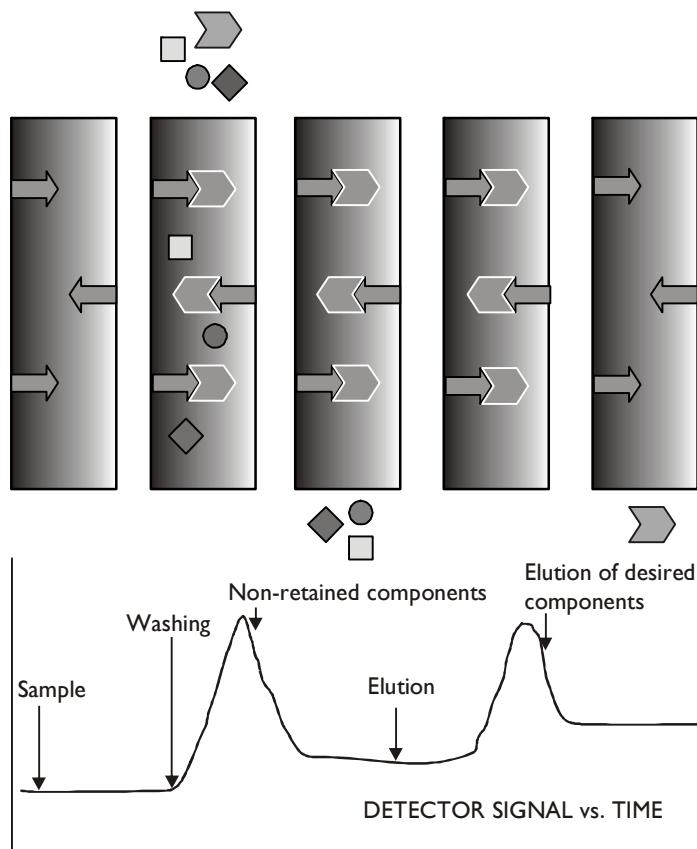


Figure 9.25 Affinity chromatography and on-line chromatogram.

The usual practice is packing of an adsorbent into a column and then introduction of sample mixture, i.e. application to the adsorbent bed that can facilitate the adsorption of the desired components.

The remaining non-complimentary species or the constituents of mixture do not recognize the ligand and pass through the column unrestrained. Later the column is washed with a suitable buffer to remove any non-specific adsorbed components of the sample leaving behind the desired counter ligand bound to the stationary phase insoluble matrix.

During the elution step, the adsorbed component is eluted or carried forward through the column in purer form by using a suitable buffer or a solvent.

In the development of chromatogram, is found that it has two peaks, the first which is due to the non-retained components of the sample and the other is due to the elution of the desired components by applying suitable solvents and buffers.

Regeneration of affinity adsorbents: It is a common practice that the adsorbents used are regenerated so that the same can be used repeatedly. This is simply for the economic reasons as the adsorbents are sometimes highly expensive. The same logic holds good for the affinity adsorbents.

Here also for the economic reasons, it is imperative that the affinity adsorbents be regenerated. The same reasoning holds valid whenever a rare ligand is employed and at the same time being expensive. In essence, the regeneration and reuse depends upon the following facts.

Firstly, the nature of sample, as within it sometimes some degradation enzymes may be present and also the second fact is the stability of the ligand and the matrix towards the conditions used during its immobilization, elution and regeneration.

The most widely accepted and used procedure for regeneration is as discussed in the succeeding lines.

Wash the adsorbent used in the affinity chromatography with several volumes each of 0.1M tris [*tris (hydroxymethyl) amino methane*], which is 99.9%, gold label and 100 grams of it costs 6.75 US dollars; or HCl buffer with a pH of 8.5 containing 0.5M NaCl followed by 0.1M sodium acetate buffer with a pH of 4.5 containing 0.5M NaCl.

The column is then equilibrated with starting buffer before it is used. Once regenerated, the adsorbent is ready for further use.

Merits of affinity chromatography: The following points explain the merits of affinity chromatography:

1. The process is highly selective and based on the molecular size, molecular charge, isoelectric point, etc. as compared to the other processes. This fact reveals the specificity of process.
2. By using this process, the purifications, recovery, etc. are extremely good and usually above 90%.

3. It imparts a high concentrating effect and is true whenever protein separations are considered.
4. The unwanted components are removed from a mixture.

Applications of affinity chromatography: Most of the applications are related to the biological components and hence the other name is bioaffinity chromatography.

They are as follows:

1. In the purification and recovery and also in the concentration of biological molecules as proteins, vitamins, hormones, etc.
2. In affinity scavenging, this refers to the selective removal of detergents or surfactants or even endotoxins from protein solutions.
3. In clinical diagnosis, and is due to high precision, accuracy, reliability, quickness and free from interferences.
4. Also used in immunoassays and immunotherapy.

Gradient chromatography: As we know that overall in a chromatographic separation, there is always a sequence of steps or procedures followed. Use of a stationary phase, mobile phase, a column, sample introduction system, washing and elution and eventually it is the development of on-line chromatogram.

A general elution problem is often observed in chromatography, when mixtures or samples to be separated are containing widely disparate components or the components that exhibit incongruity.

It is also realized that if the elution conditions are adjusted so as to obtain the resolution acceptable to the components having short retention times, then the elution of those components which have long retention times, shall be retained inside the column for too long a period for practical analysis.

On the other hand, if the elution conditions are adjusted so that they are acceptable to the resolution of those components with longer retention times, then the components with shorter retention times hardly get separated as they are found in the downstream end of the process.

In order to overcome such problems an appropriate method is suggested and that is to change the elution conditions during the development of the chromatogram. This is appropriately referred to as Gradient chromatography.

Gradient elution involves the change of eluent composition during the development either continuously or stepwise, so that the partition coefficient values of each component are changed with respect to time.

Consequently, a gradient is formed with respect to the percentage composition of a mixture of eluents or pH or ionic strength of the eluent.

This is contrary to isocratic elution method in which the eluent composition is kept constant. In such cases, the retention times of the

components in a mixture and thereby the separation depends on the partition coefficients of the individual components.

Gradient elution technique is useful for the samples that are not separated by isocratic elution.

A mobile phase gradient is formed by mixing two or more solvents either incrementally, i.e. stepwise or continuously. The most widely used gradients are binary solvent systems with a linear, concave and convex increase in the percentage volume fraction of the stronger solvent. With this the exhibited gradients are linear, concave and convex and are expressed as shown below.

$$\text{Linear Gradient, LG} = t/tg$$

$$\text{Concave Gradient, CcG} = 1 - (1 - t/tg)^n$$

$$\text{Convex Gradient, CvG} = (t/tg)^n$$

where t is the time after the gradient started, tg is the total gradient time and n is the integer for controlling the gradient called the *gradient control* and are expressed in terms of volume fraction of the stronger solvent.

9.6 FINAL PRODUCT ISOLATION

The final production isolation complies with the finishing and formulation operations. Biotechnological products require varying degrees of purification depending upon different end uses. These concepts are achieved by the following operations:

1. Crystallization, to remove closely related impurities from the desired product

It basically refers to the formation of solid crystals from a homogeneous solution by attaining super saturation. There are several ways of attaining the state of super saturation. Some important ones are adiabatic evaporation and cooling also called vacuum cooling, salting, and simple cooling process that merely involve the reduction in the temperature values.

Crystallization affords a practical method of obtaining pure chemical substances in a satisfactory condition of the product for final packaging and storing. In a process industry manufacturing the products in solid forms, crystallization is usually the last step preceding the drying process. This aids in producing highly purified products such as antibiotics.

Crystallization operates at low temperatures, and certainly minimizes the threat of thermal denaturation and degradation of the so called heat labile materials. In addition to this, these operations are conducted at high concentrations succeeding generally evaporation and the costs are low and separations factors very high.

It also aids in the formation of solid particles of specific size, uniformity and shape with exceptional purity. Formation of uniform sized and shaped crystals facilitate separation by filtration or centrifugation and drying in the later stages. Consequently, a crystalline product also has a better appearance and wide-end user acceptance.

The shape of crystalline products may be selectively modified by using habit modifiers. These habit modifiers, for example, raffinose which occurs naturally in beet sugars, can be added intentionally or may be naturally present, concentration of about 1% in sugar syrups can modify the sucrose crystals into thin narrow plates. Also use of potassium ferrocyanide to brine shall modify crystals of sodium chloride to dendrite type also called *needle like* or *whisker like growing crystals*. Use of habit modifiers in crystallization is more selective and used only when it is required.

2. Drying, to remove any solvent including water that may be present

In drying, the conditions required are very important. To select these, the physical properties of the product, heat sensitivity and desired final moisture content are to be considered.

There are several factors that affect drying process. Moisture content of the material, nature and form of material to be dried, temperature of drying, time and heat transfer parameters are some of the important ones.

Different types of driers are in use and their applications depend upon the above factors mentioned. Vacuum dryer, rotary drum dryer, spray dryers, pneumatic conveyor dryers, fluidized and spouted beds, etc. are some.

Drying operations are carried out for several reasons that are listed below.

- To purify a crystalline product so as to remove the solvent adhering to it.
 - Storage of dry solid as compared to the wet is easier.
 - The cost of transportation for dry material would be reduced.
 - Drying furnishes certain definite properties to the product material as is the case with pharmaceuticals.
 - Overall the shelf life of a product increases.
3. Formulation, to meet the customer requirements and ensure the product stability

We will see here the much used process in the drying of temperature sensitive materials by using *freeze drying*.

9.6.1 Freeze Drying

Freeze drying is also called sublimation drying or lyophilization process. As said, the materials that are temperature sensitive and are thermally unstable like foodstuffs, pharmaceuticals, biologicals, etc. are handled by freeze drying.

The method involves freezing of materials by exposure to cold air followed by sublimation of ice in vacuum from the frozen state to produce a dried product. Freezing leaves a mass having high structural rigidity preventing collapse during the sublimation of ice and when water is added later, the rehydrated product retains almost the original structural form.

The biggest advantage of this process is that the aroma and the flavour of the foodstuffs is not lost, although it is an expensive process.

During lyophilization, it is seen that the material will be composed of a frozen core and as and when ice sublimes, i.e. transformation of solid ice directly into vapour form takes place, the plane of sublimation recedes from outer surface thereby leaving a porous structure of the material. The heat of sublimation which is about 2800 kJ/kg of ice is conducted inward through the layer of dried material and the water vapour is also transferred through the layer of it. Thus it can be seen as a simultaneous heat and mass transfer during such process. This vapour pressure difference required for sublimation is attainable by maintaining total pressure in the drying chamber and should be of the order of 0.1 to 2.0 torr. In addition to it, a condensing system is also provided to remove water vapour formed along with heating system to provide the required latent heat of sublimation to the frozen material.

Equipments like batch freeze driers are employed in practice and are realized the following added advantages. These are porous structure of substance which is due to the removal of water by sublimation, shape and size retained, shrinkage and case hardening are almost negligible and above all the heat damage is minimized.

9.6.2 Formulation

Formulation is required particularly for pharmaceuticals in the form of tablets, capsules, injectibles, creams, powders, etc. Also it is needed in other products like, baker's yeast, industrial proteins and enzymes, etc. depending upon their end uses.

Formulation of a product should ensure the product stability, and more importantly the requirements of the consumer or end user. The succeeding lines with examples illustrate the importance of formulation step.

As we know baker's yeast is produced by aerobic fermentation process and is later concentrated by centrifuging followed by filtration using rotary vacuum filter to a dry matter content of about 35%. Later this concentrated yeast is extruded into long filamentous mass segmented to pieces and subjected to drying using fluidized bed mechanism employing temperatures between 40 to 60°C. This dried yeast formulation is stable and amenable for long-term storage and transport. Before using it is soaked in water which helps in restoring its biological activity.

In all the biochemical processes that make use of enzymes called *biocatalysts* for continuous operation of industrial processes, these enzymes are needed to be formulated or immobilized on suitable supports or matrices for long-term stability and use inside the packed bed or fluidized bed reactors.

Various techniques are discussed in Chapter 5 that describe the importance of immobilization of enzymes. The following examples with applications shall depict the importance of enzyme formulation.

Proteases, the protein splitting enzymes are used in the detergent industry while detergents contain about 0.3 to 0.6% of proteases. In this the formulation process must ensure the stability and activity of enzymes in the presence of other aggressive components of detergents and also operating conditions of high alkalinity and temperatures.

9.7 MEMBRANE SEPARATION PROCESSES

Membrane separation comprises different processes operating on a variety of physical principles and applicable to a wide variety of separations of miscible components. Membrane separation processes have several advantages. They are as follows:

1. Whenever a desired product material is thermally sensitive, or when the desired product is a clear liquid, the membrane separation can act as an alternative to the evaporation process in concentrating a dilute feed. This membrane separation is a low energy utilizing process.
2. In membrane separations, no phase changes are involved, thereby the chemical and mechanical stresses on the membrane as well as product are minimum.
3. The concentration and purification of product are achieved in a single step and of course selectivity towards the desired product is good.
4. The energy requirements are modest and processes are amenable for easy scale-up.

Membrane separations involve the separation of immiscible components such as the recovery of extracellular products and biomass from fermentation broths. The extracellular products include proteins, enzymes, etc. These cannot be used for the feeds that contain a high concentration of low molecular weight components because of high osmotic pressure or when the feed has high solid content because of the problems in pumping.

Membrane separations processes are attracting a lot of attention, since they cover an astonishing range of difficult separations, using different types of membranes.

Desirable attributes of membranes: It is not that all the membranes processed and synthesized can be used for separations. There are a selective few and which should exhibit the following attributes to be used in separations. The desired attributes are:

1. Good permeability.
2. High selectivity.
3. Chemical and mechanical compatibility with processing environment.
4. Stability, freedom from fouling and a reasonable useful life.
5. Amenability to fabrication and packaging, and
6. Ability to withstand large pressure differences across the membrane thickness.

Operational requirements of membranes: The membrane should also possess certain significant features that are important during the actual operation which are defined as the operational requirements or necessities of membranes. They are as listed below:

1. The membranes should exhibit high selectivity and separation efficiency.
2. Intrinsically, these membranes should offer high permeation flux rates.
3. They should be able to withstand operational stresses, i.e. should have high mechanical and physical strength.
4. During the synthesis of membranes care is to be taken to see that the final membrane exhibits high durability and consistency in performance for longer periods, so that frequent replacement of membranes is averted.
5. Membranes usually come across the feed mixtures which are corrosive in nature. Therefore, the membranes should have the ability to withstand the corrosion, i.e. should be corrosion resistant.
6. The availability of membranes is in different modules, which signifies that membranes can be moulded in appropriate shapes during fabrication, i.e. no difficulty in fabrication.

Membrane separation processes that are commonly used in a bioprocess industry are as follows:

- Reverse osmosis
- Dialysis
- Ultra-filtration
- Micro-filtration
- Nano-filtration

Membrane separation processes can be broadly classified into three categories based on the driving force, which facilitates the mass transfer across the membrane.

In the first category, the driving force is hydrostatic pressure and the separation methods include (i) reverse osmosis, (ii) ultra-filtration, and (iii) micro-filtration.

In the second category, the driving force is the concentration gradient and the method is called dialysis.

Electrodialysis belongs to the third category in which the driving force is an applied electric field.

The components of a typical membrane separation unit are shown in Figure 9.26.

The membrane separation plant includes the following:

- Reservoirs for the feed
- Permeate and reject solutions
- High pressure pump

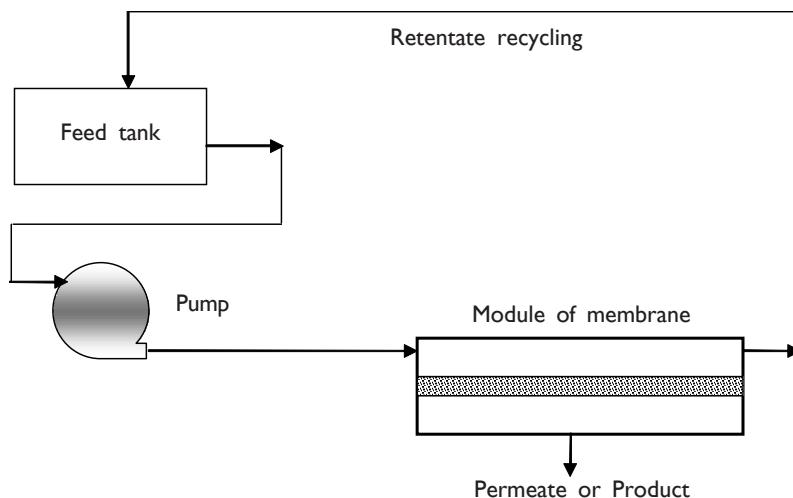


Figure 9.26 Membrane separation plant.

- Membrane modules of suitable configuration
- High pressure regulators and gauges

Membrane modules: In designing different membrane modules, the following criteria are employed:

1. To minimize the space requirement and the capital cost, a high membrane surface to volume ratio is provided.
2. To withstand stresses and operating pressures, adequate structural supports are provided to the thin polymeric membranes.
3. Low pressure drop on the concentrate side of the membrane to maintain driving force for permeation.
4. To delay the onset on concentration polarization and subsequent fouling of membranes, turbulent conditions on concentrate side.
5. Provision for back flushing and replacement of membrane, if required.

The different membrane modules such as flat sheet, spiral wound, shell and hollow fibre are shown in Figure 9.27.

Membrane modules

(a) **Flat sheet:** These are easy to construct and also are amenable to replacement of membranes whenever required. The channel width formed between the membranes can be altered to reduce the plugging problems during the process. In between is placed a support that helps to operate at higher pressures.

These are also called flat-plate systems. Their features include low membrane surface-to-volume ratio. Feed is given from one side and the product permeates exit from the other.

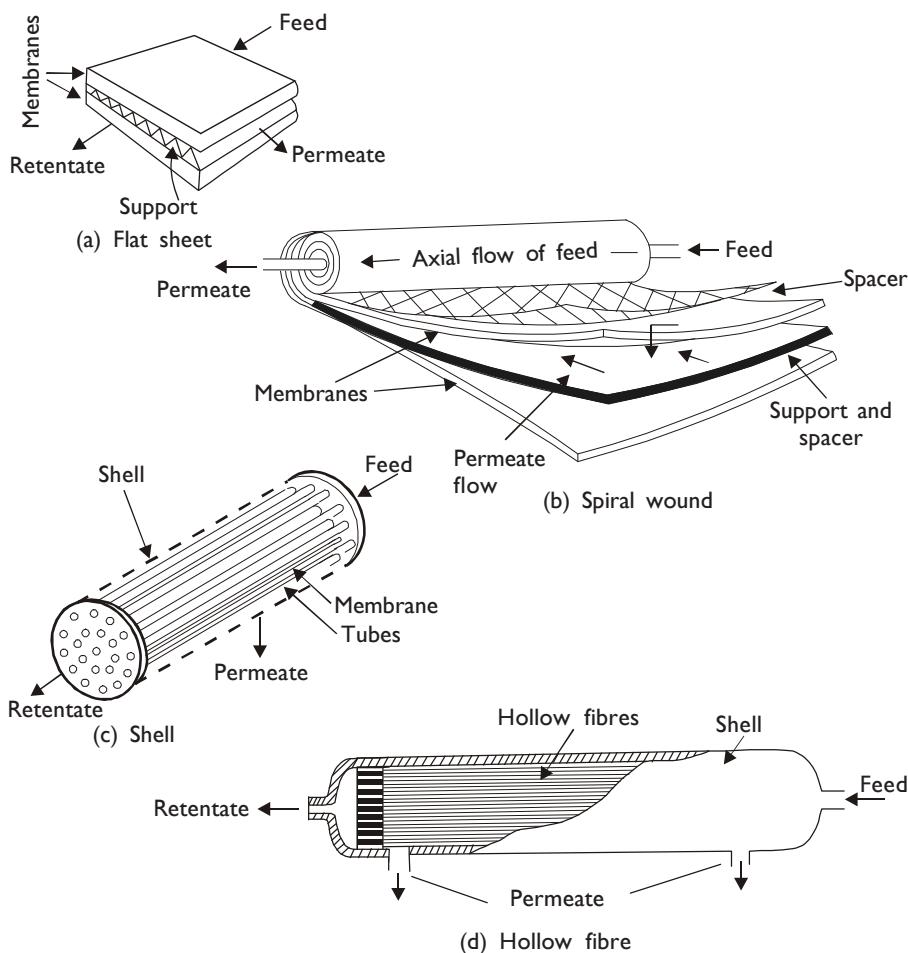


Figure 9.27 Different membrane modules.

The features of flat sheet are as follows:

The packing density of flat sheet is $3-500 \text{ m}^2/\text{m}^3$. Its resistance to fouling is good. Maintenance of flat sheet is easy. The cost per square metre of membrane surface of flat sheet is high. Flat sheet exhibits low pressure drops but cannot be employed for high pressure applications which is a major disadvantage.

(b) Spiral wound: Spiral wound membranes contain rolled membranes and in between are placed the support and spacer. These are also called *spiral cartridges* and are essentially flat-plate systems configured to increase the surface-to-volume ratio.

The flow of the feed is axial and permeates exit at the other side of it. Their characteristic features include:

The packing density of spiral wound membranes is $200\text{--}800\text{ m}^2/\text{m}^3$. Their resistance to fouling is moderate and their maintenance is not that easy. The cost per square metre of membrane surface is moderate to low. They exhibit moderate pressure drops and can be used for high pressure applications, viz. reverse osmosis, gas permeation, dialysis, ultra and microfiltration processes.

(c) Shell and tube: Shell and tube membrane modules resemble shell and tube heat exchanger modules. In these, the membranes are placed on the tube side and the feed is given on the shell side. They are also called *tubular membranes*.

The characteristic features are as follows:

The packing density of shell and tube membrane modules is $30\text{--}200\text{ m}^2/\text{m}^3$. Their resistance to fouling is very good and it is easy to maintain. Cost per square metre of membrane surface is low to moderate. Their pressure drops is very low and can be used for high pressure applications such as reverse osmosis, ultra filtration and gas permeation processes.

(d) Hollow fibre: Owing to the possible blockages and plugging in the flat plate systems and spiral wound systems, these hollow fibre systems are in use.

The characteristic features of hollow fibre systems are as follows:

They have packing density of $500\text{--}9000\text{ m}^2/\text{m}^3$, but are very poor in resistance to fouling. Their maintenance is not easy. Cost per square metre of membrane surface is very low and their pressure drop is very high and can be used for high pressure applications.

Transport mechanism in membranes: Certain models and hypotheses have been proposed to explain explicitly the mechanism of transport in porous and non-porous membranes. These are the two basic models of mass transport across the membranes that help explain the selectivity of membranes.

The two models are:

1. Capillary flow model or pore flow model.
2. Solution diffusion model.

The capillary flow model: Figure 9.28(a) depicts the capillary flow model and the features of this model are mentioned below:

- (i) Membrane is considered to be loose and microporous and capable of retaining the solutes or particles of sizes larger than 10 \AA .
- (ii) The flow of feed occurs through the pores and mainly due to the convective flow, except in an impermeable layer.
- (iii) A filtering or sieving type mechanism occurs wherein the solvent moves through the micropores in essentially a viscous flow and the solute molecules small enough to pass through the pores are carried by convection with the solvent.

- (iv) The passage of the larger molecules is prevented by the size of the micropores.
- (v) Capillary flow dominates in micro-filtration and ultra-filtration processes.

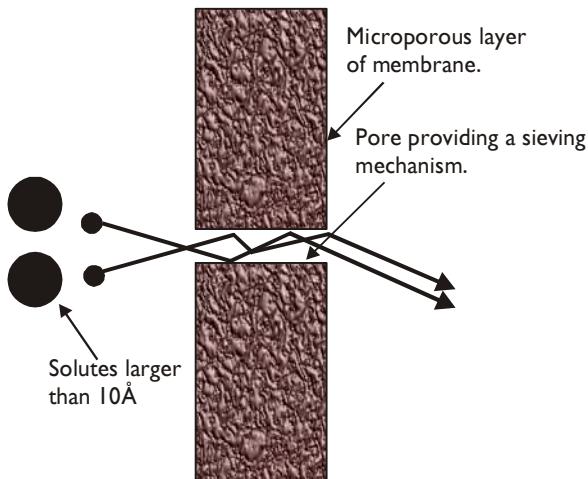


Figure 9.28(a) Pore flow model.

In Figure 9.28(a) it is seen that the pores are available for the transport of solutes through the membranes. Overall it is seen that the permeants are transported by pressure driven convective flow through tiny pores.

The separation takes place due to the permeants getting excluded from some of the pores in the membrane through which the other permeants move.

It is seen that in the microporous Knudsen flow gas separation membranes, transport occurs by pore flow or capillary flow.

The solution diffusion model: The features of this model are mentioned below:

1. This is applicable to the membranes which are non-porous or dense in structure.
2. There is always the diffusion of solutes or molecular species to be transported in the membrane material and subsequently follows the molecular diffusion across the barrier following the Fickian type of diffusion.
3. It is due to the applied pressure, a concentration gradient is set up that acts as a driving force.
4. The permeants are separated because of the differences in the solubilities of the materials in the membrane and also the differences in the rates of migration or the rates at which the materials diffuse through the membrane.

5. This model tries to explain the selectivity of membranes based on the above feature, i.e. 4.
6. Membrane is considered to be tight and capable of retaining the solutes of about 10\AA in size or less than the specified.

Dense solution diffusion membranes separate because of the differences in the solubilities and mobilities of the permeant in the membrane material.

Figure 9.28(b) illustrates the mechanism of solute dissolution in membrane, so called solution diffusion model.

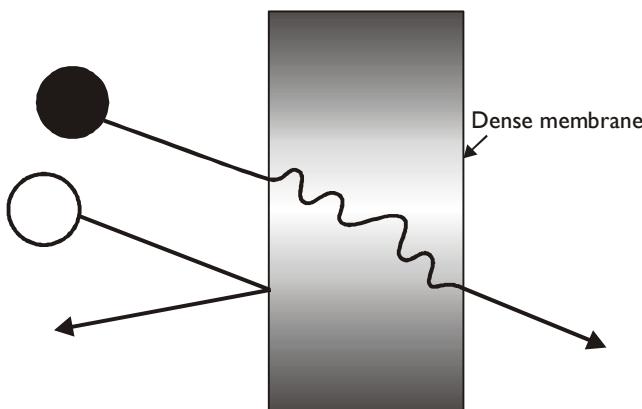


Figure 9.28(b) Solution diffusion model.

Factors influencing the membrane separation processes: The overall performance and efficiency of membrane separation process relies on two important factors that can affect or influence the selectivity nature of membranes and the flux or permeation rates through them. They are:

1. Concentration polarization at the membrane surface.
2. Fouling.

In the succeeding paragraphs, we shall find out the reasons and results of these two phenomena finally affecting the membrane performance.

Concentration Polarization (CP): It is always seen that the membrane separation process is designated with two zones across it. The first zone is upstream and the second is the downstream zone.

On the upstream zone or side, the gas or liquid mixture is in contact with the membrane surface which is always a feed side. Further, the separation occurs resulting into permeate and the retentate.

The permeate enriched in one of the components of the mixture is withdrawn from the downstream side or the withdrawal side of the membrane.

Since the feed mixture components permeate at different rates, concentration gradients are developed in the fluids on both sides of the

membrane. This mechanism tries to establish some sort of polarity in terms of concentration values called *concentration polarization*.

It is also seen that during the process, the non-permeating species are carried towards the membrane surface due to the convective flux of the feed, but the component species remain on the upstream side of the membrane.

As a result of this, the concentration of these component species elevates gradually at the membrane surface and ultimately becomes greater than their concentration in the bulk liquid thereby setting up the concentration polarization at the membrane surface.

The effect of concentration polarization is that it reduces the permeating components concentration gradient across the membrane by which it was earlier diffusing and later leading to lowering of fluxes and membrane selectivity.

The importance of concentration polarization depends upon the membrane separation process.

For instance, in reverse osmosis, CP significantly affects the membrane performance, but usually well controlled in industrial systems. On the other hand, membrane performance in ultra-filtration (UF), electrodialysis (ED), and pervaporation processes is seriously affected by concentration polarization.

In most of the membrane processes, there is a bulk flow of the liquid or gas through the membrane and the permeate-side composition depends only on the ratio of the components permeating the membrane. When this is the case, concentration gradients only form on the feed side of the membrane.

In order to explain the effect of concentration polarization, two approaches are used.

1. In the first approach, so called the resistance in series approach, it is found that during the separation, the resistance to permeation of solutes across the membrane surface and the resistance occurring in the fluid layers that are adjacent to the membrane are all treated as the resistances in series.

No such consideration is given to the thickness of various layers or the transport mechanisms taking place.

Assuming that the concentration polarization occurs only on the feed side, the flux J_i , across the combined resistances of the feed side boundary layer and the membrane can be written as

$$J_i = K (C_{ib} - C_{ip}) \quad (9.39)$$

where K is overall mass transfer coefficient $\text{kmol}/\text{m}^2\text{-sec}$.

C_{ib} is the concentration of component i in bulk feed solution.

C_{ip} is the concentration of component i in bulk permeate solution.

The flux across the boundary layer is also written as

$$J_i = k_{bl} (C_{ib} - C_{i0}) \quad (9.40)$$

where k_{bl} is mass transfer coefficient for fluid boundary layer

C_{ib} is concentration of component i in bulk feed

C_{i0} is the concentration of component i in the fluid at the feed or membrane interface.

Now the flux across the membrane can be written as

$$J_i = k_m (C_{i0} - C_{ip}) \quad (9.41)$$

where the terms in the brackets have a usual significance.

Since the overall concentration drop is (combining all the above expressions)

$(C_{ib} - C_{ip}) = (C_{ib} - C_{i0} + C_{i0} - C_{ip})$, only concentration gradients are considered.

Therefore, a resistance-in-series model can be applied and the resultant equation is as shown below:

$$1/K = 1/k_{bl} + 1/k_m \quad (9.42)$$

Further, when the value of k_{bl} is large, $1/k_{bl}$ is small and becomes an insignificant fraction of the total resistance to permeation.

Therefore, the overall resistance is equal to membrane resistance. When k_{bl} is small, $1/k_{bl}$ is large and becomes a significant fraction of the total resistance to permeation. Finally, we have $1/K = 1/k_{bl}$.

It is seen that the overall resistance increases or overall mass transfer coefficient decreases and becomes very small and hence the flux decreases.

The boundary layer mass transfer coefficient is thus an arithmetical fix used to correct the membrane permeation rate for the effect of concentration polarization. Nothing is revealed and known about the causes of concentration polarization phenomenon and this could be a limitation of this approach.

2. The second approach is the boundary layer model approach and is more of a realistic one. In this a thin layer of unmixed fluid of thickness δ exists between the membrane surface and the well-mixed bulk solution. It is seen that the concentration gradients that control the concentration polarization are formed in this layer. This model oversimplifies the hydrodynamics occurring in the membrane modules and still has one flexible and adjustable parameter, the boundary layer thickness δ .

The mechanism pertaining to this approach is depicted in Figure 9.28(c).

The solute concentration at the membrane, based on the mass balance on the solute can be estimated as discussed below:

Carrying out the mass balance on the solute getting transferred through the membrane is given as:

Rate of convection of solute towards the membrane = Rate of diffusion back into the liquid + Rate of solute permeation through the membrane.

Accordingly we have,

$$J_s C = D \frac{dC}{dx} + J_s C_p \quad (9.43)$$

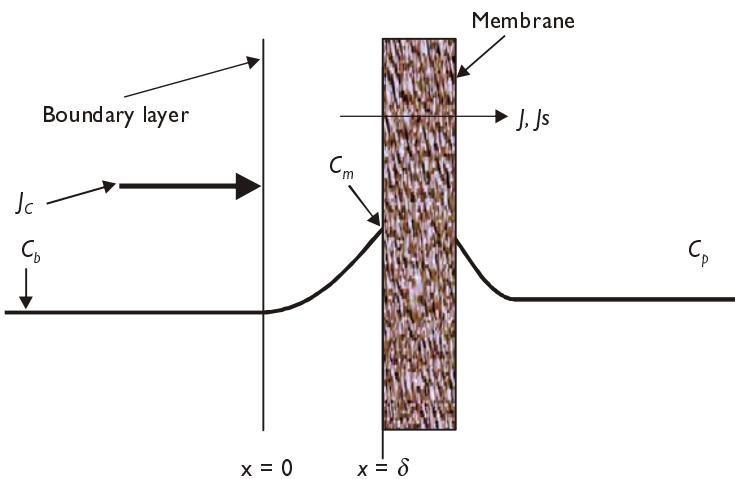


Figure 9.28(c) Boundary layer film model.

On rearranging, we have

$$\frac{dC}{(C - C_p)} = \frac{J_s}{D} dx \quad (9.44)$$

Integrating the above equation, using the limits at $x = 0$, $C = C_b$ and at $x = \delta$, $C = C_m$, we get

$$J_s = k' \ln \left(\frac{C_m - C_p}{C_b - C_p} \right) \text{ where } k' = D/\delta \text{ the mass transfer coefficient.} \quad (9.45)$$

Here if no solute passes through the membrane, then we get,

$$J_s = k' \ln \left(\frac{C_m}{C_b} \right) \quad (9.46)$$

The ratio C_m/C_b increases if the flux J_s is high or k' is low. The low values of k' may be due to the following reasons related to one another. They are the low values of D , diffusivity which is due to the high molecular weight or due to the high viscosity of the solution or also due to the large values of δ , the boundary film thickness seen due to the low magnitude of turbulence. Indeed, the values for k' are to be determined empirically.

Fouling: We know that in most of the membrane separations, flux declines with time. It is suggested that a decline to 80% of the initial output can take minutes or days and even months, depending upon the membrane characteristics as pore size, porosity and nature of membrane. The most important cause is fouling of membrane.

Fouling is an irreversible decline in the output resulting from interactions with components in the feed. Irreversible does not mean it is permanent, but

signifies that decline cannot be reversed by restoring the previous set of process conditions.

Cleaning of membranes is a common way of restoring much or all of a fouled membranes earlier output. Some forms of fouling are permanent, such as those that change the structure of membranes. Compaction resulting from the polymer creep is permanent but is not fouling.

This is probably the most frequently encountered problem with membrane-based separations and in particular those which handle liquid feeds. The coating of the membrane surface or blocking of the pores with a solid or gelatinous material thereby, creating a barrier through which the permeating components can pass. The consequences seen are the reduction in the pore size distribution and the flux through the membrane.

These fouling materials enter the module through the feed as particulates, gels or soluble, high molecular weight species or they may also precipitate from solution as part of the feed permeates. Since there is a flux toward the membrane surface caused by the flow of the material through the membrane, these fouling substances migrate through the surface of the membrane. Fouling can be reduced but can not totally be prevented by adopting the following procedures.

1. Feed should be prefiltered before giving it to the module. This is to remove the particulates of high concentration.
2. To use often relatively dead-end filtration than cross flow filtration pattern.
3. Selecting the appropriate module, for example, hollow fibre module is more susceptible to fouling while plate and frame is not.
4. Maintaining high velocities of feed on the feed side of the membrane but should not exceed the modules velocity limit.

Bio fouling: This refers to the growth of bacteria on the membrane surface. It is seen that the susceptibility of membrane to biological fouling is a strong function of membrane composition. For example, cellulose acetate membranes are ideal nutrient for bacteria and can be completely destroyed by a few weeks of uncontrolled bacterial attack. For this reason the feed water must be sterilized prior to feeding to the cellulose acetate membrane. Polyamide hollow fibres are also susceptible to bacterial attack but thin film composite membranes are resistant.

Use of bactericidal agents, i.e. the agents that kill bacteria are incorporated in the process that controls bio fouling. With this we can conclude that controlling bacteria for cellulose acetate membranes is a must and desirable for polyamides and other composite membranes. In sterilizing feed water, chlorination is a common and much used method and as cellulose acetate membranes can tolerate up to 1 ppm chlorine, sufficient chlorination is used to maintain 0.2 ppm free chlorine. Utmost care is to be taken to see that the residual chlorine is removed as sometimes membranes are chlorine sensitive. In this regard, dechlorination is done by adding sodium metabisulphite.

9.7.1 Reverse Osmosis

Reverse osmosis is also called hyper-filtration. Reverse osmosis (RO) uses the membranes of pore size in the range of 0.0001–0.001 mm, that is permeable to water but not to the dissolved salts of even low molecular weight. In this case, the direction of normal osmotic flow of a solvent across the semi-permeable membrane is reversed due to an applied pressure which is greater than the osmotic pressure of the liquid feed. Refer to Figure 9.29.

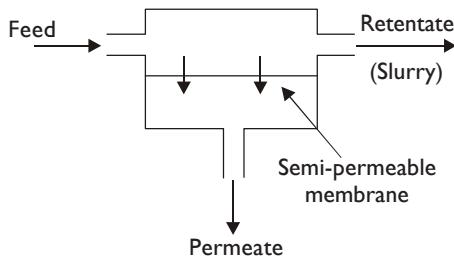


Figure 9.29 Flow diagram of reverse osmosis.

In the normal osmosis, there is always a diffusion through a membrane from a region of low concentration to a region of high concentration. Here a solvent diffuses through a membrane, separating the solution which has low molecular weight solutes and the pure solvent, for example pure water as a pure solvent and salt water as a solution. In the case of RO, a reverse pressure difference of 20–100 bar is imposed on the membrane, virtually non-porous; that causes the flow of solvent from the solution side to the pure solvent side. This in fact concentrates the solution.

Reverse osmosis is applied in separation of low molecular weight products such as salts which are 3.5% by weight in sea water by way of desalination. It is used in separation of sugar or organic acids from aqueous solutions.

It is also used in concentrating fruit juices, vegetable juices, milk and in the purification of water.

Figure 9.30 gives schematic diagrams of osmosis and reverse osmosis. When an ideal semi-permeable membrane acts as a barrier between a solution and its pure solvent, the solvent molecules pass rapidly across the membrane from the solvent to the solution. The solvent molecules pass from a region of lower concentration to a region of higher concentration. In this, the driving force for the flow is the difference in the chemical potential on the two sides of the membrane. The phenomenon is called *osmosis*, and continues until the fluid pressure in the concentrated solution is high enough to prevent the passage of further solvent molecules. At equilibrium, the chemical potentials on either sides of the membrane are equal and this fluid pressure is called *Osmotic pressure* of the solution. It governs the osmosis process.

When the process is reversed by applying the pressure P greater than the osmotic pressure π , the direction of the flow is also reversed and now it is called *reverse osmosis*.

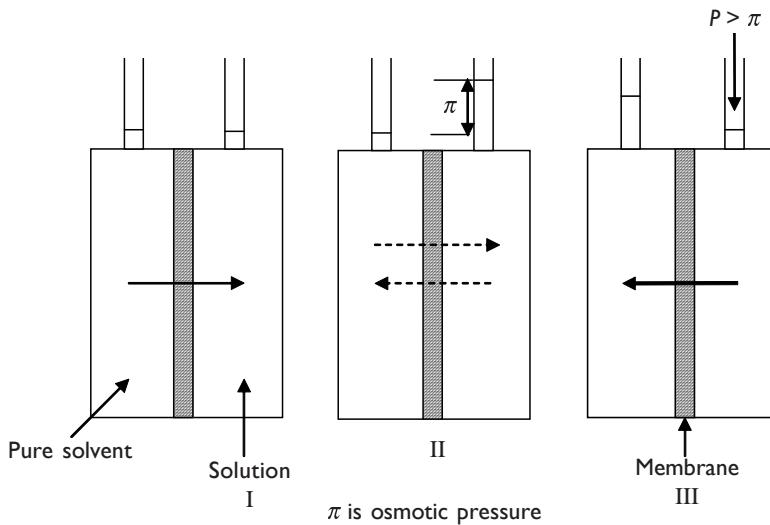


Figure 9.30 Osmosis and reverse osmosis.

Figure 9.31(a) illustrates the process taking place across the membrane which is considered to be thin film composite or asymmetric membrane.

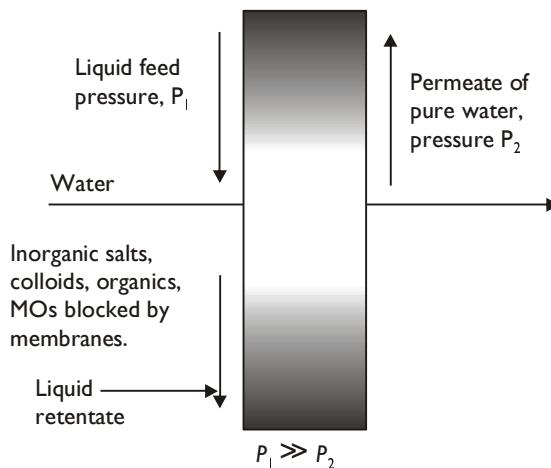


Figure 9.31(a) Thin film composite or asymmetric membrane for RO.

The feed is a liquid at a high pressure P_1 , containing solvent (water) and solubles, viz. inorganic salts, colloidal matter, etc. No sweep liquid is used, but the other side of the membrane is maintained at a much lower pressure P_2 . The membrane being perm selective, for the solvent, as acetate or aromatic polyamide can be employed.

In order to withstand the large pressure drops, the membrane must be thick for which composite structures or asymmetrical membranes that have a dense skin or layer on a thick porous support is used.

The products of RO are a permeate of almost pure solvent and a retentate of solvent depleted feed. However, a perfect separation between the solvent and the solute is not achieved since only a fraction of the solvent in the feed is transferred to the permeate.

Concentrations and fluxes in reverse osmosis: This discussion gives an insight to the concentrations and the fluxes prevailing during the process of reverse osmosis.

A mathematical treatment is provided to gain a better understanding of the profiles both for the concentration and the flux, when a feed is given to the dense membranes as in RO.

Fluxes are represented by J terms, both with respect to the water (solvent) and the salt (solute). In addition to this, the C terms referring to the concentration of solute both in the feed and the permeate form an integral part of the discussion. The pressure differences are applied across the membrane as shown in Figure 9.31(b).

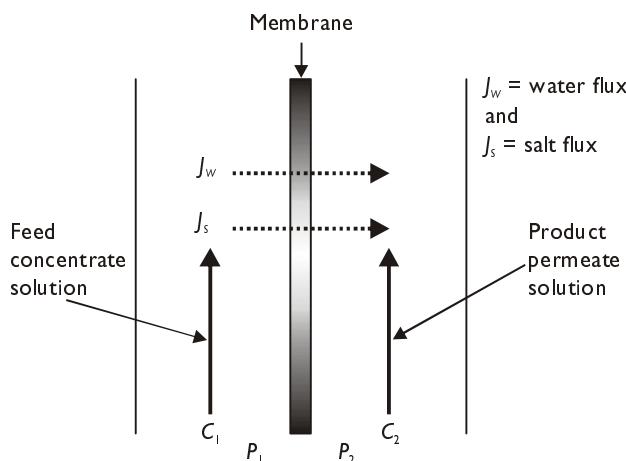


Figure 9.31(b) Concentrations and fluxes in RO process.

In the process of RO, dense membranes are used. Therefore, solution diffusion model applies.

Here in the schematic shown above, J_s is the salt or solute flux and J_w is the water or solvent flux through the membrane. The pressure differences applied on the two ends are P_1 and P_2 . Also the terms C_1 and C_2 shown are the feed concentrate solution concentration and product permeate solution concentration respectively. The water flux is given as

$$J_w = \frac{P_w}{L_m} (\Delta P - \Delta \pi) \quad (9.47)$$

where ΔP = the pressure difference across the membrane

$\Delta \pi$ = the osmotic pressure difference across the membrane

P_w = the solvent permeability; kg-solvent/sec-m-atm

L_m = the membrane thickness in m
and J_w = the flux; kg/m²-sec

The above expression can also be written as

$$J_w = A_w (\Delta P - \Delta\pi) \quad (9.48)$$

where $A_w = P_m/L_m$ and is the solvent permeability constant, kg-solvent/m²-sec-atm.

According to Eq. (9.48), at low applied pressure, when ΔP is lesser than $\Delta\pi$, water flows from the dilute side to the concentrated side by way of osmosis, while if $\Delta P = \Delta\pi$, there is absolutely no flow.

Further if $\Delta P > \Delta\pi$, there will be the water flow from concentrated side towards the dilute side which is in fact the process called reverse osmosis.

Below are defined the quantities ΔP and $\Delta\pi$ for more clarity.

ΔP is actually the hydrostatic pressure difference given as $\Delta P = P_1 - P_2$, where P_1 is the pressure exerted on the feed side and P_2 is the pressure on the permeate side.

$\Delta\pi$ is the osmotic pressure difference between the feed solution and product solution given as $\Delta\pi = \pi_1 - \pi_2$.

Similarly, the salt or solute flux J_s across the membrane is given as

$$J_s = \frac{D_s K_s}{L_m} (C_1 - C_2) \quad (9.49)$$

or can be written as

$$J_s = A_s (C_1 - C_2) \quad (9.50)$$

The terms in the above equations refer to the following:

D_s is diffusivity of solute in membrane m²/sec

K_s is C_m/C , the ratio of concentration of solute in the membrane to the concentration of solute in the solution. It is also as good as partition coefficient or distribution coefficient and L_m is the membrane thickness in metres.

From Eq. (9.50) it is seen that C_2 is usually lower than C_1 as the salt concentration in permeate is less. Therefore, we can write the above equation as

$$J_s = A_s (C_1) \quad (9.51)$$

and this is independent of P .

From the equations it is evident that the water flux is proportional to the applied pressure, but the salt flux is independent of pressure.

Selectivity of RO membranes: The selectivity 'R' of RO membranes is measured as salt rejection coefficient which describes the performance of membrane in rejecting the solutes or salts, finally giving the clear permeate.

It is given mathematically as

$$R = \left(1 - \frac{C_2}{C_1}\right) 100 \quad (9.52)$$

Similarly, the salt concentration on the permeate side can be related to the membrane fluxes as

$$C_2 = \frac{J_s \times \rho_w}{J_w} \quad (9.53)$$

where ρ_w is the density of water.

Combining all the expressions, we finally arrive at the expression for the rejection coefficient as given below.

$$R = \left(1 - \frac{\rho_w \cdot A_s}{A_w(\Delta P - \Delta \pi)} \right) 100 \quad (9.54)$$

Finally, the rejection coefficient is expressed in percentage.

Concentration polarization effects in reverse osmosis: The concentration polarization is particularly important on the feed side of the RO membrane than the permeate side.

The effect of concentration polarization on the performance of RO process is explained in the succeeding pages [Figure 9.31(c)].

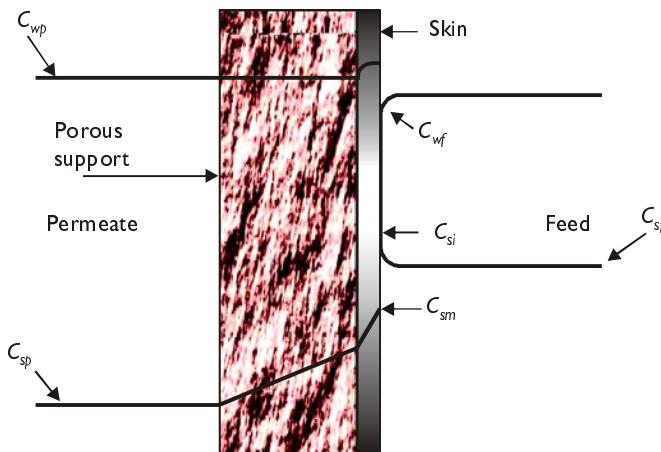


Figure 9.31(c) Effects of concentration polarization in RO.

In Figure 9.31(c), C_s refers to the concentration of salt and C_w the concentration of water in the solution to be processed.

On the feed side as the pressure applied is high, the activity of water is somewhat higher than the near pure water on the permeate side, thus providing the necessary driving force for the transport of water through the membrane.

The flux of water to the membrane carries with it the salt and is mainly due to the bulk flow. As salt cannot permeate the membrane, the concentration of the same in the liquid adjacent to the surface of the membrane, C_{si} is greater than that in the bulk of the feed C_{sf} . This difference

causes mass transfer of salt by diffusion from the membrane surface back to the bulk feed.

The back rate of salt diffusion depends upon the mass transfer coefficient for the film or boundary layer on the feed side. The lower the value of mass transfer coefficient, the higher is the value of C_{si} .

This C_{si} has a greater significance as it fixes the osmotic pressure and thus influences the driving force for water transport through the membrane.

In general, it is seen that due to the onset of concentration polarization in the RO, membranes develop fouling and overall the performance is affected.

The major applications of RO in wastewater management are as follows:

- Desalination of industrial wastewater after the secondary treatment
- Water recovery in a dye house effluent
- Recovery of water from domestic sewage for industrial use
- In pollution control, for the removal of certain hazardous organic pollutants present in wastewater.

9.7.2 Ultra-filtration

The process of ultra-filtration is operated in the range of 2–10 bar pressure. The separation process occurs across the membrane that discriminates solute molecules on the basis of their size. Figure 9.32 shows a flow diagram of ultra-filtration.

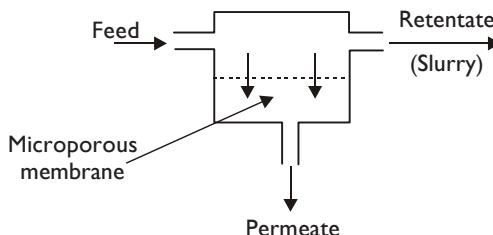


Figure 9.32 Flow diagram of ultra-filtration.

It is useful in the separation of high molecular weight products such as polymers, proteins and colloidal materials from low molecular weight solutes. It is also used in food industry to concentrate fruit juices. In dairy industry, the ultra-filtration method is used to recover whey proteins during cheese manufacture.

In pharmaceutical industry, it is used to concentrate cell-free fermentation broths containing complex products like monoclonal antibodies (MABs). This is even used to separate vaccines, enzymes, etc.

The method is said to be highly energy efficient and economical and assures a high degree of purity. The ultra-filtration membranes are made

from polysulphone or other polymers and is finely microporous and asymmetric.

The liquid flow through the membrane is by viscous flow through the pores due to the moderate applied pressure. The size range of the molecules is 10 \AA – $500/1000 \text{ \AA}$.

This process primarily is a size exclusion based on the pressure driven membrane separation process. The ultra-filtration membranes have pore size in the range of 10 – 1000 \AA and are capable of retaining the species in the molecular weight range of 500 – $500,000$ daltons.

Typical rejected solutes employing ultra-filtration include sugars, biomolecules, polymers, colloidal particles and high molecular weight organic substances depending upon their molecular weight, molecular size and also the shape.

The system design for ultra-filtration is shown below Figure 9.33(a). It is the simplest type of UF system and is a batch type.

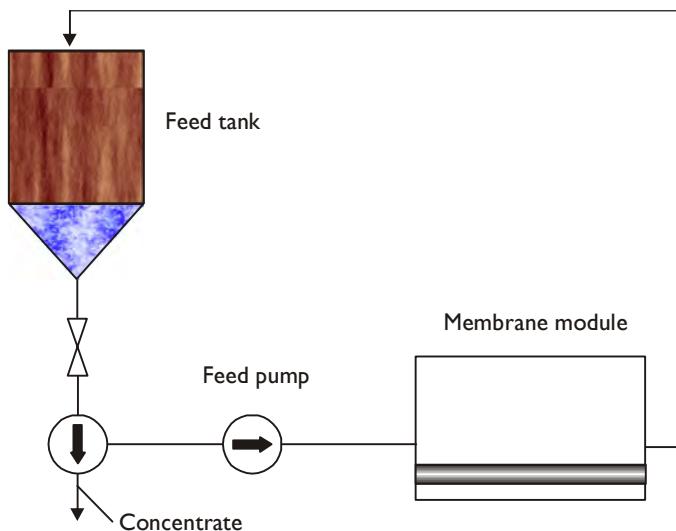


Figure 9.33(a) Batch ultra-filtration module.

It comprises a feed tank, a feed pump and a membrane module, that preferably works on the cross flow mechanism.

In this a limited volume of feed solution is circulated through a module at a high flow rate. The process continues until the required separation is achieved after which the concentrate solution is drained from the feed tank and the unit is ready for second batch of solution to be separated.

Batch processes are well-suited for small-scale operations common in biotechnology and pharmaceutical industries. Such systems no doubt can be adopted for a continuous use, but requires an automatic control system, being expensive and unreliable.

Continuous systems in ultra-filtration require modules which are to be arranged in series to get the separation required in a single pass, which are common. This is because high feed solution flow rates are required to control concentration polarization; a single pass process would not achieve the desired removal under such conditions. Solution velocities in UF modules are 5-10 times higher than in RO. For these reasons feed-and-bleed systems are commonly used in UF plants.

Concentration polarization and fouling in UF: An important factor in estimating the performance of UF membranes is the concentration polarization, which causes membrane fouling due to the deposition of retained colloidal and molecular material on the membrane surface.

Figure 9.33(b) depicts the fouling of a UF membrane.

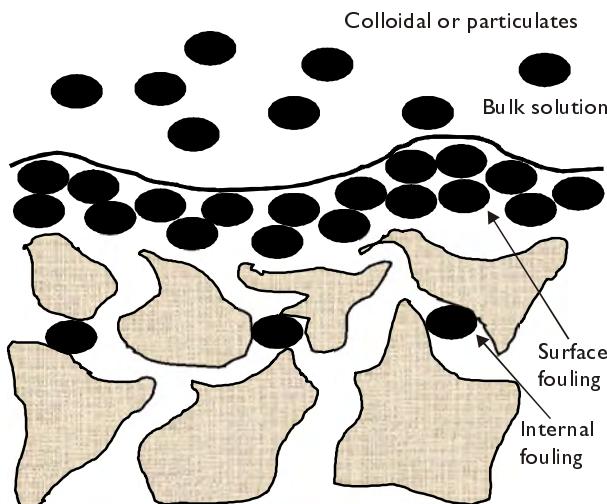


Figure 9.33(b) Fouling of a UF membrane.

Surface fouling is the deposition of solid material on the membrane surface that consolidates over time. This fouling layer can be controlled by high turbulence, regular cleaning and using hydrophilic or charged membranes to minimize the adhesion to the membrane surface.

Surface fouling is generally reversible while internal fouling is caused by the penetration of solid material into the membrane resulting in plugging of pores is generally irreversible.

These solutes or particles become so concentrated at the membrane surface that a gel layer is formed and becomes a secondary barrier to the flow of solutes through the membranes.

In the succeeding discussion is shown the formation of gel layer which is due to concentration polarization [Figure 9.33(c)].

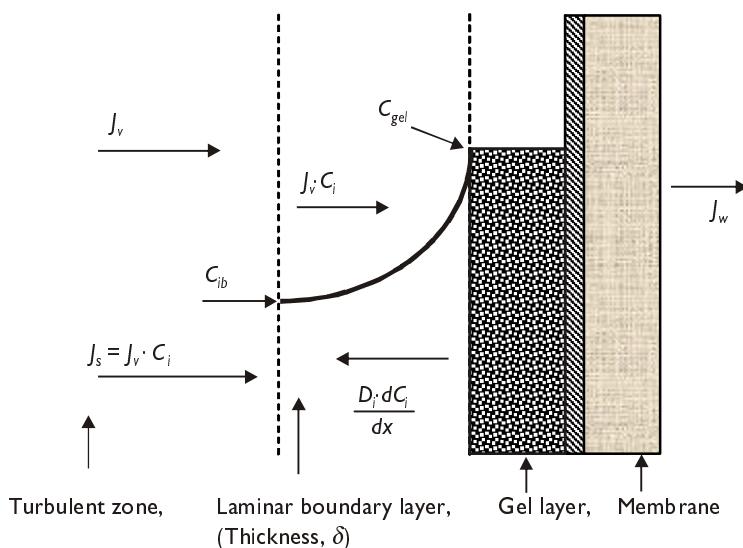


Figure 9.33(c) Formation of gel layer in UF.

At any point within the boundary layer, the convective flux of solute to the membrane surface is given by volume flux, J_v of the solution through the membrane multiplied by the concentration of the solute C_i .

At steady state, this convective flux within the laminar boundary layer is balanced by the diffusive flux of the retained solute in opposite direction as shown in Figure 9.33(c).

$$J_v C_i = D_i \frac{dC_i}{dx} \quad (9.55)$$

where D_i is the diffusivity of macromolecules viz. proteins in the boundary layer. Once a gel layer is formed the concentrations of solute at both the surfaces of the boundary layer are fixed. At one surface the concentration is the feed solution concentration C_{ib} and at the other it is the concentration at which the solute forms an insoluble layer of gel, i.e. C_{gel} .

Integrating Eq. (9.55) using the boundary conditions as at $x = 0$, $C = C_{ib}$ and at $x = \delta$, $C = C_{gel}$, we get

$$\left(\frac{J_v}{D_i} \right) \delta \int_0^\delta dx = \int_{C_{ib}}^{C_{gel}} \frac{dC_i}{C_i} \quad (9.56)$$

$$\frac{C_{gel}}{C_{ib}} = \exp \left(\frac{J_v \cdot \delta}{D_i} \right) \quad (9.57)$$

Using the above equations, we can calculate the concentration of gel layer formed in ultra-filtration process.

The major applications of ultra-filtration include the following:

- Selective concentration of milk constituents (fat and true protein)
- Concentration of fruit juices
- Separation, clarification and selective concentration of liquid foods
- Soya bean milk purification
- Animal food concentration
- Cheese making and treatment of whey
- Removal of colloidal particles from surface water
- Purification of drinking water; removal of bacteria, viruses, colour and odour
- Applicable in textile industry, pharmaceutical industry, leather industry, pesticide industry, etc.

9.7.3 Micro-filtration

Micro-filtration is also called *microporous filtration* or *cross-flow filtration* (Figure 9.34). Though it resembles conventional filtration, the filter medium being a microporous membrane, it is capable of filtering particulate matter or suspended solids rather than dissolved solute molecules.

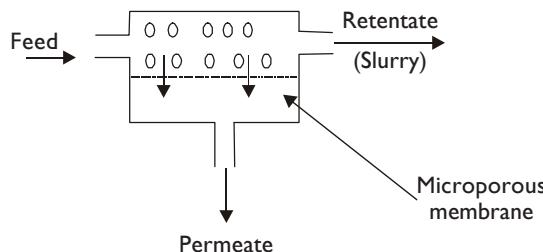


Figure 9.34 Flow diagram of micro-filtration.

The process is useful in harvesting microbial cells from fermentation broths and for separating blood cells and plasma from whole blood.

For micro-filtration, the three basic configurations used in the industry are plate and frame, spiral wound and hollow fibre modules. The commercially available membranes used in micro-filtration are synthetic organic polymer (polypropylene or poly tetrafluoro ethylene, PTFE) as well as inorganic (alumina or zirconia) membranes that have well-defined pores with high thermal and chemical stability.

Micro-filtration is used to separate species like bacteria, yeast, etc. from fermentation broth. It is also used in pharmaceutical and food industries.

The disadvantage of micro-filtration is frequent fouling of the membrane due to the solute deposition on membranes which necessitates purging of the unit periodically. Another disadvantage is that, concentrated slurry, and not a dry cake is obtained. That is why, micro-filtration is always followed by the conventional filtration or centrifugation so as to remove solids completely.

It is essentially a sterile filtration with pores ($0.1\text{--}10.0\ \mu$) so small that the microorganisms cannot pass through them. Microfiltration is a process that can be configured in two forms. One form is a *cross flow filtration* and the other is *dead end filtration*.

In the cross flow filtration, a fluid stream runs parallel to the membrane surface and there exists the pressure difference across the septum. Due to this some of the fluid passes through the membrane while the rest continues across it thereby cleaning the membrane. In the dead end filtration, also called *perpendicular filtration*, all of the fluid passes through the membrane and all of the particles that cannot pass through the pores of membrane are detained.

In addition to the applications mentioned earlier, microfiltration is also used in separating material of colloidal size and larger than the true solutions. Also, it is used to sterilize the solutions as they are prepared with pores smaller than $0.3\ \mu$, the diameter of *pseudomonas diminuta*, the smallest bacterium. With the significant features available with microfiltration process, the potential future applications may be:

1. In the concentration of biomass and separation of soluble products as a part of biotechnology industry.
2. Displacement of diatomaceous earth, an adsorbent.
3. Non-sewage waste treatment for removing intractable particles in oily fluids, aqueous waste that consists of particulates, toxins and stack gas.
4. In the separation of solvents from pigments as a part of application in a paint industry.

9.7.4 Dialysis

Dialysis is also called *hemodialysis* in the medical field, as it is required to purify the blood of the patients suffering from renal failure. It also has potential applications in the field of bioseparations, for example, separation of alcohol from beer.

Figure 9.35 shows a flow diagram of dialysis. Dialysis involves the separation of solutes by diffusion across the membrane from one liquid phase to another liquid phase, on the basis of molecular size and the molecular weight ($100 < \text{MW} < 500$).

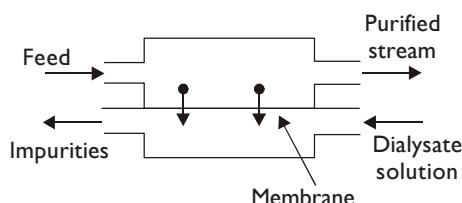


Figure 9.35 Flow diagram of dialysis.

The membranes in the dialysis process have non-porous characteristics of RO membranes. Of course, the process is very slow as compared to the UF and RO.

Dialysis is also used to remove salts from a protein solution.

Additional applications include:

1. Recovery of NaOH in cellulose processing.
2. Recovery of acids from metallurgical liquors.
3. Removal of products from a culture solution in fermentation, desalting of cheese whey solids, etc.

Electrodialysis: The method of electrodialysis, was developed for the desalination of brackish water into potable water. The process principle is as follows. It consists of a series of anion and cation exchange membranes arranged in an alternate pattern between an anode and a cathode and the positively-charged anions migrate toward the anode.

The cations pass easily through the negatively-charged anion-exchange membrane. Likewise, the negatively-charged anions pass through the anion-exchange membrane and are retained by the cation-exchange membrane.

The overall result is an ion concentration increase in the alternate compartments, while the other compartments simultaneously become depleted of ions. The depleted solution is generally referred to as *diluate* and the concentrated solution as *brine* or *concentrate*.

The electrodialysis unit consists of a stack of compartments formed by alternate cationic and anionic ion exchange membranes (Figure 9.36)

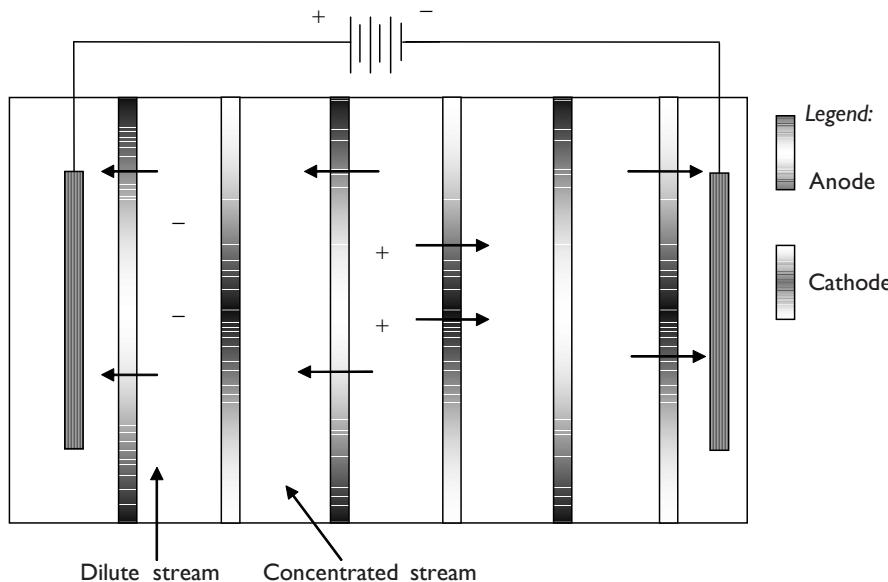


Figure 9.36 Electrodialysis unit.

In electrodialysis separation occurs due to the imposed potential difference across the ion selective cationic and anionic ion exchange membranes.

The driving force is an applied electric field that induces the current-driven flux across the compartments. This is comparatively an expensive process but efficient.

It is used widely in the production of potable water from sea water or brackish water, electroplating rinse recovery, desalting of cheese, whey, production of ultrapure water for semiconductor industry. In recent trends, it is also exploited for treating municipal effluent, industrial effluent like chemical pharmaceuticals, food and beverages, etc.

9.7.5 Nano-filtration

Nano-filtration is also a type of membrane separation process based on size, solution diffusion, etc. The pores of membranes of nano-filtration are smaller in size as compared to those of ultra-filtration membranes. Therefore, most of the organic compounds barring very low molecular linear chain organics are rejected while monovalent cations combined with monovalent anions from a compound or salts pass through the permeator.

The membranes employed in nano-filtration are low pressure RO membranes with very high rejections and high permeates of salt at low concentrations, but lose their selectivity at salt concentrations above 1000–2000 ppm of salt in water. For this reason, membranes are used to remove low levels of salt from already relatively clean water. They operate at very low pressure of 50–200 psig.

Major applications of nano-filtration are as follows:

1. Treatment of dairy wastewater
2. Decolouring of spent mineral acids
3. Removal of heavy metals from industrial effluents
4. In paint industry, dye industry, pulp industry, etc.

9.7.6 Electrophoresis

Electrophoresis is a method of separation of charged molecules applying an electric field. This technique of separation was first enunciated by A. Tiselius in the year 1937.

It refers to a technique which involves the movement of charged particles or ions in an electric field resulting in their migration towards the oppositely charged electrodes, i.e. when the charged molecules are placed in an electric field they migrate towards the oppositely charged electrodes.

This migration depends upon the net charges, size, shape and the applied electric potential.

It is more a technique that is normally used in the separation of charged biomolecules.

The basic principle of electrophoresis depends on the fact that, in an applied electric field, the drag force on a charged particle is exactly balanced by the electrostatic forces when the particle is moving with a constant terminal velocity.

This fact yields an expression suggesting the balancing of the forces which is given below.

$$qE = 3\pi\mu D_p U_t \quad (9.58)$$

or

$$U_t = \frac{qE}{3\pi\mu D_p} \quad (9.59)$$

or

$$U_t = \frac{qE}{F} \quad (9.60)$$

where $F = 3\pi\mu D_p$ and is a net fractional coefficient which is function of the mass and shape of the molecule, E is the electric field density, D_p is the diameter of the particle, μ is the viscosity of the fluid, U_t is the terminal settling velocity and q is the charge on the particle.

Molecules with a net positive charge (cations) move towards the negative cathode while those with a net negative charge (anions) migrate towards the positive anode.

Electrophoresis is a widely used analytical tool for the separation of biological molecules, such as plasma proteins, lipoproteins and immunoglobulins.

Types of electrophoresis: Originally, *moving boundary electrophoresis* as developed by Tiselius is a less frequently adopted technique for separation. It was in use for the separation of proteins. The same is described in the succeeding lines.

Moving boundary electrophoresis or flow electrophoresis comprises a U tube filled with protein solution to be separated and the buffer solution overlaid. With the application of electric field, the proteins move in solution depending on their sizes, mass and charges forming boundaries which can be later identified by way of refractive index method.

This identification serves the composition of proteins separated at different levels of pH as shown in Figure 9.37.

In general, since each protein molecule will have a different net charge q , the application of electric field will lead to different protein velocities.

Therefore, a protein sample can be separated into its individual components. It is also seen that the variation in pH values of the electrophoretic medium, will result in the changes in the velocities of protein components.

On the basis of the support medium, electrophoresis is categorized into different types. These are as follows:

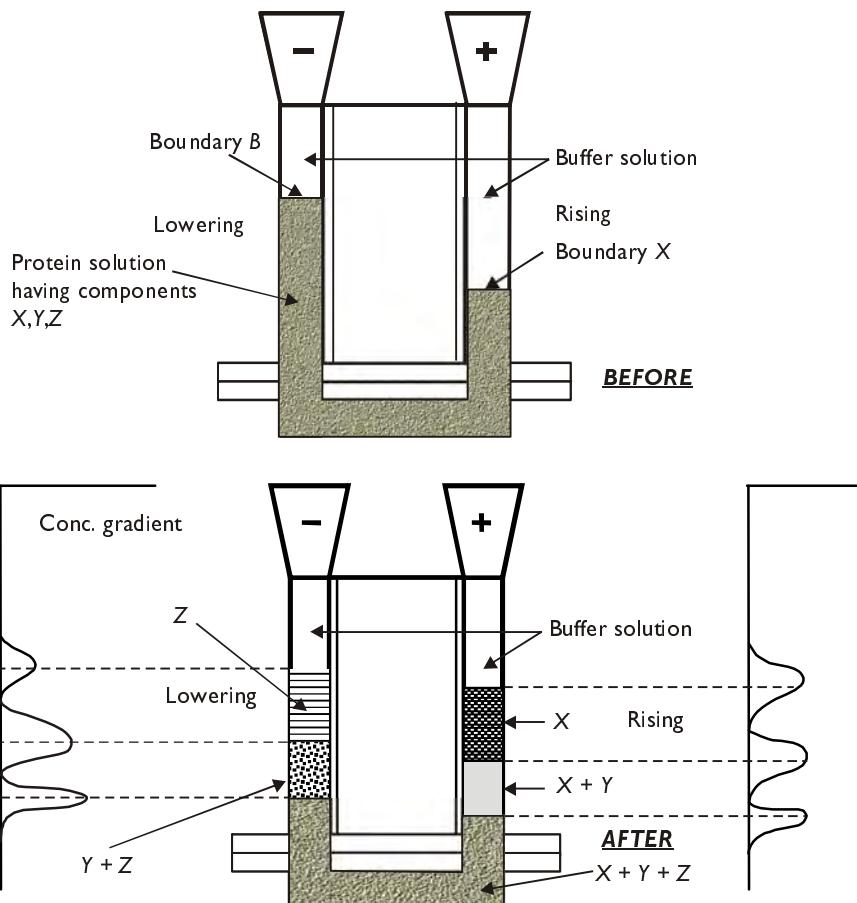


Figure 9.37 Location of protein components in an electrophoresis cell before and after application of electric field.

1. **Paper electrophoresis:** In this, a strip of paper usually Whatman filter paper or cellulose acetate paper is used as a support to separate the macromolecules on the basis of their varying sizes. The sample is applied on the strip of paper and wetted with the desired buffer solution. The ends of a strip are later dipped into the buffer reservoirs in which the electrodes are placed. Electric current is applied allowing the molecules to migrate for sufficient time.

Further, the spots developed on the filter paper are stained with a specific dye that colours the components to be detected. Later the spots are severed and dissolved in solvent for separation and analysis.

2. **Starch gel electrophoresis:** In this, starch is partially hydrolyzed in buffer to prepare a solution. It is then heated and cooled to get starch gel. This formed gel will act as a molecular sieve to separate molecules. The molecules of differing sizes migrate in gel and get separated.

3. **Immuno-electrofocussing:** Also referred to as immuno electrophoresis, is used for the separation of proteins based on the charge: mass ratio and their antigenicity. This is also employed for the analysis of complex mixtures of antigens and antibodies (Figure 9.38).

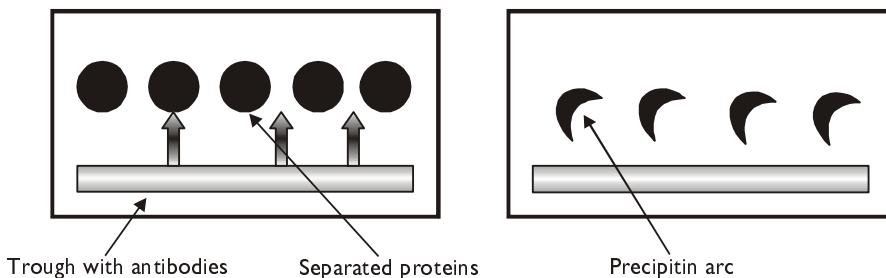


Figure 9.38 Diagrammatic representation of immuno electrophoresis.

The complex mixtures of biological samples, for instance the human serum are subjected to electrophoresis as shown above.

The antibody is then applied in a trough parallel to the electrophoretic separation. The antibodies diffuse and as and when they contact the antigens, precipitations are observed. These result in the formation of *precipitin bands* which can be later identified.

4. **Agarose gel electrophoresis:** It employs the agarose gels which are more porous than polyacrylamide gels. Also have comparatively larger pore size.

It is used in the separation of large sized macromolecules such as DNA. The diagrammatic representation is given in Figure 9.39.

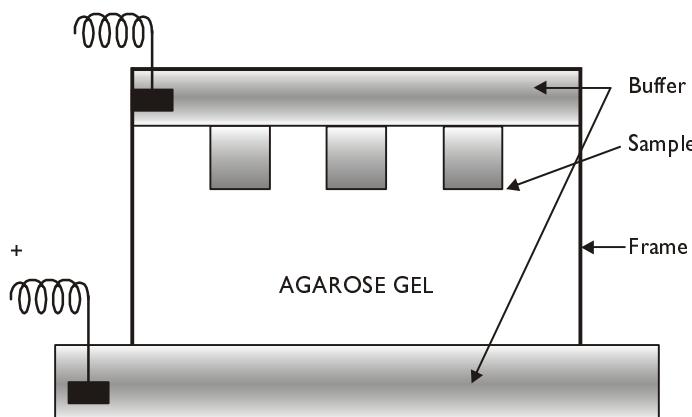


Figure 9.39 Agarose gel electrophoresis.

In the process, it is seen that at neutral pH, the negatively charged DNA migrates towards the anode after the application of electric field across the gel.

The resolution is much higher in this technique. The other gels in use are polyacrylamide, sodium dodecyl sulphate (SDS) etc.

5. Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE): In certain situations like proteins having similar charges to mass ratio, there is no separation. Therefore, in such cases, the proteins are treated first with an ionic detergent or surfactant called *sodium dodecyl sulphate* before the commencement and during the process.

In this the identical proteins are denatured by SDS resulting in their monomeric subunits. The polypeptide chains unwind, get opened and extended. On the basis of their mass but not the charge the molecule are separated.

The overall mechanism is depicted in Figure 9.40.

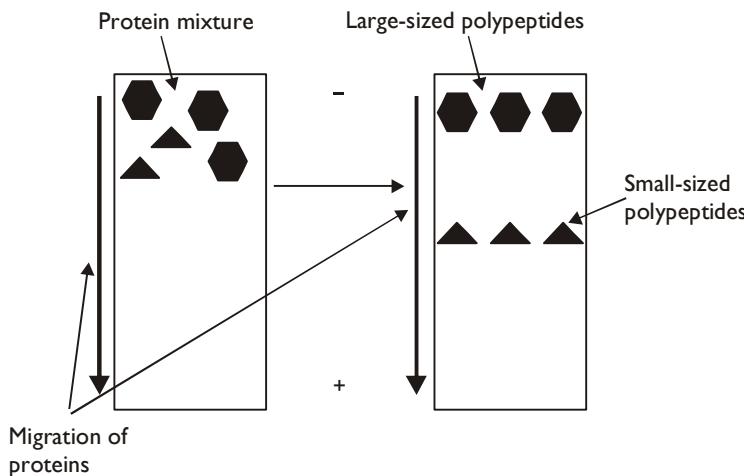


Figure 9.40 SDS-PAGE electrophoresis.

The proteins are denatured and have a negative charge with a uniform charge to mass ratio when treated with SDS reagent. This SDS reagent is a surfactant and reduces the surface tension of the components in question.

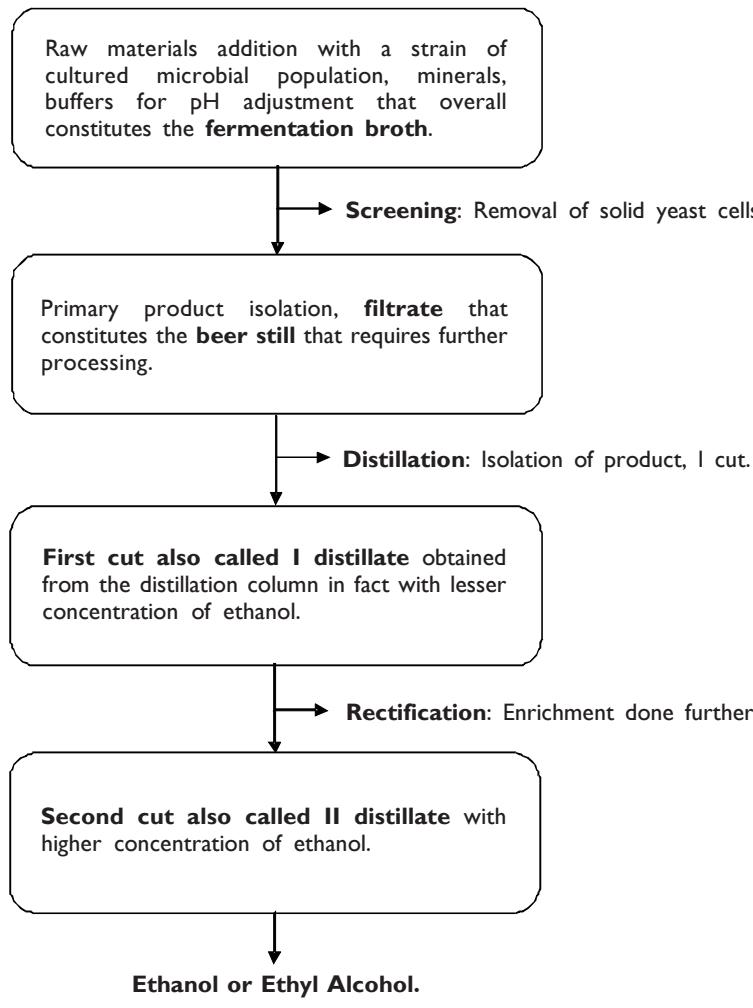
Proteins migrate towards anode at alkaline pH through PAGE gel during the process. The smaller peptides move faster followed by the larger polypeptides.

Therefore, the inherent charge on proteins is damped in SDS-PAGE. Consequently, the separation is based on the size.

9.8 CASE STUDIES

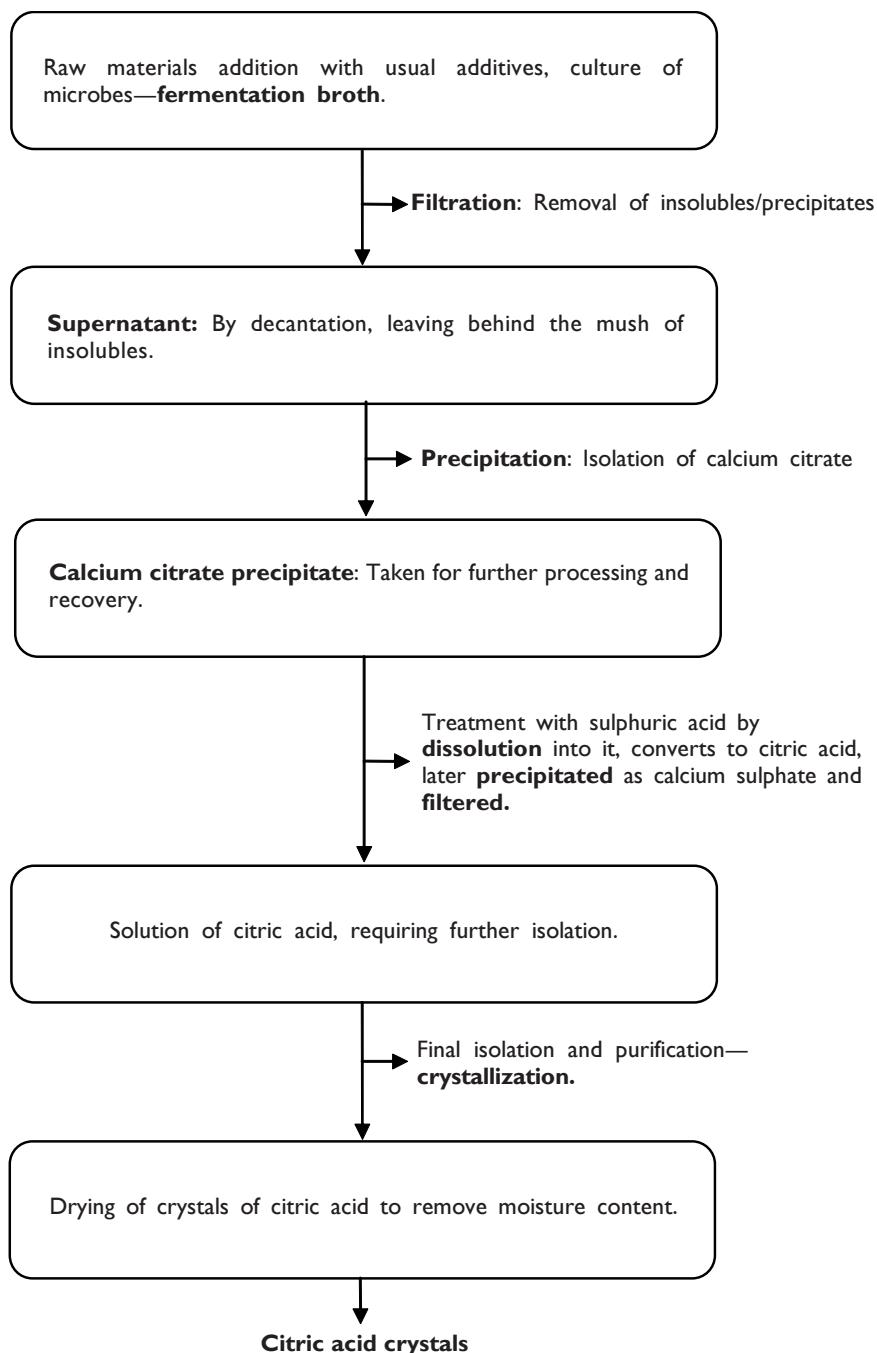
Here are a few case studies that will help to understand the complexities of the separation processes and the features of the steps involved in them.

- **Ethanol fermentation**



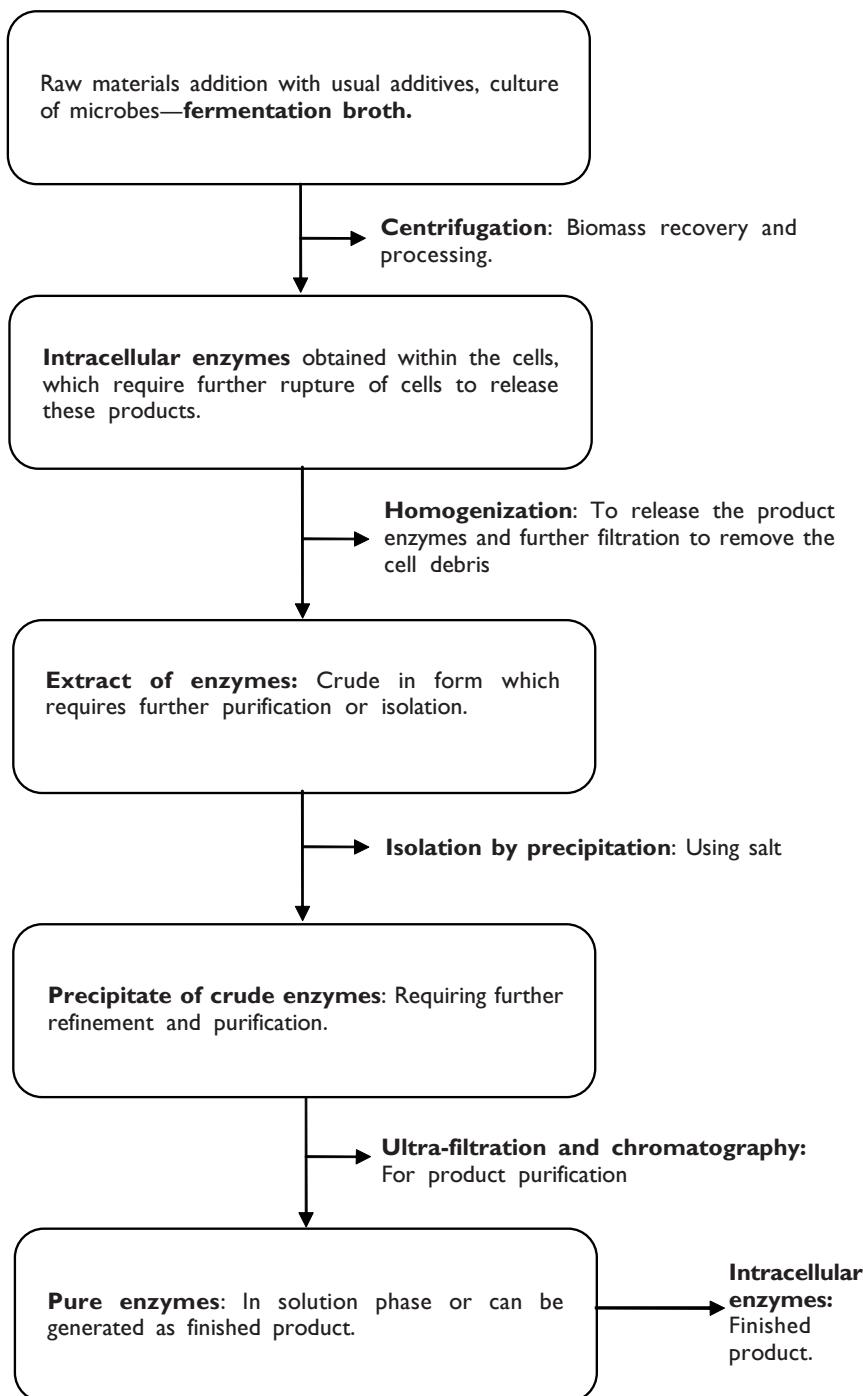
(a) Downstream processing in ethanol fermentation.

- Manufacture of citric acid



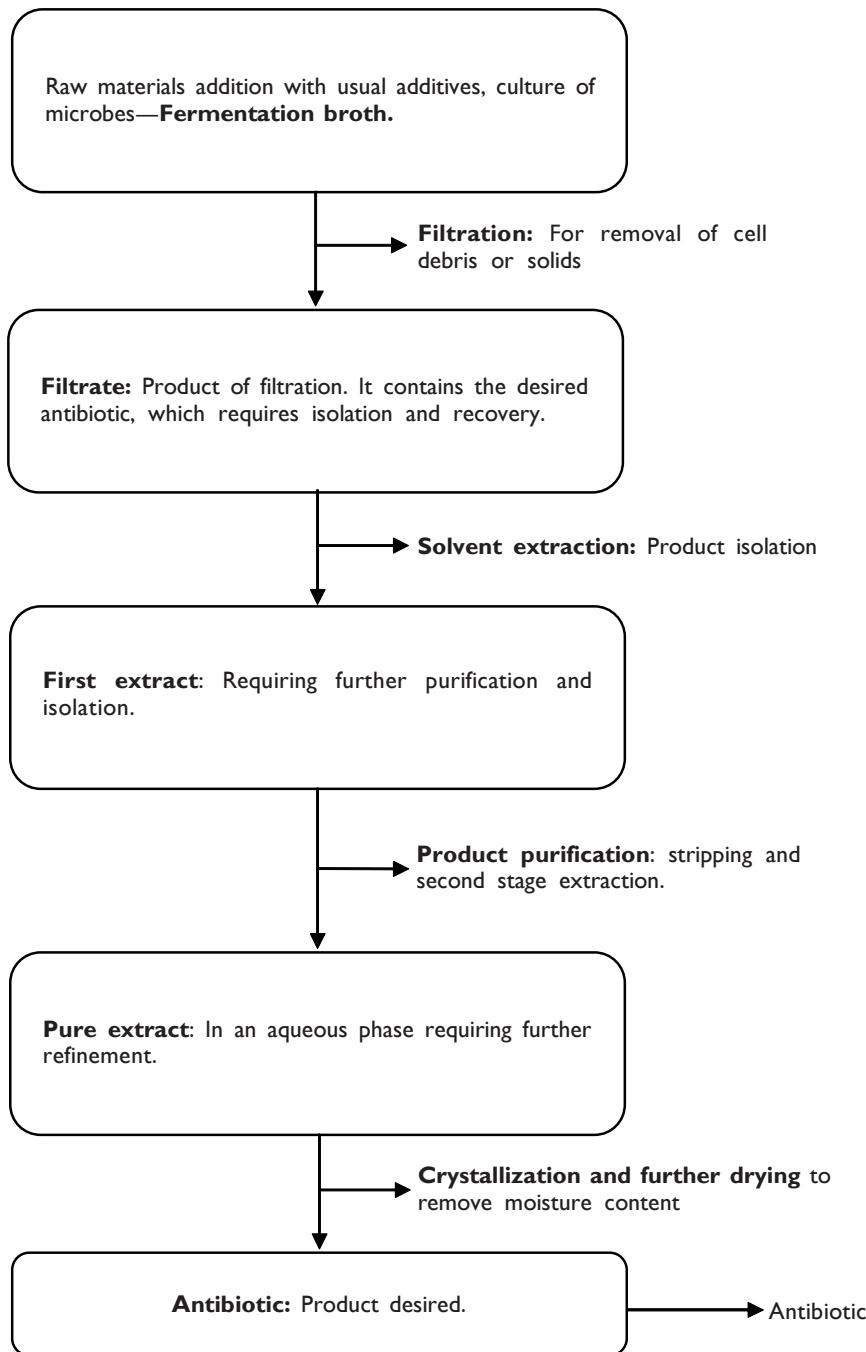
(b) Downstream processing in citric acid recovery.

- Intracellular enzymes production and recovery



(c) Downstream processing and recovery for intracellular enzymes.

- Antibiotic production and recovery



(d) Downstream processing and recovery in antibiotic manufacture.

SUMMARY

In this chapter, we learnt the following:

- The recovery and purification of products at the end of the fermentation process is an essential step.
- The product separation can be accomplished either integrated with or next to the fermentation step.
- Separation of biomass or other insoluble products, concentration or primary isolation of product and purification are the major categories of separation.
- For the product biomass, the separation strategy is relatively simple and involves filtration and centrifugation followed by coagulation/flocculation of the cells.
- For soluble products like antibiotics, enzymes, organic acids, etc., the separation strategy depends on whether the products are extracellular or intracellular.
- The separation strategy is simpler for extracellular products as compared to the intracellular products as cell disruption is not required.
- Proteins require a special strategy for separation such as isoelectric focusing or electrophoresis.
- Membrane, adsorptive, or extraction separation schemes can be used for the simultaneous separation of products during fermentation process.
- Affinity chromatography is one in which one or more components in the sample mixture to be analyzed show an affinity or attraction towards their counterparts. This makes the process overall highly specific and efficient compared to the others.
- Gradient elution involves the change of eluent composition during the development either continuously or stepwise, so that the partition coefficient values of each component are changed with respect to time.
- Crystallization operates at low temperatures, and certainly minimizes the threat of thermal denaturation and degradation of the so, called heat labile materials.
- The two models to understand the transport mechanisms in membranes are:
 - Capillary flow model or pore flow model and solution diffusion model.
 - Electrophoresis refers to a technique which involves the movement of charged particles or ions in an electric field resulting in their migration towards the oppositely charged electrodes, i.e. when the charged molecules are placed in an electric field they migrate towards the oppositely charged electrodes.

- Different types of electrophoresis are:
 1. Paper electrophoresis
 2. Starch gel electrophoresis
 3. Immuno-electrofocussing
 4. Agarose gel electrophoresis, and
 5. Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

EXERCISES

- 9.1** Discuss the importance of downstream processing in a bioprocess industry.
- 9.2** Explain in detail the physical methods of cell separation.
- 9.3** What is cell permeabilization? What is its significance?
- 9.4** Discuss the steps involved in the product isolation and purification of an enzyme.
- 9.5** What are the advantages of liquid-liquid extraction? Give an account of the theoretical principles and steps involved in the aqueous two-phase extraction of an enzyme.
- 9.6** What is supercritical fluid extraction? Discuss the principles and the characteristics of the same.
- 9.7** Enlist the advantages of supercritical fluid extraction.
- 9.8** Give a schematic diagram of a membrane separation unit and identify the components.
- 9.9** With principle explain in detail the following membrane separation processes:
 - (a) Electrodialysis
 - (b) Dialysis
 - (c) Ultra-filtration
 - (d) Reverse osmosis
 - (e) Micro-filtration.
- 9.10** Give an account of the steps involved in large scale precipitation of proteins.
- 9.11** Give a broader classification of chromatographic separation techniques.
- 9.12** Describe the chemical and enzymatic methods of cell disruption.
- 9.13** Write a note on the following:
 - (a) Thermolysis
 - (b) Osmotic shock
 - (c) Ultrasonication

- (d) Detergent solubilization
- (e) Membrane modules
- (f) Lyophilization
- (g) Sorption
- (h) Fractional precipitation

9.14 Enunciate on the following:

- i(i) Filtration theory and practice.
- (ii) Centrifugation theory and practice.

9.15 For constant pressure filtration of a suspension of cell culture, the following data was recorded.

Vol. of filtrate, $V \text{ m}^3 \times 10^{-3}$	2.04.5	6.0	8.5	10.0
Time, t in seconds	2060	115	180	270

It was found that the weight of solids per unit volume of filtrate was 50 kg/m^3 , filter medium has an area of 1 m^2 , the pressure drop being 2 bar and the viscosity of the filtrate was $1.2 \times 10^{-3} \text{ kg/m-s}$. On the basis of the above information, estimate the specific cake resistance and the filter medium resistance.

- 9.16** Explain the single stage and multistage solvent extraction processes.
- 9.17** Enlist the applications of supercritical fluid extraction processes.
- 9.18** With principle, theory and practice and applications, explain bioaffinity chromatography.
- 9.19** How regeneration of affinity adsorbents is achieved? Explain. Further, write on the merits of affinity chromatography.
- 9.20** Discuss on the gradient elution chromatography.
- 9.21** Enlist on the operational requirements and desirable attributes of membranes.
- 9.22** To explain the transport mechanisms in membranes what are the approaches or the models used? Explain.
- 9.23** Elaborate on the following:
 - (i) Electrophoresis and its principle
 - (ii) Types of electrophoresis.

Chapter 10

The Control of Microorganisms

The word ‘control’ refers to the reduction in numbers and/or the activity of total microbial flora. There are several reasons for controlling microorganisms. They are as follows:

1. To prevent the transmission of diseases and infection
2. To prevent the contamination by or the growth of undesirable organisms
3. To prevent the deterioration and spoilage of materials by organisms

Microorganisms can be removed, inhibited, or killed by various physical agents, physical processes, or chemical agents. A variety of techniques and agents are available; and each has its own limits of application. This chapter focuses on various aspects of controlling microorganisms, with the techniques and the concepts applied, so that the process upsets and contaminations are at best avoided.

At the end of the chapter is discussed the configurations of sterilization reactors that operate continually.

10.1 CONTROL FUNDAMENTALS

As we know that the term ‘death’ is defined as the irreversible loss of ability to reproduce. Viable organisms are capable of multiplying but dead organisms do not multiply or grow. The determination of death requires the laboratory techniques that indicate whether the growth occurs when a sample is inoculated into a suitable medium.

The failure of an organism in response to growth is an indication that the organism is no longer able to reproduce, and the failure to do so is the criterion of death. At the same time the response of the organism may differ in all media. For example, a suspension of *E. coli*, bacteria, exposed to heat

treatment may yield a greater number of survivors if a plating medium of trypticase soy agar is used in place of a medium containing the bile salts such as deoxycholate agar.

10.2 CONDITIONS INFLUENCING ANTIMICROBIAL ACTION

Microorganisms are not simple physical targets. There are many biological characteristics that influence the rate at which the microorganisms are killed or inactivated by various agents.

Many factors must be considered in the application of any physical or chemical agent used to inhibit or destroy microbial population. It is not that one agent can be effective in all the cases. This is because, it depends on several factors like the process situations, biological characteristics of the cells, and the environmental conditions that can influence the efficacy of the antimicrobial agents. Certain important factors are discussed as follows.

Environment: The environment medium or a material carrying the microorganisms with their physical and chemical properties can have a profound influence over the rate as well the efficacy of destruction of microbes. It is seen that the effectiveness of steam (thermal means) is far greater in acidic materials than the basic.

The capacity of an antimicrobial agent to penetrate will depend on the consistency of the material and the medium, be it aqueous or thick.

Generally, a very high concentration of carbohydrates in a medium will increase the thermal resistance to the microorganisms. Even the presence of extraneous organic matter significantly decreases the efficacy of an antimicrobial agent by inactivating it or protecting the microorganism from contact with it.

Microorganism type: The species of microorganisms differ in their susceptibility to physical and chemical agents. Spore-forming bacteria are more resistant to adverse conditions whereas the growing vegetative cells are much more susceptible to these adverse conditions.

Physiological state of cells: The physiological state also influences the susceptibility of the cells to an antimicrobial agent. The young, actively metabolizing cells are apt to be more easily destroyed than old, dormant cells in the case of an agent that causes damage through the interference with metabolism but non-growing cells would not be affected.

10.3 MODE OF ACTION OF ANTIMICROBIAL AGENTS

Many processes and the substances that use the antimicrobial agents manifest their activity in one or several ways. A great deal of research has been performed to determine the specific site of action of various agents. In

general, one may view the possible sites of action of an antimicrobial agent by recalling certain features of the microbial cell.

The manner in which the antimicrobial agents inhibit or totally destroy the microorganisms can be attributed to the following actions:

1. Cell wall is damaged or cell wall synthesis is inhibited.
2. The permeability of cytoplasmic membrane is altered.
3. Proteins and nucleic acids and their physical states are altered.
4. Enzyme action is inhibited.
5. Synthesis of protein and nucleic acid is totally prevented.

10.4 THE CONTROL OF MICROORGANISMS BY PHYSICAL AGENTS

The major physical agents or the processes used for the control of microorganisms are temperature (high and low), desiccation, osmotic pressure, radiation and filtration.

10.4.1 High Temperature

We know that the microorganisms can grow over a wide range of temperatures, from a very low temperature, characteristic of psychrophilic to a very high temperature, characteristic of thermophilic. Although each type has an optimum, minimum, and maximum growth temperatures, the temperatures above the maximum generally kill, while those below the minimum usually produce the stasis, a condition where there is an inhibition of metabolism, as such considered a preservative.

High temperatures combined with high moisture are considered to be one of the most effective methods of killing microorganisms. Here we shall try to differentiate moist heat and dry heat. The moist heat kills the microorganisms by coagulating their proteins and is much a rapid method. The dry heat destroys the microorganisms by oxidizing their chemical constituents. Examples that illustrate the differences between these two are as follows. Spores of *Clostridium botulinum* are killed at a temperature of 120°C in 4–20 minutes, whereas a time of 2 hours for the exposure to dry heat are required at the same temperature. Spores of *B. anthracis* are destroyed in 2–15 minutes by moist heat at 100°C, but with the dry heat at 150°C it requires 1–2 hours to achieve the same result.

The application of moist heat for inhibiting or destroying the microorganisms is given as follows.

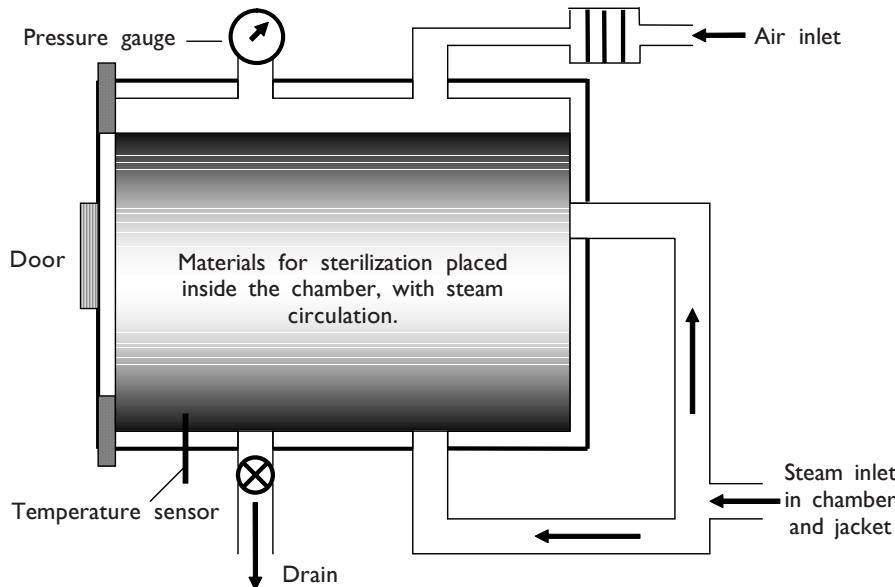
Steam under pressure: Heat in the form of saturated steam under pressure is the most practical and dependable agent for sterilization. Steam under pressure provides temperatures above those obtainable by boiling as shown in Table 10.1.

Table 10.1 Temperatures of steam under pressure

Steam pressure (lb/in ²)	Temperature (°C)
0	100.0
5	109.0
10	115.0
15	121.5
20	126.5

In addition to this, it has the advantages like rapid heating, penetration, and moisture in abundance that facilitates the coagulation of proteins.

The laboratory apparatus designed for this purpose is called an *autoclave* (Figure 10.1), which uses the steam under the regulated pressure. An autoclave also called *pressure steam sterilizer* is essentially a double-jacketed

**Figure 10.1** Autoclave.

steam chamber equipped with devices that permit the chamber to be filled with the saturated steam and maintained at the designated temperatures and pressure for any period of time. In the operation of an autoclave, it is absolutely essential to remove the air and replace it by steam. This is because, the presence of air will reduce the temperature obtained within the chamber substantially below that which would be realized if pure saturated steam were under the same pressure. In reality, it is not the pressure of the steam, but the temperature of the steam that kills the microorganisms.

Various media, discarded cultures, solutions and contaminated materials are the materials that are routinely sterilized by using an autoclave.

Generally, the operating pressure and the temperature of an autoclave are 15lb/in² and 121°C respectively.

Fractional sterilization: There are some materials that cannot be heated above 100°C without being damaged, for instance, some microbiological media, solutions of chemicals and biological materials. If, however, they can withstand the temperature of free-flowing steam (100°C), it is possible to sterilize them by the process called *fractional sterilization*, also known as *tyndallization*. It involves heating the materials at 100°C for three successive days with the incubation periods in between. Resistant spores germinate during the incubation periods; on subsequent exposure to heat, the vegetative cells will be destroyed. If the spores do not germinate during the incubation periods, the material will not be sterilized.

An autoclave can be used whenever free-flowing steam is employed.

Boiling water: It is not a method of certainty in killing microbes, because during the process the boiling water no doubt kills the microbes or the vegetative cells within a few minutes, but if the bacterial spores are formed, they can withstand the temperatures and the prevailing conditions for many hours. The practice of exposing the instruments for shorter periods of time in boiling water is more likely to bring about disinfection, a process of destruction of vegetative cells of disease-producing microbes, rather than the sterilization.

Pasteurization: Pasteurization refers to a controlled heat treatment that kills the microorganisms of certain types but does not destroy all organisms. That is why pasteurized milk is not sterilized milk. Milk, cream, alcoholic beverages, etc. are the substances that can be pasteurized.

Dry heat: Dry heat refers to hot air sterilization and is recommended whenever steam under pressure making direct or complete contact with the materials to be sterilized is unlikely or is undesirable. Substances like powders, oils, glass wares, Petri dishes, etc. can be sterilized by dry heat.

Incineration: Incineration refers to the destruction of microorganisms by burning. For example, the incineration of the used transfer needles is done by burning them in flame of a Bunsen burner.

Incineration is used in the destruction of carcasses, infected laboratory animals, and other infected materials to be disposed of. During the process care is taken to see that the fumes do not carry particulate matter that can contain the viable microorganisms into the atmosphere.

10.4.2 Low Temperatures

The rate of metabolic activity declines at temperatures below the optimum for growth, whereas at very low temperatures virtually the growth and the metabolic activity ceases.

This is the reason as to why low temperatures are favoured for the preservation of cultures as the microorganisms have a unique feature of surviving at extreme cold.

For instance, the agar slant cultures of some bacteria, yeasts, and moulds are normally stored for longer periods at a refrigeration temperature of about 4–7°C. Many bacteria and viruses are maintained at deep freeze temperatures that range from –20 to –70°C.

Microorganisms maintained at the freezing or the sub-freezing temperatures may be considered dormant; they perform no detectable metabolic activity. Of course, this condition is useful in the preservation of food materials. From the practical point of view, high temperatures may be considered as microbicidal and low temperatures as the microbistatic.

10.4.3 Desiccation

Desiccation refers to the cessation of the metabolic activity of a microbial cell. It is followed by the decline in the total viable population of the microorganisms. The process depends upon the following factors:

1. The microorganism type, i.e. whether filamentous or non-filamentous, bacterial origin, etc.
2. The material in which the organisms are dried.
3. The totality of drying process.
4. The physical conditions to which the dried organisms are exposed. The conditions are light, temperature, and humidity.

Lyophilization: It is also called *freeze drying*, in which the organisms are exposed to extreme dehydration in a frozen state and later sealed in vacuum. This makes the organism to stay in a dormant state in the desiccated cultures and remain viable for years.

10.4.4 Osmotic Pressure

When two different solutions with different concentrations, say pure water and salt solution, are separated by a semi-permeable polymeric membrane, there is always a flow of pure water through the membrane which is in the direction of increasing concentration. The trend is towards equalizing the concentration of solute on either side of the membrane. In the case of microbial cells, the solute concentrations are nearly 0.95%. Thus, if the cells are exposed to solutions with higher concentrations of solutes, water is withdrawn out of the cells. The cells shrink and the condition is called *plasmolysis*.

The reverse process, that is flow of water from a low solute concentration into the cells, causes the cells to swell and is called *plasmoptysis*. The pressure is built up in the cell as a result of this water intake and is called *osmotic pressure*.

In this case, the antimicrobial effect is the same as that caused by the process of desiccation. The changes are only found in the cytoplasmic membrane and particularly the shrinkage in the protoplast from the cell wall whereas the cell wall does not exhibit any distortions.

10.4.5 Radiations

In the context of radiations, the most significant radiations are the electromagnetic radiations, for example, light rays and X-rays. Gamma rays are more penetrating than any other rays.

When such radiations are passed through cells, they create free hydrogen radicals, hydroxyl radicals and some peroxides, which in turn can cause different types of intracellular damage. Due to this, the ionizing radiations are not that specific. This is called the method of cold sterilization. Hence, it can be used in the sterilization of temperature sensitive materials like food materials and pharmaceuticals.

Even sound waves are used in the process of sterilizing biological materials.

10.4.6 Filtration

Seitz filters having asbestos, Berkefeld filters having diatomaceous earth, Chamberland–Pasteur filters, etc. are some of the biological filters that are used in the removal of microorganisms from gases and liquids.

In these devices it is not a mere ordinary filtration that can be seen as mechanical sieves and porosity is not the only factor preventing the passage of organisms, but there are also other factors like the electric charge of the filter, the electric charge carried by the organisms, and the nature of the fluid being filtered that can influence the efficiency of the filtration process.

Membrane filters are used more recently and extensively both in the laboratory and in the industry to sterilize materials. It is customary to force the fluid through the filter by applying a negative pressure to the filter flask by use of a vacuum or the water pump or to impose a positive pressure above the fluid in the filter chamber, thus forcing it through.

On the completion of the filtration, precautions are taken to prevent the contamination of the filtered material when it is transferred to other containers.

Of late, more advanced filters are developed called *High Efficiency Particulate Air* (HEPA) filters that produce dust and bacteria free air.

Applications of physical agents for the control of microorganisms are summarized in Table 10.2.

10.5 THE CONTROL OF MICROORGANISMS BY CHEMICAL AGENTS

Most of the chemical compounds have the ability of inhibiting the growth and the metabolism of microorganisms and finally kill them. In many different situations various commercial products are available to be used as antimicrobial agents. For example, solutions of some chemical compounds are used to reduce the microbial flora of the oral cavity and to reduce the microbial flora in the dust of hospital floors.

Table 10.2 Applications of physical agents for the control of microorganisms

<i>Method/Device used</i>	<i>Recommended usage</i>	<i>Limitations</i>
Using moist heat: Autoclave is the device.	For sterilizing the following: Utensils, surgicals, culture media and other liquids.	Cannot be used for heat-labile or sensitive materials and is found to be ineffective against the organisms inside the materials impervious to steam.
Saturated steam or using boiling water	Used to sanitize beddings, dishes and clothings. Also used to destroy non-spore forming bacteria.	Not reliable as it cannot be assured in terms of sterilization by mere exposure.
Using dryheat: Hot air oven is the device.	To sterilize the following: Glass, sharp instruments, metals, etc. In general, those materials which are damaged by moisture.	Cannot be used for heat sensitive materials and exposed for a long duration.
Incineration: Incinerators are the devices	To dispose of the objects which are contaminated and cannot be reused.	Potential threat to air pollution.
Using radiations: Use of ultraviolet light	To disinfect surfaces from microorganisms and is control of air-borne infections.	Penetrability is low. Causes irritation to eyes and skin.
Use of X-rays, gamma rays, etc.	To sterilize heat-labile materials, surgicals, etc.	Proven to be an expensive process and requires special facilities for use.
Using filtration: Use of membrane filters	Used in sterilization of heat-labile materials; biologicals and also used to disinfect air.	Expensive process. Fluids should be free of suspended matter as it can clog the membranes.
Use of fibre glass filter (HEPA)	Used in the process of air disinfection.	Very expensive process.
Using physical cleaning: Use of washing process employing ultrasonic waves	Used to purify delicate instruments and is an effective method.	Expensive process as it necessitates the use of other processes adjunct to it.

No single agent is the best for any and all purposes. As a result, several classes of chemical compounds and substances have been identified that have destructive effects in terms of their suitability for practical applications.

10.5.1 Characteristics of an Antimicrobial Chemical Agent

Any antimicrobial chemical agent should have the following characteristics:

Antimicrobial activity: The antimicrobial activity refers to the capacity of the substance to kill or inhibit microorganisms, and of course is the first requirement. The chemical at a low concentration should have a broad spectrum of antimicrobial activity.

Solubility: For effective use, the substance used should be readily soluble in water or any other solvent to the maximum extent.

Stability: An antimicrobial chemical agent should be stable in the sense that it should have minimum changes upon standing and should not exhibit the significant loss of germicidal action.

Non-toxicity to humans and other animals: Ideally speaking, the compounds should be toxic or lethal to microbes and non-injurious to humans and others.

Capacity to penetrate: Unless the substance can penetrate through surface, its action is limited to solely to the site of application. Of course, sometimes the surface action is all that is required.

Non-corroding and non-staining: The chemical agent should not rust, disfigure the metals, nor stain or damage the fabrics.

Detergent action: The chemical agent also should work as a cleaning agent or as good as a disinfectant.

Deodorizing capacity: Deodorizing while disinfecting is a desirable attribute. Ideally, the disinfectant itself should be odourless or should have a pleasant smell.

Toxicity to microbes at room or body temperature: The chemical agent should exhibit its action at room temperature so that the necessity of rising temperature should not arise.

Homogeneity: The preparation should be uniform in composition so that the active ingredients are present in each application. Pure chemicals are uniform, but the mixtures may lack in homogeneity.

10.5.2 Definitions of Terms

Some of the terms that are used in describing the mechanism to control microbes are as follows:

Sterilization: Sterilization is the process of destroying microbes of all forms. A sterile object, in the microbiological sense, is free of all microbes.

The terms 'sterile', 'sterility', and 'sterilization' refer to the complete destruction or the absence of all forms of microbes and should not be used in the relative sense, i.e. more sterile or less sterile. Because once sterilized refers to the total absence of all forms of microorganisms.

Disinfection and disinfectant: Disinfection is the process of making clean and free from infection. A disinfectant refers to the use of an agent that kills

the growing forms but not the resistant spore forms of disease-producing microbes. Disinfectants are normally used on inanimate objects.

Antiseptic: An antiseptic is a substance that opposes the sepsis. It prevents the growth or action of microbes either by destroying them or by inhibiting their growth and metabolism.

Sanitizer: A sanitizer refers to an agent that reduces microbial population to safe levels as judged by the public health requirements. It usually kills growing bacteria to the extent of about 99.9 per cent. Sanitizers are usually applied to inanimate objects. They are also applied in daily care of equipment and utensils in dairies, food plants, restaurants, etc. Sanitization implies with the sanitary condition while disinfection does not necessarily imply.

Germicide (Microbicide): A germicide is an agent that kills the growing forms but not necessarily the resistant spore forms of germs. In practical sense, a germicide is the same as a disinfectant. For any application, germicides are commonly used for killing all kinds of germs.

Bactericide: A bactericide is an agent that kills bacteria.

Fungicide: A fungicide is an agent that kills fungi.

Virucide: A virucide is an agent that kills viruses.

Sporicide: A sporicide is an agent that kills spores.

Bacteriostasis: Bacteriostasis refers to a condition in which the growth of bacteria is prevented.

The agents that have in common the ability to inhibit the growth of microbes are collectively called *microbistatic agents*.

Antimicrobial agent: An antimicrobial agent is the one that interferes with the growth and metabolism of microbes. There are certain antimicrobial agents that are used to treat infection and are called *chemotherapeutic agents*.

10.5.3 Selection of a Chemical Agent for Practical Applications

The major factors that need to be assessed in the process of selecting the most appropriate chemical agent for the practical purposes are as follows:

- Nature of the material to be treated
- Type of microorganisms
- Environmental conditions

Nature of material to be treated: Any specific chemical used to disinfect contaminated utensils might be quite unsatisfactory from the point of view of safety to humans. The chemical may cause serious injuries to the skin and its cells. As a result, the substance selected must not only be compatible with the material to which it is applied, but also be safe for handling by humans.

Type of microorganisms: The differences in action also exist between the strains of the same species. Therefore, the agent selected must be known to be effective against the type of the organism to be destroyed.

Environmental conditions: The successful application of an antimicrobial agent requires an understanding of the influence of the conditions like pH, temperature, time, concentration and the presence of an extraneous organic material on the particular agent, so that it can be employed under the most favourable circumstances.

10.5.4 Major Groups of Antimicrobial Agents and Their Applications

The following are the major groups of antimicrobial agents:

- Phenol and phenolic compounds
- Alcohols
- Halogens
- Heavy metals and their compounds
- Dyes
- Detergents
- Quaternary ammonium compounds
- Aldehydes
- Gaseous agents

Table 10.3 lists the applications of antimicrobial agents.

Table 10.3 Applications of antimicrobial agents

<i>Chemical agent</i>	<i>Recommended usage</i>	<i>Limitations</i>
Phenol and its compounds	Used in general as disinfectant	It has only a limited antimicrobial activity.
Alcohols: ethyl and isopropyl	Used as an antiseptic to treat wounds	Only as an antiseptic.
Iodine	Used as skin disinfectant.	It is found to be irritating to mucous membranes.
Chlorine	Used to disinfect potable water	It imparts objectionable odour and taste to water.
Glutaraldehyde	Used as a fumigant and also in sterilization of instruments	It is said to have a limited stability, i.e. unstable.
Formaldehyde	Used as a fumigant and also in sterilization of instruments	It is highly corrosive and the permeability properties are poor.
Ethylene oxide	Used to sterilize heat-labile materials	It is flammable and use in purer form is dangerous as it explodes.
Mercurials	Used to treat skin inflammation and used as a skin disinfectant	Very slow in its action but toxic.
Silver nitrate	Used to treat burns and scalds	Possibly it produces irritation when applied on skin
Quaternaries	Used as a skin disinfectant	It does not kill spores, i.e. non-sporicidal.

10.6 MATERIALS AND METHODS OF STERILIZATION

Table 10.4 lists materials and methods of sterilization.

Table 10.4 Materials and methods of sterilization

<i>Material</i>	<i>Method of sterilization or disinfection</i>
Metallic inoculating wires	By using red heat or Bunsen burner
Soiled dressings, beddings, animal carcasses called infective materials	By high temperature burning called incineration
Glass ware, oily fluids and powders	By using hot air oven, i.e. dry heat for 1 hour at a temperature of 180°C
Serum, vaccines and body fluid	By using water bath (for serum and body fluids) for 1 hour and 56°C temperature and vaccine bath (for vaccines) at 60°C for 1 hour duration
Culture media	Using autoclave (moist heat) at 121°C for 15 minutes
Culture media with egg, serum or sugar	Tyndallization process used, i.e. steaming for 30 minutes on consecutive days
Rubber, plastics and polythene tubes (disposable syringes)	Use of glutaraldehyde, but it is unstable. Also ethylene oxide gas can be used
Dressings, aprons, catheters and other surgicals except sharp ones	By using autoclave (moist heat). If sharp instruments are to be sterilized, use of 5% cresol is made besides autoclaving.
Woolen blankets, wool and hides	By using formaldehyde gas which is highly corrosive
Operation theatres	By using formaldehyde gas with a composition of 50 ml formalin per 100 ft ³ air space.
Polythene tubing, fabrics, heart-lung machine	By using ethylene oxide
Toxin serum antibiotic solutions	By using membrane filtration process specifically using Seitz filter.
Faeces, urine, vomitus, sputum.	By using bleaching powder, cresols, formalin and also by burning.

10.7 STERILIZATION REACTORS

We know that liquids (aqueous) are sterilized by any of the following methods:

- Radiations (UV rays, X rays)
- Sonication
- Filtration
- Heating
- Chemical addition

Of the above, heating and chemical addition methods are widely used in large-scale processes. Sensitive vitamins and complex molecules are sterilized by passage through porous membranes.

The requirements for destruction of viable microbes and viruses vary widely upon the material and its intended uses. For example, in the biological treatment of wastewater, using trickling filters or activated sludge process; microbes are naturally present in the process fluid and are responsible for desirable reactions. But in the manufacture of alcohol, vinegar and silage, inhibitors for the growth of unwanted microorganisms are rapidly evolved. Here also the sterilization requirements are not extreme. In the pasteurization of milk, all the microorganisms are not killed. In fact, severe treatment leads to degradation.

On the contrary, in pure culture fermentation, tissue culture, food products, and canning industry, stringent sterilization requirements are to be met with. Essentially all contaminants are to be excluded. But the degree of perfection may vary. The degree of perfection is based on the Contamination Probability (CP), an index used to measure the sterilization. If CP ($1-P_0$) is of 10^{-2} ; (where P_0 = extinction probability during sterilization), then it can be taken for batch fermentation. On a large scale, one batch out of hundred is lost due to contamination, it means, sterilization requirements though are stringent, they cannot be met totally. So $(1-P_0)$ is the fraction of sterilizations expected to fail to produce a contaminant-free product.

Sterilization can be taken up in batch or continuous fashion. For batch processes, the methods are as follows:

- Steam sparging
- Electrical heating
- Steam (Heat exchanger)
- Coolant (Heat exchanger)

The only merit that we find here is these are relatively simple processes.

The demerits are as follows:

- Time needed for heating and cooling is more.
- Extent of thermal damage to desirable compounds.
- Vitamins are destroyed by heating.
- Proteins denature at elevated temperatures.

Therefore the sterilization process should operate at the Highest Feasible Temperature and for the Shortest Time (HTST) that is necessary for the microbial death.

10.7.1 Continuous Sterilization Reactors

In order to overcome the limitations found in batch sterilizations, reactors are so designed that the process of sterilization is carried out in a continuous fashion.

Continuous sterilization, particularly at high temperature and short exposure time, can achieve complete sterilization with much less damage to the medium. Both the heat-up and cool-down periods are very fast.

Continuous sterilizations are easier to control and reduce down time in the fermentors. But there are certain limitations as well, for example dilution of the medium with steam injection and foaming.

The flow pattern inside the pipe is critical, since the fluid residence time near the wall can be different from that in the centre.

There are two types of continuous sterilization reactors—continuous injection type and continuous plate exchanger type (Figure 10.2).

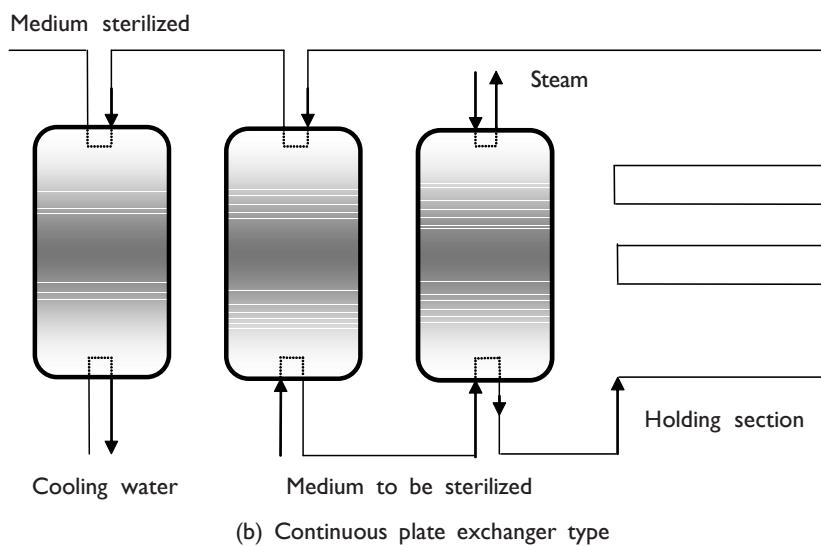
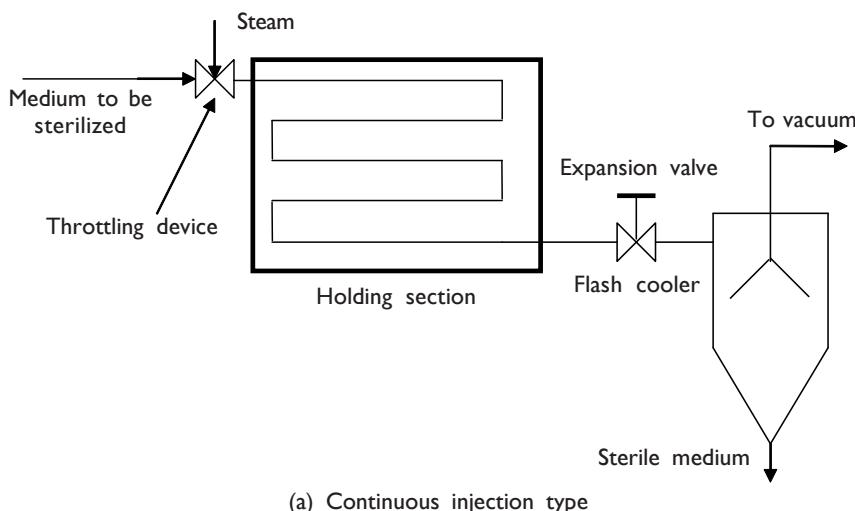


Figure 10.2 Continuous sterilization reactors.

In the continuous injection type, there is a process of direct steam injection. Raw medium needed to be sterilized is sent in along with the steam into a throttling device. It flows into the holding section that serves to indicate the time for sterilization. From the holding section it flows into the expansion valve where the sterilized material flashes into the drum giving the sterile medium at the down end of the flash cooler.

In the continuous plate exchanger type, there is an indirect heating process and features a high ratio of heat exchange surface to process volume. It provides relatively rapid heating and cooling so that the HTST conditions are realized.

SUMMARY

In this chapter, we learnt the following:

- Microorganisms can be removed, inhibited or killed by various physical agents, physical processes or chemical agents.
- The major physical agents or the processes used for the control of microorganisms are high temperature, low temperature, desiccation, osmotic pressure, radiations and filtration.
- Sterilization refers to the complete elimination of microorganisms while disinfection does not.
- The major groups of antimicrobial agents are phenols and phenolic compounds, dyes, halogens, detergents, heavy metals and their compounds aldehydes, etc.
- Large-scale bioreactors place heavy demand on processes to sterilize fluids entering the bioreactor.
- Liquid streams are thermally sterilized or filter sterilized. Steam sterilization is preferred, but the process must not damage the ability of the medium to support the growth.
- Continuous sterilization protects the medium components from degradation better than batch sterilization.
- Air streams are typically filter sterilized. Gases are sterilized by using surface filters which are of membrane cartridge type.
- There are two types of continuous sterilization reactors—continuous injection type and continuous plate exchanger type.

EXERCISES

10.1 Define the following terms:

- (a) Sterilization
- (b) Disinfection
- (c) Lyophilization
- (d) Desiccation
- (e) Sanitizer

- 10.2** What are the conditions that influence the antimicrobial action?
- 10.3** Explain the mode of action of antimicrobial agents.
- 10.4** Describe the control of microorganisms by using chemical agents.
- 10.5** Write about the control of microorganisms by physical agents.
- 10.6** Explain the process of fractional sterilization.
- 10.7** Write about the characteristics of antimicrobial chemical agents.
- 10.8** What are the major groups of chemical antimicrobial agents?
- 10.9** Write a short note on the following:
- (a) Dry heat
 - (b) Incineration
 - (c) Osmotic pressure
 - (d) Disinfectant
- 10.10** Discuss sterilization reactors in detail.

Appendix **A**

Process Kinetics and Reactor Analysis

A.1 REACTOR FUNDAMENTALS

A reactor is a volume defined by the specific boundaries and in which treatment processes take place. The treatment processes can be like wastewater treatment in the reactors.

The factors that are important in the wastewater treatment processes in a reactor are changes in the composition and the concentration of materials. These changes are due to the hydraulic transport of the materials into and out of the reactors and also due to the reactions inside the reactors.

The rates of change and the extent of the changes are important. Rates of changes are, in the sense, the rates at which the various components are removed from wastewater and the rate at which the biomass is produced in the reactor.

A.2 REACTION RATES

We know that chemical reactions are based on the following:

- Number of molecules reacting to form products
- Kinetics of the reaction

Of the above two, the kinetics of the reaction is more reliable and dependable.

The reaction rates can be expressed as follows:

The rate of reaction is proportional to the concentration of the reactants or the substrates. Therefore, the rate of reaction can be given as:

$$\text{Rate of reaction} = (\text{Concentration})^n$$

where n = the reaction order.

Taking log on both the sides, we can write

$$\log (\text{rate}) = n \log (\text{concentration}) \quad (\text{A.1})$$

Equation (A.1) can be used to interpret the rate and the order of a reaction.

A.3 REACTION ORDER

Using the $\log (\text{rate})$ values versus the $\log (\text{concentration})$ values, we can plot the graph as shown in Figure A.1.

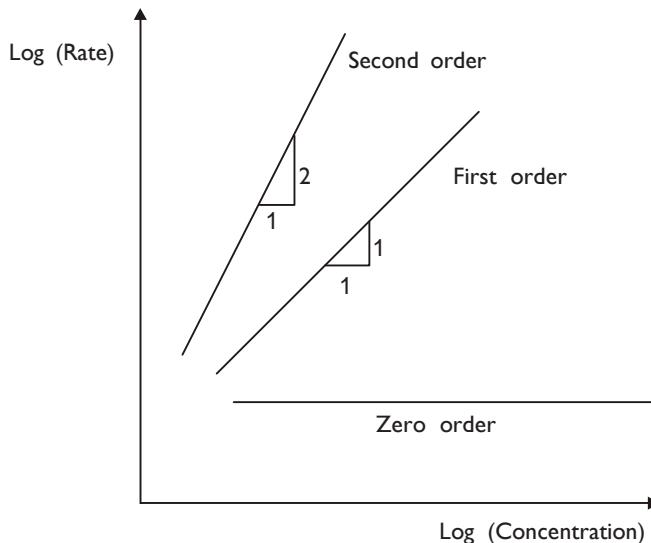
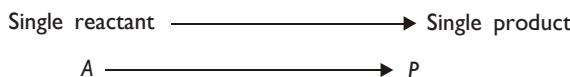


Figure A.1 Reaction order.

A.3.1 Zero Order Reactions

Referring to Figure A.1, the line is horizontal in the case of zero order reactions. The rate of reaction is concentration independent or the same at any reactant concentration.

Let us take an example, conversion of a single reactant to a single product.



Conversion following the zero order kinetics, the rate of disappearance of A is given as

$$-\frac{d[A]}{dt} = k[A]^0 = k$$

where k = reaction rate constant.

If C is the concentration of A at any time t , then the rate equation becomes

$$-\frac{dC}{dt} = k \quad (\text{A.2})$$

Rearranging and integrating, we get

$$-dC = kdt$$

or

$$dC = -kdt$$

$$C = -kt + \text{constant of integration} \quad (\text{A.3})$$

Let $C = C_0$ at $t = 0$. Eq. (A.3) becomes

$$C_0 = -k(0) + \text{constant of integration}.$$

Therefore, we get finally, after putting the value of C_0 in Eq. (A.3), as

$$C - C_0 = -kt \quad (\text{A.4})$$

The graphical representation of Eq. (A.4), i.e. plot of concentration vs time for a zero order reaction is shown in Figure A.2.

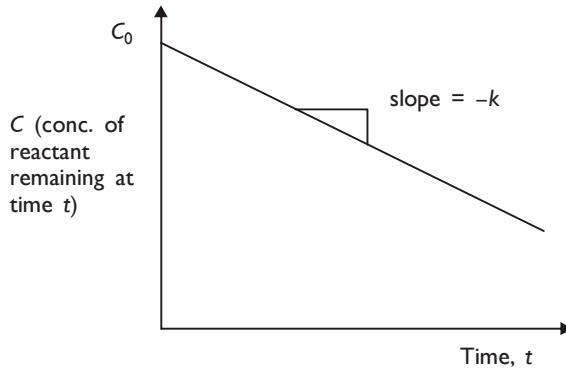


Figure A.2 Zero order reaction.

A.3.2 First Order Reactions

In the case of first order reactions, the rate of reaction is directly proportional to the concentration of a reactant, i.e.

$$\text{Rate} \propto (\text{concentration of a single reactant})$$

The rate is proportional to the concentration of a reactant and the reactant concentration changes with time. So a plot of reactant concentration at time t versus time t will not be a straight line as shown in Figure A.3.

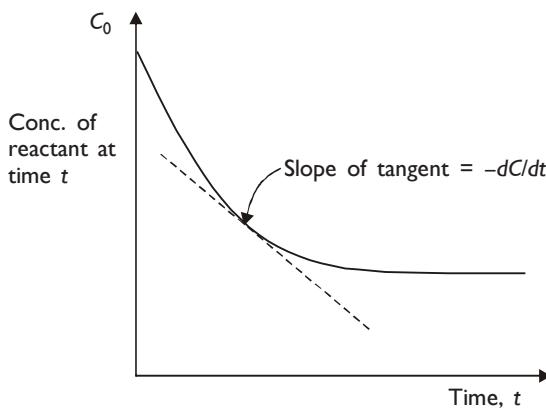
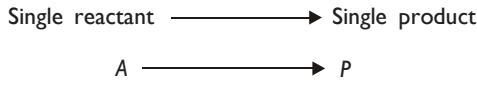


Figure A.3 First order reaction.

For example, consider the conversion of a single reactant to a single product.



If the first order kinetics is followed, then the rate of disappearance of A is given as

$$-\frac{d[A]}{dt} = k[A]'$$

If C is the concentration at any time t , then

$$\frac{-dC}{dt} = k(C)' = kC \quad (\text{A.5})$$

Unit of k , which is reaction rate, is per time (time⁻¹).

Integrating Eq. (A.5), we get

$$-\ln \left(\frac{C}{C_0} \right) = -kt$$

or

$$\ln \left(\frac{C_0}{C} \right) = kt$$

or

$$\log \left(\frac{C_0}{C} \right) = \frac{kt}{2.303}$$

Plotting $\log C$ versus time t , we get the graph as shown in Figure A.4.

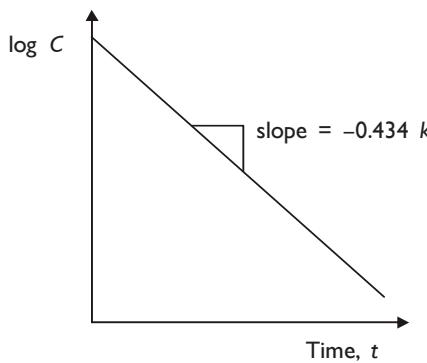


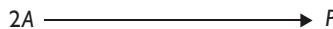
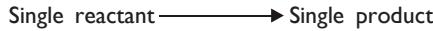
Figure A.4 Log C vs time t .

A.3.3 Second Order Reactions

In the case of second order reactions the rate of reaction is proportional to the square of concentration of reactants, i.e.

$$\text{Rate} \propto (\text{concentration})^2$$

For example, consider the conversion of a single reactant to a single product.



The rate of disappearance of A is

$$r = \frac{-d[A]}{dt} = k[A]^2$$

If C is the concentration of A at any time t , then

$$r = \frac{-dC}{dt} = kC^2 \quad (\text{A.6})$$

where k = reaction rate constant and is given as volume/mass-time.

Rearranging and integrating Eq. (A.6), we get

$$\begin{aligned} -\frac{dC}{C^2} &= kdt \\ \frac{1}{C} - \frac{1}{C_0} &= kt \end{aligned} \quad (\text{A.7})$$

Plotting the above equation, i.e. $1/C$ versus time t , we get the graph as shown in Figure A.5.

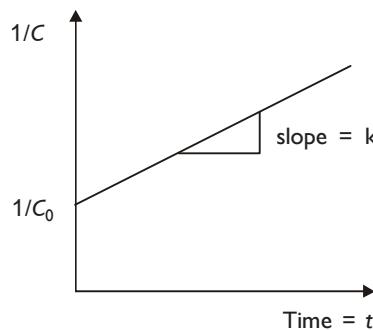


Figure A.5 Second order reaction.

A.4 REACTOR ANALYSIS

Reactor analysis refers to the combination of the rate law expressions with the hydraulic characteristics of a particular reactor system to develop the mathematical expressions that can predict the extent of the reaction and also in computing the volume of the reactor.

We come across normally different configurations of the reactors. They are as follows:

1. Completely Mixed Batch Reactor (CMBR)
2. Continuous Flow Stirred Tank Reactor (CFSTR)
3. Plug Flow Reactor (PFR)
4. Plug Flow with Dispersion Reactor (PFDR)

Here we shall discuss the first two. As a matter of fact the features and the rate expressions based on the material balances shall be considered.

A.4.1 Completely Mixed Batch Reactor

A completely mixed batch reactor is schematically shown in Figure A.6.

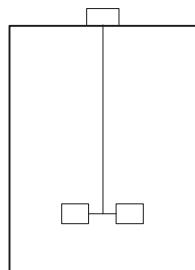


Figure A.6 Completely mixed batch reactor.

The features of a CMBR are as follows:

1. It forms a closed system.
2. The reactants are added to the empty vessel.
3. The reaction takes place accordingly.
4. The contents of the vessel are withdrawn after the reaction has proceeded to the desired degree of completion.
5. Composition in such systems varies with time. However, the composition can be regarded as uniform throughout the reactor at any time.
6. No flow into or out of the reactor during the reaction period.

For such systems the material balance can be written as shown for a specific reactant,

Rate of change in the mass of reactant A within the reactor = Rate of reaction of A within the reactor.

Let C represent the concentration of A at any time t and V represent the volume of the reactor.

The rate of reaction described by the first order kinetics is

$$r \propto \text{concentration of } A$$

or

$$r = kC_A$$

We can further write it as

$$V \left(\frac{dC_A}{dt} \right)_{\text{net}} = V \left(\frac{dC_A}{dt} \right)_{\text{reaction}} = V(kC_A)$$

or

$$\left(\frac{dC_A}{dt} \right)_{\text{net}} = \left(\frac{dC_A}{dt} \right)_{\text{reaction}}$$

or

$$\frac{dC}{dt} = kC$$

Integrating the above equation, we get the final form after rearrangement as

$$t = \frac{1}{k} \ln \left(\frac{C_0}{C_d} \right)$$

where C_0 is the initial concentration at time $t = 0$ and C_d is the desired concentration at $t = t$.

Note: The use of these batch reactors is seen in bench-scale experiments.

A.4.2 Continuous Flow Stirred Tank Reactors

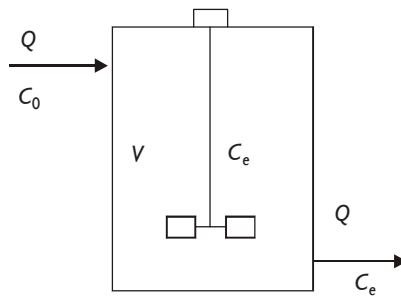


Figure A.7 Continuously flow stirred tank reactor.

The features of a CFSTR are as follows:

1. It operates at steady state conditions.
2. In this the properties do not change with time.
3. The reactants flow continuously into the reactor.
4. The products flow continuously out of the reactor.
5. Uniformity in concentration is maintained throughout the reactor volume.

Let Q be the volumetric flow rate in and out of the reactor, C_0 be the initial reactant concentration at the inlet or influent, and C_e be the desired concentration at the outlet or effluent.

For a CFSTR, the reactant concentration in the exit or effluent is the same as the reactant concentration at any point within the reactor volume.

A CFSTR represents the extreme case of back mixing or the longitudinal dispersion. The initial high driving force, i.e. the concentration of the reactant at the inlet is reduced to a final low driving force present at the reactor exit. The complete mixed batch reactor has a mean driving force somewhere between a high initial value and a low final value. This means that a CFSTR should have high volume than that of a complete mixed batch reactor to achieve the same amount of reaction.

The material balance in this case can be written as follows:

[Net rate of change in the mass of reactant A within the reactor]

$$= [\text{Rate of increase in the mass of } A \text{ due to its presence in the influent}] \\ - [\text{Rate of decrease in the mass of } A \text{ due to the removal in the effluent}] \\ - [\text{Rate of decrease in the mass of } A \text{ due to reaction in the reactor}]$$

Mathematically,

$$V \left(\frac{dC}{dt} \right)_{\text{net}} = QC_0 - QC_e - V \left(\frac{dC}{dt} \right)_{\text{reaction}}$$

or

$$V \left(\frac{dC}{dt} \right)_{\text{net}} = QC_0 - QC_e - V k C_e \quad (\text{A.8})$$

At steady state, $\left(\frac{dC}{dt} \right)_{\text{net}} = 0$

Therefore, Eq. (A.8) becomes

$$0 = QC_0 - QC_e - V k C_e \quad (\text{A.9})$$

Divide the above equation by Q to get

$$C_e - C_0 = \frac{V \cdot k C_e}{Q} \quad (\text{A.10})$$

Divide the above equation by C_e and arrange to get

$$\frac{C_e}{C_0} = \frac{1}{1 + \left(\frac{V}{Q} \right) k}$$

where (V/Q) is called nominal hydraulic retention time in CFSTR, i.e. t_{CFSTR} .

Therefore,

$$t_{\text{CFSTR}} = \frac{1}{k} \left(\frac{C_0}{C_e} - 1 \right) \quad (\text{A.11})$$

CFSTRs in series: Two CFSTRs connected in series and are considered to be of equal volumes (Figure A.8).

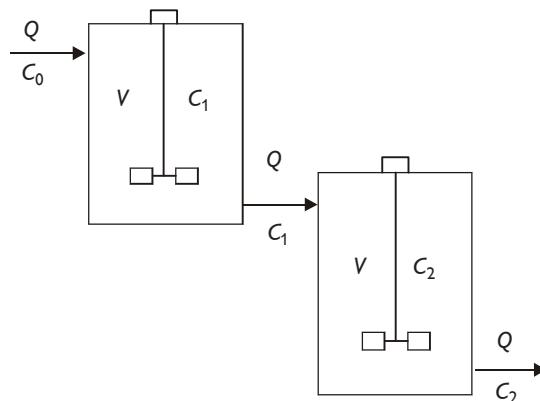


Figure A.8 CFSTRs in series.

$$\text{First reactor: } \frac{C_1}{C_0} = \frac{1}{1 + kt_{\text{CFSTR}}} \quad (\text{A.12})$$

$$\text{Second reactor: } \frac{C_2}{C_1} = \frac{1}{1 + kt_{\text{CFSTR}}} \quad (\text{A.13})$$

Multiply Eqs. (A.12) and (A.13) to get

$$\frac{C_2}{C_0} = \left(\frac{1}{1 + kt_{\text{CFSTR}}} \right)^{n=2} \quad (\text{A.14})$$

Therefore, for n number of equal size reactors in series, we can write

$$\frac{C_n}{C_0} = \left(\frac{1}{1 + kt_{\text{CFSTR}}} \right)^n$$

or

$$nt_{\text{CFSTR}} = \frac{n}{k} \left[\left(\frac{C_0}{C_n} \right)^{1/n} - 1 \right] \quad (\text{A.15})$$

Appendix **B**

Bioenergetics

Glycolysis, the process of breakdown of glucose for the production of pyruvic acid using the path ways, and the Krebs cycle are some of the processes which are part of bioenergetics.

Bioenergetics deals with energy-related processes in organisms. In order to obtain kinetic energy, all organisms carry out catabolic processes in which organic substances are broken down into either inorganic substances or simpler organic compounds having lower level of energy status. The phenomenon of liberation of energy from organic substances in a catabolic process is called *respiration*. The process of respiration also provides important intermediates that are required in the synthesis of several important organic compounds.

The energy released during respiration is available for the synthesis of ATP and ADP and inorganic phosphate, and the process is called *oxidative phosphorylation*.

Respiration can be divided into two forms—*aerobic respiration* and *anaerobic respiration*. In aerobic respiration the usual mode or mechanism is considered while in anaerobic respiration, also termed *fermentation* it is obligatory in only a few microorganisms. During this process the respiratory substrate usually glucose is incompletely oxidized to form the intermediates, later producing ethanol and carbon dioxide.

B.I METABOLISM

We know that the life support activity of even simple organisms involves a set of complex biochemical reactions. One such complex biochemical reaction is the process of metabolism.

Metabolism is a process that is the sum total of such biochemical reactions taking place inside the living cell. It is a combination of two different processes, one called *catabolism* and the other *anabolism* (Figure B.1).

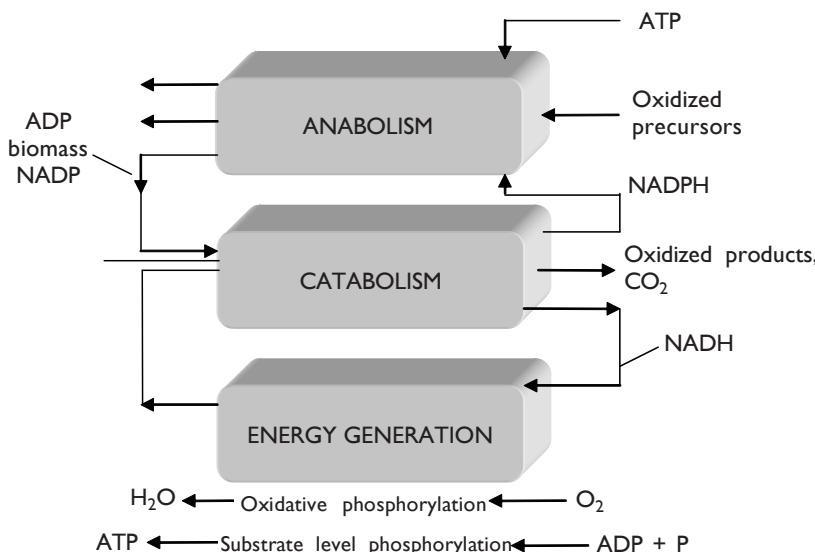


Figure B.1 Metabolism processes with energy generation.

Catabolism by definition is a process wherein the complex organic compounds are broken down into the simpler forms with the release of energy during the process itself. Anabolism is the process where the released energy is utilized to synthesize new molecules. The molecules that can be synthesized are proteins from amino acids, carbohydrates, i.e. from sugars to polysaccharides, etc.

Catabolic reactions furnish the energy required to carry out anabolic reactions. This process of coupling of energy requiring reactions and electron releasing reactions is made possible by the molecules called *Adenosine Triphosphates* (ATP) which are usually called the energy currencies of the cell.

B.2 CATABOLISM

Glycolic pathway for the production of pyruvic acid from glucose can be taken as the best example of catabolism. Most organisms oxidize carbohydrates of which the most common is glucose, which is usually an energy source. Respiration and fermentation are the two processes taken up by organisms in producing energy from glucose molecules. Both respiration and fermentation start with the glycolic pathway wherein glucose is broken down to pyruvic acid in series of steps as shown in Figure B.2. Glycolysis is also called EMP pathway.

Glycolysis or EMP pathway is common to both kinds of respiration and takes place in the cytoplasm of the cell. It consists of two steps.

Step 1. Conversion of carbohydrate or glucose to fructose-1,6-diphosphate also biphosphate

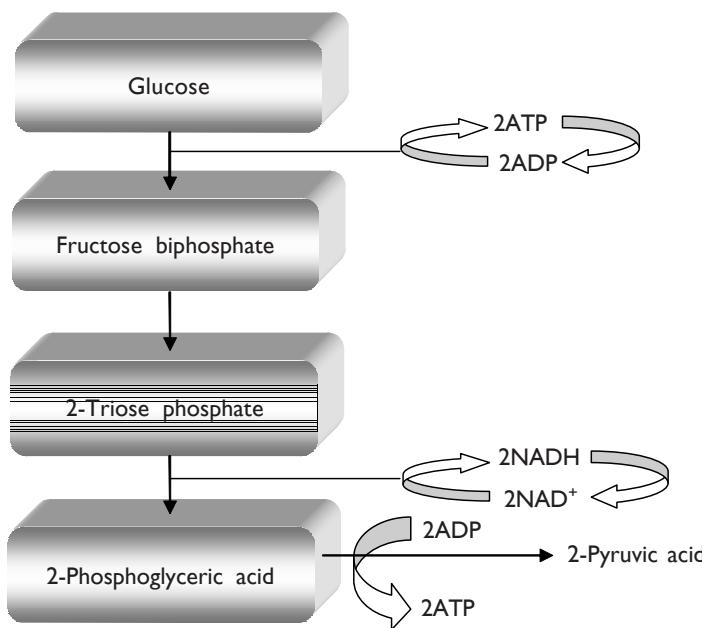


Figure B.2 Glycolysis, production of pyruvic acid from glucose.

Step 2. Conversion of fructose-1,6-diphosphate into 2 molecules of pyruvic acid.

Significance of glycolysis

1. Glycolysis forms the prelude to both aerobic and anaerobic kinds of respiration.
2. From one molecule of glucose, two molecules of pyruvic acid and two molecules of ATP are formed.
3. A total of 8 ATP molecules are yielded during the process of glycolysis.

B.2.1 Anaerobic Oxidation of Pyruvic Acid (Fermentation)

Pyruvic acid undergoes fermentation to produce lactic acid as shown in Figure B.3. The process is also termed *Lactic acid fermentation*.

Here pyruvic acid is converted into lactic acid by the enzyme lactate dehydrogenase. The co-enzyme NADH₂ is oxidized.

The other fermentation is alcoholic fermentation in which pyruvic acid is first decarboxylated to acetaldehyde in the presence of carboxylase. Acetaldehyde is then reduced to ethyl alcohol (Figure B.4) by the enzyme alcohol dehydrogenase while the co-enzyme NADH₂ is oxidized.

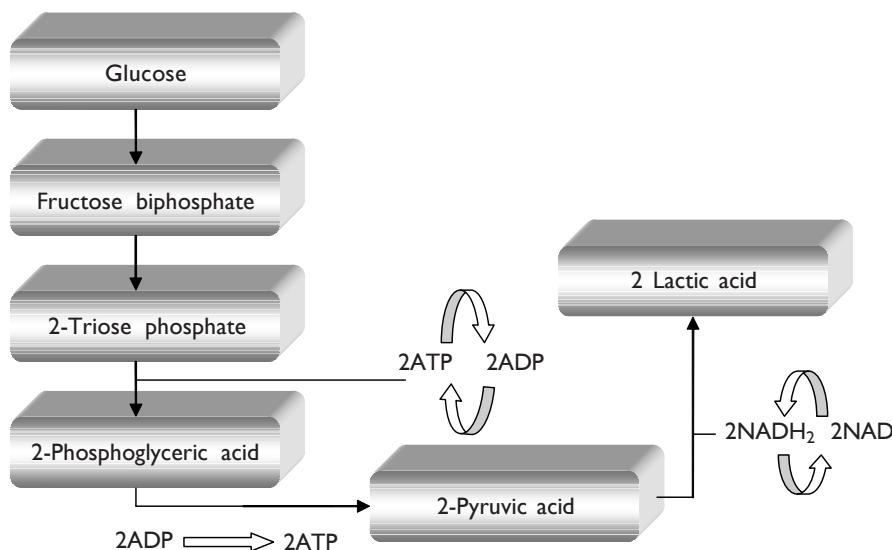


Figure B.3 Transformation of pyruvic acid to lactic acid.

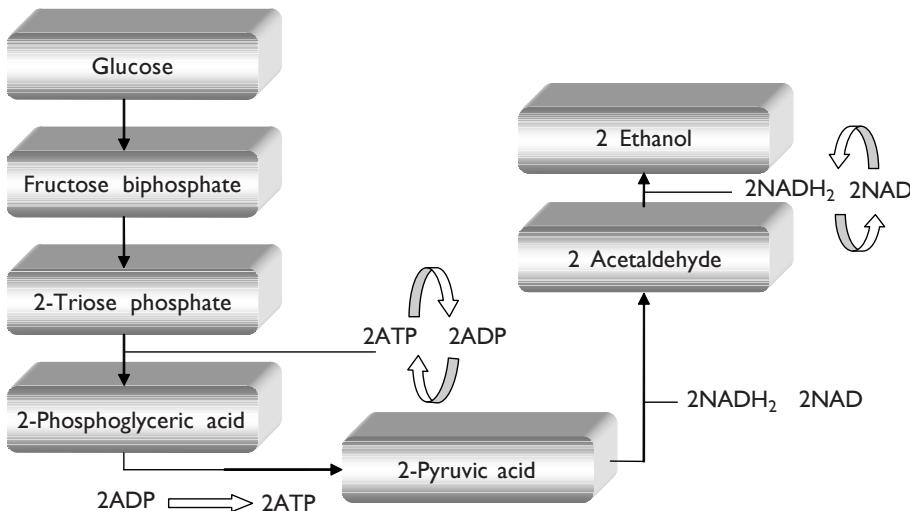


Figure B.4 Pyruvic acid to ethanol (alcoholic fermentation).

B.2.2 Aerobic Oxidation of Pyruvic Acid (Krebs Cycle)

In the presence of molecular oxygen, the pyruvic acid which is produced in glycolysis is completely oxidized to carbon dioxide and water. In 1940, Krebs gave a scheme for oxidation of pyruvic acid by means of a cycle of organic acids. This is also known as *tricarboxylic acid cycle* (TCA) and *citric acid cycle* (CAC). It takes place inside the mitochondria where all the necessary enzymes are present. Figure B.5 represents the mechanisms taking place in the cycle.

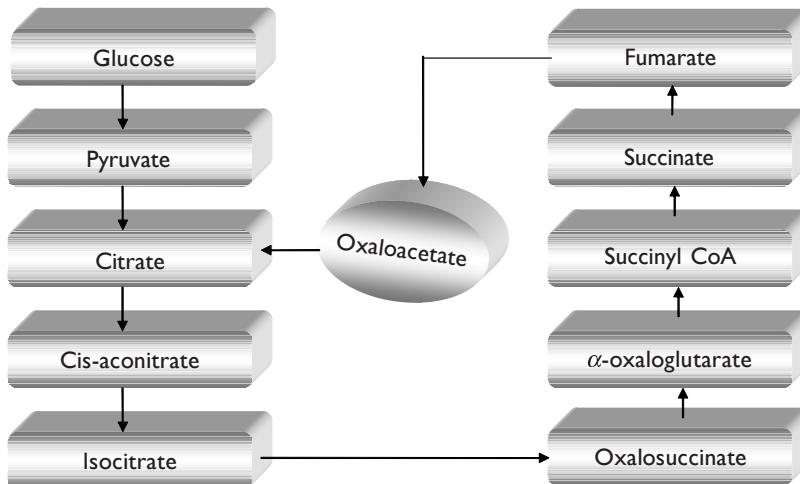


Figure B.5 TCA cycle or Krebs cycle.

B.2.3 HMP Pathway

Hexose monophosphate or HMP pathway is also known as *pentose phosphate shunt*, phosphogluconate shunt and direct oxidation pathway. It is an alternate pathway of oxidation of glucose taking place in the cytoplasm of the cell. Figure B.6 represents the mechanism followed in this pathway.

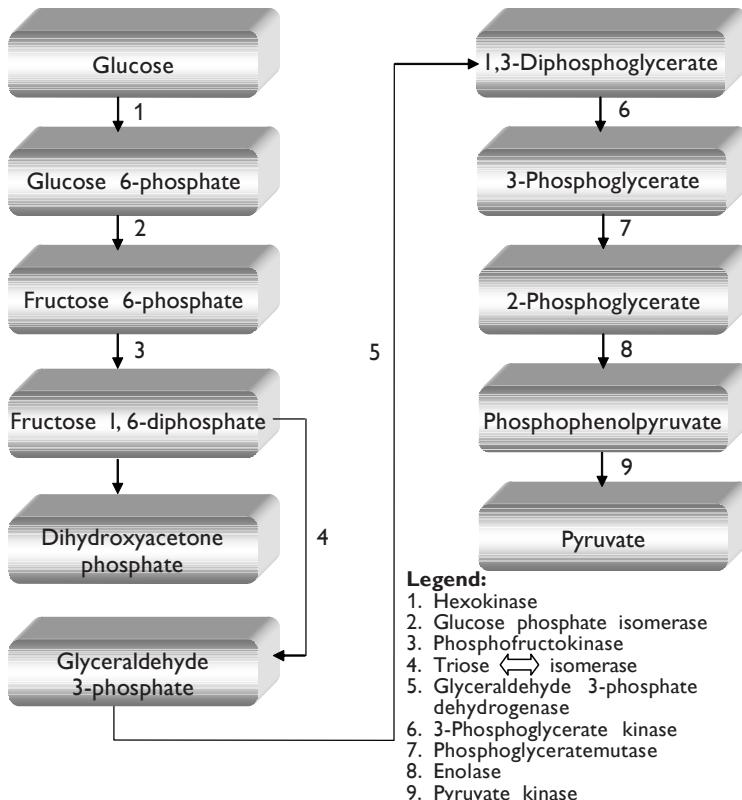


Figure B.6 Pentose phosphate pathway.

B.3 ANABOLISM

Anabolism is the process where the released energy is utilized to synthesize new molecules. The molecules that can be synthesized are proteins from amino acids, carbohydrates, i.e. from sugars to polysaccharides, etc.

The metabolic oxidation of glucose to carbon dioxide and water is considered effective, while some amount of energy produced is lost as heat and rest is utilized for anabolic processes.

B.4 MOLECULAR GENETICS AND RECOMBINANT DNA TECHNOLOGY

The techniques for the study of certain genetic disorders by biochemical assays or chromosomal analysis have been established for many years. The advancement in genetic detection during the last decade has been the use of the techniques of molecular biology to study changes at the DNA level.

B.4.1 The Human Genome

The human genome contains as many as 50–100,000 genes, ranging in size from less than 1 kilo base to more than 2 mega bases. A typical gene is organized into regions of DNA that encode specific amino acids (exons) interspersed with larger regions of non-coding DNA (introns). The function of introns is largely unknown. The great variation in the size of genes reflects differences in the number and size of introns.

Gene expression: In the production of proteins from amino acids, a gene is transcribed and then translated. Figure B.7 depicts both transcription and translation of genes.

A gene is first transcribed to produce a copy in the form of mRNA. After modification, this is translated to produce a polypeptide chain, which is further modified into the functional gene product.

Transcription: When genes are transcribed, one strand of the DNA acts as a template for the production of messenger RNA (mRNA). The primary transcript is a direct copy of the DNA, including exons and introns. Later this is modified before leaving the cell nucleus; the introns are excised and the exons are spliced together to form the processed mRNA molecule.

Translation: In the cytoplasm, mRNA is translated to form the gene product. Each set of three bases (codon), encodes a particular amino acid. Amino acids are assembled to form a polypeptide chain, which is often further modified to produce the functional gene product.

Regulation: Besides exons and introns, the genome contains regions of DNA that flank genes and have an important role in the regulation of the rate of

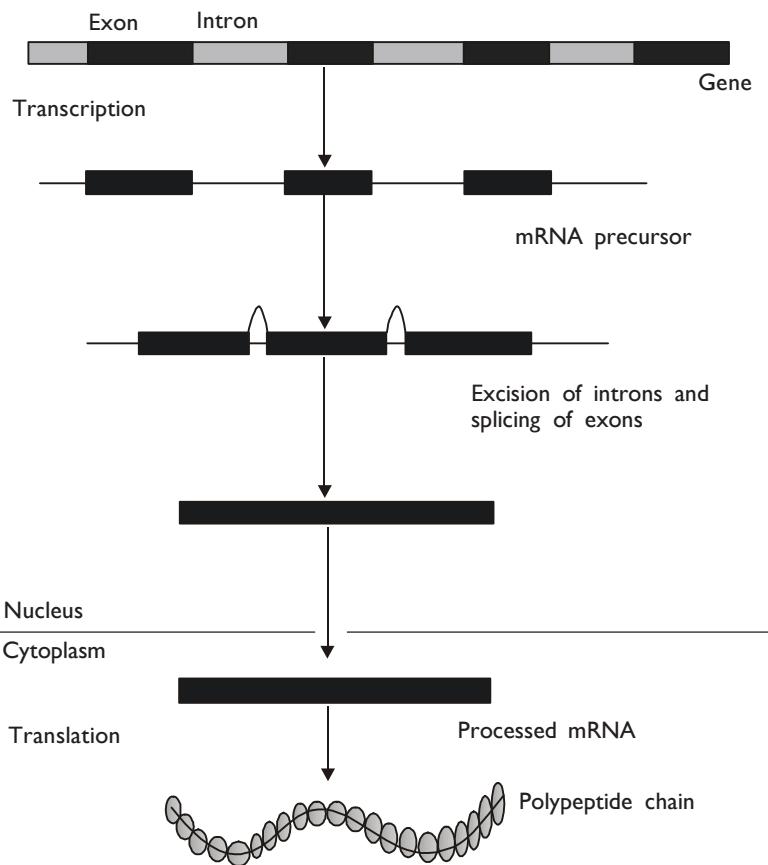


Figure B.7 Transcription and translation.

gene transcription. Other sequences are significant in the control of gene expression in various tissues at different stages of development.

B.5 DNA ANALYSIS

Because of rapid advances in molecular biology, it is possible to map and study the disease-causing genes. The three most common used methods in a diagnostic molecular genetics laboratory are DNA hybridization, DNA amplification and DNA sequencing.

B.5.1 DNA Hybridization

We know that the double-stranded DNA is held together by the mutual attraction of paired bases, $A = T$ and $G \equiv C$. The technique of DNA hybridization is based on these attractions. Under the controlled conditions,

a fragment of single-stranded DNA (a probe) will anneal to its complementary strand. A probe is usually a fragment of DNA cloned into a bacterial vector. Figure B.8 depicts DNA hybridization.

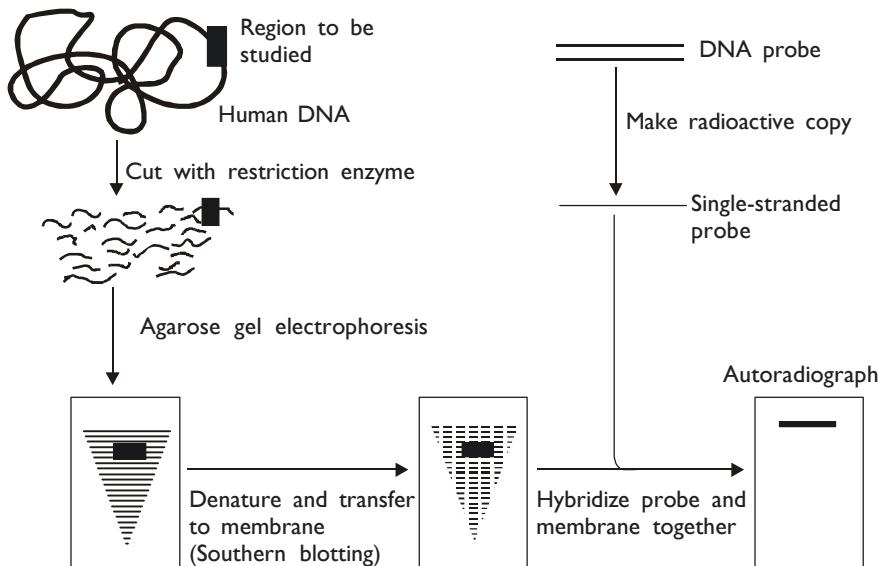


Figure B.8 DNA hybridization.

Testing for hybridization: The probe DNA is labelled, usually radioactively, and is denatured by heating. Denaturation means making a single stranded DNA. The DNA to be analyzed is also denatured and is immobilized on a nylon membrane. The membrane is soaked in a solution containing the radioactive probe, which hybridizes to its complementary strand on the membrane. The presence of hybridized probe on the membrane is detected by auto radiography. This process is also known as *Southern blotting*.

Uses: Uses of DNA hybridization include the following:

- To check for the presence of the probe DNA in a patient.
- To determine whether DNA probe has undergone any rearrangement.
- In molecular cytogenetic technique known as fluorescent *in situ* hybridization, in which a fluorescently labelled DNA probe is hybridized to a standard chromosome preparation on a microscopic slide.

B.5.2 DNA Amplification

DNA amplification involves producing many copies of a target sequence of DNA using the polymerase chain reaction (PCR). The method has three stages (Figure B.9):

1. Denaturation, where DNA is heated to produce two single strands.
2. Annealing, where the DNA is cooled and two specific oligonucleotide primers that flank the region of interest anneal—hybridize to their respective strands.
3. Synthesis, where the primers initiate the synthesis of two new strands of DNA by a thermostable polymerase.

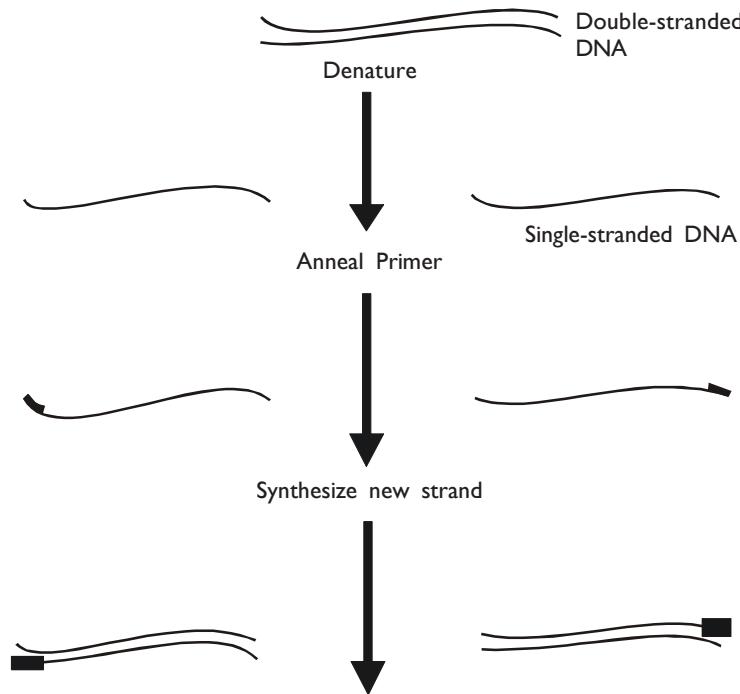


Figure B.9 DNA amplification (polymerase chain reaction, PCR).

Further, amplification leads to the target sequence and can be analyzed by gel electrophoresis and staining. DNA amplification is fast and efficient and when applied allows DNA analysis of a particular sample within 24–48 hours.

The method can also be used to study RNA by first making a DNA copy of the mRNA using the enzyme reverse transcriptase; this complimentary DNA (cDNA) can then be amplified as above.

PCR is an extremely powerful technique; small amounts of tissue can be typed, and even a single cell can be analyzed. The potential is enormous but needs a pair of specific oligonucleotide primers. It can be applied only to the regions of DNA that have been cloned and sequenced.

B.5.3 DNA Sequencing

DNA sequencing is used to determine the specific order of the bases in a given fragment of DNA. The sequence of an uncharacterized section of DNA can be ascertained or the sequence of a PCR product can be compared to a published sequence to identify possible mutations.

The procedure is semi-automated, up to 400 bp of sequence can be read at one time and the data are analyzed by computer.

B.6 MONOCLONAL ANTIBODIES (MABs)

MABs are ultra pure antibodies manufactured by hybridoma cells. Hybridoma cells are fused lymphocytes and malignant myeloma cells. The normal immune system can manufacture a million different kinds of antibodies to combat and inactivate foreign proteins or other antigens that may invade the body. A malignant myeloma cell synthesizes only a single type of antibody, an immunoglobulin protein that may be anyone of the almost innumerable proteins possible. The technique for production of MABs consists of three steps:

1. Production of hybridomas by hybridizing lymphocytes with myeloma cells which would secrete specific antibodies.
2. Separating different hybrid myelomas from each other by cloning.
3. Continuous propagation of hybridomas producing specific monoclonal antibody.

Figure B.10 depicts the production of MABs.

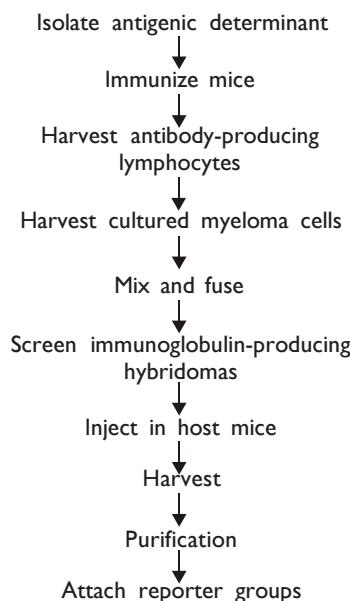


Figure B.10 Production of MABs.

B.6.1 Applications of MABs

The following are the applications of MABs.

1. The utility of MABs has been envisaged for the labelling and precise identification of specialized cells such as neurons.
2. They are useful in determining the structure of cell membranes and can be used as standard reagents to identify molecules present in cell membranes and to distinguish between cell populations.
3. They are used in serotherapy as well as in the preparation of very specific vaccines active against viral strains.
4. They are used in the neutralization of the action of lymphocytes responsible for the rejection of grafts and destroy the auto-antibodies produced in auto-immune diseases.
5. MABs find applications in tumour and cancer therapy.
6. They are useful for identification and classification of major histocompatibility complex products for tissue typing in transplantation and population genetic studies.
7. They are useful for serogenetic classification of infectious micro-organisms and protozoan and metazoan parasites.
8. They are used as a tool in enzyme genetics also called immunogenetics.
9. They are also employed in the process of enzyme purification.

A summary of applications of MABs and DNA probes is shown in Figure B.11.

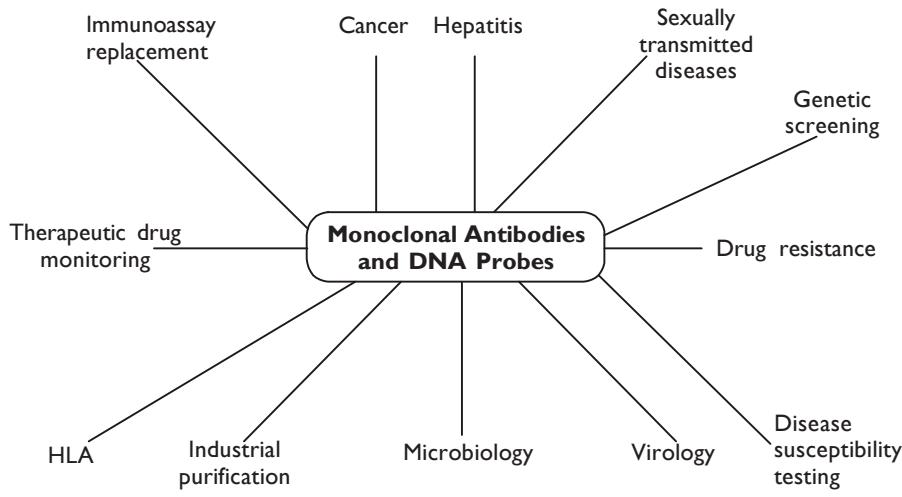


Figure B.11 Applications of MABs and DNA probes.

Concepts in Environmental Microbiology

C.I BACTERIAL NOMENCLATURE

Bacterial classification is not only based on the Gram-staining reactions as Gram-positive and Gram-negative but is also based on.

1. their application as autotrophic, saprophytic or heterotrophic and the usual mode of commercial usage and action, either in the presence of air or in its absence as aerobic or anaerobic respectively.
2. a logical and systematic way of their segregation that has gained a lot of prominence in the field of microbiology.

This mode of classification has been illustrated in Bergey's *Manual of Systematic Bacteriology* that deals with the study of the forms and the features of bacteria exclusively. According to this the world of bacteria is understood to have the following categories:

1. The World of Bacteria I: Ordinary Gram-Negative Bacteria
2. The World of Bacteria II: Ordinary Gram-Positive Bacteria
3. The World of Bacteria III: Bacteria with Unusual Properties
4. The World of Bacteria IV: Gram-Positive, Filamentous Bacteria of Complex Morphology.

In this section are explained the selective forms of bacteria that appear in one of the above stated categories.

C.I.I Rickettsias

These are said to be 'ordinary' Gram-negative bacteria and are obligate parasites. The parasitic features show that they truly depend on the others that are normally referred to as the hosts. These grow within the host cells that are said to be disease causing or pathogenic.

Rickettsias belong to the order Rickettsiales and the family Rickettsiaceae. The important features of rickettsias are as follows:

1. Metabolic patterns and the metabolic activities are said to be very complex. They synthesize ATP (adenosine triphosphate), the energy carriers during the metabolism of cells.
2. Developmental forms and cycles are simple, that is, they are said to be lacking in the complex development cycles.
3. Always said to be associated with the hosts, the arthropods serve as major carriers or vectors for the transmission of diseases to vertebrates.
4. Interaction or association is said to be *mutualistic* with arthropods. For example, they are associated mutually with lice, fleas and mites.
5. They have a tendency to multiply or grow within the cytoplasm of vertebrates called the targets for the transmission of diseases and specifically in the endothelial cells or in the erythrocytes in blood of animals.
6. Rickettsias are pathogenic to humans and are liable to be cultured in the laboratory within the hosts such as guinea pigs and mice.

Some of the diseases caused by rickettsias are: Rocky Mountain spotted fever by ticks, classical typhus fever by lice, rickettsial pox through mites, etc.

C.1.2 Mycoplasmas

Mycoplasmas are 'ordinary' Gram-negative bacteria and are said to belong the order Mycoplasmatales and the family Mycoplasmataceae.

The distinctive features of mycoplasmas are as follows:

1. They lack cell walls that can certainly act as shape giving structures to the microbes. But they do have outer cytoplasmic covering or membrane that predominantly helps in the ingress and the egress of the nutrients.
2. Shapes are not rigid or rather flexible as they lack cell walls. They can assume different shapes and forms from spheres to the branched filaments.
3. These are susceptible to lysis by osmotic shock, that is, sudden change in the cell nutrient and the surrounding conditions may be due to dilution of the cell suspension or medium with water.
4. Even higher levels of penicillin cannot inhibit as they lack cell walls. However, they are inhibited by antibiotics like tetracyclines and chloramphenicols that can influence in one way or the other the process of protein synthesis.
5. They have the ability of cultivating *in vitro* on the non-living media as facultative anaerobes or obligate anaerobes.

6. They also have parasitic features and mucous membranes, and the joints of animals are the regions that host these microbes. For their growth, they require cholesterol and can cause various diseases like pneumonia and urogenital disorders.

C.I.3 Archaeobacteria

Archaeobacteria are the bacteria with unusual properties, one of the four categories as stated earlier.

These can be Gram-positive or can be Gram-negative. The important features are as follows:

1. Archaeobacteria are phylogenetically different from eubacteria.
2. Methanogenic or methane-producing bacteria, extreme halophiles and thermoacidophiles are the three types of archaeobacteria which have a tendency to withstand extreme acidic and high temperature conditions.
3. Methane producers require high levels of NaCl for growth while others grow at a low pH and at a high temperature.

Generally, they are employed in the treatment of wastes that generate methane gas as a fuel.

As referred to section 1.7, environmental microbiology pertains to the study of microbes that interact with the various means of environment as soil, water and air. Their reactions, influence, physical and chemical changes that can be brought upon due to some kind of transformations are the real concepts of environmental microbiology. Also it has been stated that it refers to the study of microbiology of water, soil, and aquatic systems, domestic and industrial wastewater. In this discussion, is also included their distribution in nature, relationship to each other and to the other living organisms. We know that microorganisms are closely associated with health and welfare of humans; but some being beneficial and some detrimental.

Environmental microbiology provides an insight to the study of microorganisms in a broader perspective and a broader spectrum that encompasses overall features like their metabolic activity, growth, aging processes and altogether their life processes when applied in the field of environmental engineering; may be air, water and soil pollution. It has been realized that one can modify the conditions of the environment to assess the metabolic activities, growth patterns and even their details of genetic pattern. All these can be done without destruction of a microbe. An environmental engineer should also assess that well before the microorganisms are exploited for various commercial applications in the treatment of wastes, etc. It is quite essential to acknowledge the basics of microorganisms and their features so that use of microbes is a boon and not a curse. All these concepts are dealt with in Chapter 1.

Characterization, classification and identification of microorganisms are

equally of significance for an engineer to know before applied for various purposes.

C.2 CHARACTERISTICS OF MICROORGANISMS

Determination of the characteristics of microorganisms is not only important and a prerequisite for the classification, but also is valid for the other reasons as well. For instance, as microorganisms are involved in essential roles in nature, as it can be justified with respect to various biogeochemical cycles, described in Chapter 1, it is therefore a desirable attribute to have an understanding of the characteristics of species that enable these activities to take place.

There are several major characteristics of microorganisms that later aid in the process of classification. All these have been assigned based on their structure, form, culture, habitat, chemical composition, metabolic patterns, antigenicity, pathogenicity, etc. They are explained in brief as follows:

1. Morphological characteristics: Determination of size, shape and structure, cell array, motility of special structures like flagellates, developmental forms, staining reactions, etc. are all morphological characteristics and requires the study of the cells individually of a pure culture.

Pure culture: A pure culture consists of a single kind of microorganism (one living species), regardless of the number of individuals, in an environment free of other microbes. This is also called *axenic culture*. In true sense a pure culture is one that is grown from a single cell.

2. Chemical composition: This refers to various chemical constituents that make up the cell. Many organic compounds, viz. lipopolysaccharides, proteins and other nitrogen-carrying compounds, etc. are present in the cells of microorganisms and can even govern the life processes of the cell.

3. Cultural characteristics: The nutritional requirements and the physical conditions, so called the surroundings, required for the growth and also the manner in which it grows are all the features. We know that each kind of microorganism is specific to a specific growth requirement.

4. Metabolic characteristics: Metabolic characteristics refer to the manner in which cells obtain their energy and later utilize it to carry out some specific chemical reactions. Some microorganisms obtain energy by absorbing light and some others by way of oxidation of various organic compounds, while most of the reactions being catalyzed by the enzymes.

5. Antigenic characteristics: These characteristics refer to special large chemical constituents of the cell, distinctive of certain microbes only.

Basically, antigens are the chemical compounds of microbial sources, and this antigenic feature has a great influence and a practical significance. An antigen reacts with an antibody.

6. Genetic characteristics: Hereditary material of the cell, DNA and occurrence and functions of other forms of DNA as plasmids are the genetic characteristics of microorganisms.

7. Pathogenic characteristics: Pathogenic characteristics basically refer to the ability to cause disease in themselves and other forms of life. As we know that relatively a few species cause diseases and are pathogenic.

8. Ecological characteristics: The habitat, the distribution of microorganisms in nature, their relations and the interactions between and among species are the ecological characteristics.

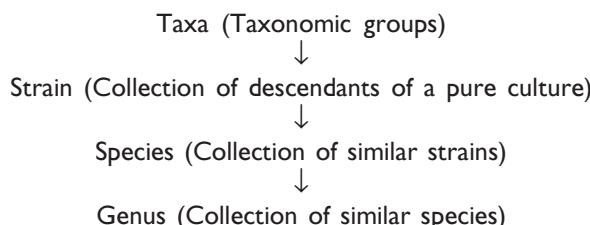
C.3 CLASSIFICATION OF MICROORGANISMS

Next to characterization, is the classification of microorganisms. Classification as such has been targeted towards the achievement of the following two qualities and are adopted by taxonomists. The very first quality or the desirable attribute is the *stability* and the other is *predictability*.

Stability is the criterion to assess the situation that is more prone to the frequent and radical changes, perhaps leading to a sort of confusion. In this regard, every attempt is made to devise classifications that need only minor changes as and when new information is available.

Predictability is the one which is useful in assigning the characteristics to the microorganisms of the same group by knowing the characteristics of one member of a particular taxonomic group. In case, if this is not feasible, then classification has little or no value. Perhaps these attributes are the goals of classification.

Taxonomists have devised the classification as shown below.



Based on the above discussion, the bacterial classification is made by using three methods:

- Intuitive method
- Numerical taxonomy, and
- Genetic relatedness.

Intuitive method: This method is based on the experience and exposure over a period of several years to the study of the properties and features of microorganisms. Based on this one can decide upon the classification in terms of one or more species or genera.

Numerical taxonomy: This is a statistical method of classification. In this method, several characteristics (usually more than 100) for each strain are studied. Each strain studied for a particular characteristic is given equal importance. Based on computations, the percentage similarity (%SIM) for each strain to every other strain is computed. It is computed using the following formula.

For any two strains,

$$\%SIM = NS/(NS+ND)$$

where NS represents the number of characteristics that are similar, may be positive or negative for the two strains, while ND represents the number of characteristics that are different or not similar. Further, those strains that have high percentage similarity (%SIM) are placed into the groups that are segregated from the dissimilar ones. In this way the classification is done.

Genetic relatedness: This method is considered to be the most reliable and accurate one. It is based on the degree of genetic relatedness between the microorganisms and on the most fundamental aspect of organism hereditary, i.e. the DNA; its mole per cent G + C values. It is quite evident that those two microorganisms that have similar mole per cent G + C values are of the same or similar species, while having different mole per cent G + C values are said to be not closely related. In this regard, there are certain precise techniques to have an accurate comparison. They are as follows.

DNA homology techniques: These are useful at the species level of classification and determined as shown in Figure C.1.

r-RNA homology experiments and r-RNA oligonucleotide cataloging: This involves the concept of r-RNA cistrons that refer to the r-RNA coding for the fraction of DNA molecule.

C.4 IDENTIFICATION OF MICROORGANISMS

Once having classified a microorganism, a few of its characteristics are selected by which it can be identified. In order to be useful for identification,

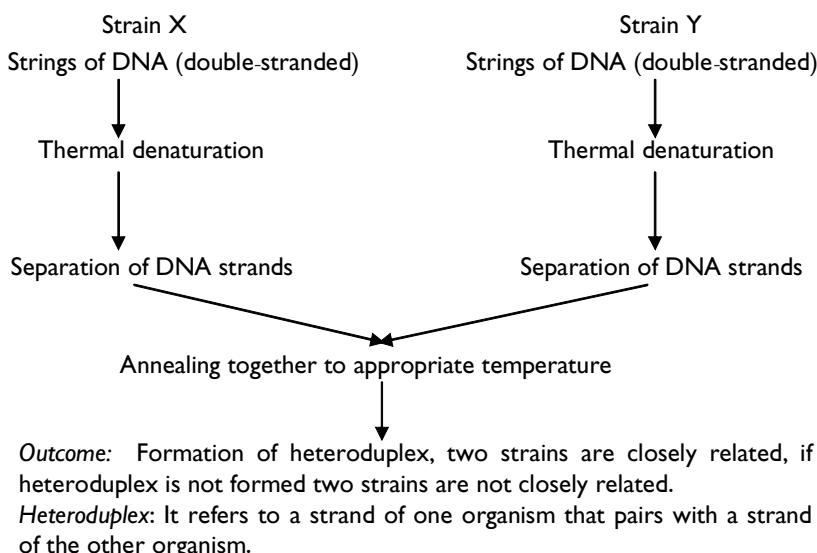


Figure C.1 DNA homology technique.

the combination of characteristics selected must occur only in that particular kind of microorganism and in no other. The chosen characteristics for the identification process such as shape, size, and staining reactions and of course the sugar fermentations should be quite easy and amenable for the determination.

In this context, many *identification keys* and *tables* are available which are in the summarized forms and are proven to be useful for identification of microorganisms.

C.5 PURE CULTURES AND CULTURAL CHARACTERISTICS

In the earlier discussion, we have defined the pure culture which is also termed *axenic culture*. With reference to pure cultures, now we shall attempt to find out as to how the isolation of pure cultures is made and what is the significance of isolation and finally the characteristics of pure cultures, more specifically the cultural characteristics.

We know that in the natural environments and the surroundings, a single kind of bacterium said to be as bacterial species, usually exists as only one component of large and complex population that contains many other species. In order to study the characteristics of one particular species, that species must be separated from all the other species, that is, it must be isolated from the rest of the species that form a pure culture. In order to achieve this, a selective method is used before isolation, which can subsequently increase the relative proportion of the desired species in the population so that it can be more easily isolated. Once obtained, a pure culture can be maintained or preserved in a culture collection.

The selective method is the most preferred one over others as it is much more specific to a particular microorganism. In the selective method of isolation, we find that there is favour made to the desired species in terms of growth while discouraging or even killing the other microorganisms present in the mixed culture.

Also there are certain other methods available like chemical methods of selection, physical methods of selection and even biological methods of selection. The use of dilute media, with very low levels of carbon or nitrogen sources as growth medium, favouring the growth of a specific microorganism and use of inhibitory chemicals like bile salts, dyes, salts of heavy metals, etc. are the chemical methods, while heat treatment methods, use of incubation temperature and use of pH medium are the physical methods. Exploiting the advantage of pathogenic properties of microorganisms and using animal as the selective medium in which the microorganism can infest and grow is the biological method of selection.

In all probability, by using one or the other method, the isolation of pure cultures can be done. There are also several known methods or techniques to isolate pure cultures. They are as follows:

- Streak-plate technique
- Pour-plate technique
- Spread-plate technique
- Roll-tube technique
- Micromanipulator technique

C.6 MAINTENANCE AND PRESERVATION OF CULTURES

Once isolated, the pure cultures are required to be stored or preserved until they are used. This, of course, is essential as there can be a lapse in the period from the time of isolation till the use of it. This is quite critical as the utmost care is to be taken to see that there are no contaminations or any undesirable growth. To achieve this target, cultures are required to be maintained and preserved. There are certain methods as stated below.

- Overlaying of cultures with mineral oil
- Lyophilization also called freeze drying; temperatures maintained at around -60 to -78°C
- Storage at low temperatures using liquid nitrogen and dimethyl sulfoxide that prevent the cell damage by forming ice crystals
- Periodic transfer to the fresh medium

With any of the above methods, preservation and maintenance of cultures is possible.

The cultural characteristics are required to be understood to ascertain the growth characteristics of strain of a bacterial culture. It is customary to

observe the features of colonies and the broth cultures. The colony characteristics are determined by the features such as size, texture, elevation, consistency, opacity and translucency called optical properties, pigmentation and colour of the colonies. The characteristics of the broth cultures are also required. The amount of growth which may be scanty, moderate or abundant and the distribution and type of growth, whether uniform, uneven, confined to the surface of the broth as a scum or pellicle or even sediment that can get accumulated during the process are all the characteristics of cultures to be determined.

C.7 MUNICIPAL WATER PURIFICATION

Domestic water is obtained from various sources such as rivers, lakes, and streams which are collectively called natural water supplies. Due to rapid urbanization and industrialization, it is realized that the natural water supplies are likely to get contaminated with domestic and industrial wastes. The disease-causing microorganisms get frequently transmitted through water and lead to water-borne diseases like intestinal tract infections, typhoid, cholera, etc.

It is also understood that these causative organisms are present in the faeces and urine of an infected person and when discharged can gain entry into the body of water that ultimately serves as drinking source. Therefore, it is necessary to employ the treatment facilities that purify the wastewater prior to disposal and also water purification methods that can provide safe drinking water.

Potable water is one that is absolutely free from disease-causing microorganisms and the chemical substances that are said to be deleterious to health. Water contaminated with either domestic or industrial wastes is non-potable water or also termed polluted water.

The following are the steps briefly discussed adopted in the purification of water seen in the municipal water purification plant (Figure C.2). The entire plant comprises the following stages:

- Sedimentation
- Coagulation
- Filtration
- Disinfection
- Storage of treated water for supply to the consumer

Sedimentation occurs in large reservoirs where the water remains for a large holding period due to allow the larger particulates settle down at the bottom of the reservoir. This process is enhanced by the addition of alum or aluminium sulphate, a coagulant that produces a sticky flocculent precipitate. Within this precipitate is found that most of the microorganisms are removed along with the finely suspended matter. This precipitate

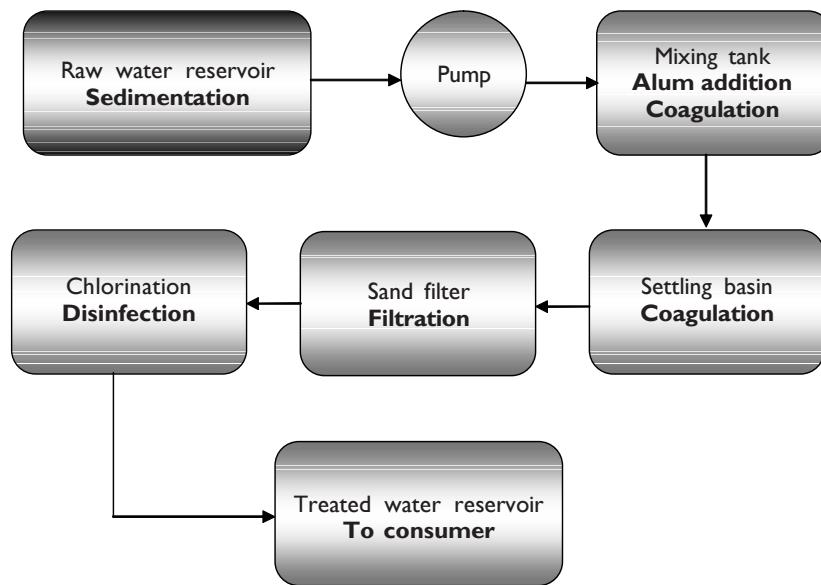


Figure C.2 Water treatment plant.

descends through the water in the settling basins. Then the water is passed through the sand filters in which 99% of bacteria are removed.

Subsequent to this water is sent for disinfection by the addition of chlorine or by means of ozonation or also by using UV radiations. This is to be done to ensure the potability of water. Chlorine dosage must be sufficient to leave a residual content of 0.2 to 2.0 mg per litre free chlorine.

Besides this, the purification processes may also include the techniques for removing the minerals that cause hardness to water, pH adjustment if water is too acidic or alkaline. With all these procedures performed, water is now said to be potable.

C.8 WASTEWATER TREATMENT

Wastewater treatment plants carry out a series of processes to accomplish water quality acceptable according to the standards prior to disposal into water bodies. The processes are summarized as follows.

1. Primary treatment: This is to ensure that the coarser solids are removed and also to accomplish the removal of settleable solids. In this a variety of mechanical techniques and devices like screening, grinding and grit chambers are incorporated.

Further, the units like tanks, basins or mechanical devices provide the means for concentrating the particulate matter referred to as the sludge and overall the process is sedimentation.

2. Secondary treatment: This is to adsorb and ultimately oxidize organic constituents of the wastewater, i.e. to reduce the BOD content. In this technique, the treatment is accomplished by oxidation of the organic material in the liquid waste by the action of microbial activity. The oxidation methods are as follows:

- Filtration by intermittent sand filters, contact filters and trickling filters
- Aeration by the use of activated sludge process or contact aerators
- Oxidation ponds

3. Advanced treatment: This is required to remove the additional impurities and objectionable substances to further reduce the BOD content and also includes the removal of nutrients such as P and N. This is in fact required when the removal of substances beyond the limits normally achieved by conventional primary and secondary processes is necessary. It is indeed the biological treatment process. The techniques adopted are as follows:

- Biological nitrification and denitrification
- Filtration by biological filters
- Carbon adsorption
- Chemical addition and downstream processing that uses reverse osmosis and ion exchange processes

4. Final treatment: The purpose is to disinfect and dispose of the liquid effluent. Chlorination, ozonation and treatment with UV radiations are done in this step. This is done to protect public health when the receiving waters are used for several purposes as downstream water supply, recreation, irrigation or shellfish harvesting.

In certain cases the dissolved oxygen content is increased with the addition of oxygen and it is possible with the use of mechanical aerators. This is also called *post-aeration process*.

5. Solids processing: It has been estimated in terms of cost factor that a major part of the investment is associated with the solids-processing techniques like thickening, stabilization, dewatering and disposal of the sludge. Solids are removed from the primary, secondary, and advanced stages of treatment. Thickening is generally employed to concentrate the sludge prior to stabilization. Gravity thickening and dissolved air flotation are the processes that are taken up for thickening.

Stabilization processes are employed, including aerobic digestion, composting, chemical addition and heat treatment. Aerobic digestion is the most common process for modern municipal treatment facilities. Later dewatering is accomplished by physical methods and is often enhanced by the addition of polymer or the other chemical coagulants. The devices used are centrifuges, filter presses, etc. for the dewatering of sludge.

Composting of solids is a process where the dewatered sludge undergoes decomposition, usually within the thermophilic range of temperatures. Dewatered sludge is mixed with the bulking agent such as wood chips to enhance the circulation of air through the sludge so as to improvise the stabilization process. This mixed sludge is placed in a tier of aerated piles where oxygen is furnished by way of forced aeration. Then the mixture is allowed to cure or biologically decompose for a certain period. It has been realized that the effective stabilization is achieved in 21 days. After 21 days the bulking agent is removed or separated from the sludge and further the sludge is allowed to cure for several weeks. Upon final curing, the sludge is transferred to a humus-type material and is suitable for use as a soil conditioner.

C.9 MICROBIAL INTERACTIONS

We know that the different microorganisms inhabit the soil and are called *microbial flora*. The microbial system of the soil includes the total microbial flora together with the physical composition and physical characteristics of the soil. In other words, it is the combination of abiotic and biotic components of the soil. Within this arena, it is amazing to see that there are many different types of interactions and associations taking place between the microorganisms. Well, in these associations some are beneficial or positive and some are detrimental or negative. The types of microbial associations or interactions taking place are as follows:

1. Neutral associations or neutralism: In neutral associating two different species of microorganisms occupy the same environment without affecting each other. In no way the activities of one microorganism are going to affect the other.

2. Positive associations: These include *mutualism* and *commensalism*. Mutualism is an example of a symbiotic relationship in which each organism benefits from the other. The manner in which benefit is derived varies. One type of mutualistic relationship involves the exchange of the nutrients between the two species. This phenomenon is called *syntrophism*. The other mutualistic relationship or association is characterized by the different metabolic products from the association as compared with the sum of the products of the separate species.

Commensalism refers to an association in which one species of microorganism pair benefits, while the other is not affected.

3. Negative associations: In the case of negative associations, one species adversely affects the environment for another species. It is said to an *antagonistic* type of association. In this, we have certain other associations: *competition* and *parasitism*.

Competition is a negative association that may result from competing for the essential nutrients between and among the species. In such situations the best adapted microbial species will predominate or in fact eliminate the other species that are dependent upon the same limited and essential nutrient.

Parasitism is defined as a relationship between the organisms in which one organism lives in or on another organism called host. One of the species so called parasite is benefited at the expense of the host.

C.10 BIODEGRADATION

Biodegradation refers to the use of microorganisms or their metabolites to treat toxic chemicals and render them harmless. There is a growing faith that once developed and proven, the process of the biodegradation is potentially less expensive than any other approach to neutralize toxic wastes. This is said to involve a low capital investment, low energy consumption and often self-sustaining operations.

In biodegradation, a microbe feeds on the toxic water, or in the presence of a particular waste, it secretes an enzyme to degrade the waste and also reduces the volume of organic matter. Today, microorganisms that degrade some of the most toxic chemicals are being isolated from the soil of hazardous waste sites. Later these organisms can be selectively breed in the laboratory and returned to the environment as more efficient scavengers.

Biodegradation techniques are versatile and can be used at different stages. Some of the potential applications of biodegradation include removal of contaminants from raw materials before processing, treatment of pipe line wastes before discharge from the installation of a factory, decontamination of soils and surface and ground water and the clean-up of dump sites. Biodegradation operations can be carried out in a number of ways.

1. Microorganisms or their active metabolites can be released into the environment which is contaminated.
2. Already being present in the environment, can be enhanced by the addition of suitable nutrients that favour the growth and their activities called bioaugmentation.
3. Microorganisms to be used in contained or semi-contained reactors.

Biodegradations are applied for herbicides and pesticides to reduce their concentration levels. Oil biodegradation is also an important application of this process. In this case oil spills and oil seeps, and their effects are reduced gradually by sequential metabolism of various classes of compounds present in oil. It is said that a species of bacteria *Pseudomonas putida* feeds on the contents of various hydrocarbon products like xylene, camphor, etc. and reduce the effects of oil spillage especially in water.

C.11 BIOLEACHING OR MICROBIAL LEACHING

Today, it is an accepted fact that all the microorganisms interact with metals. Leaching of metals from their ores and concentrating metals from large amounts of wastes or dilute mixtures are the two important sections that are of potential significance. Mineral leaching is shown by several bacterial species. The process is gaining a lot of prominence over the conventional means for the following reasons.

1. Their ability to treat low grade ores as richer mineral deposits is getting depleted.
2. The traditional method, smelting, is said to be a concern as it is causing pollution problems.

Microorganisms are able to improvise both these situations. For instance, *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* when grown in the presence of copper ores produce acid and effect oxidation of ore with subsequent precipitation of metal. This is microbial leaching or also called *bioextractive metallurgy*.

Self-Assessment Exercises

ATTEMPT THE FOLLOWING QUESTIONS

1. Microbiology is a study of living microorganisms concerned to those which are minute in size and include structure, reproduction methods, physiology, metabolic activities, etc. along with their detailed classification and as well as their relationship with each other and their effects on humans, plants or animals. (True/False)
2. All microorganisms are harmful. (True/False)
3. Kingdom protista is one which includes
 - (a) all unicellular organisms
 - (b) all unicellular organisms that are neither plants nor animals
 - (c) unicellular which are plant like
 - (d) unicellular which are animal like.
4. Three principal modes of nutrition are....., and
5. Bacteria are lower protists. (True/False)
6. The smallest known cell is
 - (a) mycoplasma
 - (b) virus
 - (c) yeast
 - (d) bacteriophage
7. Plastids and mitochondria contain type of ribosomes.
 - (a) 80 S
 - (b) 70 S
 - (c) 60 S
 - (d) 77 S

8. The cell organelle connected with cell plate formation is
 - (a) endoplasmic reticulum
 - (b) golgi complex
 - (c) polysome
 - (d) lysosome
9. Which one of the following is absent in autotrophic prokaryotes?
 - (a) cell wall
 - (b) cytoplasm
 - (c) photosynthetic pigment
 - (d) chloroplast
10. Which of the following is a heteropolysaccharide?
 - (a) cellulose
 - (b) heparin
 - (c) chitin
 - (d) pectin
11. The difference between unsaturated fatty acids and saturated fatty acids is that the former has
 - (a) a long chain
 - (b) two carboxylic groups
 - (c) one or more double bonds
 - (d) essential fatty acids only in its molecule
12. The primary structure of proteins is maintained by
 - (a) hydrogen bonds
 - (b) ionic bonds
 - (c) disulphide bonds
 - (d) peptide bonds
13. The ring structure, perhydroxy cyclo pentanophenanthrene is found in
 - (a) fatty acids
 - (b) steroids
 - (c) carotenoids
 - (d) carbohydrates
14. Sheet structure of protein
 - (a) contains only peptide bonds
 - (b) is exhibited by keratin
 - (c) usually contains two or more peptide chains
 - (d) are characteristic of globular proteins
15. In competitive inhibition of enzyme activity, the rate of reaction would increase when
 - (a) concentration of enzyme is increased
 - (b) inhibitor concentration is increased
 - (c) substrate concentration is increased
 - (d) rate of reaction in such inhibition cannot be increased

16. A competitive inhibitor of an enzyme
- (a) decreases K_m without affecting V_{max}
 - (b) increases V_{max} without affecting K_m
 - (c) increases K_m without affecting V_{max}
 - (d) decreases both K_m and V_{max}
17. Two proteins having similar amino acid constituents may differ because
- (a) sequence of amino acids is different
 - (b) they are synthesized by two different cells
 - (c) they are formed by different ribosomes
 - (d) one protein is dependent on the other for its function
18. The rate of reaction increases if the substrate concentration is increased in competitive inhibition because
- (a) substrate molecules have increased chance of binding to the active site
 - (b) substrate molecule prevents binding of competitor to the allosteric site
 - (c) more substrate molecules inactivate the inhibitor
 - (d) there occurs no competition for active site
19. All the statements regarding competitive inhibition are true except
- (a) the inhibitor binds reversibly with the enzyme forming enzyme inhibitor complex
 - (b) rise in inhibitor concentration lowers the substrate affinity towards the enzyme
 - (c) degree of inhibition is independent of the relative concentration of the substrate and the inhibitor
 - (d) the inhibitor bears a close structural resemblance with the substrate
20. First class proteins
- (a) are easily digestible
 - (b) contain all essential amino acids
 - (c) have a few of the essential amino acids
 - (d) are easily available as food
21. Aspergillus niger is a useful fungus. (True/False)
22. The unique nitrogenous base found in RNA is
23. The functional unit of enzyme is called
24. Polysaccharides are normally amorphous, insoluble in water, are tasteless and also referred to as non sugars. (True/False)
25. Enzyme E can hold substrate S at certain angles and positions to enhance the reaction rate and is called
26. The Turn Over Number in enzyme catalyzed reactions also called ranges between and

27. Two industrially important moulds are and
28. In commercial practice under adverse conditions, some bacteria are able to produce stable, dormant and resting forms called
.....
29. *Lactobacillus*, *Streptococcus* and *Leuconostoc* are Gram positive, non-photosynthetic and found in plants. (True/False)
30. The cyclic steroid nucleus found generally in steroids is
.....
31. In the nitrogen cycle, the amount of nitrogen in the atmosphere is balanced between the processes that withdraw nitrogen from it called.....and those which add nitrogen to it called
.....
32. Entrapment of enzymes within insoluble gel matrix is.....
.....method of immobilization.
(a) chemical
(b) physical
(c) both chemical and physical
(d) none of these
33. Cell culture medium is said to be of synthetic type when the chemical composition is well defined. (True/False)
34. In a stationary phase during transient growth, the net growth rate is zero but still the cell is metabolically active and produces secondary metabolites. It is called endogenous metabolism. (True/False)
35. For most of the enzymes, the temperature quotient Q_{10} is
..... between 0°C and 40°C .
36. In the process of cell fractionation, centrifuging supernatant in an ultracentrifuge for 10 minutes and at 600 g releases
..... and
37. In an eukaryote, the DNA base ratios (C+G) present are around 40%. (True/False)
38. The vegetative structures in moulds are called
39. Endospores are the dormant forms of bacteria that can resist adverse conditions. (True/False)
40. Generally lipids are regarded as non-informational biopolymers. (True/False)
41. The unique nitrogenous base found in RNA is
42. The functional unit of enzyme is called

43. Polysaccharides are normally amorphous, insoluble in water, are tasteless and also referred to as non sugars (True/False)
44. Enzyme E can hold substrate S at certain angles and positions to enhance the reaction rate and is called
45. Induced fit theory is also called
46. In the reversible uncompetitive inhibition it is found that both K_m and V_{max} decrease. (True/False)
47. Entrapment of enzymes within insoluble gel matrix is..... method of immobilization.
(a) chemical
(b) physical
(c) both chemical and physical
(d) none of these
48. The operational sequence through which a bioreactor broth must pass for a highly purified product is
(a) purification, primary isolation, removal of particulates and final product isolation
(b) removal of particulates, primary isolation, purification and final product isolation
(c) removal of particulates, purification, primary isolation and final product isolation
(d) purification, removal of particulates, primary isolation and final product isolation
49. Downstream processing is also called product recovery operation. (True/False)
50. The response of bacteria to a rapid staining test is Gram staining test. (True/False)
51. Enzyme cross linking by multifunctional reagents is method of immobilization.
52. The RNA molecules that have catalytic properties are called
53. The sites of protein synthesis in a cell which are dark grainy spots are called.....
54. The mode of nutrition in kingdom fungi is
55. The cell membrane plays a vital role in controlling the ingress and egress of nutrients. (True/False)
56. The response of bacteria to a rapid staining test is called Milon's test. (True/False)

57. The loss of a three dimensional structure of a protein is called
.....
58. Glycogen is a heteropolysaccharide. (True/False)
59. Enzymes that occur in several molecular forms, but catalyze the same reaction are called
60. In the Michaelis-Menten expression when K_m is far greater than S, the reaction rate becomes first order. (True/False)
61. In a stationary phase during transient growth, the net growth rate is zero but still the cell is metabolically active and produces secondary metabolites. This is called
62. Enzyme cross linking by multifunctional reagents is.....
..... method of immobilization.
63. The specific sequence followed in a typical batch growth curve is
(a) lag phase, stationary phase, acceleration with exponential phase and death phase
(b) stationary phase, lag phase, acceleration phase and death phase
(c) lag phase, acceleration with exponential phase, stationary phase and death phase
(d) acceleration with exponential phase, lag phase, stationary phase and death phase
64. Two industrially important moulds are.....
and.....
65. Lipids that have both polar and non polar groups are called
(a) glycolipids
(b) lipo proteins
(c) amphipathic lipids
(d) neutral lipids
66. In the formation of lipid micelle, the overall energy of the resultant i.e. micelle and the solution is
(a) higher than that of the original solution
(b) lower than that of the original solution
(c) same as that of the original solution
(d) none of these
67. Vitamin C and pantothenic acid are water soluble vitamins. (True/False)
68. Vitamins K and E are soluble in organic solvents and insoluble in water. (True/False)
69. Snake venom and immuno globulins are
(a) storage proteins
(b) defense proteins
(c) contractile proteins
(d) genetic proteins

70. Amino acids, arginine, and isoleucine are
- (a) sour in taste
 - (b) tasteless
 - (c) bitter in taste
 - (d) salty
71. At the isoelectric pH, the molecule exists as a zwitterions, i.e.
- (a) it carries positive charge
 - (b) it carries negative charge
 - (c) carries no net charge
 - (d) none of these
72. Quaternary structure applies only to proteins which are multimeric. (True/False)
73. The content of nitrogen in proteins on an average is
- (a) 20%
 - (b) 16%
 - (c) 12%
 - (d) 10%
74. Protein denaturation refers to the loss ofstructure.
- (a) secondary
 - (b) primary
 - (c) tertiary
 - (d) quaternary
75. Starch consists of two components i.e. amylose around 10–30% and amylopectin around 70–90%. (True/False)
76. Which of the following statements is true to enzymes *lyases*
- (a) catalyze transfer of functional groups
 - (b) specialized in the addition or removal of H_2O , NH_3 , etc.
 - (c) used in isomerisation reactions
 - (d) bring about hydrolysis of compounds.
77. Which of the following is the correct sequence
- (a) holoenzyme = apoenzyme + coenzyme
 - (b) apoenzyme = holoenzyme + coenzyme
 - (c) coenzyme = holoenzyme + apoenzyme
 - (d) none of these
78. The RNA molecules that have the catalytic effect are called
- (a) apoenzymes
 - (b) ribozymes
 - (c) coenzymes
 - (d) isozymes

79. In an enzymatic reaction, the regions of substrates are close to each other and also to the active site of an enzyme molecule. It is called
- (a) orientation effect
 - (b) proximity effect
 - (c) catalytic effect
 - (d) none of these
80. The number of catalytic events taking place per second per enzyme molecule also called turn over number varies between
- (a) 100 and 3×10^{10}
 - (b) 100 and 3×10^6
 - (c) 100 and 3×10^{12}
 - (d) 10 and 10^5
81. Which of the following theories are more realistic and operative in explaining the ES complex formation.
- (a) lock and key model
 - (b) substrate strain
 - (c) induced fit
 - (d) combination of (b) and (c)
82. As per the steady-state approximation in enzyme catalyzed reactions, the true equilibrium is never established during the fast step because subsequent step is constantly removing the intermediate complex. (True/False)
83. In comparing the enzyme catalyzed reactions with uncatalyzed reactions the following fact is valid.
- (a) standard free change is more in uncatalyzed than catalyzed reactions
 - (b) standard free change is less in uncatalyzed than the catalyzed reactions
 - (c) standard free change is the same in (a) and (b)
 - (d) none of these
84. In the enzyme catalyzed reactions it is seen that the energy of activation is
- (a) lowered
 - (b) increased
 - (c) may increase or decrease
 - (d) no change
85. The rate of enzyme catalyzed reactions attains zero order when
- (a) the active centres on the enzyme are fully occupied by substrates at high concentrations
 - (b) the active centres on the enzyme are fully occupied by substrates at low concentrations
 - (c) the active centres on the enzyme are fully occupied by substrates at saturation
 - (d) the active centres are not fully occupied by substrates

86. In the evaluation of kinetic parameters of Michaelis-Menten parameters by Eadie-Hofstee method following values are plotted.
- (a) $1/r$ vs $1/[S]$
 - (b) r vs $r/[S]$
 - (c) $[S]/r$ vs $[S]$
 - (d) $1/r$ vs $r/[S]$
87. The changes in the pH value of the medium in which an enzyme is present may result in the changes in the three dimensional structure of an enzyme. (True/False)
88. Most of the enzymes (higher organisms) show the optimum activity at pH between
- (a) 3.0 and 6.0
 - (b) 6.0 and 8.0
 - (c) 6.0 and 10.0
 - (d) 3.0 and 7.0
89. In thermal denaturation of enzymes, it is seen that variations in temperature
- (a) may affect K_m
 - (b) may affect V_{max}
 - (c) may affect both K_m and V_{max}
 - (d) no change in K_m and V_{max}
90. Exposure of enzymes to radiations like UV, beta or gamma or X-rays leads to loss of structure
- (a) primary
 - (b) secondary
 - (c) tertiary
 - (d) quaternary
91. Active sites are small regions on the enzyme surfaces to which the substrates binds and participates in the process of catalysis. These are due structures of the proteins.
92. Enzyme inhibitions may be reversible or irreversible. (True/False)
93. Which of the following statements is true to the reversible non-competitive inhibition process.
- (a) K_m remains unchanged, V_{max} is lowered
 - (b) K_m lowers, V_{max} increases
 - (c) K_m increases, V_{max} decreases
 - (d) K_m remains unchanged, V_{max} unchanged
94. Which of the following statements is true to the reversible uncompetitive inhibition process.
- (a) K_m increases, V_{max} decreases
 - (b) K_m increases, V_{max} increases
 - (c) K_m decreases, V_{max} decreases
 - (d) K_m remains unchanged, V_{max} remains unchanged

95. enzyme is responsible for hydrolyzing sucrose into glucose and fructose.
96. High substrate concentrations may cause inhibition in enzymatic reactions. (True/False)
97. Proteins containing two or more topologically distinct binding sites which interact functionally with each other are called.....
.....
(a) allosteric proteins
(b) fibrous proteins
(c) globular proteins
(d) multimeric proteins
98. Allosteric inhibitors and activators are collectively called
-
99. In comparing Michaelis–Menten and Allosteric kinetics on rate vs substrate concentration it is observed that
(a) Allosteric profile is above the MM curve
(b) Allosteric profile is coinciding with MM curve
(c) Allosteric profile is below the MM curve
(d) None of these
100. Half life period of an enzyme molecule is given by the following equation
(a) $t_{0.5} = 0.693 \times k_{de}$
(b) $t_{0.5} = 0.693/k_{de}$
(c) $t_{0.5} = 0.693 + k_{de}$
(d) $t_{0.5} = 0.693 - k_{de}$
101. The binding of S and P to E to form complexes is sometimes possible in enzymatic reactions. Such complexes are called
-
102. Feedback inhibition is also called
103. Esterases act upon glycerides to give
(a) glucose + oligosaccharides
(b) methanol + polygalacturonic acids
(c) glycerol + fatty acids
(d) none of these
104. Urease hydrolyzes urea into carbondioxide and ammonia. (True/False)
105. Galactose and glucose are liberated when
(a) lactase acts upon lactose
(b) maltase acts upon maltose
(c) amylase acts upon starch
(d) fructosidase acts upon sucrose

106. Proteolytic enzymes, if attack on terminal group of poly amino acids, they are called exopeptidases. (True/False)
107. Proteolytic enzymes are used in
(a) meat processing industry
(b) dairy industry
(c) tanning industry
(d) all of these.
108. In the desugaring of eggs enzyme is used.
109. Starch liquefying amylase among the following is
(a) Pectinase
(b) Protease
(c) Taka diastase
(d) Bromelain
110. Penicillinase is produced by
(a) *Bacillus coagulans*
(b) *Bacillus cereus*
(c) *Bacillus licheniformis*
(d) *Bacillus subtilis*
111. *Saccharomyces cerevisiae* produces
(a) cellulase
(b) invertase
(c) amylase
(d) pectinase
112. In enzyme stabilization following strategies are used
(a) addition of stabilizing compounds
(b) chemically modifying soluble proteins
(c) immobilising proteins on or within insoluble solid matrix
(d) all of these
113. α -amylase is stabilized by the addition of 50 to 70 per cent sorbitol which finally imparts better storage property and thermal stability. (True/False)
114. Of the following supports, pick the synthetic support for immobilizing enzymes
(a) cellulose
(b) dextran
(c) agarose
(d) biogel
115. Of the following, pick the chemical method of immobilization
(a) cross linked enzyme matrix
(b) microencapsulation
(c) cross linking by multi functional reagents
(d) both (a) and (c)

116. Damkohler number is defined as
- (a) the ratio of maximum rate of reaction to maximum rate of diffusion
 - (b) ratio of maximum rate of diffusion to maximum rate of reaction
 - (c) ratio of minimum rate of reaction to minimum rate of diffusion
 - (d) ratio of minimum rate of reaction to maximum rate of diffusion
117. Effectiveness factor is defined as
- (a) ratio of reaction rate without diffusion limitation to reaction rate with diffusion limitation
 - (b) ratio of reaction rate with diffusion limitation to reaction rate without diffusion limitation
 - (c) reaction rate with diffusion limitation—reaction rate without diffusion limitation
 - (d) none of these
118. In the formulation and characterization of immobilized cell biocatalysts, the methods employed to prepare porous polymer beads are
- (a) Polymer precipitation
 - (b) Polycondensation
 - (c) Polymerization
 - (d) All of these
119. Cells are immobilized by using the method of
- (a) attachment
 - (b) entrapment
 - (c) containment and aggregation
 - (d) all of these
120. In the perspective of cell population kinetics, the ideal case is
- (a) Structured and segregated
 - (b) Structured and unsegregated
 - (c) Unstructured, unsegregated
 - (d) Unstructured and segregated
121. In balanced growth the average cellular composition is
- (a) affected by the proliferation of population
 - (b) unaffected by the proliferation of population
 - (c) affected by the non proliferation of population
 - (d) none of these
122. Ideal continuous stirred tank reactors are also called chemostats. (True/False)
123. In the kinetics of balanced growth it is seen that
- (a) dilution rate is not equal to specific growth rate
 - (b) dilution rate is equal to specific growth rate
 - (c) dilution rate is less than specific growth rate
 - (d) dilution rate is more than the specific growth rate

124. In the multiple lag phases during growth multiple carbon sources are utilized by the micro organisms. (True/False)
125. Yield coefficients for organisms growing aerobically on glucose range between
- 0.9 and 1.4 g/g
 - 0.4 and 0.9 g/g
 - 0.9 and 1.0 g/g
 - none of these
126. CSTRs or CSTFs can be operated as turbidostats which refer to the feed metered in such a way that
- biomass concentration decreases
 - biomass concentration is constant
 - biomass concentration increases
 - none of these
127. At high dilution rates it is seen that the substrate concentration and biomass concentration at steady state
- increases and decreases respectively
 - decreases and increases respectively
 - both increase
 - both decrease
128. Specific rate of production is also called cell productivity. (True/False)
129. As dilution rate D nears maximum specific growth rate μ_{\max} and X becoming infinitesimally small, this condition is called
.....
130. High fructose corn syrup (HFCS) is a low calorie sweetener with a higher level of fructose (55%). (True/False)
131. Prednisone, cortisone, dexamethasone, corticosteroids commonly known as steroids are manufactured by using the raw material, complex alcohols. (True/False)
132. Dextran is commercially produced using an organism *Leuconostoc mesenteroides*. (True/False)
133. Primary metabolites as amino acids, nucleotides and proteins are produced during the growth phase and are needed for growth. (True/False)
134. Antibiotics are generally secondary metabolites. (True/False)
135. A decanter centrifuge is also called
136. In the process of cell disruption, this is not a physical method.
- thermolysis
 - osmotic shock
 - lipid solubilisation
 - ultrasonication

137. Precipitation as a means of downstream process, can be brought about by
- (a) Adding a precipitant
 - (b) Isoelectric pH
 - (c) Reduction of medium dielectric constant
 - (d) All of the above
138. The counter ligand for antibody as a ligand in affinity chromatography is
- (a) substrate analogue
 - (b) antigen
 - (c) virus
 - (d) both (b) and (c)
139. Freeze drying is also called and is generally used for materials.
140. In reverse osmosis a reverse pressure difference applied on the membrane is
- (a) 20–100 bars
 - (b) 10–100 bars
 - (c) 20–200 bars
 - (d) none of these
141. Ultra filtration generally is operated in the pressure range between
- (a) 2 and 10 bar
 - (b) 2 and 20 bar
 - (c) 20 and 50 bar
 - (d) above 50 bar
142. Dialysis involves the separation of solutes by diffusion across the membrane from one liquid phase to another on the basis of and
143. The process useful in harvesting microbial cells from fermentation broth is
- (a) dialysis
 - (b) ultra filtration
 - (c) microfiltration
 - (d) reverse osmosis
144. The word *control of microorganisms* refers to the reduction in the numbers and or the activity of microbial flora and is used
- (a) to prevent the transmission of diseases and infection
 - (b) to prevent the contamination by or the growth of undesirable organisms
 - (c) to prevent the deterioration and food spoilage
 - (d) all of these
145. Which of the following statements is true to microorganism type
- (a) spore forming bacteria are more resistant to adverse conditions
 - (b) vegetative cells are more susceptible to adverse conditions

- (c) both are susceptible to adverse conditions
 - (d) both (a) and (b)
146. Antimicrobial agents inhibit or destroy the microorganisms and their activity by
- (a) damaging the cell wall
 - (b) altering the cytoplasmic membrane permeability
 - (c) preventing the synthesis of proteins and nucleic acids
 - (d) all of these
147. High temperatures combined with high moisture are considered to be one of the most effective methods of killing microorganisms. (True/False)
148. Autoclave is also called high pressure steam sterilizer. (True/False)
149. Desiccation that refers to cessation of microbial metabolic activity depends on
- (a) type of microorganism
 - (b) physical conditions to which they are exposed
 - (c) the material in which organism is dried
 - (d) all of these
150. Which of the following is not a characteristic of antimicrobial chemical agent:
- (a) insolubility
 - (b) capacity to penetrate
 - (c) detergent action
 - (d) deodorizing capacity
151. Germicide is an agent that kills the growing forms but not necessarily the resistant spore forms of germs. (True/False)
152. Culture medium can be sterilized by
- (a) tyndallization
 - (b) using red heat
 - (c) using autoclave (moist heat) at 121°C for 15 minutes
 - (d) incineration
153. In continuous sterilization, using highest feasible temperature for short time, HTST it is seen that
- (a) both heat-up and cool-down periods are very fast
 - (b) heat-up period is fast and cool-down period is slow
 - (c) heat-up period is slow and cool-down period is fast
 - (d) both are slow
154. Bacteriostasis is a condition in which the growth of bacteria is not prevented. (True/False)
155. The membranes employed in NF are low pressure RO membranes with very high rejections and high permeates of salt at low concentrations, but lose their selectivity at salt concentrations above 1000–2000 ppm of salt in water. (True/False)

Glossary

Acclimation: Automatic adjustment of microorganisms to the surroundings.

Acetyl choline: Most common neurotransmitters.

Acetyl Co A: The entry compound from the Krebs cycle in cellular respiration, formed from a fragment of pyruvate attached to a coenzyme.

Actin: A globular protein that links into chains, two of which twist helically about each other to form micro-filaments in contractile muscles.

Active site: Specific part of an enzyme that attaches to the substrate by means of weak chemical bonds.

Aerobic respiration: Harvesting chemical energy in the form of ATP from food molecules, with oxygen as an electron acceptor.

Allosteric site: A specific receptor site on an enzyme molecule away from the active site.

Alpha (α) helix: Form of secondary structure of proteins arising from a specific hydrogen bonding giving a spiral shape.

Amino acid: An organic molecule having both carboxyl and amino groups, and are monomers of proteins.

Anaerobic respiration: Respiration that occurs in a few groups of bacteria leaving in anaerobic environments such as soil. In this case sulphates and nitrates are the electron acceptors.

Antibiotic: A chemical that kills or inhibits the growth of bacteria, usually by transcriptional or translational regulation.

Antibody: An antigen binding immunoglobulin produced by B cells and functions as effector in an immune response.

ATP (Adenosine Triphosphate): An adenine containing nucleoside triphosphate that releases free energy when its phosphate bonds are hydrolyzed. It is called an energy currency of the cell.

ATP synthetase: A protein complex that produces ATP.

Auto radiography: A method for localizing radioactive atoms in microscopic preparations of biological materials by exposing a photographic film emulsion to radioactive atoms incorporated in the biological specimen.

B cells: A type of lymphocyte that develops in the bone marrow and later produces antibodies that mediate humoral immunity.

Bacterium (Plural, Bacteria): A unicellular microbe also known as a prokaryote and has no true nucleus. Classified into two groups based on difference in cell walls as determined by Gram staining.

Beta (β) pleated sheet: A form of secondary structure of proteins having hydrogen bonds between polypeptide segments running in opposite directions giving a zigzag shape.

Binary fission: Type of cell division by which prokaryotes reproduce giving daughter cells that receive a copy of single parental chromosome.

Biofuel: Fuel derived from organic waste and is an alternate source of fuel.

Biogeochemical cycles: The various nutrient circuits, which involve both biotic and abiotic components of ecosystems.

Biomass: The dry weight of organic matter comprising a group of organisms in a particular habitat.

Biosphere: Entire portion of earth having life.

Biotechnology: The industrial use of living organisms or their components to improve human health and food production.

Budding: An asexual means of reproduction giving protrusions during growth.

cDNA: Complimentary DNA.

Carbohydrate: A sugar or one of its dimers or polymers.

Carboxyl group: Functional group in organic acids consisting of a single carbon atom double bonded to an oxygen atom and also bonded to a hydroxyl group.

Catabolic pathway: A metabolic pathway that releases energy by breaking down complex molecules into simpler ones.

Cell fractionation: The disruption of a cell and separation of its organelles by centrifugation.

Cell wall: Unique to plant cells having cellulose fibres and is rigid.

Cellular respiration: The most efficient catabolic pathway for the production of ATP.

Cellulose: Structural polysaccharide of cell walls consisting of glucose monomers held by glycosidic bonds.

Chitin: Structural polysaccharide found in many fungi and in the exoskeletons of all arthropods.

Cholesterol: A steroid forming an essential component of animal cell membranes and can act as a precursor molecule for the synthesis of other important steroids.

Coenzyme: An organic molecule serving as a cofactor. Many vitamins function as coenzymes in important metabolic reactions.

Cofactor: Non-protein molecule that is needed for proper functioning of an enzyme.

Competitive inhibitor: A substance that reduces the activity of an enzyme by competing with a substrate.

Cyanobacteria: Photosynthetic, oxygen producing bacteria also called blue green algae.

Cyclic AMP (cAMP): Cyclic adenosine monophosphate.

Cytoskeleton: Network of micro-tubules, micro-filaments that serve a variety of mechanical and transport functions.

Denaturation: A process in which a protein loses its native conformation, thereby becoming biologically inactive.

DNA: Deoxyribonucleic acid, double stranded and can replicate in transmission of characters.

DNA polymerase: An enzyme that catalyzes the elongation of new DNA during replication.

DNA probe: A chemically synthesized, radioactively labelled segment of nucleic acid used to find a gene of interest.

Enzymes: A class of proteins serving as catalysts.

Essential nutrient: A chemical element required by animals and plants and cannot be synthesized within itself.

Exons: The coding regions of a eukaryotic gene that are expressed and are separated from each other by introns.

Fat (Triacylglycerol): A biological compound consisting of three fatty acids linked to one glycerol molecule.

Fatty acid: A long carbon chain carboxylic acid.

Fermentation: A catabolic process that makes a limited amount of ATP from glucose without an electron transport chain and produces ethyl alcohol or lactic acid.

Gel electrophoresis: Separation of nucleic acids or proteins on the basis of their size and electric charge, by measuring their rate of movement through an electric field in a gel.

Gene: DNA fragment.

Gene amplification: The selective synthesis of DNA, which results in multiple copies of a single gene, thereby enhancing expression.

Genome: The complete compliment of an organism's genes.

Glycolysis: One of the metabolic pathways in which glucose is converted to pyruvate.

Half life: The average amount of time it takes for one half of a specified quantity of a substance to decay or disappear.

Hormone: One of many types of circulating chemical signals in all multicellular organisms.

Hydrolysis: A process that splits the molecules by the addition of water.

Hydrophilic: Having an affinity for water.

Hydrophobic: Having an aversion to water.

Immunoglobulins: The class of proteins comprising antibodies.

Induced fit: The change in shape of the active site of an enzyme, so that it binds more snugly to the substrate, induced by entry of the substrate.

Introns: The non-coding, intervening sequence of coding region (exon) in eukaryotic genes.

Krebs cycle: A chemical cycle involving eight steps, which completes the metabolic breakdown of glucose molecules to carbon dioxide and occurs in mitochondria. Also called TCA cycle.

Lipid: Family of biopolymers that comprises fats, phospholipids and steroids that are insoluble in water.

Lipoprotein: A protein bonded to a lipid.

Lysozyme: An enzyme in perspiration, tears and saliva that attacks bacterial cell walls.

Messenger RNA (mRNA): A type of RNA synthesized from DNA in the genetic material that attaches to ribosomes in the cytoplasm and specifies the primary structure of a protein.

Metabolism: The totality of an organism's chemical processes, consisting of catabolic and anabolic pathways.

Micelle: Complexes of bile salts, fatty acids, monoglycerides and cholesterol that are highly soluble.

Mould: A rapid growing, asexually reproducing fungus.

Monoclonal antibody (MABs): A defensive protein produced by cells descended from a single cell; an antibody that is secreted by a clone of cells and consequently is specific for a single antigenic determinant.

Mycelium: A densely-branched network of hyphae in a fungus.

Myeloma: A tumor of the antibody producing B-lymphocyte; it is used to produce MABs.

Non-competitive inhibitor: A substance that reduce the activity of an enzyme by binding to a location far away from the active site changing the conformation.

Nucleic acid: A biological molecule such as DNA or RNA that allows organisms to reproduce.

Nucleoside: An organic molecule consisting of a nitrogenous base with a pentose sugar.

Nucleotide: The building block of nucleic acid, consisting of pentose sugar covalently bonded to a nitrogenous base and a phosphate group.

Peptide bond: The covalent bond between two amino acids formed by condensation synthesis.

Polymer Chain Reaction (PCR): A technique for amplifying DNA *in vitro* by incubating with special primers, DNA polymerase molecules and nucleotides.

Polysaccharide: A polymer of up to over a thousand monosaccharides, formed by condensation synthesis.

Primary structure: The level of protein structure referring to the sequence of amino acids.

Primer: An existing DNA chain bound to the template DNA to which nucleotides must be added during the DNA synthesis.

Protein: A three-dimensional biological polymer having a set of 20 different amino acids.

Quaternary structure: The particular shape of a complex, aggregate protein, defined by the characteristic three-dimensional arrangements of its subunits, each a polypeptide.

Recombinant DNA: A technique in which gene segments from different sources are recombined *in vitro* and transferred into cells, where the DNA may be expressed.

Restriction enzyme: A degradative enzyme that identifies and cuts up DNA that is foreign to a cell.

Reverse transcriptase: An enzyme encoded by some viruses that uses RNA as a template for DNA synthesis.

Ribonucleic acid (RNA): A single-stranded nucleic acid molecule involved in protein synthesis and the structure of which is given by DNA.

Ribozyme: Enzymatic RNA molecule and catalyzes reactions during RNA splicing.

RNA polymerase: An enzyme that links together the growing chain of ribonucleotides during transcription.

Secondary structure: The localized, repetitive folding of the polypeptide backbone of a protein due to hydrogen bond between peptide linkages.

Steroids: A class of lipids characterized by a carbon skeleton consisting of four rings with various functional groups.

Substrate: A substance on which an enzyme acts.

Tertiary structure: Irregular contortions of a protein molecule due to interactions of side chains involved in hydrophobic interactions, ionic bonds and disulphide bridges.

Transcription: The transfer of information from a DNA molecule into an RNA molecule.

Transfer RNA (tRNA): An RNA molecule that acts between nucleic acid and protein language by selecting specific amino acids and identifying the appropriate codons in mRNA.

Translation: The transfer of information from an RNA molecule into a polypeptide, involving a change of language from nucleic acids to amino acids.

Unsaturated fatty acid: The fatty acid possessing one or more double bonds between carbons in the hydrocarbon tail.

Vitamin: An organic molecule required in the diet in very small quantities; serve as a coenzyme or part of a coenzyme.

Yeast: A unicellular fungus that lives in liquid or moist habitats reproducing asexually or by budding of a parent cell.

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