

BIOTECHNOLOGY OF MICROBIAL ENZYMES

PRODUCTION, BIOCATALYSIS AND INDUSTRIAL APPLICATIONS

Edited by
GOUTAM BRAHMACHARI

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Applications

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Dedication

*To all those who are working
with the microbial world*

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Preface

This single volume, titled *Biotechnology of Microbial Enzymes: Production, Biocatalysis, and Industrial Applications*, is an endeavor focusing on the recent cutting-edge research advances in the field of microbial enzymes, very particularly emphasizing their production, modifications, and industrial applications. This book, comprising a total of 20 chapters written by active researchers and leading experts in microbial enzymes from several countries, in response to our personal invitation, brings together an overview of current discoveries and trends in this remarkable field. We are most grateful to the contributors for their generous and timely response in spite of their busy and tight schedules with academics, research, and other responsibilities.

Enzymes are potential biocatalysts produced by living cells to bring about specific biochemical reactions, linked with the metabolic processes of the cells. Due to the unique biochemical properties of enzymes, the demand for industrial enzymes is continuously rising driven by a growing need for sustainable solutions. Microorganisms have provided, and continue to provide, an impressive number of such biocatalysts with a wide range of applications across several industries such as food, animal feed, household care, technical industries, biofuels, fine chemicals, and pharmaceuticals. As mentioned, the unique properties of enzymes, such as high specificity, fast action, and biodegradability, allow enzyme-assisted processes in industry to run under mild reaction conditions, with improved yields and reduced waste generation. However, despite these advantages, natural enzymes do not often fulfill all process requirements and need further tailoring or redesign in order to fine-tune key catalytic properties.

Recent advances in *omics* technologies (eg, genomics, metagenomics, proteomics), efficient expression systems, and emerging recombinant DNA techniques, facilitate the discovery of new microbial enzymes from nature, or by creating (or evolving) enzymes with improved catalytic properties. Combinations of engineered and de novo designed enzymes, coupled with chemistry, have been successful in generating more (and even new) chemicals and materials from cheaper and renewable resources, thereby opening a new window to establishing a bio-based economy and achieving low carbon “green” growth. The ongoing progress and interest in enzymes provide further success in many areas of industrial biocatalysis. In addition, the application of one-pot multistep reactions using multifunctional catalysts, or new and improved enzyme immobilization techniques, is also receiving growing interest in biocatalysis.

Many of the technologies and strategies mentioned above are gathered together in this book. In the introductory chapter (see chapter: Useful Microbial Enzymes—An Introduction), Sanchez and Demain have offered an overview on the importance and significance of useful microbial enzymes in both academia and industry. “Production, Purification, and Application of Microbial Enzymes” by Pandey and his coauthors deals with the production, purification, and applications of microbial enzymes focusing on their industrial benefits. “Solid State Fermentation for Production of Microbial Cellulases” by Ray and Behera is devoted to solid state fermentation techniques for production of microbial cellulases from various lignocellulosic substrates. The authors have critically analyzed the pros and cons of this viable technique along with its future openings and prospects. Koga and his group in “Hyperthermophilic Subtilisin-Like Proteases From *Thermococcus kodakarensis*” have described in detail the unique maturation and stabilization mechanisms of two subtilisin-like proteases, Tk-subtilisin and Tk-SP, isolated from *Thermococcus kodakarensis*, highlighting the prospect of their applications in novel medical and industrial fields. In “Enzymes from Basidiomycetes—Peculiar and Efficient Tools for Biotechnology,” Peralta and coauthors have offered an excellent overview on the potential biotechnological applications of basidiomycete-produced enzymes in the degradation of the complex structure of lignocelluloses, thereby opening a useful window towards the exploration of such lignocellulosic biomass for the production of fuel ethanol and other value-added commodity chemicals. Liu and his group have overviewed, in “Microbial Production and Molecular Engineering of Industrial Enzymes: Challenges and Strategies,” the commonly used strategies for microbial enzyme production and molecular engineering, emphasizing their opportunities and challenges in this remarkable field. “Metagenomics and the Search for Industrial Enzymes” by Ferrer and coauthors highlights the metagenomic techniques and possible applications in achieving collections of sequences encoding enzymes applicable in industrial sectors. Alcalde and his group have elaborated in detail (see chapter: The Pocket Manual of Directed Evolution: Tips and Tricks) the recent research advances in the field of *directed evolution*, a robust approach to tailor enzymes with improved attributes. Useful applications, opportunities, challenges, and future scopes of such *Tips and Tricks* are examined thoroughly by the authors in their discussion. “Insights into the Structure and Molecular Mechanisms of β-Lactam Synthesizing Enzymes in Fungi” by Martín and Liras deals with the structure and molecular mechanisms of β-lactam synthesizing enzymes in fungi. They have offered insights into the molecular characterization of the enzymes involved in penicillin biosynthesis and the early steps of cephalosporin formation, so as to have an idea of their molecular mechanisms. Recent studies on the cellulosome, a supramolecular assembly of microbial biomass-degrading enzymes, have provided researchers with an insight into how microbial enzymes can exist in the form of a mysterious extracellular organelle and how abundant water-insoluble plant biomass is degraded in nature by such a well-organized protein complex. Chow and Wu

have outlined these aspects in “The Cellulosome: A Supramolecular Assembly of Microbial Biomass-Degrading Enzymes,” highlighting the composition, structure, and assembly of the cellulosome of *Clostridium thermocellum*, a thermophilic and anaerobic bacterium.

Liu and Kokare have overviewed the classification, microbial resources, microbial production, and industrial applications of a group of industrially-used microbial enzymes, such as carbohydrases, proteases, and lipases, in “Microbial Enzymes of Use in Industry.” This chapter also focuses on the challenging potential of enzyme technology required to redesign naturally occurring enzymes in order to fine-tune their substrate specificity and thermostability for proper utilization on the industrial scale. Glucokinases (GKs) are responsible for glucose phosphorylation utilizing diverse phosphoryl donors such as ATP, ADP, and/or polyphosphate. Sanchez and coauthors have highlighted the significance of such microbial glucokinases in “Significance of Microbial Glucokinases,” emphasizing their physicochemical and biochemical characteristics, as well as their potential applications. Microbial enzymes, today, find useful applications as biocatalysts in a wide array of organic transformations with a handful of advantages; Brahmachari has offered a recent update on lipase-catalyzed organic transformations focusing on the biocatalytic efficacy of the enzyme in carrying out various types of organic reactions, including esterification, transesterification, additions, ring-closing, oxidation, reduction, amidation, and many others, in “Lipase-Catalyzed Organic Transformations: A Recent Update.” “Enzymatic Biocatalysis in Chemical Transformations: A Promising and Emerging Field in Green Chemistry Practice” by Blamey et al. elaborates the chemo-enzymatic uses of a variety of *green* biocatalysts in chemical transformations, yielding fine chemicals with potential applications, both in academia and industry. Microbial enzymes carry out many targeted chemical reactions with ease and specificity satisfying many aspects of green chemistry as well, and the authors have offered a useful discussion herein. Desmet and his group have outlined one such important chemical transformation, that is, glycosylation of useful chemical entries, with the help of microbial enzyme-catalysis, in “Microbial Enzymes for Glycoside Synthesis: Development of Sucrose Phosphorylase as a Test Case,” focusing on recent research outcomes in optimizing the glycosylation potential of sucrose phosphorylase as a test case. In “Industrial Applications of Multistep Enzyme Reactions,” Honda overviews the current status of commercially exploited multistep enzymatic reactions and ongoing challenges for expanding the industrial applicability of multienzyme systems. Advancing gene- and enzyme-engineering technologies have provided more versatile and less expensive approaches to prepare a variety of enzymes, leading to more flexible design of multistep enzyme cascades; the chapter focuses on such developments with representative examples of such reactions and the current state-of-the-art for developing further advanced multienzyme systems in detail. “Biocatalysis for Industrial Production of Active Pharmaceutical Ingredients (APIs),” by Barredo and coauthors, presents an excellent overview on biocatalysis for industrial

production of active pharmaceutical ingredients, with examples of the application of isolated enzymes (wild-type or mutant) and whole cells, either in soluble or immobilized form, in the synthesis of such chemical entities, at both the industrial and laboratory scales. The authors also discuss the biocatalytic production of enzyme inhibitors with therapeutic effects, and drugs that modify the transport across membranes, along with those that interact with membrane and intracellular receptors as well. “Agro-Industrial Residues and Microbial Enzymes: An Overview on the Eco-Friendly Bioconversion into High Value-Added Products” by Valdo Madeira Jr et al. provides an updated and succinct overview of agro-industrial residues, applications of enzymes in lignocellulosic materials, and production of chemical compounds with potential applications from such biomass. Fernandes and Carvalho have underlined the fruitful applications of microbial enzymes for the food industry in “Microbial Enzymes for the Food Industry.” “Productive Chain of Biofuels and Industrial Biocatalysis: Two Important Opportunities for Brazilian Sustainable Development” by Ferreira-Leitão and her collaborators offers insight into the current situation of the two main biofuels in Brazil, that is, ethanol and biodiesel, and introduces a discussion of opportunities and bottlenecks in the exploitation of lignocellulosic and oleaginous materials, focusing on the important role of enzymatic and microbial processes to support a sustainable industry.

Representation of facts and their discussions in each chapter are extensive, authoritative, and deeply informative; hence, the book serves as a key reference for recent developments in the frontier research on the biotechnological developments of microbial enzymes and their prospective industrial applications. The broad interdisciplinary approach of this book will surely make the work very interesting to scientists deeply engaged in the research and/or use of microbial enzymes. We would like to express our sincere thanks to all the contributors for their excellent reviews in this remarkable area. It is their participation that made our effort to organize such a book possible. Their masterly accounts will surely provide the readers with a strong awareness of current cutting-edge approaches in this remarkable field of research.

We would also like to express our deep sense of appreciation to all the editorial and publishing staff members associated with Elsevier Inc. for their keen interest in publishing this book, as well as their all-around help to ensure that the highest standards have been maintained in publishing this book.

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Chapter 1

Useful Microbial Enzymes— An Introduction

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1.1 THE ENZYMES: A CLASS OF USEFUL BIOCHEMICALS

According to the International Union of Biochemistry (IUB), and based on their nature of reaction, enzymes are divided into six classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. The use of enzymes in industrial processes has been of crucial importance since they can eliminate the use of high temperatures, extreme pH values, organic solvents, and at the same time, offer high substrate specificity, low toxicity, product purity, reduced environmental impact, and ease of termination of activity. Microorganisms constitute the major source of enzymes as they produce high concentrations of extracellular enzymes. Screening for the best enzymes is simple, allowing the examination of thousands of cultures in a short period of time. Microorganisms used for enzyme production include around 50 GRAS bacteria and fungi. Bacteria are mainly represented by *Bacillus subtilis*, *Bacillus licheniformis*, and various *Streptomyces* species. Fungi are usually represented by *Aspergillus*, *Mucor*, and *Rhizopus*. Microorganisms can be cultured in large quantities in a relatively short period by established methods of fermentation. Microbial enzyme production is economical on a large scale due to inexpensive culture media and short fermentation cycles.

There are more than 3000 different enzymes known but only 5% are commercially used (Binod et al., 2013). Over 500 commercial products are made using enzymes (Johannes and Zhao, 2006). In regard to the total enzyme market, its global figures depend on the consulted source. In one case, the market reached \$5.1 billion in 2009 and is predicted to rise 6.45 per annum to grasp \$6.9 billion in 2017 (The Freedonia Group, Inc., 2014). In a second report, it was estimated to be \$3.3 billion in 2010 and to reach \$4.4 billion by

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2015 (BBC Research, 2011; Binod et al., 2013). The major technical enzymes are used in bulk form for manufacture of detergents, textiles, leather, pulp, paper, biofuels, and the market for these enzymes reached \$1.2 billion in revenues in 2011 and is still on the rise. Other applications include household care, foods, animal feed, fine chemicals, and pharmaceuticals. Enzymes have unique properties such as rapid action, high specificity, biodegradability, high yields, ability to act under mild conditions, and reduction in generation of waste materials. These properties offer flexibility with respect to operating conditions in the reactor.

Sales of feed enzymes are expected to reach \$730 million in 2015. They are used to increase nutrient digestibility, and to degrade unacceptable components of feed. Included, mainly for poultry and swine, are proteases, phytases, glucanases, alpha-galactosidases, alpha-amylases, and polygalacturonases. Recent emphasis has been on development of heat-stable enzymes, economical and rapid assays that are more reliable, improvement of activity, and discovery of new nonstarch polysaccharide-degrading enzymes.

Enzymes for food and beverage manufacture are a major part of the industrial enzyme market, reaching sales of almost \$1.2 billion in 2011. Lipases constitute a major portion of the usage, targeting fats and oils. In order to maximize flavor and fragrance, control of lipase concentration, pH, temperature, and emulsion content is necessary. Lipases are potentially useful as emulsifiers for foods, pharmaceuticals, and cosmetics. *Aspergillus oryzae* is used as a cloning host to produce fungal lipases, such as those from *Rhizomucor miehi*, *Thermomyces lanuginosus*, and *Fusarium oxysporum*.

Important detergent additives include proteases, lipases, oxidases, amylases, peroxidases, and cellulases which catalyze the breakdown of chemical bonds upon the addition of water. The useful ones are active at thermophilic temperatures (c. 60°C) and alkalophilic pH (9–11), and in the presence of components of washing powders.

Over 60% of the worldwide enzyme market is devoted to proteases. These enzymes are involved in the manufacture of foods, pharmaceuticals, leather, detergents, silk, and agrochemicals. Their use in laundry detergents constitute 25% of global enzyme sales. They include (1) the *B. licheniformis* alkalase Biotex, (2) the first recombinant detergent lipase called Lipolase, made by cloning the lipase from *Humicola lanuginose* into *A. oryzae*, (3) the *Pseudomonas mendocina* lipase (Lumafast), and (4) the *Pseudomonas alcaligenes* lipase (Lipomax).

Natural enzymes are often unsuitable for use as industrial biocatalysis and need modifications for industrial use. The production strains are usually modified by genetic manipulation to gain improved properties, including high production levels. With the introduction of recombinant DNA technology, it has been possible to clone genes encoding enzymes from microbes and expressing them at levels tens and hundreds times higher than those produced by unmodified microorganisms. Because of this, the enzyme industry rapidly accepted the

technology and moved enzyme production from strains not suited for industry into industrial strains (Galante and Formantici, 2003). Genomics, metagenomics, proteomics, and recombinant DNA technology are employed to facilitate the discovery of new enzymes from microbes in nature, and to create or evolve improved enzymes. A number of new and useful enzymes have been obtained by metagenomics (Ferrer et al., 2007).

Directed evolution of proteins includes DNA shuffling, whole genome shuffling, heteroduplex, random chimeragenesis of transient templates, assembly of designed oligonucleotides, mutagenic and unidirectional reassembly, exon shuffling, Y-ligation-based block shuffling, nonhomologous recombination, and the combination of rational design with directed evolution (Yuan et al., 2005; Siehl et al., 2005; Bershteyn and Tewfic, 2008; Reetz, 2009). Directed evolution has yielded increased activity, stability, solubility, and specificity of enzymes. For example, it increased the activity of glyphosate-N-acetyltransferase 10,000-fold and, at the same time, its thermostability by 5-fold.

1.2 MICROBIAL ENZYMES FOR INDUSTRY

According to their applications, microbial enzymes have been applied to make numerous biotechnology products and in processes commonly encountered in the production of laundry, food and beverages, paper and textile industries, clothing, etc. The use of enzymes as detergent additives represents a major application of industrial enzymes. The detergent market for enzymes has grown strongly in the last 25 years. In the year 2003, it was around \$0.79 billion, with proteases as the major detergent enzyme product. The detergent industry uses more than 25% of the total enzyme production.

Proteases, lipases, amylases, oxidases, peroxidases, and cellulases are added to the detergents where they catalyze the breakdown of chemical bonds on the addition of water. For this purpose, they must be active under thermophilic (60°C) and alkalophilic (pH 9–11) conditions, as well as in the presence of various components of washing powders (Stoner et al., 2005). The market share of detergent proteases is estimated to be at 72% of the global detergent enzyme market (Maurer, 2015). The first detergent containing a bacterial protease was introduced in 1956, and in 1960, Novo Industry A/S introduced alcalase produced by *B. licheniformis* (“Biotex”). Cellulase from *Bacillus* sp. KSM-635 has been used in detergents because of its alkaline pH optimum and insensitivity to components in laundry detergents (Ozaki et al., 1990). Later, Novozymes launched a detergent using a cellulase complex isolated from *Humicolla insolens* (Celluzyme). Certain microorganisms called extremophiles grow under extreme conditions such as 100°C, 4°C, 250 atm., pH 10, or 5% NaCl. Their enzymes, which act under such extreme conditions, are known as extremozymes. One such enzyme, called Cellulase 103, was isolated from an alkaliophile and commercialized because of its ability to break down microscopic fuzz of cellulose fibers which trapped dirt on the surface of cotton textiles.

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It has been used for over 10 years in detergents to return the “newness” of cotton clothes, even after many washings. As early as the mid-1990s, virtually all laundry detergents contained genetically-engineered enzymes (Stoner et al., 2005). Over 31% of the enzymes used in detergents are recombinant products (McAuliffe et al., 2007).

The major application of proteases in the dairy industry is for the manufacturing of cheese. Four recombinant proteases have been approved by FDA for cheese production. Calf rennin had been preferred in cheese-making due to its high specificity, but microbial proteases produced by GRAS microorganisms, such as *Mucor miehei*, *Mucor pusilis*, *B. subtilis*, and *Endothia parasitica*, are gradually replacing it. The primary function of these enzymes in cheese-making is to hydrolyze the specific peptide bond (Phe105-Met106) that generates para-k-casein and macropeptides. Nearly 40,000 U/g bran of milk-clotting activity are produced by *A. oryzae* at 120h by solid state fermentation (Vishwanatha et al., 2009). For many years, proteases have also been used for production of low allergenic milk proteins used as ingredients in baby milk formulas (Gupta et al., 2002).

Proteases can also be used for synthesis of peptides in organic solvents. Thermolysin is used in this way to make aspartame (Oyama et al., 1981). Aspartame sales reached \$1.5 billion in 2003 (Baez-Viveros et al., 2004). In 2004, the production of aspartame amounted to 14,000 metric tons. The global sugar substitute market is the fastest growing sector of the sweetener market.

Fungal alpha-amylase, glucoamylase, and bacterial glucose isomerase are used to produce “high fructose corn syrup” from starch in a business amounting to \$1 billion per year. Fructose syrups are also made from glucose by “glucose isomerase” (actually xylose isomerase) at a level of 15 million tons per year. The food industry also uses invertase from *Kluyveromyces fragilis*, *Saccharomyces cerevisiae*, and *Saccharomyces carlsbergensis* for manufacture of candy and jam. Beta-galactosidase (lactase), produced by *Kluyveromyces lactis*, *K. fragilis*, or *Candida pseudotropicalis*, is used to hydrolyze lactose in milk or whey and alpha-galactosidase from *S. carlsbergensis* is employed in the crystallization of beet sugar.

Microbial lipases catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids. They are commonly used in the production of a variety of products ranging from fruit juices, baked foods, pharmaceuticals, and vegetable fermentations to dairy enrichment. Fats, oils, and related compounds are the main targets of lipases in food technology. Accurate control of lipase concentration, pH, temperature, and emulsion content is required to maximize the production of flavor and fragrance. The lipase mediation of carbohydrate esters of fatty acids offers a potential market for use as emulsifiers in foods, pharmaceuticals, and cosmetics. Another application of increasing importance is the use of lipases in removing pitch (hydrophobic components of wood, mainly triglycerides and waxes). A lipase from *Candida rugosa* is used by Nippon Paper Industries to remove up to 90% of these compounds (Jaeger and Reetz, 1998). The use of

enzymes as alternatives to chemicals in leather processing has proved successful in improving leather quality and in reducing environmental pollution. Alkaline lipases from *Bacillus* strains, which grow under highly alkaline conditions in combination with other alkaline or neutral proteases, are currently being used in this industry. Lipases are also used in detergent formulations for removal of lipid stains, fatty food stains, and sebum from fabrics (Hasan et al., 2010). Alkaline yeast lipases are preferred because they can work at lower temperatures, as compared to bacterial and fungal lipases. Cold-active lipase detergent formulation is used for cold washing which reduces energy consumption and wear and tear of textile fibers. It is estimated that every year, about 1000 tons of lipases are added to approximately \$13 billion tons of detergents (<http://www.aukbc.org/beta/bioprop2/introduction.html>).

In other enzyme applications, laccases oxidize phenolic and nonphenolic lignin-related compounds as well as environmental pollutants (Rodríguez-Couto and Toca-Herrera, 2006). They are used to detoxify industrial effluents of the paper and pulp, textile, and petrochemical industries, as a medical diagnostic tool, for bioremediation of herbicides, pesticides, and explosives in soil, cleaning agent for water purification systems, catalyst in drug manufacture, and as ingredients in cosmetics.

Enzymes are also used in a wide range of agro-biotechnological processes, and the major application is the production of feed supplements to improve feed efficiency. A recent advance in feed enzymes involves the application of phytases in agriculture as an animal feed ingredient and also in foods to improve plant phosphorous uptake by monogastric animals (Vohra and Satyanarayana, 2003). Phytate phosphorus is often unavailable to farm animals and chelates valuable minerals. Phytase allows liberation of phosphorus from plant feedstuffs, which contain about 2/3 of their phosphorus as phytate. Hydrolysis of phytate prevents its passage via manure into the soil where it would be hydrolyzed by soil and water microbes causing eutrophication. Therefore, the use of phytase in the food industry involves removal of phytic acid which acts as an antinutritional factor. The annual market of phytase is about \$500 million. The enzyme is made by many bacteria, yeasts, and filamentous fungi. Production is controlled by phosphate. Cloning of the phytase-encoding gene *phyA* from *Aspergillus niger* var. *awamori* and reintroduction at a higher dosage increased phytase production by sevenfold (Piddington et al., 1993). Recombinant *Hansenula polymorpha* produced 13 g/L of phytase (Mayer et al., 1999). New fungal phytases with higher specific activities or improved thermostability have been recently identified (Haefner et al., 2005).

In the paper and textile industries, enzymes are being increasingly used to develop cleaner processes and reduce the use of raw materials and production of waste. An alternative enzymatic process in the manufacture of cotton was developed based on a pectate lyase. The process for removing pectin and other hydrophobic materials from cotton fabrics is performed at much lower temperatures and uses less water than the classical method. Applying Gene

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Site Saturation Mutagenesis™ technology on DNA encoding for pectinolytic enzymes (selected from more than 100 environmental DNA libraries), single site mutants, exhibiting improved thermotolerance, were produced. In addition, variants with improved thermotolerance were produced by Gene Reassembly™ technology ([Solvak et al., 2005](#)). The best performing variant (CO14) contained eight mutations and had a melting temperature 16°C higher than the wild-type enzyme while retaining the same specific activity at 50°C. Optimal temperature of the evolved enzyme was 70°C, which is 20°C higher than the wild-type. Scouring results obtained with the evolved enzyme were significantly better than the results obtained with chemical scouring, making it possible to replace the conventional and environmentally harmful chemical scouring process ([Solvak et al., 2005](#)). Furthermore, alkaline pectinases are used for treatment of pectic wastewaters, degumming of plant bast fibers, paper making, and coffee and tea fermentations.

In the chemical industry, enzymes are used to replace chemical processes if they successfully compete on a cost basis. They sometimes require less energy, yield a higher titer with enhanced catalytic efficiency, produce less catalyst waste and by-products, and lower volumes of wastewater streams. They often involve hydrolases and ketoreductases that are stable in organic solvents. They also can be used to produce valuable compounds such as L-amino acids. For example, L-tyrosine has been made from phenol, pyruvate, pyridoxal phosphate, and ammonium chloride with a thermostable and chemostable tyrosine phenol lyase from *Symbiobacterium toebii*. The amino acid was produced at 130 g/L in 30 h with continuous substrate feeding. About 150 biocatalytic processes are used in the chemical industry and this number will increase with the application of genomics and protein engineering.

Enzymes are also important in the pharmaceutical industry. They are used in the preparation of beta-lactam antibiotics such as semisynthetic penicillins and cepalosporins. This antibiotic group is extremely important, making up 60–65% of the total antibiotic market. Enzymes are also involved in the preparation of chiral medicines, that is, complex chiral pharmaceutical intermediates. For example, esterases, proteases, lipases, and ketoreductases are used to prepare chiral alcohols, carboxylic acids, amines, and epoxides.

1.3 IMPROVEMENT OF ENZYMES

Certain enzymes face problems such as poor stability, substrate/product inhibition, narrow substrate specificity, or enantioselectivity. To solve these problems, genetic modification is often carried out using recombinant DNA techniques. In some cases, this has improved activity by 100-fold. Modification of the enzyme is carried out by (1) rational redesign of the biocatalyst and/or by (2) combinatorial methods in which the desired functionality is searched for in randomly generated libraries. The rational design approach is carried out by site-directed mutagenesis to target amino acid substitutions. It requires knowledge about the

three-dimensional structure of the enzyme and the chemical mechanism of the reaction. It often fails but successes have been achieved ([Beppu, 1990](#); [Van den Burg et al., 1998](#)). Combinatorial methods include directed evolution which does not require extensive knowledge about the enzyme. Here, a large number of variants are created for screening for catalytic efficiency, enantioselectivity, solubility, catalytic rate, specificity, and enzyme stability. It is rapid and inexpensive. It includes a range of molecular biological methods which allow the achievement of genetic diversity, mimicking mechanisms of evolution in nature. Random mutagenesis of the protein-encoding gene is carried out by various techniques such as (1) error-prone polymerase chain reaction (PCR), (2) repeated oligonucleotide directed mutagenesis, or (3) action of chemical agents. Error-prone PCR introduces random point mutations in a population of enzymes. Molecular breeding techniques, such as DNA shuffling, allow in vitro random homologous recombination, usually between parental genes with homology above 70% ([Ness et al., 2000](#)). After cloning and expression, a large collection of enzyme variants, that is, about 10^4 to 10^6 , is generated and subjected to screening or selection.

1.4 DISCOVERY OF NEW ENZYMES

Screening of natural microbes for enzymes suffers from the fact that less than 1% of the microbes inhabiting the biosphere can be cultivated in the laboratory by standard techniques. New enzymes can be obtained from nature by three techniques, that is, metagenomic screening, genome mining, and taking advantage of the diversity of extremophiles.

Metagenomic screening involves preparation of a genomic library from environmental DNA and the systematic screening of the library for open reading frames potentially encoding novel enzymes ([Uchiyama and Miyazaki, 2009](#); [Gilbert and Dupont, 2011](#)). Metagenomic screening of particular habitats (arctic tundra, cow rumen, volcanic vents, marine environments, and termite guts) has yielded enzymes such as lipase, oxidoreductase, amidase, amylase, nitrilase, decarboxylase, epoxide hydrolase, and beta-glucosidase. Although *Escherichia coli* has been the usual host for screening of foreign genes, the system has been improved by the use of alternative hosts and expression systems such as *Streptomyces lividans*, *Pseudomonas putida*, and *Rhizobium leguminosarum*.

Genome mining involves exploring genome sequence databases for genes encoding new enzymes. An example of a useful database is the NCBI database ([NCBI Microbial Genomes, 2013](#)) which includes more than 2000 genome sequences and draft assemblies. Two methods are used for the discovery of new enzymes. One of these, that is, genome hunting, involves the search for open reading frames in the genome of a particular microbe. Those sequences that are annotated as putative enzymes are subjected to subsequent cloning, overexpression, and activity screening. A second approach, called data mining, is based

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on homology alignment among all sequences deposited in databases. Using bioinformatic tools such as BLAST, the search for conserved regions between sequences yields homologous protein sequences that are then considered candidates for further study.

Extremophiles can survive under extreme conditions. These include temperature (-2°C to 12°C , 60°C to 110°C) pressure, radiation, salinity (2–5 M NaCl), and pH (<2, >9). They contain extremely stable enzymes. Genera such as *Clostridium*, *Thermotoga*, *Thermus*, and *Bacillus* contain extreme thermophiles growing at 60 – 80°C , whereas hyperthermophiles are members of *Archaea*, for example, *Pyrococcus*, *Methanopyrus*, and *Thermococcus*. An example of an extremely useful enzyme is the Taq DNA polymerase from the thermophile *Thermus aquaticus*, which had sales of \$500 million in 2009 (De Carvalho, 2011). Industry already uses thermophilic cellulases, amylases, and proteases.

Psychrophiles are already supplying cold-active enzymes, such as proteases, amylases, and lipases, for future development of detergents to reduce wear and tear of textile fibers. Cold-active cellulases and xylanases are of interest in the pulp and paper industry and for production of second generation biofuels via saccharification of pre-treated lignocellulosic biomass. They are also potentially useful for extraction and clarification of fruit juices, improvement of bakery products, bioremediation of waters contaminated with hydrocarbons or oils, and polishing and stone-washing of textiles. Halophilic xylanases, proteases, amylases, and lipases have been isolated from halophiles, such as species of *Halobacillus*, *Halobacterium*, and *Halothermothrix* (Van den Burg, 2003).

Also of interest are microbes surviving under extreme pH conditions that could be useful for isolation of thermoalkiphilic proteases and lipases as additives in laundry and dishwashing detergents (Shukla et al., 2009).

1.5 CONCLUDING REMARKS

Microbial enzymes have long been used by industrial product makers as major catalysts to transform raw materials into end products. Over 500 commercial products are made using enzymes. They are economically produced by different microorganisms and are quickly broken down when they have done their job. New technical tools to use enzymes as crystalline catalysts, for ability to recycle cofactors, and engineering enzymes to function in various solvents with multiple activities are important technological developments, which will steadily create new applications.

The industrial enzyme market will grow steadily mainly due to improved production efficiency resulting in cheaper enzymes, new application fields, new enzymes from screening programs, and by engineering properties of traditional enzymes. Tailoring enzymes for specific applications will be a future trend with continuously improving tools, further understanding of structure-function relationships, and increased searching for enzymes from exotic environments. New applications are to be expected in the field of textiles and new animal diets, such

as ruminant and fish feed. It can be expected that breakthroughs in pulp and paper applications will materialize. The use of cellulases to convert waste cellulose into sugars and further to ethanol or butanol by fermentative organisms has been a major study topic for years. Increasing environmental pressures and energy prices will make this application a real possibility in the future.

Enzymes should never be considered alone but rather as a part of a biocatalyst technology. Recent developments in the fields of genetic engineering and protein chemistry are bringing ever more powerful means of analysis to bear on the study of enzyme structure and function, that will undoubtedly lead to the rational modification of enzymes to match specific requirements, and also the design of new enzymes with novel properties. Techniques such as protein engineering, gene shuffling, and directed evolution will enable the development of enzymes better suited to industrial environments. These tools will also allow the synthesis of new biocatalysts for completely novel applications, resulting in the production and commercialization of new enzymes, thus seeding a second explosive expansion to the current multibillion dollar enzyme industry.

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Chapter 2

Production, Purification, and Application of Microbial Enzymes

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2.1 INTRODUCTION

Enzymes are the active proteins (except RNase) that can catalyze biochemical reactions. These are biomolecules required for both syntheses as well as breakdown reactions by living organisms. All living organisms are built and maintained by these enzymes, which are truly termed as biological catalysts having the capability to convert a specific compound (as substrate) into products at higher reaction rates. Like chemical catalysts, enzymes increase the reaction rate by lowering its activation energy (E_a), hence, products are formed faster and reactions reach their equilibrium state more rapidly. The rates of most enzymatic reactions are millions of times faster than those of the uncatalyzed reactions. They can perform conversions in minutes or even in seconds which otherwise may take hundreds of years (Dalby, 2003; Otten and Quax, 2005). Enzymes are known to catalyze about 4000 biochemical reactions in living beings (Bairoch, 2000). For example, lactase is a glycoside hydrolase that is able to hydrolyze lactose (milk sugar) into its constituent galactose and glucose monomers. It is produced by various microorganisms and also in the small intestine of humans and other mammals helping to digest milk completely. Enzymes are also enantioselective catalysts, which can be used either in the separation of enantiomers from a racemic mixture or in the synthesis of chiral compounds.

Humans recognized the importance of enzymes thousands of years ago; clarification and filtration of wines and beer being the earliest examples of the application of industrial enzymes. Enzymes have been used in brewing, baking, and alcohol production since prehistoric times; however, they did not call

them enzymes. One of the earliest written references to enzymes is found in Homer's Greek epic poems dating from about 800 BC, where it was mentioned that enzymes were used in the production of cheese. The Japanese have also used naturally-occurring enzymes in the production of fermented products like sake, Japanese schnapps brewed from rice, for more than a thousand years. Some enzymes have been designed by nature to form complex molecules from simpler ones while others have been designed for breaking up complex molecules into simpler ones, also a few modify molecules. These reactions involve the making and breaking of the chemical bonds in the components. Owing to their "specificity," a property of an enzyme that allows it to recognize a particular substrate that they are designed to target, they are useful for industrial processes and are capable of catalyzing the reaction between particular chemicals even if they are present in mixtures with many chemicals. These enzymes are environmentally safe, natural, and are applied very safely in food and even pharmaceutical industries. Still, enzymes are proteins, which like any protein can cause and have caused in the past allergic reactions, hence, protective measures are necessary in their production and applications.

Enzyme technology is an ever evolving branch of "Science and Technology." With the intervention and influence of Biotechnology and Bioinformatics, continuously novel or improved applications of enzymes are emerging. With novel applications, the need for enzymes with improved properties are also emerging simultaneously. Development of commercial enzymes is a specialized business which is usually undertaken by companies possessing high skills in:

- Screening for new and improved enzymes
- Selection of microorganisms and strain improvement for qualitative and quantitative improvement
- Fermentation for enzyme production
- Large-scale enzyme purifications
- Formulation of enzymes for sale

Enzyme technology offers industries and consumers an opportunity to replace processes using aggressive chemicals with mild and environmentally friendly enzyme processes. About 3000 enzymes are known of which only 150–170 are being exploited industrially. At present only 5% of chemical products are produced through a biological route in this green era. However, economically feasible and eco-friendly enzymatic processes are emerging as alternatives to physico-chemical and mechanical processes. Based on the different application sectors, industrial enzymes can be classified as: (1) Enzymes in the food industry, (2) Enzymes for processing aids, (3) Enzymes as industrial biocatalyst, (4) Enzymes in genetic engineering, and (5) Enzymes in cosmetics.

Today, enzymes are envisaged as the bread and butter of biotechnology because they are the main tools for several biotechnological techniques (gene restriction, ligation, and cloning, etc.), bioprocesses (fermentation and cell culture), and in analytics in human and animal therapy as medicines or as drug

targets. Furthermore they find applications in several other industries, such as food and feed, textiles, effluent and waste treatment, paper, tannery, baking, brewing, dairy, pharmaceuticals, confectionary, etc. (Pandey et al., 2006).

The enzymes utilized today are also found in animals (pepsin, trypsin, pancreatic, and chymotrypsin) and plants (papain, bromelain, and ficin), but most of them are of microbial origin, such as glucoamylase, α -amylase, pectinases, etc. The advantage of using microbes for enzyme production is their higher growing abilities, higher productivity, and their easier genetic manipulation for enhanced enzyme production, etc. Enzymes produced from microbial origins are termed as microbial enzymes. Microbes are mainly exploited in industries for enzyme production. Moreover, microbial enzymes are supplied, well standardized, and marketed by several competing companies worldwide. Depending on the type of process, enzymes can be used in soluble form (animal proteases and lipases in tannery) and in immobilized form (isomerization of glucose to fructose by glucose isomerase).

2.2 PRODUCTION OF MICROBIAL ENZYMES

Bacteria and fungi produce most industrial enzymes. Naturally occurring microorganisms are the most productive producers of enzymes. This knowledge has been exploited by industry for more than 50 years. Bacteria and fungi are the microorganisms best suited to the industrial production of enzymes. They are easy to handle, can be grown in huge tanks without light, and have a very high growth rate.

Bacterium *Bacillus subtilis* and the fungus *Aspergillus oryzae* are the most employed microorganisms for enzyme production by the global biotechnology company Novozymes. Both have immense capacity for producing enzymes and are considered completely harmless for humans (<http://www.novozymes.com/en/about-us/our-business/what-are-enzymes/Pages/creating-the-perfect-enzyme.aspx>).

The ideal microorganism grows quickly and produces lots of the desired enzyme at mild temperatures whilst consuming inexpensive nutrients. However, like most things in life, the ideal microorganism is hard to come by. Most microorganisms found in the wild are not well suited to domestication in large fermentation tanks. Some only produce tiny quantities of enzyme or take a long time to grow. Others can produce undesired by-products that would disturb industrial processes. So for industrial production a perfect microorganism is the foremost requirement. Table 2.1 shows the list of microorganisms involved for the production of enzymes along with their industrial applications.

2.2.1 Enzyme Production in Industries

Different microorganisms have been employed for industrial enzyme production, varying from eukaryotic systems, such as yeast and fungi, to prokaryotic system involving both Gram positive and Gram negative bacteria.

TABLE 2.1 Industrial Application of Enzymes

Enzyme	Source organism	Method of production	Industrial application
Amylase (α and gluco)	Bacteria (<i>Bacillus amyloliquifaciens</i> , <i>Bacillus licheniformis</i> , <i>Bacillus coagulans</i>)	SmF	<ol style="list-style-type: none">1. Mashing for beer making2. Sugar recovery from scrap candy in candy industry3. Starch modification for paper coating in paper industry4. Cold swelling laundry starch in starch and syrup industry5. Wall paper removal6. Desizing of fabrics in textiles7. Degradation of protein, causing stains in detergent industry
	Fungi (<i>Aspergillus oryzae</i> , <i>Aspergillus niger</i> , <i>Rhizopus</i> sp.)	SSF and SmF	<ol style="list-style-type: none">1. Precooked baby foods and breakfast foods in cereals industry2. Sugar recovery from scrap candy in candy industry3. Removal of starch, clarification, oxygen removal for flavour enhancement4. Starch removal from pectin in fruits and fruit juices5. Corn syrup in starch and syrup industry6. Production of glucose in starch and syrup industry7. Bread baking in baking and milling industry8. Digestive aids in clinics and pharmaceuticals9. Liquefying purees and soups
Protease	Bacteria (<i>B. amyloliquifaciens</i>)	SmF	<ol style="list-style-type: none">1. Chillproofing in beer industry2. For condiment in food industry3. Milk protein hydrolysate making in dairy industry4. Unhairing and bating in leather industry5. Recovery of silver from films in photography6. Degradation of fat, causing stains in detergent industry

	Fungi (<i>A. oryzae</i> , <i>A. niger</i> , <i>Pseudomonas</i> sp., <i>Penicillium chrysosporium</i> , <i>R. oligosporus</i> , <i>Actinomycetes</i> strain)	SSF and SmF	<ol style="list-style-type: none"> 1. Bread baking in baking and milling industry 2. Chillproofing in beer industry 3. For condiment in food industry 4. Milk protein hydrolysate making in dairy industry 5. Evaporated milk stabilization in dairy industry 6. Spot removal in dry cleaning, laundry industry 7. Digestive aids in clinics and pharmaceuticals 8. Unhairing and tanning in leather industry 9. Meat tenderizing, tenderizing casings, condensed fish soluble 10. Resolution racemic mixture of amino acids
Glucose oxidase	Fungi (<i>A. niger</i> and <i>Penicillium</i> sp.)	SSF and SmF	<ol style="list-style-type: none"> 1. Oxygen removal in beer and beverages 2. Dried milk, oxygen removal in dairy industry 3. Oxygen and oxygen removal, mayonnaise in dried and egg industry 4. Paper test strips for diabetic glucose in pharmaceuticals 5. Oxygen removal for flavours enhancement in fruits and juices 6. In tooth paste to convert glucose into gluconic acid and hydrogen peroxide as both acts as disinfectant
Pectinases	Fungi (<i>A. niger</i> , <i>Penicillium</i> sp.)	SSF and SmF	<ol style="list-style-type: none"> 1. Coffee bean fermentation, coffee concentrates in coffee industry 2. Clarification, filtration, concentration in fruits and fruit juices 3. Pressing, clarification, filtration in wine industry
Lactase	Yeast (<i>Kluyveromyces</i>)	SmF	<ol style="list-style-type: none"> 1. Whole milk concentrates 2. Ice cream and frozen desserts 3. Whey concentrates 4. Lactose hydrolysis in dairy industry
Cellulase	Fungi (<i>Trichoderma reesei</i> , <i>Trichoderma viride</i> , <i>Penicillium</i> sp., <i>Humicola grisea</i> , <i>Aspergillus</i> sp., <i>Chrysosporium lucknowense</i> , <i>Acremonium</i> sp.)	SSF and SmF	<ol style="list-style-type: none"> 1. Deinking of papers for recycling in paper and pulp industry 2. Bio stonewashing denim in textile industry 3. Hydrolyzing cellulosic biomass to generate glucose for ethanol production in biofuel industry 4. Loosening of cellulose fibers to easily remove dirt and color in detergent industry

(Continued)

TABLE 2.1 Industrial Application of Enzymes (Continued)

Enzyme	Source organism	Method of production	Industrial application
Xylanase	Fungi (<i>Myceliophthora thermophila</i> , <i>Bacillus</i> sp., <i>A. oryzae</i> , <i>Trichoderma</i> sp.)	SSF and SmF	<ol style="list-style-type: none">1. Biobleaching in paper and pulp industry2. Fiber solubility in animal feed industry
Lipase and proteinase	<i>A. oryzae</i> , <i>A. terreus</i> , <i>Pseudomonas</i> ssp., <i>Alcaligena</i> ssp., <i>Staphylococcus</i> sp., <i>Candida albicans</i> , <i>Rhizopus</i> sp., <i>Mucor</i>	SSF	<ol style="list-style-type: none">1. Contact lens cleaning2. Brightening in detergent industry3. Ripening of cheese in dairy industry
Phytase	<i>Aspergillus</i> sp., <i>A. ficuum</i> , <i>P. funiculosum</i> , <i>Bacillus</i> sp., <i>Pseudomonas</i> , <i>Xanthomonas oryzae</i>	SSF	<ol style="list-style-type: none">1. Release of phosphate in animal feed industry
Dextrinase	Fungi	SSF	<ol style="list-style-type: none">1. Corn syrup preparation in starch and syrup making
Invertase	Yeast (<i>Saccharomyces</i>)	SmF	<ol style="list-style-type: none">1. Soft center candies and fondants2. High test molasses
Laccases and peroxidase	<i>Aspergillus nidulans</i> , <i>Aspergillus</i> sp., <i>Basidiomycetes</i>	SSF	<ol style="list-style-type: none">1. Polymerize materials with wood based fibers in paper and pulp industry
Hemicellulase	Fungi (<i>A. niger</i> , <i>T. reesei</i> , <i>Penicillium</i> sp.)	SSF and SmF	<ol style="list-style-type: none">1. Coffee concentrates2. Hydrolyzing hemicellulosic biomass to generate glucose for ethanol production in biofuel industry
Catalase	<i>Aspergillus</i> sp.		<ol style="list-style-type: none">1. Biopolishing and bleach clean-up in textile industry

SSF, solid-state fermentation; SmF, submerged fermentation.

The first enzyme industry emerged for producing subtilisin, an alkaline protease, naturally secreted by *Bacillus licheniformis* to break down proteinaceous substrate, and used in detergent. An industry for alpha amylase production was also based on *Bacillus licheniformis* that naturally secretes a highly thermostable α -amylase capable of breaking down starch to easily digestible oligosaccharides. Hence, strains of *Bacillus* were regarded as workhorses of enzyme production for decades because of their ability to overproduce subtilisin and α -amylase. Amylase from *Bacillus* has long been used to liquefy starch. For the complete breakdown of starch into its monosaccharide other enzymes called glucoamylases are required. The most widely used glucoamylases for complete hydrolysis of starch into glucose are produced by a fungal strain of the genus *Aspergillus* (Sonenshein et al., 1993). Overproducing strains have been isolated over the years leading to high producing glucoamylases. Likewise, an acidic cellulase complex is secreted by a fungal strain of *Trichoderma*. Initially it was thought that this enzyme complex would convert cellulosic substrate to glucose in a similar way to the starch degrading enzymes but this application was not initially commercialized due to its slow action. Instead it has found an application in the treatment of textiles and as an additive in detergents. The potential of cellulases for biomass hydrolysis for bioethanol production was ignored for some years, but recently its potential for cellulose hydrolysis has regained attention from researchers all over the world, mainly for the application of bioethanol production using cellulosic biomass which is the most abundant material available for use to mankind. Several efforts were made to improve the enzyme complex and its expression. At present there are several enzyme companies preparing a cellulase cocktail for bioethanol application, for example, Genencor and Novozymes.

Glucose isomerase catalyzes the conversion of glucose into fructose; resulting in a product sweeter in taste. This particular enzyme was produced by a species of *Streptomyces* which led to the development of an industry based on the above application.

All of the strains described above that have been employed for industrial application are capable of differentiation. For example, *Bacillus* shows the tendency to survive adverse environmental conditions by forming spores which are dormant yet viable. The spore remains dormant until it reaches a favorable environment where it can germinate and multiply.

This differentiation is highly associated with the regulation of enzyme production by microorganisms. This type of highly complex behavior makes modeling studies difficult for process development. Protease production by *Bacillus* is highly regulated with differentiation and it is similarly the case for *Aspergillus* and *Trichoderma*. *Streptomyces* does not truly sporulate, although it differentiates by forming filaments unlike isolated single cells. This property exerts an effect on the production as well as the physical properties of fermentation broth. An organism can be considered as a metabolic system capable of utilizing substrates to produce cell mass and by-products. Enzymes catalyze the

various reactions that are vital for an organism's growth and metabolic activities. Each cell is equipped with a mechanism that regulates the synthesis of enzymes in an economical way, enabling the cell to respond adequately to environmental changes.

The basic mechanism of enzyme synthesis includes transcription, translation, and posttranslational processing which is highly conserved (Rhem and Reed, 1985). However, several differences exist between various classes of organisms, as well as some fundamental differences between prokaryotic and eukaryotic organisms. The enzymes themselves differ in their molecular structure, number of polypeptide chain, degree of glycosylation, and isoelectric point. Although all the differences influence the synthetic pattern, the basic enzyme synthesis mechanisms are similar enough to allow a general treatment of the microbiological production process. However, differences exist between the production kinetics of different enzymes by different microorganisms because of their varied physical characteristics and growth pattern, which necessitates the optimization of each production process separately.

2.2.2 Industrial Enzyme Production Technology

Fermentation technologies have been employed exclusively for the production of industrial enzymes, preferably by microorganisms such as bacteria or fungi under carefully controlled conditions due to their ease of multiplication and handling. Microorganisms employed are GRAS (Generally Recognized as Safe) strains due to their application in the food and feed industries (Pandey et al., 2008; Singhania et al., 2010). Currently, researchers are locating extremophile organisms from different parts of the world, ranging from the rain forest to arid regions to the bottom of the ocean, that may produce enzymes with a promising industrial nature. In practice, the great majority of microbial enzymes come from a very limited number of genera, of which *Aspergillus* species, *Trichoderma* species, *Bacillus* species, *Streptomyces* species, and *Kluyveromyces* species predominate. Most of the strains used have either been employed by the food industry for many years or have been derived from such strains by mutation and selection (Sarrouh et al., 2012).

Selection of the strain for industrial enzyme production is a very important factor for a successful industrial process. Ideally a strain producing an extracellular enzyme should be selected as it makes the purification and recovery far easier than when the enzyme is produced intracellularly. Different organisms may also differ in their suitability for fermentation; process characteristics, such as viscosity or recoverability, or legal clearance of the organism should also be considered before selection. The industrial strains typically produce up to 50 g/L of extracellular protein.

Fermentation process design is interdisciplinary and requires knowledge of both chemical engineering and microbial physiology to be successfully scaled up. Submerged fermentation (SmF) and solid state fermentation (SSF) are the

two important fermentation technologies available. Both of these technologies offer several benefits and have their own limitations. Most industries employ SmF for enzyme production; however, there has been a resurgence in popularity of SSF for few applications and specific industries (Pandey, 2003; Singhania et al., 2009; Thomas et al., 2013b).

2.2.2.1 *Submerged Fermentation*

Fermentation carried out in the presence of an excess of free water is termed as submerged fermentation. The use of an aerobic submerged culture in a stirred-tank reactor is a typical industrial process for enzyme production involving microorganisms that produce an extracellular enzyme. It is the preferred technology for industrial enzyme production due to the ease of handling at a large scale when compared to SSF. Large scale fermenters for SmF, varying in volume from thousands to a hundred thousand liters, are well developed and offer online control over several parameters such as, pH, temperature, DO (dissolved oxygen), and foam formation; and moreover there is no problem of mass transfer and heat removal. Thus, these are some of the benefits which make this production technology superior to SSF and widely accepted for industrial metabolite production. The medium in the SmF is liquid which remains in contact with the microorganisms. A supply of oxygen is essential in the SmF, which is done through a sparger. Stirrers and impellers play an important role in these fermenters for mixing gas, biomass, and suspended particles.

There are four main ways of growing the microorganisms in the SmF. These are batch culture, fed batch culture, perfusion batch culture, and continuous culture. In the batch culture, the microorganisms are inoculated in fixed volume of medium. In the case of fed batch culture, the concentrated components of the nutrient are gradually added to the batch culture. In the perfusion batch culture, the addition of the culture and withdrawal of an equal volume of used cell-free medium is performed. In the continuous culture, fresh medium is added into the batch system at the exponential phase of the microbial growth with a corresponding withdrawal of the medium containing the product. The continuous cultivation gives a near-balanced growth, with little fluctuation of the nutrients, metabolites, cell numbers, or biomass.

Some enzymes are produced more as a secondary metabolite, and specific productivity may then be an inverse function of growth rate, that is, nongrowth-associated production. Here a recycling reactor may be most suitable. A recycling reactor is similar to the continuous culture, but a device is added to return a significant fraction of the cells to the reactor. Low growth rates with high cell concentration can often be achieved in such systems.

In practice scale-up effects are more pronounced for the aerobic process than the anaerobic process. To achieve aeration, agitation is maintained during the scale-up in the fermenter to maintain constant oxygen supply. Scale-up complications arise from cell responses to distributed values of dissolved oxygen,

temperature, pH, and nutrients. For enzyme production, economy of scale leads to the use of fermenters with a volume of 20–200 m³. The concomitant problems of mass and heat transfer are usually neglected in small fermenters and at low cell densities. However, in industrial microbiology, with the above-mentioned fermenter volumes and the economic necessity of using the highest possible cell densities, transport processes must be considered. These can limit the metabolic rates, for example, oxygen limitation leads the microorganisms to respond with changes in their physiological pattern. In these conditions the desired control of microbial metabolism is lost. In controlled operation of an industrial process, metabolic rates must be limited to a level just below the transport capacity of the fermenter. Therefore, the highest possible productivity in a fermenter is obtained at maximal transport capacity.

2.2.2.2 Solid State Fermentation

Current developments in biotechnology are yielding new applications for enzymes. SSF holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented products may be used directly as enzyme sources. This system offers numerous advantages over the SmF system, including high titer, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipment, less trained labor, etc. ([Pandey et al., 2007](#)).

A large number of microorganisms, including bacteria, yeast, and fungi, produce different groups of enzymes. Selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. For example, it has been reported that while a strain of *Aspergillus niger* produced 19 types of enzymes, α -amylase was being produced by as many as 28 microbial cultures ([Pandey et al., 1999](#)). Thus, the selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and the environmental conditions. Generally, hydrolytic enzymes, for example, cellulases, xylanases, pectinases, etc., are produced by fungal cultures, since such enzymes are used in nature by fungi for their growth. *Trichoderma* spp. and *Aspergillus* spp. have been used most widely for these enzymes. Also, amylolytic enzymes are commonly produced by filamentous fungi and the preferred strains belong to the species of *Aspergillus* and *Rhizopus*. Although commercial production of amylases is carried out using both fungal and bacterial cultures, bacterial α -amylase is generally preferred for starch liquefaction due to its high temperature stability. In order to achieve high productivity with less production cost, genetically modified strains apparently will hold the key to enzyme production.

Agro-industrial residues are generally considered the best substrates for the SSF processes, and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce enzymes. Some of the substrates that have been

used include sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soy hull, sago hampas, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pretreated willow, starch, etc. ([Pandey et al., 1999](#)). Wheat bran however holds the key, and has most commonly been used, in various processes.

The selection of a substrate for enzyme production in an SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In an SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in suboptimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement externally with these nutrients. It has also been a practice to pretreat (chemically or mechanically) some of the substrates before their use in SSF processes (eg, ligno-cellulose), thereby making them more easily accessible for microbial growth.

Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size, initial moisture level, and water activity are the most critical. Generally, smaller substrate particles provide a larger surface area for microbial attack and, thus, are a desirable factor. However, too small a substrate particle may result in substrate agglomeration, which may interfere with microbial respiration/aeration, and therefore result in poor growth. In contrast, larger particles provide better respiration/aeration efficiency (due to increased interparticle space), but provide limited surface area for microbial attack. This necessitates a compromise in particle size for a particular process.

Over the years, different types of fermenters (bioreactors) have been employed for various purposes in SSF systems. Pandey et al. (2009) have shown the aspects of the design of a fermenter in SSF processes. Laboratory studies are generally carried out in Erlenmeyer flasks, beakers, petri dishes, roux bottles, jars, and glass tubes (as column fermenters). Large-scale fermentation has been carried out in tray-, drum- or deep-trough type fermenters. The development of a simple and practical fermenter with automation is yet to be achieved for the SSF processes.

SSF processes are distinct from SmF culturing, since microbial growth and product formation occur at or near the surface of the solid substrate particle having low moisture contents. Thus, it is crucial to provide optimized water content, and control the water activity (a_w) of the fermenting substrate, because the availability of water in lower or higher concentrations adversely affects microbial activity. Moreover, water has a profound impact on the physico-chemical properties of the solids and this, in turn, affects the productivity of the overall process.

The major factors that affect microbial synthesis of enzymes in an SSF system include: selection of a suitable substrate and microorganism; pre-treatment of the substrate; particle size (interparticle space and surface area) of the substrate; water content and a_w of the substrate; relative humidity; type and size of the inoculum; control of temperature of fermenting matter/removal of metabolic heat; period of cultivation; maintenance of uniformity in the environment of the SSF system; and the gaseous atmosphere, that is, oxygen consumption rate and carbon dioxide evolution rate.

Ideally, almost all the known microbial enzymes can be produced under SSF systems. A literature survey reveals that much work has been carried out on the production of enzymes of industrial importance, such as proteases, cellulases, ligninases, xylanases, pectinases, amylases, glucoamylases, etc.; and attempts are also being made to study SSF processes for the production of inulinases, phytases, tannases, phenolic acid esterases, microbial rennets, aryl-alcohol oxidases, oligosaccharide oxidases, tannin acyl hydrolase, a-L-arabinofuranosidase, etc.

2.3 STRAIN IMPROVEMENTS

It is well recognized that the large majority of the naturally occurring microorganisms do not produce enzymes at industrially appreciable quantities or often do not possess desirable properties for applications. Hence, huge efforts have been made in improving the strains using classical or molecular tools to obtain hyperproducing strains or developing the required characteristics ([Pandey et al., 2010](#)).

2.3.1 Mutation

Most of the strains used for industrial enzyme production have been improved by classical selection. There are four classes of mutations: (1) spontaneous mutations (molecular decay), (2) mutations due to error prone replication bypass of naturally occurring DNA damage (also called error prone translation synthesis), (3) errors introduced during DNA repair, and (4) induced mutations caused by mutagens. Scientists may also deliberately introduce mutant sequences through DNA manipulation for the sake of scientific experimentation. Mutagenesis by UV radiation or chemical mutagens have been applied to quickly find the useful variants. Many cells are subjected to mutation and the resulting mutants are selected for the desired combination of traits. Usually, mutation causes changes of protein structure which results in deterioration of function. Rarely, changes in structural components by mutation result in improvements unless the specific loss of function is required for production purposes, for example, when a loss of regulatory function results in enhanced enzyme production. Mutation and selection are directed primarily toward higher overall productivity rather than

mutation of a specific function, but a loss of regulatory function is highly probable. There are several examples of mutant strains which are known as hyper-producers such as *Trichoderma reesei* RUT C-30, which has been one of the best cellulase producers for decades.

2.3.2 Recombinant DNA (rDNA) Technology

Some microorganisms have the capability of producing the perfect enzyme. Others could win the Olympic gold medal in growth and enzyme production. By combining the best from each organism, one could obtain a microorganism that grows very quickly on inexpensive nutrients, whilst at the same time producing large quantities of the right enzyme. This is done by identifying the gene that codes for the desired enzyme and transferring it to a production organism known to be a good enzyme producer.

Industrial enzymes need to be perfectly suited to the tasks that they perform, but sometimes the perfect enzyme for a specific job is impossible to find. This does not mean, however, that we can't make an enzyme for the job. Normally, scientists can find a naturally-occurring enzyme that is almost perfect, and using modern biotechnology it can be upgraded to the desired efficiency. This is done by altering small parts of the genes in the microorganism which code for the production of the enzyme. These tiny alterations only alter the structure of the enzyme very slightly, but this is normally enough to make a good enzyme into a perfect enzyme.

Microorganisms isolated from diverse environments represent sources of enzymes that can be used for industrial process chemistry. Though the use of high-throughput screening (HTS) methods have enabled us to find novel and potent enzymes from microorganisms, many of those microorganisms are not easily cultivated in laboratory conditions or their enzyme yield is too low for economical use. Using DNA technology, cloning the genes encoding these enzymes and heterologous expression in commonly used industrial strains has become a common practice.

The novel enzymes suitable for the specific conditions may be obtained by genetically modifying the microorganism. Industrial production of insulin is produced by genetically modified *E. coli*. Recombinant DNA technology enables the production of enzymes at levels 100-fold greater than the native expression, making them available at low cost and in large quantities ([Shu-Jen, 2004](#)). As a result, several important food processing enzymes such as amylases and lipases with properties tailored to particular food applications have become available. Several microbial strains have been engineered to increase the enzyme yield by deleting native genes encoding the extracellular proteases. Moreover, certain fungal production strains have been modified to reduce or eliminate their potential for the production of toxic secondary metabolites ([Olempska-Bier et al., 2006](#)).

Although the use of DNA technology significantly lowers the cost of enzyme production, the applications of enzymes are still limited. Most chemicals with industrial interest are not natural substrates for these enzymes. If a desired enzyme activity is found, the yield is often low. Moreover, enzymes are not usually stable in harsh reaction conditions, such as pH higher or lower than physiological pH 7, high temperature, or the presence of organic solvents required to solubilize many substrates. This approach precludes the transfer of any extraneous or unidentified DNA from the donor organisms to the production strain.

2.3.3 Protein Engineering

Recent advances in PCR technology, site-specific and random mutagenesis are readily available to improve enzyme stability in a wider range of pH and temperature and tolerance to a variety of organic solvents. Since a large quantity of enzyme can be obtained by recombinant expression, X-ray crystallography can facilitate the understanding of the tertiary structure of an enzyme and its substrate binding/recognition sites. This information may assist a rational design of the enzyme, predicting amino acid changes for altering substrate specificity, catalytic rate, and enantioselectivity (in the case of chiral compound synthesis). To engineer a commercially available enzyme to be a better industrial catalyst, two different approaches are presently available: a random method called directed evolution and a protein engineering method called rational design.

Protein engineering is a method of changing a protein sequence to achieve a desired result, such as a change in the substrate specificity, or increased stability to the temperature, organic solvents, and/or extremes of pH. Many specific methods for protein engineering exist, but they can be grouped into two major categories: those involving the rational design of the protein changes, and the combinatorial methods which make changes in a more random fashion.

Protein engineering or rational methods, such as site-directed mutagenesis, require targeted amino acid substitutions, and therefore, require a large body of knowledge on the biocatalyst being improved, including the three-dimensional structure and the chemical mechanism of the reaction. The main advantage of rational design is that a very small number of protein variants are created, meaning that very little effort is necessary to screen for the improved properties. The combinatorial methods, on the other hand, create a large number of variants that must be assayed; however, they have the advantage of not requiring such extensive knowledge about the protein. In addition, often nonobvious changes in the protein sequence lead to large improvements in their properties, which are extremely hard to predict rationally, and thus, can only be identified by the combinatorial methods.

Several enzymes have already been engineered to function better in the industrial processes. These include the proteinases, lipases, cellulases, α -amylases, and glucoamylases. Xylanase is a good example of an industrial

enzyme, which needs to be stable at high temperature and active at the physiological temperature and pH when used as the feed additive and in the alkaline conditions when used in the bleaching in the pulp and paper industry. One of the industrial production organisms of the xylanases is *Trichoderma* sp. Its xylanase has been purified and crystallized. By designed mutagenesis, its thermal stability has been increased by about 15°C. The mutational changes increased the half-life in the thermal inactivation of this enzyme from approximately 40 s to approximately 20 min at 65°C, and from less than 10 s to approximately 6 min at 70°C (Fenel et al., 2004).

By designed mutagenesis its thermal stability has been increased about 2000 times at 70°C and its pH optimum shifted towards alkaline region by one pH-unit. The most successful strategies to improve the stability of the *Trichoderma* xylanase include the stabilization of the alpha-helix region and the N-terminus.

The above strategies for strain improvement and production process optimization may lead to reduction of the cost of enzymes to an extent, however, downstream processing and product formulation for reasonably purified and highly stable enzymes are the key step towards the success of any enzyme industry.

2.4 DOWNSTREAM PROCESSING/ENZYME PURIFICATION

The goal of the fermentation processes is to produce a final formulated enzyme product and it also includes many postfermentation unit operations. Still the maximum production rate could be the most important factor. However, the lowest unit production cost could also be an important driving force. Optimization of each individual unit operation does not always lead to an optimal overall process performance, especially when there are strong interactions between unit operations (Groep et al., 2000). An understanding of these interactions is crucial to overall process optimization. For instance, product concentration or purity in the fermentation broth can significantly impact downstream purification unit operation. If the fermentation is optimized for productivity without taking into account its effect on the purification step, the overall process productivity can be negatively affected. The use of antifoaming agents in the fermentation process is another example of such a trade-off. By reducing foaming in the fermentation, a higher working volume can be used to optimize the fermentation unit operations. Care has to be taken since antifoams could have adverse effect on filtration unit membranes. Thus it is important to have knowledge of how the fermentation process is going to affect the other unit operations of downstream processing.

Usually the aim of the purification process is to achieve maximum possible yield, maximum catalytic activity, and the maximum possible purity. Most of the industrial enzymes produced are extracellular and the first step in their purification is separation of cells from the fermentation broth. For intracellular enzymes, disruption of cells by mechanical or nonmechanical methods are required. Filtration, centrifugation, flocculation, floatation, and finally concentration methods lead to the development of a concentrated product. Salting out and solvent

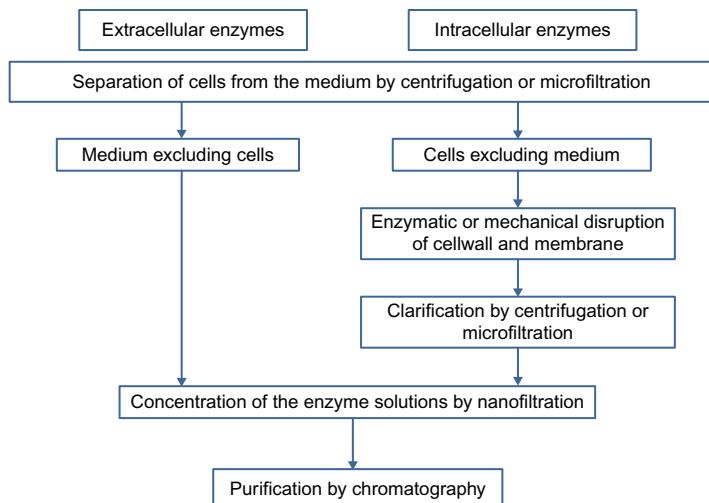


FIGURE 2.1 Downstream processing of industrial enzymes.

precipitation methods could be employed for protein concentration in industries. Acetone precipitation is a popular method of protein concentration in industries as acetone can be recycled. Ultrafiltration, electrophoresis, and chromatography lead to a highly purified product. Fig. 2.1 shows the basic steps followed during downstream processing of the microbial enzyme production process and verifies that extracellular enzymes are more desirable for industrial applications being more economical as per downstream processes. However, the number of steps and economic viability is highly associated with the degree of purity required, which is, in turn, associated with the end application of the enzyme.

Thus, it is very clear that extent of purification required is based on its end application. For pharmaceutical and food industries purification is critical, whereas in textile industry, detergent industry, and biofuel industry generally cocktails are preferred. The purification steps and their principles will not be uniform for all enzyme products. There could be a set of steps for purification of particular enzyme and also a particular set of steps can be employed for several enzymes. High volume low value enzymes should minimize the extent of purification steps to be economically viable. Cellulase is one among many industrial enzymes and has several applications including in the textile industry, detergent industry, and in cellulosic biomass degradation for bioethanol production. Cellulases are available commercially in a liquid stage as well as a powder stage and both were found to be quite stable. These are normally produced by SmF but can be produced also by SSF and are generally concentrated by acetone precipitation. After concentration, principal separation methods are employed based on the properties of the enzyme to be separated, these are listed in Table 2.2.

TABLE 2.2 Principal Separation Methods Used in Purification of Enzymes

Property	Method	Scale
Size or mass	Centrifugation	Large or small
	Gelfiltration	Generally small
	Dialysis, Ultrafiltration	Generally small
Polarity • Charge • Hydrophobic character	Ion-exchange chromatography	Large or small
	Chromatofocusing	Generally small
	Electrophoresis	Generally small
	Isoelectric focusing	Generally small
	Hydrophobic chromatography	Generally small
Solubility	Change in pH	Generally large
	Change in ionic strength	Large or small
	Decrease in dielectric constant	Generally large
Specific binding sites or structural features	Affinity chromatography	Generally small
	Immobilized metal ion chromatography	Generally small
	Affinity elution	Large or small
	Dye-ligand chromatography	Large or small
	Immunoadsorption	Generally small
	Covalent chromatography	Generally small

2.5 PRODUCT FORMULATIONS

The primary task of formulation is to minimize losses in enzymatic activity during transport, storage, and usage. So enzymes are sold as stabilized liquid concentrates or as particulate solids. Enzymes are often exposed to humid, hot, or oxidative environments in industrial applications such as detergents, textile formulations as well as food and beverage processing. Chemical stabilizers are available to protect the thermolabile enzymes thermally and chemically to an extent. So, it is preferred to select enzymes that are structurally more stable or resistant to oxidation during screening itself.

Formulations enhance stability by counteracting the primary forces of deactivation:denaturation, catalytic-site deactivation, and proteolysis ([Becker et al, 1997](#)). Denaturation occurs by physical unfolding of an enzyme's tertiary protein structure under thermal or chemical stress. Once an enzyme begins to unfold it becomes dramatically more vulnerable to deactivation and proteolysis.

In order to minimize unfolding, the formulator can alter the protein's environment so as to induce a compact protein structure. This is done most effectively by adding water associating compounds such as sugars, polyhydric alcohols, and lyotropic salts which via "preferential exclusion" detach water molecules from the protein surface. The best ways to combat active site inactivation are to ensure sufficient levels of any required cofactors, to add reversible inhibitors, and to exclude reactive or oxidizing species from the formulation. There are several key secondary requirements, besides enzymatic stability, a formulation should meet; these include preservation against microbial contamination, avoidance of physical precipitation or haze formation, minimizing the formation of sensitizing dusts or aerosols, and the optimization of aesthetic criteria, such as color and odor.

Many of these problems are best addressed by focusing as far "upstream" as possible, including the choice of raw materials in the fermentation or enzyme recovery process. Downstream operations such as diafiltration, adsorption, chromatography, crystallization, and extraction can be used to remove impurities responsible for color, odor, and precipitation (Becker, 1995). By formulating the enzyme near to its isoelectric point with hydrophilic solvents such as glycerol or propylene glycol, the risk of physical precipitation could be minimized. Moderate levels of solvating salts can also be added to avoid either salting-out or "reverse salting-in." A combination of filtration, acidification, and the minimization of free water; biocides could be effective to prevent microbial contamination. The range of acceptable chemicals for controlling or killing microbes should only be used when circumscribed by health and safety regulations. Dry granular enzyme formulations for powdered laundry detergents and textile formulations are the results of motivation towards workers safety. Enzyme granules have become increasingly resistant to physical breakage and formation of airborne dusts upon handling. Two processes producing the most attrition-resistant granules to date are high-shear granulation and fluidized-bed spray coating. These processes use various binders, coatings, and particle morphologies to produce nonfriable particles which protect enzymes during storage and at the same time allow for their ready release in solution during application.

2.6 GLOBAL ENZYME MARKET SCENARIOS

In 2008, the \$2.1 billion industrial enzymes market covers a wide variety of product applications ranging from food and beverages to detergents, diagnostics, animal feed, renewable biofuels to greener production of tires and adhesives. It is expected to exceed \$3 billion by 2015. The market is highly competitive, dominated by large companies, and highly sensitive to production costs (http://www.idiverse.com/html/target_industrial_enzymes.htm). Fig. 2.2 shows the proportion of industrial enzymes sales by sector and Fig. 2.3 shows the world enzyme market by application sector.

In a recent report published by BBC Research (BBC Research, 2011) regarding the industrial enzyme market, it stated that the global market for industrial

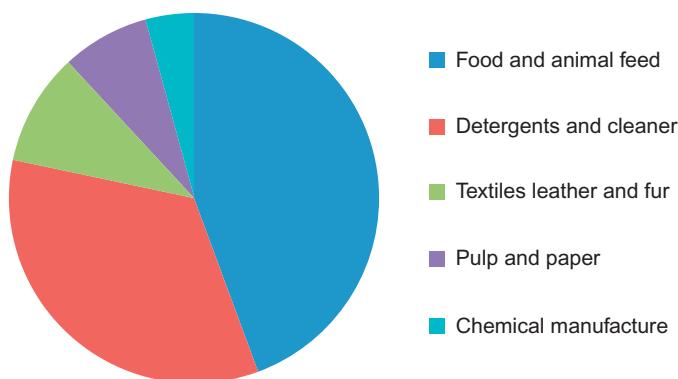


FIGURE 2.2 Industrial enzyme sales by sector.

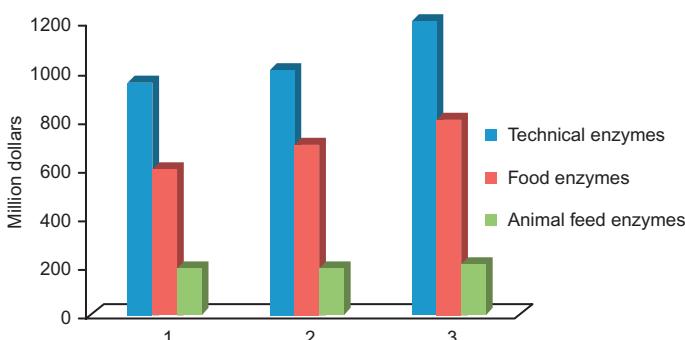


FIGURE 2.3 World enzyme market by application sectors.

enzymes was estimated to reach a value of \$3.3 billion in 2010. This market is expected to reach \$4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% over the 5-year forecast period.

According to the report on industrial enzymes ([Global Industry Analysts, 2011](#)), the global market for industrial enzymes was fairly immune to the recent turmoil in the global economy and grew moderately during 2008–2009. In mature economies such as the United States, Western Europe, Japan, and Canada, the market was relatively stable while the developing economies of Asia-Pacific, Eastern Europe, and Africa emerged as the fastest growing markets for industrial enzymes. The United States and Europe collectively command a major share of the world industrial enzymes market, whereas Asia-Pacific growth was stagnant with just an 8% compounded annual growth rate in 2008–2012 ([Sarrouh et al., 2012](#)).

Major enzyme producers are located in Europe, the United States, and Japan. The major players in the enzyme market are Novozymes (45%) and

Danisco (17%) in Denmark, Genencor in USA, DSM in the Netherlands, and BASF in Germany (Binod et al., 2008; Binod et al., 2013; BCC-Business Communications, 2009). The pace of development in the emerging markets suggested that companies from India and China can join this restricted party in the very near future (Carrez and Soetaert, 2005; Chadel et al., 2007; Research and Markets, 2011 (India); Research and Markets, 2011 (China)). Another recently published research report on the enzymes market (Global Industry Analyst, 2011) highlighted the fact that Proteases constitutes the largest product segment in the global industrial enzymes market, and the Carbohydrases market is projected to be the fastest growing product segment, with a CAGR of more than 7.0% over the analysis period. Lipases represent the other major product segment in the global industrial enzymes market with high growth potential.

Sectors such as pharmaceuticals and bioethanol have succeeded in drawing significant attention of the investors and are self-sufficient in undertaking new product development activities and in launching novel and unique products in the market, thus offering new opportunities to the industrial enzyme manufacturers. However, segments such as waste water treatment chemicals and paper and pulp lack sufficient funding for carrying out new product developments (Global Industry Analyst, 2011).

According to Pitman (2011) the use of enzymes in the cosmetic industry is estimated to grow by 5% CAGR every year up to 2015. Market research highlights the fact that industrial demands for enzymes is being driven by new enzyme technologies and increased use of organic compounds in place of petrochemical-based ingredients.

The global industrial enzymes market consists of various enzymes, which can be broadly categorized as carbohydrases, proteases, lipases, and others. Carbohydrases industrial enzymes are further classified as amylases, cellulases, and other carbohydrases which are projected to grab the market at a fast pace (Industrial enzymes market by types, 2014).

2.7 INDUSTRIAL APPLICATIONS OF ENZYMES

It was very well realized that on applying enzymes to biological processes the rate of the reaction could be enhanced and the production process could be performed with cheaper raw materials within a fraction of the time taken at a lower temperature and pressure. The enzyme industry is in an ongoing search for sustainable processes that enables higher yields with improved efficiency and dynamic nature. From creating lactose-free dairy products to fast-acting laundry detergents, innovation is the key in engineering; improved, cost-effective end-products for textiles, foods, detergents, animals, biofuels, and more. Table 2.1 shows large-scale enzyme applications; however, there are other applications too which are not listed in the table, such as therapeutic and specialty enzymes, which are not required in bulk but where purity is the major concern as these need to be free from other enzyme activities.

2.7.1 Food Industry

2.7.1.1 Starch Industry

The use of starch degrading enzymes was the first large-scale application of microbial enzymes in the food industry. There are two main enzymes that carry out the conversion of starch to glucose: alpha-amylase and glucoamylase ([Pandey, 1995; Pandey et al., 2000](#)).

Sometimes additional debranching enzymes, such as pullulanase, are added to improve the glucose yield. Beta-amylase is commercially produced from barley grains and used for the production of the disaccharide maltose ([Selvakumar et al., 1996](#)).

Studies have been carried out on the application of transglutaminase as a texturing agent in the processing of sausages, noodles, and yoghurt, where cross-linking of proteins provides improved viscoelastic properties of the products ([Kuraishi et al., 2001](#)). In the United States large volumes of glucose syrups are converted by glucose isomerase after Ca^{2+} removal (alpha-amylase needs Ca^{2+} for activity but it inhibits glucose isomerase) to fructose containing syrup. This is done by bacterial enzymes, which need Mg^{2+} ions for activity. Fructose is separated from glucose by large-scale chromatographic separation and crystallized. Alternatively, fructose is concentrated to 55% and used as a high fructose corn syrup in the soft drink industry.

2.7.1.2 Baking Industry

Alpha-amylases have been most widely studied in connection with improved bread quality and increased shelf life. Both fungal and bacterial amylases are used. The added amount needs to be carefully controlled as overdosage may lead to sticky dough. One of the motivations to study the effects of enzymes on dough and bread qualities comes from the pressure to reduce other additives. In addition to starch, flour typically contains minor amounts of cellulose, glucans, and hemicelluloses, such as arabinoxylan and arabinogalactan. There is evidence that the use of xylanases decreases the water absorption and thus reduces the amount of added water needed in baking. This leads to more stable dough. In particular, xylanases are used in wholemeal rye baking and dry crisps common in Scandinavia.

Proteinases can be added to improve dough-handling properties; glucose oxidase has been used to replace chemical oxidants and lipases to strengthen gluten, which leads to more stable dough and better bread quality.

2.7.1.3 Brewing Industry

Enzymes have many applications in the drinks industry. Chymosin is used in cheese-making to coagulate milk protein. Another enzyme used in the milk industry is β -galactosidase or lactase, which splits milk-sugar lactose into glucose and galactose. This process is used for milk products that are consumed by lactose intolerant consumers.

2.7.1.4 *Fruit Juice Industry*

Enzymes are used also in fruit juice manufacturing. The addition of pectinase, xylanase, and cellulase improves the liberation of the juice from the pulp. Pectinases and amylases are used in juice clarification. Similarly, enzymes are widely used in wine production to obtain a better extraction of the necessary components and thus improve the yield. Enzymes hydrolyze the high molecular weight substances like pectin. Enzymes can be used to help starch hydrolysis (typically alpha-amylases), solve filtration problems caused by beta-glucans present in malt (beta-glucanases), hydrolyze proteins (neutral proteinase), and control haze during maturation, filtration, and storage (papain, alpha-amylase, and beta-glucanase).

2.7.2 **Textile Industry**

The use of enzymes in the textile industry is one of the most rapidly growing fields in industrial enzymology. Amylases are used for desizing of textile fibers. Other important enzymes used in the textile industry are the cellulases. Due to their ability to modify the cellulosic fibers in a controlled and desired fashion so as to improve the quality of fabrics, these (neutral or acidic) offer an excellent replacement for stone-washing of blue denim garments as it eliminates the disadvantages caused due to use of the stones, such as damage to the washers and garments, handling and environment problems. The enzymatic stonewashing allows up to 50% higher jean load and yields the desired look and softer finish. The neutral cellulase is the enzyme of choice for stonewashing because of the reduction in back staining and its broader pH profile. This latter property reduces the need for the rigid pH control of the wash, resulting in a more reproducible finish from wash to wash. Fuzz formation and pilling are the common problems associated with fabrics comprising of cotton or other natural fibers; the cellulases are utilized for digesting off the small fiber ends protruding from the fabric, resulting in a better finish.

Catalase is used to degrade excess peroxide, as hydrogen peroxides are used as bleaching agents to replace chlorine-based chemicals. Another recent approach is to use oxidative enzymes directly to bleach textiles. Laccase—a polyphenol oxidase from fungi—is a new candidate in this field. It is a copper-containing enzyme, which is oxidized by oxygen, and which, in an oxidized state, can oxidatively degrade many different types of molecules like dye pigments.

2.7.3 **Detergent Industry**

The detergent industry is the largest single industry for the use of enzymes, using about 25–30% of the total of industrial enzymes. About half of the detergents available in the market contain enzymes in their formulations; however, information is rarely published about the formulations.

Dirt in clothes could be protein, starch, or lipid in nature. It is possible to remove most types of dirt using detergents in water at high temperatures with vigorous mixing but the cost of heating the water is high and lengthy mixing or beating would be required, which shortens the life of clothes. The use of enzymes allows lower temperatures to be employed and shorter periods of agitation are needed, often after a preliminary period of soaking. In general, enzyme detergents remove protein from clothes soiled with blood, milk, sweat, grass, etc. far more effectively than nonenzyme detergents. Cellulases are employed for loosening the fibers so as to remove the dirt easily and also it gives a finishing touch by digesting fine fibers during washing. At present only proteases, amylases, cellulases, and lipases are commonly used in the detergent industry.

Enzymes are used in surprisingly small amounts in most detergent preparations, only 0.4–0.8% crude enzyme by weight (about 1% by cost). The ability of enzymes to withstand the conditions of use is a more important criterion than its cost. Now, second generation detergent enzymes are available and employed with enhanced activity at low temperature and alkaline pH.

2.7.4 Pulp and Paper Industry

Intensive studies have been carried out during the last 20 years to apply many different enzymes in the pulp and paper industry. Xylanases are applied in pulp bleaching, which liberate lignin fragments by hydrolyzing residual xylan (Thomas et al., 2013a). This reduces considerably the need for chlorine-based bleaching chemicals. Cellulases are used for deinking the cellulose fibers during recycling.

In paper making, amylases are used especially in the modification of starch, which improves the strength, stiffness, and erasability of paper. The starch suspension must have a certain viscosity, which is achieved by adding amylase enzymes in a controlled process. Pitch is a sticky substance, composed of lipids present mainly in softwoods. It causes problems for the paper machine when mechanical pulps of red pine are used as a raw material. The pitch can be removed by lipases.

2.7.5 Animal Feed Industry

Enzyme addition in animal feed was intensively started in the 1980s to reduce viscosity, increase absorption of nutrients, liberate nutrients either by hydrolysis of nondegradable fibers or by liberating nutrients blocked by these fibers, and reduce the amount of faeces. They are added as enzyme premixes (enzyme-flour mixture) during the feed manufacturing process, which involves extrusion of wet feed mass at high temperature (80–90°C). Therefore, the feed enzymes need to be thermotolerant during the feed manufacturing and operative in the animal body temperature.

The first commercial success was the addition of β -glucanase into barley-based feed diets. Barley contains β -glucan, which causes high viscosity in the

chicken gut. The net effect of enzyme usage in feed has been increased animal weight gain with the same amount of barley resulting in increased feed conversion ratio. The addition of xylanase to wheat-based broiler feed is capable of increasing the available metabolizable energy by 7–10%.

Another important feed enzyme is phytase, which is a phosphoesterase and liberates the phosphate from the phytic acid. Phytic acid is commonly present in the plant-based feed materials. The supplementation of the phytase results in a reduced amount of the phosphorous in the faeces, which in turn results in reduced environmental pollution. It also minimizes the need to add phosphorus to the feed. Currently phytase from fungal sources are potent feed enzymes ([Pandey et al., 2001](#)). Usually a feed-enzyme preparation is a multienzyme cocktail containing glucanases, xylanases, proteinases, and amylases.

2.7.6 Leather Industry

The leather industry uses proteolytic and lipolytic enzymes in leather processing. The use of these enzymes is associated with the structure of animal skin as a raw material. Enzymes are used to remove unwanted parts. Alkaline proteases are added in the soaking phase. This improves water uptake by the dry skins, removal and degradation of protein, dirt, and fats, and reduces the processing time. In some cases pancreatic trypsin is also used in this phase.

Proteases are used in dehauling and dewooling of leather, and improve its quality (cleaner and stronger surface, softer leather, fewer spots). Lipases are used in this phase or in the bating phase to specifically remove grease. The use of lipases is a fairly new development in the leather industry.

2.7.7 Biofuel From Biomass

Perhaps the most important emerging application of the enzymes currently being investigated actively is in the utilization of the lignocellulosic biomass for the production of biofuel. Biomass represents the most abundant renewable resource available to mankind for effective utilization. However, the lack of cost-effective enzyme conversion technologies has made it difficult to realize. This is basically due to the high cost of the cellulases and also the lack of specificities for various lignocellulosic substrates. The strategy employed currently in bioethanol production from biomass is a multistep process in which enzymatic hydrolysis is a crucial step ([Singhania et al., in press](#)). In the effort to develop efficient technologies for biofuel production, significant research has been directed towards the identification of efficient cellulase systems and process conditions, as well as studies directed at the biochemical and genetic improvement of the existing organisms utilized in the process. Dupont and Novozymes have been actively involved in cellulases research and have significantly reduced the cost of the enzyme and improved the efficiency of the enzyme leading to the development of economically feasible enzyme production.

In recent time, bioethanol from biomass via the enzymatic route has become a reality with several biofuel industries emerging. Advanced Biofuels, LLC, in Scotland (SD, USA, since 2008), BetaRenewables in Rivalta (Italy, since 2009), Inbicon in Kalundborg (Denmark, since 2009), and Clariant in Munich (Germany, since 2009) are some of the proofs of the dream turned to reality. Beta Renewables constructed the world's first commercial-scale cellulosic ethanol plant in Crescentino, Italy, with a capacity of 20 MGY. This plant began operation at the end of 2012, and it uses the PROESA process to convert agricultural nonfood wastes to ethanol ([Gusakov, 2013](#)).

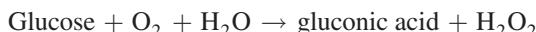
2.7.8 Enzyme Applications in the Chemistry and Pharma Sectors

An important issue in the pharma sector is the large number of compounds that must be tested for biological activity to find a single promising lead. The combinatorial biocatalysis has received much attention here, as it could add a level of the complexity to the diversity of existing chemical libraries or could be used to produce the libraries de novo ([Rich et al., 2002](#)). An example is the use of glycosyltransferases to change the glycosylation pattern of the bioactive compounds. Only a few commodity chemicals, such as acrylamide, are now produced by the enzyme technology (annual production scale 40,000 tons). Nonetheless, this success has demonstrated that the bioconversion technology can be scaled up. Many other chemicals, including chiral compounds ([Jaeger et al., 2001](#)), are also produced by the biocatalysis on a multiton scale.

2.7.8.1 Speciality Enzymes

In addition to large volume enzyme applications, there are a large number of specialty applications for enzymes. These include the use of enzymes in clinical analytical applications, flavor production, protein modification, personal care products, DNA-technology, and in fine chemical production. Contrary to bulk industrial enzymes these enzymes need to be free from side activities, which requires an emphasis to be placed on elaborate purification processes.

Alkaline phosphatase and peroxidases are used for immunoassays. An important development in analytical chemistry is biosensors. The most widely used application is a glucose biosensor involving a glucose oxidase catalyzed reaction:



Several commercial instruments are available which apply this principle for the measurement of molecules like glucose, lactate, lactose, sucrose, ethanol, methanol, cholesterol, and some amino acids.

2.7.8.2 Enzymes in Personal Care Products

Personal care products are a relatively new area for enzymes and the amounts used are small but worth mentioning as a future growth area. One application

is contact lens cleaning. Proteinase- and lipase-containing enzyme solutions are used for this purpose. Hydrogen peroxide is used in the disinfection of contact lenses. The residual hydrogen peroxide after disinfection can be removed by a heme-containing catalase enzyme, which degrades hydrogen peroxide.

Glucoamylase and glucose oxidase are used in some toothpaste, as glucoamylase liberates glucose from starch-based oligomers produced by alpha-amylase and glucose oxidase converts glucose to gluconic acid and hydrogen peroxide, which both function as disinfectants. Dentures can be cleaned with protein degrading enzyme solutions. Enzymes, such as chitinase, are being studied also for applications in skin and hair care products.

2.7.8.3 *Enzymes in DNA-Technology*

The DNA modifying enzymes play a very crucial role in DNA-technology, which has revolutionized traditional as well as modern biotechnology. They can be divided into two classes:

1. Restriction enzymes: They recognize specific DNA sequences and cut the chain at these recognition sites.
2. DNA modifying enzymes: These synthesize nucleic acids, degrade them, join pieces together, and remove parts of the DNA.

Restriction enzymes produce cleavage after recognizing a specific code sequence in the DNA. These enzymes are essential in gene technology. DNA-polymerases synthesize new DNA chains using a model template which they copy. Nucleases hydrolyze the phosphodiester bonds between DNA sugars. Kinases add phosphate groups and phosphatases remove them from the end of DNA chain. Ligases join adjacent nucleotides together by forming phosphodiester bonds between them.

In the cell these enzymes are involved in DNA replication, degradation of foreign DNA, repairing of mutated DNA, and in recombining different DNA molecules. The enzymes used in gene technology are produced like any other enzyme but their purification needs extra attention.

The use of enzymes in industrial applications has been limited by several factors such as high cost of the enzymes, their availability in small amounts, and their instability. Also the enzymes are soluble in aqueous media and it is difficult as well as expensive to recover them from reactor effluents at the end of the catalytic process. This limits the use of soluble enzymes to batch operations, followed by disposal of the spent enzyme-containing solvent.

2.8 CONCLUDING REMARKS

Though enzyme technology is a well established branch of science, it is still passing through a continuous phase of evolution. Our society is moving towards an eco-friendly technology replacing several chemical-based technologies so

as to protect our environment for the future generation. Searches for novel enzymes based on a potential application for a known enzyme are moving ahead simultaneously. Enzymes have already shown a tremendous capacity to guide us towards biological processes as biocatalysts. Several chemical processes have been replaced by biological processes with several benefits, such as mild operating conditions, specificity, and environmental feasibility. Enzymes have made interventions in almost all the major commercial sectors, especially in the pharmaceutical and food industries. Enzymes will continue with their potential and beneficial roles in a more intensified manner in the future.

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Chapter 3

Solid State Fermentation for Production of Microbial Cellulases

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3.1 INTRODUCTION

Lignocelluloses are the most abundant renewable carbon resource in the world and they are mainly composed of cellulose (35–50%), hemicelluloses (25–30%), and lignin (25–30%) (Anwar et al., 2014). By hydrolysis, cellulose and hemicellulose can be degraded into sugars, which are further processed into value-added products, such as biochemicals, biofuels, and biopolymers (Hu et al., 2011; Aguiar et al., 2013). Improvement of microbial and enzymatic processes was initially investigated several decades back for the bioconversion of biomass that gave way to research in the industrial applications of cellulases in animal feed, food, fuels, textiles and detergents, and in the paper industry (Zhang and Sang, 2012).

The successful utilization of lignocellulosic wastes or cellulosic materials as renewable carbon sources is dependent on the development of economically feasible process technologies for cellulase production (Kricka et al., 2015). Cellulases are produced by several types of microorganisms, such as fungi, bacteria, and actinomycetes (Liu et al., 2012; Pathak et al., 2014). These microorganisms are exploited to utilize lignocellulosic wastes to produce cel lulolytic enzymes (Pathak et al., 2014). Moreover, cellulases have the ability to hydrolyze the β -1,4-glucosidic linkages of cellulose to low molecular weight products, including hexoses and pentoses (Singhania et al., 2013). However, a cellulosic enzyme system has a complex organization, consisting of three major components: endo- β -glucanase (EC 3.2.1.4), exo- β -glucanase (EC 3.2.1.91),

and β -glucosidase (EC 3.2.1.21). The mode of action of each of these is as follows:

- Endo- β -glucanase, 1,4- β -D-glucan glucanohydrolase, CMCCase, Cx: “random” scission of cellulose chains yielding glucose and cello-oligosaccharides.
- Exo- β -glucanase, 1,4- β -D-glucan cellobiohydrolase, Avicelase, C1: exo-attack on the nonreducing end of cellulase with cellobiose as the primary product.
- β -glucosidase, cellobiase: hydrolysis of cellobiose to glucose.

Several groups of researchers proposed that exo- β -glucanase causes a disruption in cellulose hydrogen bonding, followed by hydrolysis of the accessible cellulose with endo- β -glucanase. Although sequential stages in cellulolysis have not been fully developed, the hypothesis depicted in Fig. 3.1 is now accepted (Saranraj et al., 2012; Juturu and Wu, 2014). According to this hypothesis, in a synergistic sequence of events, endo- β -glucanase acts randomly on the cellulose chain, while exo- β -glucanase acts on exposed chain ends by splitting off cellobiose or glucose. Cellobiose is subsequently hydrolyzed by β -glucosidase

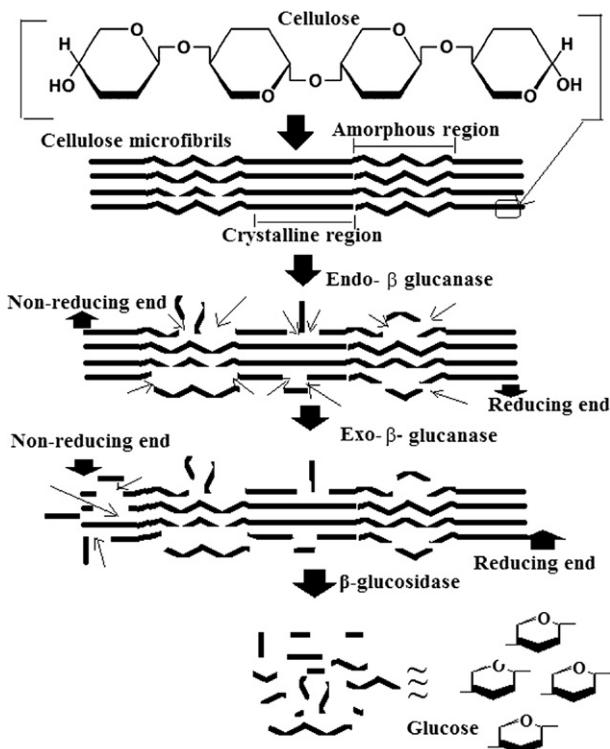


FIGURE 3.1 Schematic representation of sequential stages in cellulolysis.

to glucose. This hypothesis is however the opposite of that proposed by [Reese \(1956\)](#) and indicates that three, rather than two enzymes are essential for the decomposition of cellulosic biomass.

3.2 SOLID STATE FERMENTATION (SSF)

SSF (also known as solid state bioprocessing) has emerged as a potential technology for the production of microbial cellulase ([Da Silva Delabona et al., 2012](#); [Coradi et al., 2013](#)). SSF refers to the process where microbial growth and product formation occurs on the surface of solid materials. This process involves the absence (or near absence) of “free” water; however, the moisture is absorbed to the solid substrate to support growth and microbial metabolism ([Ang et al., 2013](#); [Pirota et al., 2014](#)).

3.2.1 Comparative Aspects of Solid State and Submerged Fermentations

Recent enzyme production technologies followed by fermentation processes are conducted either in a liquid phase (SmF) or using solid substrates (SSF). A comparison between the two fermentation technologies is summarized below:

- Most of the cellulases are produced by SSF using wild-type microbial strains that are considered advantageous microorganisms. However, almost 90% of all industrial enzymes are produced by SmF, often using genetically-modified microorganisms ([Adsul et al., 2011](#); [Singhania et al., 2015](#)).
- SSF provides an environmental-friendly approach with many advantages, such as prevention of environmental pollution, and avoidance of agricultural waste or by-product accumulation ([Kumar and Kanwar 2012](#)).
- In SSF, the agro-industrial residues are used as solid substrates, acting both as carbon and energy sources ([Philipoussis et al., 2011](#); [Carvalho et al., 2013](#)). [Herculano et al. \(2011\)](#) evaluated the production of β -glucosidase (β G), total cellulase (FPase), and endoglucanase (CMCase) by *Aspergillus japonicus* URM5620 in SSF, using castor bean meal (*Ricinus communis* L.) as the carbon and energy source.
- SSF is considered advantageous for certain microorganisms, such as filamentous fungi, since the solid medium simulates their natural habitat ([Binod et al., 2010](#); [Sarkar et al., 2012](#)). [Liang et al. \(2012\)](#) isolated a novel cellulase-producing strain, that is, *Aspergillus* sp. SEMCC- 3.248 under SSF using rice grass (*Spartina* spp.) as the main substrate. Under optimized conditions (initial moisture content 70%, initial pH 5.0, incubation temperature 32°C and fermentation period 5 days), the cellulase activity could reach 1.14 FPIU/gram dry substrate (gds).

To summarize, the SSF process is an attractive method to produce cellulase from microorganisms that is economical due to its lower capital investment, lower operating expenses, simpler equipment, and higher productivity per reactor volume (Hafiz Muhammad Nasir et al., 2011; Iqbal et al., 2013; Anwar et al., 2014).

In this context, several groups of researchers examined the production of microbial cellulases in SmF and SSF processes (Kumar et al., 2011; Cunha et al., 2012; Agrawal et al., 2013). Kumar et al. (2011) studied the comparative evaluation of SmF and SSF for the optimum yield of both pectinase and cellulase by *Aspergillus niger* NCIM 548 using different carbon sources and claimed that the SSF fermentation conditions yielded more enzymes than did SmF. A sequential SSF and SmF cultivation with sugarcane bagasse as substrate for cellulase production by *A. niger* A12 was assessed by measuring endoglucanase activity. An endoglucanase productivity of 57 ± 13 IU/L/h was achieved in SSF (bubble column reactor), an approximately 3-fold improvement as compared with the conventional SmF fermentation (Cunha et al., 2012). However, more recently, Florencio et al. (2015) developed a cost-effective and novel sequential solid state and submerged fermentation method validating the use of *Trichoderma reesei* Rut-C30, which is considered a reference strain for cellulase production. The new sequential cultivation methodology using sugarcane bagasse as substrate was shown to be favorable for endoglucanase (EGase) production, resulting in up to 4.2-fold improvement compared to conventional SmF.

3.2.2 Cellulase-Producing Microorganisms in SSF

In nature, lignocellulosic biomass is degraded with the cooperation of many microorganisms that mainly include diverse fungal and bacterial genera producing a variety of cellulolytic and hemicellulolytic enzymes under aerobic and anaerobic conditions (Kumar et al., 2011). The major microorganisms employed in cellulase production vide SSF are shown in Table 3.1.

3.2.3 Extraction of Microbial Cellulase in SSF

The extraction processes of cellulase from microbial cells (fungi, bacteria, and actinomycetes) in SSF were reported by several researchers (Singhvi et al., 2011; Da Silva Delabona et al., 2012; Li et al., 2013a,b; Trivedi et al., 2013; Cunha et al., 2014). In brief, for the extraction of the microbial enzyme, the fermented substrate is suspended in sodium acetate buffer. The broth is mixed properly by gentle shaking before extracting cellulase. The extract is filtered through double-layered muslin cloth, then centrifuged at 7000 rpm at 4°C for 15 min. The clear supernatant obtained is considered as a crude enzyme mix and used for cellulolytic assay (Trivedi et al., 2015).

3.2.4 Measurement of Cellulase Activity in SSF

Cellulase activity in SSF is determined by cellulolytic enzyme assays, such as filter paper activity (FPase), endoglucanase (CMCase), xylanase, and β -glucosidase activities.

TABLE 3.1 Major Microorganisms^a Employed in Cellulase Production via Solid State Fermentation

Major groups	Microorganisms	
	Genus	Species
Fungi	<i>Aspergillus</i>	<i>A. niger</i> ; <i>A. nidulans</i> ; <i>A. oryzae</i> ; <i>A. fumigates</i> ; <i>A. phoenicis</i>
	<i>Fusarium</i>	<i>F. solani</i> ; <i>F. oxysporum</i>
	<i>Humicola</i>	<i>H. insolens</i> ; <i>H. grisea</i>
	<i>Melanocarpus</i>	<i>M. albomyces</i>
	<i>Paecilomyces</i>	<i>P. themophila</i>
	<i>Penicillium</i>	<i>P. brasiliandum</i> ; <i>P. decumbans</i> ; <i>P. occitanis</i>
	<i>Phanerochaete</i>	<i>P. chrysosporium</i>
	<i>Trichoderma</i>	<i>T. reesei</i> ; <i>T. longibrachiatum</i> ; <i>T. harzianum</i> ; <i>T. viride</i>
	<i>Cladosporium</i>	<i>C. sphaerospermum</i>
	<i>Phanerochaete</i>	<i>P. chrysosporium</i>
	<i>Trametes</i>	<i>T. versicolor</i> ; <i>T. trogii</i> ; <i>T. pubescens</i> ; <i>T. hirsute</i> ; <i>T. ochracea</i>
	<i>Pleurotus</i>	<i>P. ostreatus</i> ; <i>P. dryinus</i> ; <i>P. tuberregium</i> ; <i>P. sajor-caju</i> ; <i>P. pulmonarius</i>
	<i>Lentinus</i>	<i>L. edodes</i> ; <i>L. tigrinus</i>
	<i>Cerrena</i>	<i>C. maxima</i>
	<i>Funalia</i>	<i>F. trogi</i>
	<i>Coriolopsis</i>	<i>C. polyzona</i>
	<i>Pycnoporus</i>	<i>P. coccineus</i> ; <i>P. sanguineus</i>
	<i>Bjerkandera</i>	<i>B. adusta</i>
	<i>Fomes</i>	<i>F. fomentarius</i>
	<i>Psedotremella</i>	<i>P. gibbosa</i>
	<i>Trichaptum</i>	<i>T. biforme</i>
	<i>Irpex</i>	<i>I. lacteus</i>
	<i>Ceriporiopsis</i>	<i>C. subvermispora</i>
	<i>Laetiporeus</i>	<i>L. sulfurous</i>
	<i>Wolfiporia</i>	<i>W. cocos</i>
	<i>Piptoporus</i>	<i>P. betulinus</i>
	<i>Gloeophyllum</i>	<i>G. trabeum</i>

(Continued)

TABLE 3.1 Major Microorganisms^a Employed in Cellulase Production via Solid State Fermentation (Continued)

Major groups	Microorganisms	
	Genus	Species
Bacteria	<i>Acidothermus</i>	<i>A. cellulolyticus</i>
	<i>Bacillus</i>	<i>B. subtilis; pumilus</i>
	<i>Clostridium</i>	<i>C. acetobutylicum; C. thermocellum</i>
	<i>Cellulomonas</i>	<i>C. fimi; C. bioazotea; C. uda</i>
Actinomycetes	<i>Streptomyces</i>	<i>S. drozdowiczii; S. lividans</i>
	<i>Thermonospora</i>	<i>T. fusca; T. curvata</i>

^aUpdated from: [Da Silva Delabona et al. \(2012\)](#); [Yoon et al. \(2014\)](#).

3.2.4.1 Filter Paper Activity (FPase)

The FPase is a relative measure of the overall cellulose-hydrolyzing capacity of microbial cellulase preparations. Reliable and comparable data may be obtained only under standardized conditions. The conditions of the FPA assay were standardized for SSF ([Da Silva Delabona et al., 2012](#); [Thomas et al., 2013](#); [Shahzadi et al., 2014](#)). In brief, FPase is assayed by incubating the diluted enzyme with buffer (citrate buffer, 50 mmol/L, pH 5.0) solution, containing filter paper (Whatman No. 1). The reaction mixture is incubated at 50°C for 60 min ([Da Silva Delabona et al., 2012](#)). The FPase activity is expressed as filter paper units. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol of reducing sugar per min.

3.2.4.2 Carboxymethyl Cellulase Activity (CMCase)

Endoglucanase activity is measured with 1% (w/v) carboxymethyl cellulose in 50 mM sodium citrate buffer pH 4.8 ([Cunha et al., 2014](#)). One unit of endoglucanase activity can be defined as the amount of enzyme that releases 1 µmol of reducing sugar per min, using the dinitrosalicylic acid ([Miller, 1959](#)) method. Similarly, 1 unit of endoglucanase activity corresponds to 1 µmol of glucose released per min.

3.2.4.3 Xylanase Activity

Xylanase activity is determined by using 0.1 mL of suitably diluted enzyme and 1% xylan solution as substrate. The solution mixture is added in citrate buffer (50 mmol/L, pH 5.0). This mixture is incubated at 50°C for 15 min. One unit of

xylanase activity corresponds to 1 μmol of xylose released per min (Da Silva Delabona et al., 2012).

3.2.4.4 β-Glucosidase Activity

β-glucosidase activity is determined by using cellobiose as substrate and quantifying the sugars released by use of an enzymatic kit for glucose measurement (Da Silva Delabona et al., 2012).

All these cellulase activities in SSF results are expressed as activity units per mass of initial dry solid substrate (IU/gds or FPU/gds).

3.3 LIGNOCELLULOSIC RESIDUES/WASTES AS SOLID SUBSTRATES IN SSF

The agro-industrial lignocellulosic residues/wastes form the most important renewable reservoir of carbon for a variety of vitally important chemical feedstocks and fuels in the overall economy of any country. Their unlimited availability, and environmental pollution potential if not disposed of properly, dictate renewed efforts for their efficient and economic utilization (Liu et al., 2011).

A number of such substrates (Table 3.2) have been employed for the cultivation of microorganisms to produce cellulase. Some of the substrates that have been used include wheat bran, wheat straw, sugar cane bagasse, oil palm trunk, apple pomace, wood chips, cassava bagasse, rice bran, maize bran, rice straw, rice husk, soy hull, grapevine trimming dust, saw dust, corncobs, coir pith, banana waste, etc. Wheat bran, however, holds the key and is the most commonly used substrate in the production of cellulase (Li et al., 2010; Deswal et al., 2011; Dhillon et al., 2011; Da Silva Delabona et al., 2012; Flodman and Noureddini, 2013). More recently, green seaweed of *Ulva fasciata* (Trivedi et al., 2015) has been used in the production of cellulase.

The selection of a substrate for cellulase production in an SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues (Ruiz et al., 2012). In an SSF process, the solid substrate not only supplies the nutrients to the microorganism(s) growing on it but also serves as anchorage for the cells. The substrate that provides all the needed nutrients to the microorganism growing on it should be considered as the ideal substrate. However, some of the nutrients may be available in suboptimal concentrations, or even absent in the substrates. In such circumstances, it would become necessary to supplement with these nutrients (Guoweia et al., 2011).

3.4 PRETREATMENT OF AGRICULTURAL RESIDUES

Many factors, such as lignin content, crystallinity of cellulose, and particle size, limit the digestibility of the hemicelluloses and cellulose present in the lignocellulosic biomass (Hendriks and Zeeman, 2009; Swain et al., 2009; Menon and

TABLE 3.2 Spectrum of Microbial Cultures Employed for Production of Cellulases in Solid State Fermentation Systems

Types of wastes/substrate	Inoculant	Nutrient and culture conditions	Activity (cellulase production)	References
Substrate as Carbon Source				
Oil palm trunk	<i>Aspergillus fumigatus</i> SK1	Cultured on potato dextrose agar (PDA) at 30°C for 5 days and maintained at -80°C for long time storage; supplementation with modified Mendel medium with 0.75 g/L peptone and 2 mL/L Tween 80	CMCase: 54.27 Units/gram dry substrate (gds);	Ang et al. (2013)
			FPase: 3.36 Units/gds;	
			β-glucosidase: 4.54 Units/gds	
Wheat bran	<i>Chrysoporthe cubensis</i>	125 mL Erlenmeyer flasks containing 5 gds moistened with 12 mL of culture media	Highest endoglucanase (33.84 U/gds), FPase (2.52 U/gds), β-glucosidase (21.55 U/gds) and xylanase (362.38 U/gds) were obtained	Falkoski et al. (2013)
Rice grass (<i>Spartina</i> spp.)	<i>Aspergillus</i> sp. SEMCC- 3.248	Rice grass (0.8–0.9 mm) 2.5 g, wheat bran 1.5 g, 4 mL of nutrient solution ((NH ₄) ₂ SO ₄ , 14 g/L, KH ₂ PO ₄ , 2 g/L, CaCl ₂ , 4 g/L, MgSO ₄ ·7H ₂ O, 0.2 g/L, soluble starch 2.62 g/L, peptone 1.5 g/L), 1 mL of spore suspension (1 × 10 ⁷ spores/mL), initial moisture content 70%, initial pH 5.0, incubation temperature 32°C and fermentation period 5 days	Cellulase activity could reach 1.14 FPU (Filter Paper Units)/gds	Liang et al. (2012)
Green seaweed (<i>Ulva fasciata</i>)	<i>Cladosporium sphaerospermum</i>	PDA at 4°C as a pure culture; 250 mL Erlenmeyer flasks containing 10 g of dried and powdered seaweed in a modified mineral salt medium	The enzyme assayed: CMCase : 0.20 ± 0.40 U/gds;	Trivedi et al. (2015)
			FPase: 9.60 ± 0.64 U/gds	

Wheat bran, sugarcane bagasse, soybean bran, and orange peel	<i>Aspergillus fumigatus</i>	Erlenmeyer flasks containing 5 g of substrate moistened with mineral mixyure to achieve 60% moisture holding capacity, sterilized, and inoculated with spores at 107 ml^{-1} . Incubated at 370°C for 5 days.	CMCase: 160.1 IU/gds; FPase: 5.0 IU/gds; β -glucosidase: 105.82 IU/gds; xylanase: 1055.62 IU/gds were achieved	Da Silva Delabona et al. (2012)
Deoiled <i>Jatropha curcas</i> seed cake	<i>Scytalidium thermophilum</i>	1% oat-spelt xylan, adjusted to pH 9.0, moisture content 1:3 w/v, inoculated with 1×10^6 spores per 5 g cake and incubated at 45°C	1455 Units of xylanase/gds deoiled seed cake was obtained	Joshi and Khare (2011)
Wheat straw	<i>Trichoderma reesei</i> CBS 439.92 (QM9123)	PDA in petri dishes at 30°C for 1 week prior to inoculation of SSF	A typical range of cellulase activities obtained in these experiments (0.75–1.2 FPU/gds)	Lever et al. (2010)
Sugarcane bagasse and corn steep liquor	<i>Bacillus</i> sp. SMIA-2	Maximum stability of avicelase and CMCase was observed at a pH range between 6.5–8.0 and 7 respectively, and the optimum temperature of avicelase and CMCase was 70°C , and both enzymes remained 100% stable until the treatment at 60°C for 1 h.	Maximum avicelase (0.83 U/mL) and CMCase (0.29 U/mL) activities	Ladeira et al. (2015)

Mixed- or Coculture System

Agricultural residues: cauliflower waste, kinnow pulp, rice straw, pea-pod waste, wheat bran	Individual and mixed cultures of <i>Aspergillus niger</i> and <i>Trichoderma reseei</i>	Mendel–Weber medium; in the tray fermentation (120 h)	Maximum cellulase activities: such as, FPase: 24.17 IU/gds; β -glucosidase: 24.54 IU/gds (mixed culture) (rice straw with supplementation wheat bran in the ratio of 3:2)	Dhillon et al. (2011)
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(Continued)

TABLE 3.2 Spectrum of Microbial Cultures Employed for Production of Cellulases in Solid State Fermentation Systems (Continued)

Apple pomace	<i>Aspergillus niger</i> (NRRL567) and <i>A. niger</i> (NRRL2001)	Fungal growth and the cellulase production were carried out at 30°C for 5 days using 500 cm ³ Erlenmeyer flasks and plastic trays containing 40g of apple pomace	Using <i>A. niger</i> NRRL 567, CMCase: 425.3 IU/gds; FPase: 383.7 IU/gds; β-glucosidase: 336.1 IU/gds; xylanase: 4868 IU/gds	Dhillon et al. (2012)
			Using <i>A. niger</i> NRRL2001, CMCase: 544.7 IU/gds; FPase: 401 IU/gds; β-glucosidase: 285.4 IU/gds; xylanase: 4580.7 IU/gds	
Kinnow (<i>Citrus reticulata</i>) waste supplemented with wheat bran using	<i>A. niger</i> , <i>T. reesei</i> independently and in combination	250 mL Erlenmeyer flasks containing 100 mL potato dextrose broth	Mixed cultures enhanced the oil recovery by 11%	Oberoi, et al. (2012b)
Wheat straw	<i>T. reesei</i> RutC30, with either the novel <i>Aspergillus saccharolyticus</i> AP, <i>Aspergillus carbonarius</i> ITEM 5010 or <i>A. niger</i> CBS 554.65	Strains were maintained in 10% glycerol at -80°C; Solid medium comprised 25.6% (w/v) wheat bran; medium was inoculated with 1 mL of 5 × 10 ⁶ /mL spore suspension and incubated as stationary culture at 25°C for 10 days	Approx. 80% efficiency of hydrolysis and 30% more avicelase activity comparable to results obtained using monocultures of <i>T. reesei</i>	Kolasa et al. (2014)
Reed	<i>T. reesei</i> RUT-C30 ATCC 56765 and <i>Clostridium acetobutylicum</i> ATCC824	40.01 g/L of glucose and 3.55 g/L xylose, the pH was adjusted to 6.5 using 2 M NaOH; medium containing carbon source and yeast extract (3 g/L) sterilized at 121°C for 15 min	Fermentation of the hydrolysate medium by <i>Clostridium acetobutylicum</i> produced 14.24 g/L of biobutanol and aceto butyl alcohol with yield 0.33 g/g	Zhu et al. (2015)

Comparative Studies of SmF and SSF				
Wheat bran, corn bran, and kinnow peel	<i>A. niger</i> NCIM 548	<i>A. niger</i> NCIM 548 under same temperature (30°C), agitation speed (170 rpm), and other media components in g/L [(NH ₄) ₂ SO ₄ , 1.0, MgSO ₄ , 5.0, FeSO ₄ . 7H ₂ O, 0.005, and KH ₂ PO ₄ , 5.0]	Production of pectinase and cellulase is 7.13 and 1.95 times higher respectively in SSF than in SmF	Kumar et al. (2011)
Sugarcane bagasse	<i>T. reesei</i> Rut-C30	Sequential solid-state and submerged fermentation; conidia suspension of 10 ⁷ spores/mL, which was transferred to Erlenmeyer flasks containing 100 mL of nutrient medium, enriched with 30 g/L of glucose	4.2-folds improvement compared with conventional submerged fermentation	Florencio et al. (2015)
Metabolic Engineering and Strain Improvements				
Sugarcane bagasse	<i>Chaetomium cellulolyticum</i> NRRL 18756	Supplemented with 1% (w/w) peptone, 2.5 mM MgSO ₄ , and 0.05% (v/v) Tween 80. Optimal moisture content and initial pH was 40% (v/v) and 5.0–6.5, respectively; medium was fermented at 40°C for 4 days	CMCase yield was 4-folds more than that of the wild type strain	Fawzi and Hamdy (2011).
Castor bean meal (<i>Ricinus communis</i> L.)	<i>Aspergillus japonicus</i> URM5620	Inoculum was prepared from spores (10 ⁷) on the malt extract agar plates in 0.05 M citrate buffer and per gram was inoculated in the substrate used for SSF; cellulases were stable in the range of pH 3–10 and temperature 30–50°C	Maximum βG, FPase, and CMCase activity was 88.3, 953.4, and 191.6 U/g dry substrate, respectively	Herculano et al. (2011)

(Continued)

TABLE 3.2 Spectrum of Microbial Cultures Employed for Production of Cellulases in Solid State Fermentation Systems (Continued)

Sequential Cultivation Methodology				
Sugarcane bagasse	<i>A. niger</i> A12	Shake flasks and a bench-scale bubble column bioreactor; potato dextrose agar medium slants incubated for 7 days at 32°C	Endoglucanase productivity of 57±13 IU/L/h was achieved	Cunha et al. (2012)
Sugarcane bagasse	<i>T. reesei</i> Rut-C30	Conidia suspension of 10 ⁷ spores/mL; enriched with 30 g/L of glucose; cultivations were performed for 72 h at 30°C and 200 rpm	4.2-folds improvement compared with conventional SmF	Florencio et al. (2015)
Bioreactor Systems				
Soybean hulls	Coculture of <i>T. reesei</i> and <i>Aspergillus oryzae</i>	Static tray fermenters; 250-mL Erlenmeyer flasks containing sterilized 100 mL potato dextrose broth supplemented with 0.1 mL Tween-80; flasks were incubated at 30°C for 5–6 days under static conditions until a mycelial mat was observed	Maximum activity of FPase of 10.7 FPU/gds and β-glucosidase of 10.7 IU/gds	Brijwani et al. (2010)
Sugarcane bagasse	<i>T. reesei</i> N RRL-6156	Packed-bed bioreactor; medium for preinoculum was composed of (g/L): 2.0 (NH ₄) ₂ SO ₄ , 0.5 MgSO ₄ , 1.0 FeSO ₄ ·7H ₂ O, 1.0 MnSO ₄ ·7H ₂ O, 10 yeast extract, 10 glucose, 150 corn steep liquor, and 1 mL/L of Tween 80	Higher for hydrolysis of sugarcane bagasse in ultrasound baths, was obtained (229 g/kg) at temperature of 43.4°C and concentration of enzymatic extract of 18.6%	Gasparotto et al. (2015)
Sugarcane bagasse	<i>Caloramator boliviensis</i>	Packed bed reactor; Inocula were prepared by cultivating <i>C. boliviensis</i> (2%, v/v) in 100-mL serum vials containing 70 mL of anaerobic mineral medium at 4°C	98% of substrates were converted; Ethanol yields of 0.40–0.46 g/g of sugar were obtained	Crespo et al. (2012)

Rao, 2012). Therefore, the main goal of pretreatment (physically, mechanically, and/or chemically) is to increase the enzyme accessibility, improve digestibility of cellulose, and make it easily accessible for microbial growth (Alvira et al., 2010; Agbor et al., 2011; Sarkar et al., 2012). In recent years, progress has been made in developing more effective pretreatment and hydrolysis processes leading to higher yield of sugars (Talebnia et al., 2010). However, several pretreatment process techniques were followed by different groups of researchers and each pretreatment has a specific effect on the cellulose, hemicellulose, and lignin fractions in lignocellulosic biomass (Jabasingh and Nachiyar, 2011). Thus, different pretreatment methods and conditions should be chosen according to the process configuration selected for the subsequent hydrolysis and fermentation steps (Alvira et al., 2010; FitzPatrick et al., 2010; Fu et al., 2011; Menon and Rao, 2012).

3.4.1 Physical/Mechanical Pretreatments

Physical pretreatment methods are the first step in using lignocellulosic biomass to improve the efficiency of downstream processing. Physical pretreatment methods include comminution (mechanical reduction in biomass particulate size).

3.4.1.1 Mechanical Comminution

The objective of the comminution is the reduction of particle size and crystallinity of lignocellulosic in order to increase the specific surface, size of pores, and reduce the degree of polymerization (Canilha et al., 2013). The physical method is considered as the first step for ethanol production from agricultural solid wastes and comprises grinding, milling, or chipping that reduces cellulose crystallinity and improves the efficiency of downstream processing (Sarkar et al., 2012).

3.4.1.2 Grinding/Milling/Chipping

Grinding or milling the agricultural residues to a small particle size markedly enhances its susceptibility to microbial influence. In addition, this process is needed to make material handling easier through subsequent processing steps (Talebnia et al., 2010; Wettstein et al., 2012). Da Silva et al. (2010) studied the effect of mechanical pretreatments such as ball milling and wet disk milling on sugarcane bagasse and straw, coupled to enzymatic hydrolysis and fermentation, using industrial C6 and C6/C5 strains of *Saccharomyces cerevisiae*. They reported that ball milling improved enzymatic hydrolysis. Glucose and xylose hydrolysis yields at optimum conditions for bagasse and straw were 78.7% and 72.1% and 77.6% and 56.8%, respectively, compared to wet disk milling. In the latter, glucose and xylose hydrolysis yields were 49.3% and 36.7%, and 68.0% and 44.9%, respectively. Ball milling decreased the crystallinity and favored the enzymatic conversion.

Size reduction may provide better results but very fine particle size generates clumps and may impose negative effects on subsequent processing such as pretreatment and enzymatic hydrolysis (Sarkar et al., 2012).

3.4.2 Physico-Chemical Pretreatments

The solubilization of lignocellulose components depends on temperature, pH, and moisture content. Steam explosion, liquid hot water, and ammonia fiber explosion are among the physico-chemical methods investigated for pretreatment of lignocellulosic biomass (eg, wheat and rice straw) (Talebnia et al., 2010). Steam explosion (autohydrolysis) is one of the most cost-effective and widely used pretreatment methods for lignocellulosic biomass (Mood et al., 2013).

3.4.2.1 Steam Explosion (Autohydrolysis)

Steam explosion is initiated at a temperature of 160–260°C with a corresponding pressure of 0.69 to 4.83 MPa for several seconds to several minutes before the material is exposed to the atmosphere for cooling (Yu et al., 2012; Mood et al., 2013).

3.4.3 Chemical Pretreatments

Chemical hydrolysis (pretreatment) is one of the feasible methods commonly used for the production of fermentable sugars from lignocellulosic biomass (Trivedi et al., 2015). Chemical pretreatment of lignocellulosic biomass employs different chemicals such as acids, alkalis, ethylamine, ammonia, and oxidizing agents, for example, peroxide and ozone (Ertas et al., 2014). There is a wide range of differences in the manner in which alkali, acid, or ammonia affect the cellulose in wood chips, rice straw or bran, and wheat straw or husk, due primarily to the extent of lignifications in the treated plant materials (Moodley and Kana, 2015; Srivastava et al., 2015).

NaOH (4%) pretreatment of wheat straw maximized the level of FPase in *T. reesei* mutants in SSF. The FPase is a relative measure of the overall cellulose-hydrolyzing capacity of microbial cellulase preparations, thus reliable and comparable data may be obtained only under standardized conditions (Velmurugan and Muthukumar, 2012). The cellulase system produced was capable of hydrolyzing over 80% of delignified wheat straw. In this context, Bansal et al. (2012) reported on the pretreatment of substrates with acids and bases for use by *A. niger* NS-2 in SSF using various agricultural materials (including corncobs, carrot peelings, composite, grass, leaves, orange peelings, pineapple peelings, potato peelings, rice husk, sugarcane bagasse, saw dust, wheat bran, wheat straw, and kitchen waste residues). The yields of cellulases were claimed to be higher (CMCase, FPase and β -glucosidase at the levels of 310, 17, and 33 U/gds, respectively) in alkali-treated substrates as compared to acid-treated and untreated substrates with the exception of wheat bran.

Combinations of NaOH pretreatment with steam explosion did not enhance the activity of the cellulase systems in two mutants of *T. reesei* (Oberoi et al., 2012a,b). Similar results were obtained with *Cellulomonas biazotea*, when kaller grass (*Leptochloa fusca*) was used as solid substrate for cellulase production

(Parmar and Rupasinghe, 2012). In contrast, Brijwani et al. (2010) reported no difference in cellulase yield from *T. reesei* QM 9414 between NaOH- treated and untreated sugarcane bagasse.

However, in large-scale production of cellulase, pretreatment of agricultural residues is not practical because of the enormous expenditure that it may incur, which would escalate the cost of enzyme production by 100% to 150% (Bansal et al., 2012). In addition, chemical hydrolysis results in the production of some nonsugar by-products, including 5-hydroxymethylfurfural, formic acid, levulinic acid, acetic acid, phenols, and heavy metals. These compounds hamper subsequent downstream fermentation and cause environmental hazards (Gupta et al., 2011; Trivedi et al., 2015).

3.4.4 Biological Pretreatment

Biological pretreatment uses microorganisms such as brown-, white-, and soft-rot fungi for selective degradation of lignin and hemicelluloses, among which white-rot fungi seems to be the most effective microorganism (Talebnia et al., 2010).

Falkoski et al. (2013) investigated the production of cellulases and hemicellulases in SSF by *Chrysoporthe cubensis*, a well-documented pathogen of various tree species, using wheat bran as the carbon source. The ability of the crude extract to hydrolyze lignocellulosic biomass (at 40°C and 50°C through 48 h of preincubation) was compared with a commercial cellulase extract. It was observed that the *C. cubensis* extract provided a higher production of glucose and xylose (320.8 mg/gds and 288.7 mg/gds respectively) as compared to commercial cellulase (250.6 mg/gds and 62.1 mg/gds of glucose and xylose, respectively).

As an alternative, using enzymes obtained from microorganisms could provide new avenues in converting complex polysaccharides into fermentable sugars (Hui et al., 2010; Trivedi et al., 2015). The enzymatic hydrolysis causes an increase in the porosity and specific surface area of lignocellulosic biomass. Although enzymatic hydrolysis indeed presents a green approach, it suffers from the high cost of commercial hydrolyzing enzymes (Trivedi et al., 2015).

3.5 ENVIRONMENTAL FACTORS AFFECTING MICROBIAL CELLULASE PRODUCTION IN SSF

Environmental factors such as water activity, moisture content, temperature, pH, oxygen levels, and concentrations of nutrients and products significantly affect microbial growth and cellulase production (Mohanty et al., 2009; Chen and Qiu, 2010). In SmF, environmental monitoring is relatively simple because of the homogeneity of microbial cell suspensions and of the solutions of nutrients and enzyme in the liquid phase. Due to the complex nature and heterogeneity of substrates, environmental monitoring is more challenging in SSF (Limayem et al., 2012; Liang et al., 2014).

3.5.1 Water Activity/Moisture Content

Moisture content is a critical factor for SSF processes because this variable influences growth and biosynthesis of cellulase (Menon and Rao, 2012). However, the moisture requirements of microorganisms must be better defined in terms of water activity (a_w), rather than moisture content of the solid substrate (Van Dyk and Pletschke, 2012). In addition, water activity affects biomass development, metabolic reactions, and the mass transfer processes. The optimum a_w for the growth of a number of fungi used in SSF processes is at least 0.96 (Buck et al., 2015).

3.5.2 Temperature

Temperature directly affects spore germination, growth of the microorganisms, and cellulase production in SSF (Farinas et al., 2011; Liu et al., 2014). In addition, the temperature level reached is a function of the type of microorganism and the porosity, particle diameter, and depth of the substrate (Yoon et al., 2014). Usually a temperature range of 25–30°C is found to be optimal for most mesophilic organisms in SSF (Farinas et al., 2011; Bhalla et al., 2013; Liu et al., 2014).

3.5.3 Mass Transfer Processes: Aeration and Nutrient Diffusion

In SSF, the mass transfer processes related to gases and nutrient diffusion are strongly influenced by the physical structure of the matrix and by the liquid phase of the system (Li et al., 2013a,b).

3.5.3.1 Gas Diffusion

Aeration essentially has two functions: (1) oxygen supply for aerobic metabolism and (2) removal of CO₂, heat, water vapor, and volatile components produced during the metabolism (He and Chen, 2013). In general, gas diffusion increases with pore size and decreases with the reduction of the diameter of the particle due to substrate packaging (Rodrigues-Zuniga et al., 2013; Chen et al., 2014).

3.5.3.2 Nutrient Diffusion

Nutrient diffusion refers to mass-transfer of nutrients and enzymes, which includes both the diffusion of intraparticulate nutrients toward the cells and the hydrolysis of solid substrates by the microbial enzymes (Guo et al., 2010). Nutrient diffusion processes are especially important in bacterial and yeast SSF processes (Zhi and Wang, 2014).

3.5.4 Substrate Particle Size

Particle size of the substrate plays a crucial role in enzyme production. The effect of particle size on growth and product formation has been studied by different authors (Xin and Geng, 2010; Membrillo et al., 2011; Schmidt and

Furlong, 2012; Wan and Li, 2012). Using different strains on different substrates (different in particle sizes), differences were reported in the production of lignocellulolytic enzymes (Membrillo et al., 2011).

Generally, smaller substrate particle size provides larger surface areas for microbial attack and thus, is a desirable factor (Zhang and Sun, 2014). However, use of too small substrate particles may result in substrate accumulation, which may interfere with microbial respiration/aeration, and therefore, result in poor growth. In contrast, larger particles provide better respiration/aeration efficiency (due to increased inter-particle space). However, they provide a limited surface for microbial attack. This necessitates a compromised particle size for a particular process as well as a particular substrate (Caceres et al., 2015).

3.5.5 Other Factors

Factors such as pH of a culture, inoculation size (optimum size of inocula) and stimulatory effects of surfactants can act as regulating agents for enzyme production by microorganisms in SSF (Caceres et al., 2015). The pH of a culture may change in response to microbial metabolic activities. The clearest reason is the secretion of organic acids such as citric, lactic, and acetic acids, particularly in fungal cultures, that will cause the pH to decrease (Parmar and Rupasinghe, 2012; Li et al., 2013a,b). The optimum size of inocula (viable spores/gds) of cultivated microorganisms on substrate is essential for cellulase production (Vastrad and Neelagund, 2011; Irshad et al., 2012). In this context, Sun et al. (2010) reported an inoculum size of 2×10^8 spores/flasks (500 mL) which was used for cellulase production by *Trichoderma* species. However, various types of surfactants such as Tween 20, Tween 60, Tween 80, Triton X-100, polyethylene glycol, sodium lauryl sulphate, sodium taurocholate, etc., can be used for cellulase production (Qi et al., 2010; Cao and Aita, 2013). Among these, Tween 80 is the most commonly used surfactant for cellulase production by several microorganisms, including *Aspergillus fumigatus* (Soni et al., 2010).

3.6 STRATEGIES TO IMPROVE PRODUCTION OF MICROBIAL CELLULASE

Spectacular successful strategies such as strain improvement, heterologous cellulase expression, coculture systems, and design of bioreactors have been extensively applied in the production of microbial cellulase.

3.6.1 Metabolic Engineering and Strain Improvement

To maximize cellulase production, strain improvement and optimization of culture conditions should be performed consequently. The improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics (Vu et al., 2011). Mutagenic

agents, such as N-methyl-N-nitro-N-nitrosoguanidine, ethyl-methane sulphonate and ultraviolet radiation have been used on several fungal strains to improve cellulase production (Dhillon et al., 2011; El-Ghonemy et al., 2014). Fawzi and Hamdy (2011) investigated the effect of various doses of gamma irradiation on the cellulose-producing fungus *Chaetomium cellulolyticum* NRRL 18756 for enhancing production of CMCase. CMCase yield was found to be 4-fold more than that of the wild type strain grown on the basal (wheat bran) medium. The mutagenic effectiveness of gamma irradiation has been reported to be higher than that of a chemical like ethyl-methane sulphonate (Dhulgande et al., 2011). For maximum production of cellulase, optimization of culture conditions is essential to design a suitable SSF process (El-Ghonemy et al., 2014).

3.6.2 Recombinant Strategy (Heterologous Cellulase Expression)

Engineering of yeast, bacteria and plant systems by coexpressing a cellobiose transporter and β -glucosidase is a novel strategy, reported recently by several groups of researchers (Lee et al., 2011; Baek et al., 2012; Ramani et al., 2015). They are of special interest, since they involve a one-step conversion of cellulose to ethanol with the capabilities of cellulose degradation and efficient fermentation. The process is known as “consolidated bioprocessing” (Njokweni et al., 2012; Kricka et al., 2015).

3.6.2.1 Yeast Expression Systems

Several yeast expression systems, including *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *Pichia pastoris*, have been developed for use in the biofuel industry and are especially of interest as consolidated bioprocessing organisms (Kricka et al., 2015). Of these, *S. cerevisiae* is the most well-characterized and commonly used yeast for heterologous gene expression that produces cellulases in sufficient quantities to hydrolyze cellulose (Garvey et al., 2013; Zhao et al., 2013a,b; Kim et al., 2014). Guo et al. (2011) developed and expressed a *BGL1* gene, encoding β -glucosidase, from *Saccharomyces fibuligera* (intracellular, secreted or cell-wall associated) in an industrial strain of *S. cerevisiae*. When β -glucoside permease and β -glucosidase were coexpressed in this strain, it could take up cellobiose and show a higher growth rate (0.11/h) on cellobiose. However, its major limitations as a commercial protein production host are its relatively low protein yields and tendency to hyperglycosylate certain heterologous proteins, which contribute to a reduced secretion rate (Zhao et al., 2013a,b).

3.6.2.2 Bacterial Expression Systems

A wide range of bacterial expression systems have been employed for the significant developments in the production of recombinant cellulase. These bacterial expression systems include *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium*

glutamicum, *Zymomonas mobilis*, etc., among which *E. coli* remains the most commonly used system for recombinant cellulase production (Graham et al., 2011; Garvey et al., 2013). However, bacterial expression systems suffer from problems associated with recombinant protein expression in bacterial species (eg, *Clostridium* sp.). It may be due to the occurrence of enzyme truncation, which may be caused by the lack of protective glycosylation of fungal cellulases produced in bacteria (Mingardon et al., 2011; Xia et al., 2013). Moreover, the above problem may be associated with codon mismatching which is another potential barrier to heterologous cellulase expression (Linger et al., 2010). Thus, bacterial systems encounter expression failures or extremely low yields. In contrast to these obstacles, recombinant production of cellulases in bacteria often leads to an increase in enzyme yield, as compared to the original host, according to several groups of researchers (Afzal et al., 2010; Lindenmuth and McDonald, 2011).

3.6.2.3 Plant Expression System

Plants are potentially ideal systems for cost-effective cellulase production, in part because of their ability to be economically produced on an industrial scale (Sainz, 2011). Moreover, plants are already being used successfully for molecular farming of enzymes, other proteins, carbohydrates and lipids (Mei et al., 2009; Dunwell 2014; Zhang et al., 2014). In addition, subcellular targeting, especially when produced in the endoplasmic reticulum, is also potentially more suitable for the accumulation of recombinant cellulases, since it corrects protein folding, glycosylation, and increases biological activity. Furthermore, plants show reduced protein degradation and increased stability of enzymes (Mei et al., 2009; Nahampun et al., 2013; Hefferon, 2015). In this context, Jung et al. (2013) developed a transgenic tobacco plant (*Nicotiana tabacum* cv. SR1) that accumulated *Thermotoga maritima* *BglB* cellulose. It was driven by the alfalfa RbcSK-1A promoter and contained a small subunit of the rubisco complex transit peptide. The improved *BglB* expression achieved in transgenic plants demonstrates the feasibility of increasing the yield of cellulolytic enzymes in plants.

3.6.3 Mixed-Culture (Coculture) Systems

Several groups of researchers utilized substrate by a single strain in pure culture-based processes for bioconversion of biomass to biochemical products of use in industrial applications. However, utilization of the substrate by a single strain in pure-culture-based processes has been limited to a narrow range of biomass materials (Lin et al., 2011). On the other hand, mixed cultures are structured by highly diverse microbes and constructed from natural inocula, which facilitate biological processes under nonsterile conditions without altering strain stability (Zhu et al., 2015). In addition, such cultures utilize mixtures of substrates with various compositions due to their high microbial diversity (Lin et al., 2011).

This facilitates the interactions between the environment and the microbial community (Brijwani et al., 2010; Dhillon et al., 2011).

Dhillon et al. (2011) performed an SSF to evaluate the potential of agricultural residues for the production of cellulase and hemicellulase using individual and mixed cultures of *A. niger* and *T. reesei*. The results of investigation showed that higher cellulase activity (24.54 IU/gds) and an optimal combination of cellulase and β -glucosidase can be achieved through mixed-culture SSF in trays. Moreover, mixed-culture SSF using rice straw supplemented with wheat bran in the ratio 3:2 resulted in higher cellulase activity (3106.34 IU/gds) as compared to the activities obtained using monocultures.

Oberoi et al. (2012a) studied SSF of kinnow (*Citrus reticulata*) waste supplemented with wheat bran using crude filtrate extracts (using *A. niger* and *T. reesei* independently and in combination for production of cellulase, protease, and pectinase for oil recovery from mustard seeds). This study indicated that pretreatment using crude enzyme obtained with mixed cultures enhanced oil recovery by 11% as compared to the control where no enzyme was used.

Cocultivation of fungi may be an excellent system for on-site production of cellulolytic enzymes in a single bioreactor (SSF). Kolasa et al. (2014) investigated mixed cultures of *T. reesei* RutC30 with either the novel *Aspergillus saccharolyticus* AP, *Aspergillus carbonarius* ITEM 5010 or *A. niger* CBS 554.65 cultivated in SSF. These cocultures were tested for cellulase (avicelase, FPase, endoglucanase, and β -glucosidase) production, as well as hydrolysis of pretreated wheat straw. The results indicated approximately 80% efficiency of hydrolysis and 30% more avicelase activity compared to results obtained using monocultures of *T. reesei*.

3.7 FERMENTER (BIOREACTOR) DESIGN FOR CELLULASE PRODUCTION IN SSF

Over the years, different types of fermenters (bioreactors) have been employed for various purposes, including cellulase production in SSF systems (Chen and Qiu, 2010). SSF processes for production of cellulase could be operated in batch, fed-batch, or continuous modes, although batch processes were the most common (Ray and Swain, 2011). Tray bioreactors, packed bed bioreactors, rotary drum bioreactors, and fluidized bed bioreactors are the most commonly used laboratory scale bioreactors for SSF (Figueroa-Montero et al., 2011; Yoon et al., 2014).

3.7.1 Tray Type Bioreactor

The tray bioreactor is the most widely employed bioreactor in laboratory, pilot, and even on the industrial scale. The tray type bioreactor consists of flat trays, where the bio-particle system is embedded, forming a layer of about 1.5 or 2 cm thickness (Figueroa-Montero et al., 2011). The trays may be incubated

in a temperature-controlled chamber with passive aeration. The application of tray bioreactors in SSF for industrial operations show the following advantages.

- Require less energy consumption, since mixing is not required during operation.
- Simple to operate, that is, no sophisticated operation technology is required on the industrial scale.
- Extensively applied in strain selection and optimization of fermentation conditions.

[Singhania et al. \(2010\)](#) reported that in a tray fermenter, the solid substrate (lignocellulosic waste from the xylose industry) could be reused in at least three batches and the highest cellulase (FPase) activity (158IU/g koji) was obtained in the second fermentation batch using the filamentous fungus, *T. reesei*. To produce cellulase on a larger scale, a deep tank fermenter with forced aeration was used and 128IU/g koji (~305IU/g cellulose) was reached after 5 days of SSF. However, in the tray bioreactor system, the transfer of gas is diffusion-limited, causing major problems in heat and mass transfer ([Ruiz et al., 2012](#)). [Brijwani et al. \(2010\)](#) performed SSF of soybean hulls supplemented with wheat bran using a coculture of *T. reesei* and *Aspergillus oryzae*. The maximum cellulase activities, that is, filter paper activity of 10.7FPU/gds and β -glucosidase of 10.7IU/gds, were obtained after a 96h incubation period in a static tray fermenter, in agreement with optimized activities at the shake flask level. Using SSF in Erlenmeyer flasks and plastic trays, with apple pomace as the solid substrate, and *A. niger* NRRL567 and *A. niger* NRRL2001 as the organisms, [Dhillon et al. \(2012\)](#) conducted an experiment on inducing and moistening the fungal cellulase and hemicellulase. The results showeded that SSF is a simple and cheap technology for high cellulase and hemicellulase (4868IU/gds for xylanase using *A. niger* NRRL 567) production using apple pomace waste.

3.7.2 Rotary Drum Bioreactor

Among the SSF reactors, rotating drum bioreactors provide relatively gentle and uniform mixing by improving baffle design, since there is no agitator within the substrate bed. The rotary drum bioreactor typically is a horizontal wire mesh cylinder, rotating slowly inside a cylindrical glass vessel containing the culture medium at the bottom of the reactor. Rotation can be intermittent or continuous, depending on the importance of mixing effects on the solid substrate. The wire mesh cylinder may contain carriers (supports) attached to the fungus. The fungus is connected to the upper part of the reactor for aeration ([Castro et al., 2015](#)). [Alam et al. \(2009\)](#) studied SSF of oil palm branches lacking fruit for cel lulase production by *T. harzianum* T2008 using two SSF systems: Erlenmeyer flask (500 mL) and horizontal rotary drum bioreactor (50L). The highest cellulase activity in the Erlenmeyer flask was 8.2 FPase/gds of empty fruit branch on

the fourth day of fermentation, while the activity with the rotary drum bioreactor was 10.1 FPase/gds on the second day of fermentation.

The problems with the rotary drum bioreactor are particle agglomeration over time, shear sensitive mycelia with high rotation speed, and difficulties in controlling heat and mass transfer inside the bed (Zhang et al., 2012).

3.7.3 Packed Bed Bioreactor

The packed bed bioreactor is composed of a jacketed glass column with a wet solid matrix of inoculated filamentous fungi (Alani et al., 2009; Thomas et al., 2013). Due to the consumption of the substrate, increases in the concentration of the fungal biomass and the cellulase production are achieved. However, fungal growth is accompanied by heat release due to respiratory activities (Chen and He, 2013). To overcome these limitations, Buck et al. (2015) developed a relevant feedback control scheme that influences and equalizes the moisture and temperature distributions along the packed-bed bioreactor during SSF. This prevents the occurrence of locally critical process conditions which may necessitate the shutdown of the bioreactor operation.

Crespo et al. (2012) evaluated the ability of an organism to ferment D-(+)-cellobiose, D-(+)-xylose, and pentose-rich sugarcane bagasse hydrolysate using an immobilized anaerobic bacterium, that is, *Caloramator boliviensis*, in a packed bed reactor operated at 60°C. Ethanol yields of 0.40–0.46 g/g of sugar were obtained when the sugarcane bagasse hydrolysate was fermented. Zanelato et al. (2012) studied the production of endoglucanase (CMCase) by SSF in a packed bed bioreactor using the recently isolated thermophilic fungus *Myceliophthora* sp. I-1D3b, cultivated in sugar cane bagasse and wheat bran. The results for CMCase activity (total filter-paper cellulase (FPase) and xylanase) was 878 U/gds at 288 h of incubation time, even though a reasonable amount (669 U/gds.) was obtained as early as 96 h, indicating a very good potential for industrial application.

Using SSF conditioned in conical flasks and validated in a packed-bed bioreactor, Gasparotto et al. (2015) studied optimal conditions for production of cellulolytic enzymes by *T. reesei* NRRL-6156 using a hydrolysate of sugarcane bagasse in water and ultrasound baths. The crude enzymatic extract was used for hydrolysis of sugarcane bagasse in ultrasound baths (229 g/kg), higher than water (224.0 g/kg) obtained at temperature of 43.4°C and concentration of enzymatic extract of 18.6% and higher yield was obtained.

Packed bed bioreactors are cost-, space-, and labor-effective and they are often employed in SSF with the aim of increasing productivity (Karimi et al., 2014). Packed beds, in which air is introduced through the packed mass of substrate, is another alternative. However, this design suffers from higher pressure drop and channeling, especially at high flow rates, which prevents its large-scale use (Fanaei and Vaziri, 2009).

3.7.4 Fluidized Bed Bioreactor

A fluidized bed bioreactor is a type of continuous reactor system, in which a fluid (gas or liquid) is passed through a granular solid material at high velocities. This process is known as fluidization. The solid substrate is typically supported by a porous plate, known as a distributor and the fluid is forced through the distributor up through the solid material (Foong et al., 2009; Saqib et al., 2013; Thomas et al., 2013). The use of fluidized bed bioreactor systems with high cell densities can improve ethanol volumetric productivities as compared with traditional batch systems. The fluidized bed bioreactors have advantages over conventional reactor systems, since they provide uniform particle mixing, and maintain a uniform temperature gradient.

Sheikhi et al. (2012) developed a sequential modular simulation approach, for prediction of the performance of three-phase fluidized bed bioreactors at different operating conditions.

3.8 BIOMASS CONVERSION AND APPLICATION OF MICROBIAL CELLULASES

Cellulases were initially investigated several decades ago for the bioconversion of biomass which led to research on the industrial applications of the enzyme for animal feed, food, textiles, detergents, and in the paper industry (Dashtban et al., 2010). With the shortage of fossil fuels and the rising need to find alternative source of renewable energy and fuels, there is a renewal of interest in the bioconversion of lignocellulosic biomass using cellulases and other enzymes (Juturu and Wu, 2014). In some fields, the technologies and products using cellulases have reached the stage where these enzymes have become indispensable.

3.8.1 Textile Industry

Cellulases are the most successful enzymes used in the textile industry (Kuhad et al., 2011). Cellulases are used in the biostoning of denim garments (jeans) and biopolishing of cotton and other cellulosic fabrics (Bera et al., 2015). Traditional stonewashing of denim garments involves the amylase-mediated removal of starch coating (desizing) and treatment (abrasion) of jeans with pumice stone in large washing machines (Menon and Rao, 2012). Cellulases are used in the biostoning of denim garments for producing softness and the faded look of denim garments, replacing the use of pumice stones which were traditionally employed in the industry (Kuhad et al., 2011). The enzymes act on the cellulose fiber to release the indigo dye used for coloring the fabric, producing the faded look of denim. *Humicola insolens* cellulase is most commonly employed in biostoning, although use of acidic cellulose from *Trichoderma* along with proteases was found to be equally good (Zhao et al., 2013a,b).

Caparros et al. (2012) analyzed the influence of the alkaline cellulose enzyme from *Bacillus* spp. (BaCel5) on the sorption properties of cotton fabrics.

They used unbleached cotton woven fabrics (24/21 ends/picks per cm and area density of 140 g/m²) as the cellulosic substrate for the enzymatic treatments. The results showed that the use of BaCel5 helps to avoid fabric damage, thus enhancing fabric care benefits. [Yu et al. \(2014\)](#) investigated the effect of soluble immobilized-cellulase on the surface morphology and structure of cotton fibers. The results showed that smooth surface cotton fibers, fiber accessibility due to limited interior hydrolysis of the cotton fibers, and an increased crystalline index of the cotton fibers, could be obtained after the immobilized-cellulase treatment.

3.8.2 Laundry and Detergents

Cellulases, in particular EGIII and CBH I variants, are commonly used in detergents for cleaning textiles, a recent innovation in the detergent industry ([Khattak et al., 2012](#)). Several groups of researchers reported that EG III variants, in particular those from *T. reesei*, are suitable for use in detergents ([Saranraj et al., 2012](#)). At present, fungi such as *Trichoderma viride* and *T. harzianum* are considered to be excellent endoglucanase and exoglucanase producers as compared to other groups of fungi, such as species of *Aspergillus*, *Penicillium*, and *Rhizopus* ([Singhania et al., 2010; Li et al., 2013a,b](#)). Generally, cellulase preparations have the potential of reforming cellulose fibrils and retaining original color to the garments. Moreover, cellulase helps in softening the garments and in removal of dirt particles trapped within the microfibril network ([Ibrahim et al., 2011; Hao et al., 2012; Gupta et al., 2013](#)). Cellulase preparations, mainly from species of *Humicola* (*H. insolens* and *H. grisea* var. *thermoidea*) that are active under mild alkaline conditions and at elevated temperatures, are commonly added to washing powders and detergents ([Saranraj et al., 2012](#)). Furthermore, in recent days, to improve the stability of cellulase, liquid laundry detergents containing surfactants, a water-soluble salt, citric acid, protease, and a mixture of propanediol and boric acid have been used ([Li et al., 2013a,b](#)).

[Ladeira et al. \(2015\)](#) reported the production of cellulase by thermophilic *Bacillus* sp. SMIA-2 using sugarcane bagasse and corn steep liquor as substrates and exploiting its potential in the detergent industry. Maximum avicelase (0.83 U/mL) and CMCase (0.29 U/mL) activities were reached after 120 h and 168 h of culturing time, respectively. The compatibility of cellulases varied with each laundry detergent tested, being more stable in the presence of Ultra Biz and less stable with Ariel. In addition, the enzyme was stable in sodium dodecyl sulfate and RENEX-95, and was inhibited by TritonX-100 and H₂O₂.

3.8.3 Food and Animal Feed

In the food industry, cellulase has a wide spectrum of applications, such as the extraction and clarification of fruit and vegetable juices, production of fruit nectars and purees, extraction of olive oil, and increase of aroma production ([Karmakar and Ray, 2010](#)). [Rossi et al. \(2009\)](#) investigated the increase of

fruity aroma production in citric pulp, a waste from the citric juice production industry, using *Ceratocystis fimbriata* in SSF. The optimal production of volatile compounds, quantified by headspace analysis in a gas chromatograph, reached 99.60 µmol/L. Best production was obtained when the citric pulp was supplemented with 50% of soya bran, 25% of sugarcane molasses (as carbon and nitrogen sources), and in mineral saline solution.

In beer manufacturing and in the wine industry, exogenous hemicellulases and glucanases are added to improve the malting of barely (Saigal and Ray, 2007; Buzrul, 2012; Khattak et al., 2013). In terms of feeding value, crude fiber is a measure of the quantity of the fibrous, poorly digested material in the feed. In this context, cellulases are used to improve the nutritive quality of forages (Brokner et al., 2012). Moreover, microbial fermentation has been used to reduce antinutritional factors, including nonstarch polysaccharides such as agalactosides, glycinin, and β-conglycinin in animal feed. Using SSF, Opazo et al. (2012) isolated selective mesophilic, aerobic, and cellulolytic bacteria (belonging to the genera *Streptomyces*, *Cohnella*, and *Cellulosimicrobium*) and assessed their ability to reduce soybean meal agalactosides and nonstarch polysaccharides. SSF resulted in a reduction of nearly 24% in total nonstarch polysaccharides, 83% of stachyose, and 69% of raffinose and an increase in protein content.

3.8.4 Pulp and Paper Industry

The technology for pulp manufacture is highly diverse, and numerous opportunities exist for the application of microbial enzymes (Nagar et al., 2011; Kumar et al., 2013). Historically, enzymes such as cellulases, hemicellulases, and xylanase are employed in the paper industry (biochemical pulping) for modification of the coarse mechanical pulp, hand sheet strength properties, and deinking of recycled fibers (Garg et al., 2011). Moreover, the pulp and paper industry processes huge quantities of lignocellulosic biomass every year (Saleem et al., 2012; Kumar et al., 2013). These enzymes are also used routinely for improving drainage and sewerage of pulp at the mill scale (Kumar et al., 2013). Furthermore, biocharacterization and biobleaching of pulp fibers are other applications where microbial cellulases are employed (Kumar and Satyanarayana 2012).

Thermophilic xylanase may be a useful replacement for chlorine and alkali used during biobleaching in the pulp and paper industry. Joshi and Khare (2011) assessed a suitable substrate, deoiled *Jatropha curcas* seed cake, for the production of xylanase by the thermophilic fungus *Scytalidium thermophilum* in SSF. This xylanase is useful in biobleaching of paper pulp and may lead to a reduced use of chlorine in the pulp and paper industry.

3.8.5 Biofuels

Probably the most significant application in recent years is in the utilization of lignocellulosic wastes for the production of biofuels (Cheng et al., 2012; Singh

and Bishnoi, 2012; Liang et al., 2014; Zhu et al., 2015). Lignocellulosic biomass holds considerable potential to meet the current energy demand of the modern world (Anwar et al., 2014). It also represents the most prolific renewable resource of second-generation biofuels as sustainable alternative transportation fuels which are in high demand (Zhu et al., 2015). A potential application of cellulase is the conversion of cellulosic materials to glucose and other fermentable sugars, which in turn can be used as microbial substrates for the production of single-cell protein or a variety of fermentation products such as ethanol (Lever et al., 2010; Martínez et al., 2015). In this context, several reports disclose that cellulase extracts, produced by SSF are sufficient to produce ethanol from ground lignocellulosic biomass (Singh and Bishnoi, 2012; Liang et al., 2014).

Economou et al. (2010) studied biotechnological biodiesel production from sweet sorghum in a semisolid state fermentation with the use of the oleaginous fungus *Mortierella isabellina*. Similarly, Lever et al. (2010) investigated the production crude unprocessed cellulase extracts by SSF using *T. reesei* in order to make ethanol from wheat straw. Using a typical range of cellulase activities obtained in these experiments (0.75–1.2 FPU/gds) and the values of cellulase loading employed in the ethanol fermentations (0.02 and 0.05 FPU/mL), it was calculated that the amount of wheat straw required as substrate for cellulase production varied from approximately 5% to 25% of the straw converted to ethanol.

Fungal saccharification followed by fermentation (SSF) can improve ethanol production from lignocellulosic biomass. Shrestha et al. (2010) evaluated the effectiveness of mild alkali and steam pretreatments of wet-milled corn fiber on SSF carried out by white-, brown-, and soft-rot fungi (*Phanerochaete chrysosporium*, *Gloeophyllum trabeum*, and *T. reesei*), and on subsequent yeast fermentation of the hydrolysates to ethanol. The ethanol yield in fungal SSF was as high as 75% of that from a commercial cellulase enzyme (Spezyme-CP) in SSF.

Reed is a potential feedstock for both biobutanol and cellulase production (Srirangan et al., 2012). Zhu et al. (2015) efficiently utilized reed for production of both cellulase and biobutanol using *Clostridium acetobutylicum* under SSF. Organsolv pretreatment can efficiently provide reed hydrolysate that can be converted to biobutanol without any usage commercial cellulase. Moreover, the fermentation of the hydrolysate medium by *C. acetobutylicum* produced 14.24 g/L of biobutanol and acetone/butanol/ethanol (ABE) with yield 0.33 g/g.

However, to develop efficient technologies for bioethanol production, significant research needs to be directed towards biotechnological and genetic improvement of the existing organisms utilized in the process (Zhao et al., 2013a,b). The use of pure enzymes in the conversion of biomass to ethanol or to fermentation products is currently uneconomical due to the high costs of commercial cellulases (Dogaris et al., 2009; Saini et al., 2015). Effective strategies are yet to be resolved and active research has to be carried out in this direction (Kao et al., 2013; Bagy et al., 2014).

3.9 CONCLUDING REMARKS

The biological aspects of SSF of cellulosic biomass have become the crux of future research involving cellulases and cellulolytic microorganisms. Critical analysis of the literature shows that production of cellulases by SSF offers several advantages, such as easy enzyme recovery, low cost of production, high product concentration, and reducing energy requirements. It is well established that cellulase titers produced in SSF systems are several-fold higher than in SmF systems. However, the problems which warrant attention are not limited to cellulase production alone. A concerted effort to understand the basic physiology of cellulolytic microorganisms is needed, coupled with engineering principles applied to SSF. The aspects open to consideration include (1) cheaper technologies for pretreatment of cellulosic biomass providing for better microbial attack, (2) designing of bioreactors, (3) better systems for process optimization leading to higher cellulase yields, (4) treatment of biomass for production of hydrolytic products, which can then serve as feedstock for downstream fermentative production of valuable primary and secondary metabolites, and (5) protein engineering to improve cellulase qualities.

ABBREVIATIONS

βG	β-glucosidase
CMCase	carboxymethyl cellulase
EGase	endoglucanase
FPase	filter paper activity
FPIU	Filter Paper International Units
FPU	filter paper units
gds	gram dry substrate
IU	International Units
SSF	solid state fermentation
SmF	submerged fermentation

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Chapter 4

Hyperthermophilic Subtilisin-Like Proteases From *Thermococcus kodakarensis*

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4.1 INTRODUCTION

Hyperthermophiles are defined as microorganisms that optimally grow at temperatures above 80°C (Stetter, 2013) or that can grow at temperatures above 90°C (Adams and Kelly, 1998). These microorganisms, predominantly distributed in the domain Archaea, have been isolated from a large variety of natural thermal environments, including hot springs and volcanoes, as well as from artificial environments. All of these organisms' proteins, DNAs, RNAs, and cytoplasmic membranes have adapted to the high temperatures necessary to thrive in such harsh environments (Imanaka, 2011; Stetter, 2013). The enzymes from hyperthermophiles generally display superior stability with respect to their mesophilic homologs, and therefore have been regarded not only as excellent models for studying protein stabilization mechanisms, but also as potential candidates for industrial applications. Thermostable enzymes are useful for industrial enzymatic processes performed at high temperatures (de Miguel Bouzas et al., 2006). Performing enzymatic reactions at high temperatures increases reaction rates and solute solubility, while simultaneously reducing the risk of contamination, resulting in high product yields. Furthermore, enhanced thermal stability often confers resistance to chemicals such as surfactants, denaturants, and oxidative reagents. Thus, hyperthermophilic enzymes allow us to apply enzymatic catalytic techniques to a huge variety of environments.

Proteases that catalyze the hydrolysis of peptide bonds in proteins and peptides are ubiquitous. Proteolytic activity is essential for many biological

processes, including development, differentiation, and immune response. Furthermore, proteases are the most important industrial enzymes, accounting for at least 65% of sales in the world market ([de Miguel Bouzas et al., 2006](#); [Rao et al., 1998](#)). Proteases are used in a multitude of market sectors, including detergents, food, pharmaceuticals, leather, diagnostics, waste management, and in silver recovery from X-ray film. Notably, the largest share of the enzyme market is held by bacterial alkaline proteases used as detergent additives. These are highly active at alkaline pHs, and enhance the washing ability of detergents by degrading protein stains. However, the stability of these proteases deteriorates in the presence of surfactants, which are usually present in detergents, and therefore they are destabilized and degraded by their own activities. Searches for more stable, thermophilic proteases have helped solve this issue, as well as the development of protein engineering methods, which can improve the stability of existing enzymes. Hyperthermophiles are an additional, attractive source of novel proteases, which are sufficiently stable even in the presence of surfactants.

Serine proteases are classified based on their amino acid sequence. The SB clan, known as the subtilisin-like protease (subtilase) superfamily, is diverse and found in archaea, bacteria, fungi, and higher eukaryotes ([Siezen and Leunissen, 1997](#); [Siezen et al., 2007](#)). The SB clan is further divided into six families, subtilisin, thermitase, proteinase K, pyrolysin, kexin, and lantibiotic peptidase. These proteases all possess a catalytic triad consisting of the amino acids serine, histidine, and aspartate. The serine triggers catalytic hydrolysis through nucleophilic attack of the peptide bond carbonyl group. Histidine is the general base that extracts a proton from the hydroxyl group of serine, while aspartate stabilizes the protonated imidazole group of histidine and orients it into the correct position. Most subtilases display maximum activity at alkaline pHs, because deprotonation of the imidazole group is required for catalysis. Prokaryotic subtilases are generally secreted into the extracellular environment, and provide nutrients by degrading extracellular proteins, whereas eukaryotic subtilases play essential roles in many, varied cellular events. Examples in human cells include PCSK9 ([Awan et al., 2014](#)) and furin ([Seidah et al., 2008](#)), which are responsible for cholesterol metabolism and the maturation of hormone precursors, respectively. Prokaryotic subtilases are present not only in mesophiles, but also in thermophiles ([Catara et al., 2003](#); [Choi et al., 1999](#); [Gödde et al., 2005](#); [Jang et al., 2002](#); [Kannan et al., 2001](#); [Kluskens et al., 2002](#); [Kwon et al., 1988](#); [Li and Li, 2009](#); [Li et al., 2011](#); [Matsuzawa et al., 1988](#); [Sung et al., 2010](#); [Wu et al., 2004](#)) and psychrophiles ([Arnórsdóttir et al., 2002](#); [Davail et al., 1994](#); [Dong et al., 2005](#); [Kulakova et al., 1999](#); [Kurata et al., 2007](#); [Morita et al., 1998](#); [Narinix et al., 1997](#)). The crystal structures of several have been reported ([Almog et al., 2003, 2009](#); [Arnórsdóttir et al., 2005](#); [Kim et al., 2004](#); [Smith et al., 1999](#); [Teplyakov et al., 1990](#)). Extracellular subtilases are often used for various biotechnological applications. The subtilisins from mesophilic *Bacillus* species have been widely

used by the detergent industry owing to broad substrate specificity and ease of large-scale active enzyme preparation (Saeki et al., 2007).

Subtilisin E from *Bacillus subtilis* (Stahl and Ferrari, 1984), subtilisin BPN' from *Bacillus amyloliquefaciens* (Wells et al., 1983), and subtilisin Carlsberg from *Bacillus licheniformis* (Jacobs et al., 1985) are representative bacterial subtilisins. The structure and maturation of these three subtilisins have been extensively studied (Bryan, 2002; Chen and Inouye, 2008; Eder and Fersht, 1995; Eder et al., 1993; Fisher et al., 2007; Hu et al., 1996; Li et al., 1995; Shinde and Inouye, 2000; Yabuta et al., 2001). Bacterial subtilisins are synthesized as an inactive precursor, termed prepro-subtilisin, containing a signal peptide (pre) and a propeptide (pro) at the *N*-terminus of the subtilisin domain. The pre-sequence assists in the translocation of subtilisin to the extracellular environment and is cleaved off through the secretion process. The subtilisin precursor is then secreted in pro-form (pro-subtilisin) and subsequently matures following three steps: (1) folding of pro-subtilisin, (2) autoprocessing of pro-peptide, and (3) degradation of propeptide (Fig. 4.1). The propeptide is essential for maturation owing to its dual function as an intramolecular chaperone that assists folding of the subtilisin domain, and as an inhibitor that forms an inactive complex with subtilisin upon autoprocessing. The subtilisin domain alone cannot fold into its native structure; subtilisin folding stops at a molten globule-like intermediate, because its folding rate is extremely low in the absence of

The diagram illustrates the maturation pathway of bacterial subtilisin:

- Prepro Subtilisin:** The gene structure shows a grey box labeled "Prepro" and a larger grey box labeled "Subtilisin" joined together.
- Expression:** An arrow points down to the protein chain.
- Secretion:** An arrow points right, showing the protein chain exiting a wavy line labeled "Cell".
- Pro-subtilisin:** The protein chain is shown with an "N" at the top and a "C" at the bottom. It is labeled "Pro-subtilisin".
- 1) Folding:** An arrow points down to a circular structure representing the folded subtilisin domain.
- 2) Autoprocessing:** An arrow points down to a complex structure where the "Propeptide" (black oval) is being cleaved from the "Subtilisin domain" (grey oval).
- 3) Degradation of propeptide:** An arrow points left to a circle with a wedge removed, representing the matured subtilisin.
- Extracellular environments:** A box labeled "Extracellular environments" is located in the bottom right corner.

FIGURE 4.1 Maturation of bacterial subtilisin in the extracellular environment.

propeptide (Eder et al., 1993). In the propeptide:subtilisin complex structure, the propeptide interacts with two surface helices and the active-site cleft of subtilisin. The C-terminus region of the propeptide binds to the active site in a substrate-like manner and competitively inhibits activity upon autoprocessing (Li et al., 1995). The propeptide of mesophilic subtilisins is an intrinsically unstructured protein (Hu et al., 1996; Huang et al., 1997; Wang et al., 1998) and therefore cleaved by subtilisin immediately upon its dissociation. The dissociation of the first propeptide molecule initiates a cascade, in which the entire propeptide in the complex is destroyed *in trans*, and subsequently active proteases are released. Hence, the release of the propeptide is the rate-determining step in the maturation reaction. This propeptide-mediated bacterial subtilisin maturation is a general maturation model for not only subtilases (Baier et al., 1996; Basak and Lasure, 2003; Jia et al., 2010; Marie-Claire et al., 2001; Shinde and Thomas, 2011) but also for other extracellular proteases (Grande et al., 2007; Marie-Claire et al., 1999; Nemoto et al., 2008; Salimi et al., 2010; Schilling et al., 2009; Tang et al., 2002; Winther and Sørensen 1991).

Subtilisin stability is highly dependent on Ca^{2+} ions. Bacterial subtilisins bind Ca^{2+} ions at two binding sites, a high affinity site (the A or Ca1 site) and a low affinity site (the B or Ca2 site), according to the crystal structures (Bott et al., 1988; Jain et al., 1998; McPhalen and James, 1988). Both sites are located far from the active site, and the Ca^{2+} ions are not involved in catalysis. The two Ca^{2+} ions in bacterial subtilisins contribute to the enzyme's stability, and their removal greatly destabilizes the structures (Gallagher et al., 1993). In contrast, pro-subtilisin can mature in the absence of Ca^{2+} ions, indicating that Ca^{2+} ions are not required for the maturation of bacterial subtilisins (Yabuta et al., 2002).

The structure and maturation of subtilisins from mesophilic bacteria have been studied in great detail, as reviewed above. However, our understanding of hyperthermophilic subtilisins is still quite limited, even though they should prove to be promising candidates for many industrial applications. Our recent studies have revealed the mechanism by which two hyperthermophilic subtilisins are matured effectively and acquire their stable structure in an extremely hot environment. In this chapter, we summarize the unique maturation and stabilization mechanisms of two subtilisin-like proteases from a hyperthermophilic archaeon, and we discuss the prospect of their application in novel medical and industrial fields.

4.2 TWO SUBTILISIN-LIKE SERINE PROTEASES FROM *THERMOCOCCUS KODAKARENSIS KOD1*

Thermococcus kodakarensis KOD1 is a hyperthermophilic archaeon isolated from a solfatara at a wharf on Kodakara Island, Kagoshima, Japan (Atomi et al., 2004; Morikawa et al., 1994). The strain grows at temperatures ranging from 60°C to 100°C, with an optimum growth temperature of 85°C. This strain offers a promising source of a wide range of commercially valuable enzymes, such as

KOD polymerase (Toyobo Co., Ltd, Japan), which has exceptionally high fidelity and efficiency in polymerase chain reaction applications, and its hyperstable thiol protease (Morikawa et al., 1994). The *T. kodakarensis* genome contains three genes encoding subtilisin-like proteases, Tk-1675 (NCBI YP_184088), Tk-1689 (NCBI YP_184102), and Tk-0076 (NCBI YP_182489) (Fukui et al., 2005). Tk-1675 and Tk-1689 are designated prepro-Tk-subtilisin (Kannan et al., 2001) and prepro-Tk-SP (Foophow et al., 2010a), respectively. These two proteases are enzymatically active when overproduced in *Escherichia coli*. Furthermore, *T. kodakarensis* cells produce these enzymes as active extracellular proteases when the genes are expressed under a strong constitutive promoter (Takemasa et al., 2011). In contrast, an active form of Tk-0076 has not yet been obtained.

The amino acid sequences of the three subtilisins from *T. kodakarensis* are compared with that of prepro-subtilisin E (Protein ID AAA22742) from *B. subtilis* in Fig. 4.2. Prepro-subtilisin E consists of a putative signal peptide [Met(-106)–Ala(-78)], a putative propeptide [Ala(-77)–Tyr(-1)], and a mature

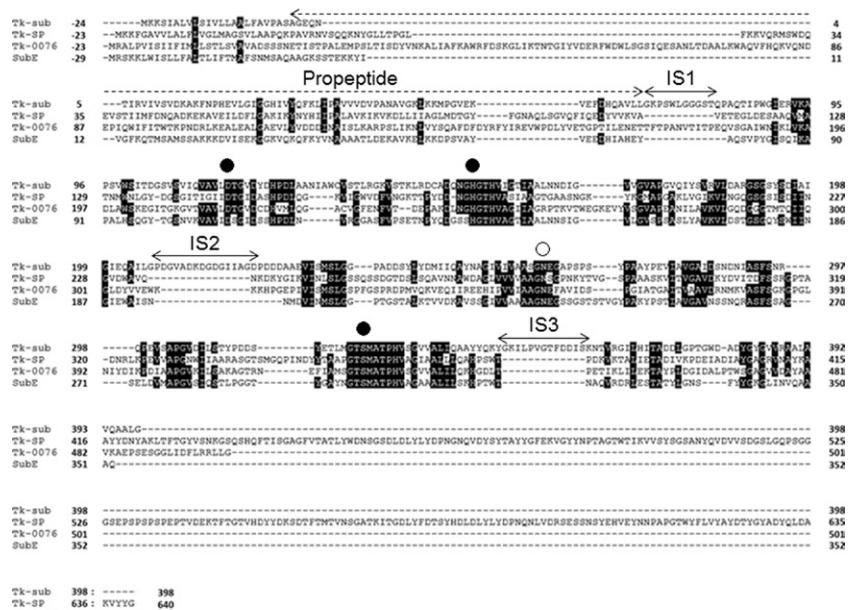


FIGURE 4.2 Sequence alignment of subtilisin-like serine proteases. The amino acid sequences of Prepro-Tk-subtilisin (Tk-sub), Prepro-Tk-SP (Tk-SP), Tk-0076, and prepro-subtilisin E (SubE) are aligned; NCBI accession numbers: YP_184088 (Tk-sub), YP_184102 (Tk-SP), YP_182489 (Tk-0076), and AAA22742 (SubE). Highly conserved residues are indicated with *white letters*. Amino acid residues that form the catalytic triad, and the asparagine residue that forms the oxyanion hole, are indicated by *solid* and *open circles*, respectively. The propeptide domain of the proteases, and the insertion sequences (IS1–IS3) of Pro-Tk-subtilisin are shown above the sequences.

domain, subtilisin E (Ala1–Gln275). Similarly, prepro-Tk-subtilisin consists of a putative signal peptide [Met(–24)–Ala(–1)], a putative propeptide, Tkpro (Gly1–Leu69), and a mature domain, Tk-subtilisin (Gly70–Gly398). Prepro-Tk-SP consists of a putative signal peptide [Met(–23)–Ala(–1)], a putative propeptide, proN (Ala1–Ala113), and a mature domain, Tk-SP (Val114–Gly640).

The pro-forms of Tk-subtilisin and Tk-SP are designated Pro-Tk-subtilisin and Pro-Tk-SP, respectively. The three amino acid residues of the subtilisin catalytic triad, and the asparagine residue that is required for the formation of an oxy-anion hole, are fully conserved in the sequences. Comparison of the Tk-subtilisin and Tk-SP sequences with that of bacterial subtilisin prepro-subtilisin E shows that Tk-subtilisin contains three insertion sequences, termed IS1 (Gly70–Pro82), IS2 (Pro207–Asp226), and IS3 (Gly346–Ser358); and that Tk-SP has a long C-terminal extension (Ala422–Gly640). Except for these inserted sequences, Tk-subtilisin and Tk-SP show 31% identity to each other, and both of them show approximately 40% identity to bacterial prepro-subtilisin E.

4.3 TK-SUBTILISIN

4.3.1 Ca²⁺-Dependent Maturation of Tk-Subtilisin

The primary structures of Pro-Tk-subtilisin (Gly1–Gly398), Tk-subtilisin (Gly70–Gly398), Tkpro (Gly1–Leu69), and their derivatives are schematically shown in Fig. 4.3. When Pro-Tk-subtilisin is overproduced in *E. coli* under the T7 promoter, it accumulates in inclusion bodies. The protein is solubilized in the presence of 8 M urea, purified in a denatured form, and then refolded by removing the urea. The refolded protein yields an approximately 45 kDa protein band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4.4, 0 min) and remains inactive unless incubated in the presence of Ca²⁺ ions. When Pro-Tk-subtilisin (Gly1–Gly398, 45 kDa) is incubated at 80°C and pH 9.5 in the presence of 5 mM CaCl₂, it starts autoprocessing into Tk-subtilisin (Gly70–Gly398, 37 kDa) and Tkpro (Gly1–Leu69, 8 kDa) within 2 min, and then Tkpro is gradually degraded by Tk-subtilisin (Fig. 4.4). Tk-subtilisin activity increases as the amount of Tkpro decreases, indicating that Tkpro acts as an inhibitor, which forms an inactive complex with Tk-subtilisin upon autoprocessing. Therefore, Tk-subtilisin is fully activated by the eventual degradation of Tkpro. The autoprocessing of Pro-Tk-subtilisin does not occur in the absence of Ca²⁺ ions. This result suggests that Pro-Tk-subtilisin requires Ca²⁺ ions for correct folding. Surprisingly, Tk-subtilisin is correctly refolded into its native structure even in the absence of Tkpro, as long as sufficient Ca²⁺ ions are present, although the refolding yield is quite low compared to that in the presence of Tkpro (Pulido et al., 2006; Tanaka et al., 2008). Bacterial subtilisins, such as pro-subtilisin E, are refolded, autoprocessed, and then activated in the absence of Ca²⁺ ions, because they do not require Ca²⁺ ions for folding, only needing the Ca²⁺ ions for stability (Yabuta et al., 2002). When pro-subtilisin E

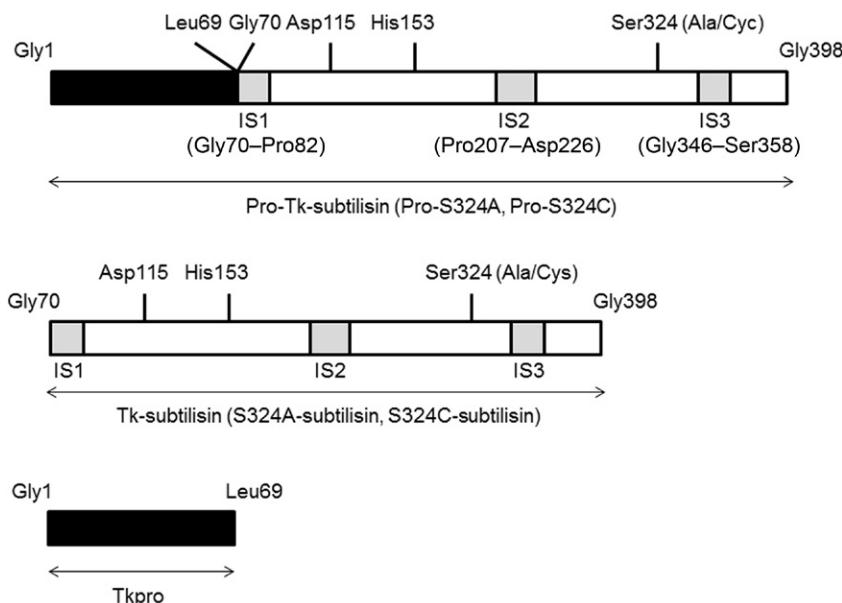


FIGURE 4.3 Schematic representation of the Pro-Tk-subtilisin, Tk-subtilisin, and Tkpro primary structures. The *dark box* represents the propeptide domain, the *open box* represents the subtilisin domain, and *gray boxes* represent the insertion sequences (IS1–IS3). The locations of the catalytic triad, Asp115, His153, and Ser324 (alanine in Pro-S324A and S324A-subtilisin, and cysteine in Pro-S324C and S324C-subtilisin), and the N- and C-terminal residues of each domain are shown. These proteins were separately produced in *E. coli*. Pro-Tk-subtilisin (Pro-S324A and Pro-S324C) and Tk-subtilisin (S324A-subtilisin) accumulate in inclusion bodies upon overproduction and are purified in a denatured form. S324C-subtilisin is obtained when Pro-S324C is autoprocessed into S324C-subtilisin and Tkpro in the presence Ca^{2+} ions. Tkpro is produced in a soluble form and purified.

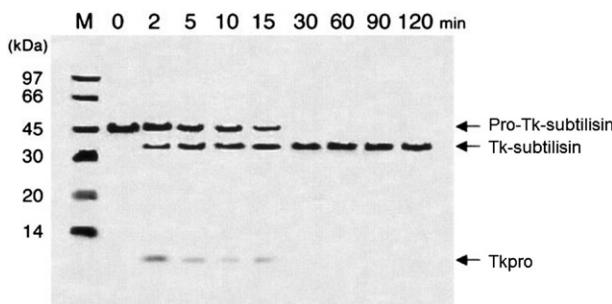


FIGURE 4.4 Autoprocessing and degradation of Tkpro at 80°C. Pro-Tk-subtilisin (300 nM) was incubated in 50 mM CAPS-NaOH (pH 9.5) containing 5 mM CaCl_2 at 80°C for the time indicated at the top of the gel. Upon incubation, all proteins were precipitated by addition of trichloroacetic acid (TCA), and subjected to 15% Tricine-SDS-PAGE. Arrows indicate Pro-Tk-subtilisin (45 kDa), Tk-subtilisin (38 kDa), and Tkpro (7 kDa), from top to bottom.

is overproduced in *E. coli*, it matures in the cells and exhibits serious toxicity (Li and Inouye, 1994). However, Pro-Tk-subtilisin does not fold in *E. coli* cells, probably owing to an insufficient Ca^{2+} concentration, and thereby accumulates in inclusion bodies. These results suggest that the maturation of Tk-subtilisin is initiated when its precursor is secreted into the Ca^{2+} -rich extracellular environment so as not to degrade cellular proteins.

The maturation rate of Pro-Tk-subtilisin greatly depends on temperature. It is fully mature within 30 min at 80°C and 3 h at 60°C, while maturation is not complete within 4 h at 40°C and below (Pulido et al., 2006). Pro-Tk-subtilisin is immediately autoprocessed, even at 20°C, in the presence of Ca^{2+} ions. Nevertheless, the subsequent degradation of Tkpro does not occur at this temperature. Circular dichroism (CD) spectroscopy shows that Tkpro is highly structured in an isolated form, unlike the propeptide of bacterial subtilisins, which are unstructured upon dissociation from the mature domain. Therefore, Tkpro is tolerant to proteolysis, and requires a high temperature for its degradation. In conclusion, Tk-subtilisin is matured from Pro-Tk-subtilisin by the same three steps, folding, autoprocessing, and degradation of Tkpro, as bacterial subtilisins are, but the maturation mechanism is unique in that it requires Ca^{2+} ions for folding, instead of its cognate propeptide (Tkpro), as well as a high temperature for the degradation of Tkpro.

4.3.2 Crystal Structures of Tk-Subtilisin

The crystal structures of Tk-subtilisin in three isoforms (unautoprocessed, autoprocessed, and mature) were determined using mutant enzymes, in which the catalytic serine (Ser324) was replaced with either alanine or cysteine (Tanaka et al., 2006, 2007a,b). Pro-Tk-subtilisin with the Ser324→Ala mutation, termed Pro-S324A, represents the unautoprocessed form of Tk-subtilisin. The Ser324→Ala mutation completely abolishes enzymatic activity, and therefore Pro-S324A is not autoprocessed upon folding. In contrast, the Ser324→Cys mutation greatly reduces activity such that Pro-S324C is autoprocessed into Tkpro and S324C-subtilisin upon folding, but further degradation of Tkpro does not occur. Pro-S324C forms a stable Tkpro:S324C-subtilisin complex after autoprocessing, and therefore represents the autoprocessed form. The mature form is prepared by the maturation of Pro-Tk-subtilisin in the presence of Ca^{2+} ions at 80°C. Mature Tk-subtilisin is inactivated by modifying the catalytic serine with a specific inhibitor, diisopropyl fluorophosphate, to prevent the autodegradation of Tk-subtilisin during crystallization processes. It is likely that this modification does not seriously affect the overall structure of Tk-subtilisin. Thus, the resultant monoisopropylphospho-Tk-subtilisin (MIP-Tk-subtilisin) represents the mature form of Tk-subtilisin. S324A-subtilisin, that is Tk-subtilisin with the Ser324→Ala mutation, was refolded in the presence of Ca^{2+} ions, and the structure was determined as another mature form (mature form 2). The preparation of these proteins is schematically described in Fig. 4.5A. These structures are

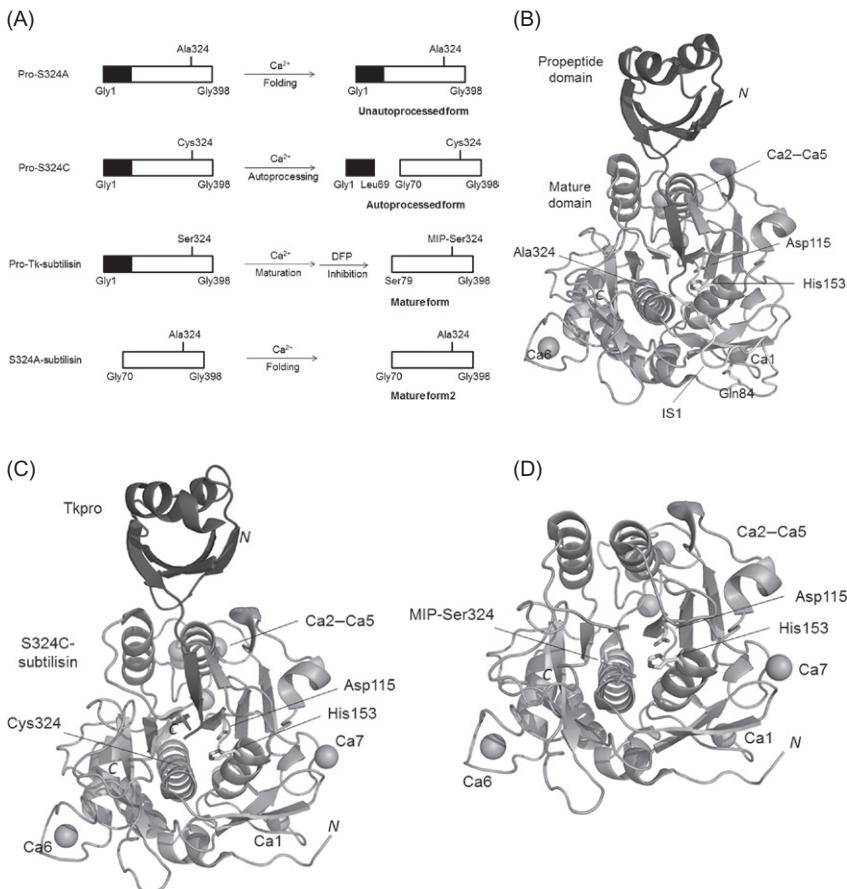
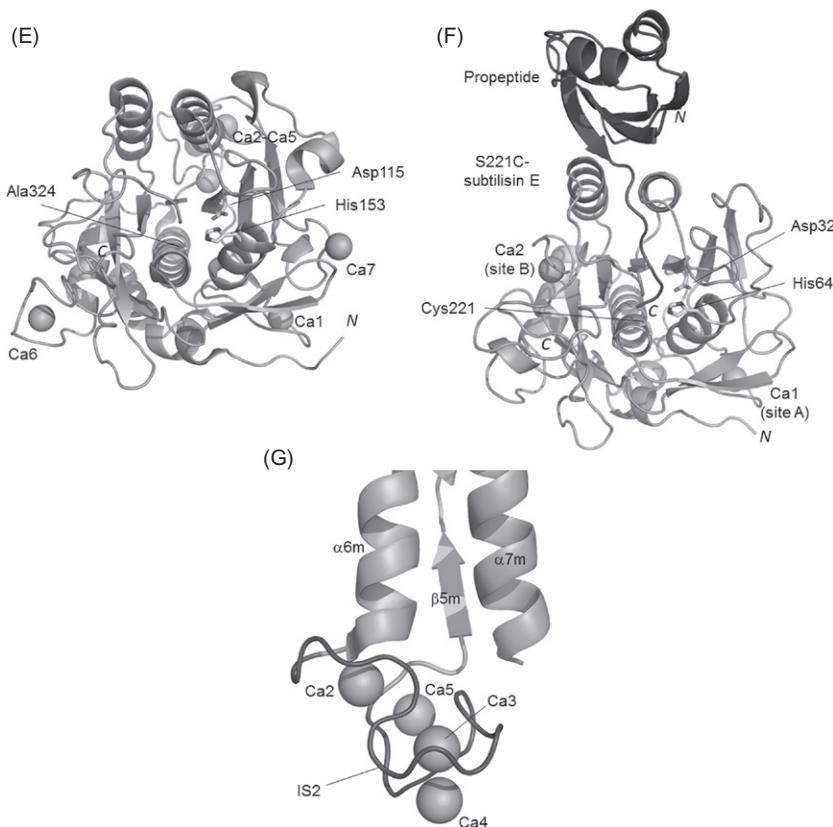


FIGURE 4.5 Structures. (A) Schematic representation of protein preparation. *Filled* and *open* boxes represent the propeptide and mature domains, respectively. MIP-Ser represents monoisopropylphospho-Ser. (B–G) Crystal structures of Tk-subtilisin and subtilisin E. The propeptide and subtilisin domains are colored *black* and *gray*, respectively. The catalytic triad, Asp115, His153, and Ser324 (or Ala324, Cys324, and MIP-Ser324) are indicated with stick models. The Ca^{2+} ions are displayed as gray spheres. N and C represent the N- and C-termini, respectively. (B) Structure of the unautoprocessed form (Pro-S324A). (C) Structure of the autoprocessed form (Pro-S324C). (D) Structure of the mature form (MIP-Tk-subtilisin). Tk-subtilisin is matured from Pro-Tk-subtilisin by incubation at 80°C in the presence of 10 mM CaCl_2 and inactivated with diisopropyl fluorophosphate. (E) Structure of S324A-subtilisin. S324A-subtilisin is refolded in the presence of Ca^{2+} ions and absence of Tkpro. Misfolded and unfolded proteins are removed by proteolysis. (F) Structure of autoprocessed pro-subtilisin E (pro-S221C-subtilisin E). (G) The Ca^{2+} binding loop of Tk-subtilisin IS2 is highlighted with *dark gray*.

**FIGURE 4.5** (Continued)

shown in Fig. 4.5B–E, compared with pro-S221C-subtilisin E, which represents the autoprocessed form of pro-subtilisin E (Fig. 4.5F).

The overall structure of autoprocessed Pro-S324C (Fig. 4.5C) highly resembles that of autoprocessed pro-S221C-subtilisin E (Fig. 4.5F), except that the mature domain contains seven, instead of two, Ca^{2+} ions, and three unique surface loops, which are formed by the insertion sequences (IS1, IS2, and IS3). The number of Ca^{2+} ions in Tk-subtilisin is the highest among those so far reported for various subtilases (Almog et al., 2003, 2009; Arnórsdóttir et al., 2005; Betzel et al., 1988; Bode et al., 1987; Bott et al., 1988; Gros et al., 1988; Helland et al., 2006; Henrich et al., 2003; Jain et al., 1998; Kim et al., 2004; McPhalen and James, 1988; Smith et al., 1999; Teplyakov et al., 1990; Vévodová et al., 2010). The Ca1 site is conserved in the members of the subtilisin, thermitase, and kexin family, while the other six, with a few exceptions for Ca6 (Almog et al., 2003, 2009), are unique in the structure of Tk-subtilisin. All Ca^{2+} binding sites are located on surface loops far from the active site. The Ca2–Ca5 sites

are localized in the unique Ca^{2+} binding loop, which is mostly formed by IS2 (Fig. 4.5G), whereas the Ca1, Ca6, and Ca7 sites are located on different surface loops.

The entire structure of the unautoprocessed form (Fig. 4.5B) and autoprocessed form (Fig. 4.5C) of Pro-Tk-subtilisin is similar, indicating that the autoprocessing reaction does not cause significant conformational changes. In contrast, the crystal structure of the unautoprocessed form of pro-subtilisin E has not yet been determined, because it is unstable owing to the absence of the Ca1 site (Yabuta et al., 2002). The Ca1 site of subtilisin E is formed only when the peptide bond between the propeptide and the mature domain is cleaved and the *N*-terminus of the mature domain leaves the active site, such that Gln2 at the *N*-terminus of the mature domain can directly coordinate the Ca^{2+} ion at the Ca1 site. The corresponding residue of Tk-subtilisin, Gln84, coordinates the Ca1 ion in the unautoprocessed form owing to the extension loop between the propeptide and the mature domain (Fig. 4.5B). Tk-subtilisin has a 13 amino acid residue insertion sequence (IS1, Gly70–Pro82) at the *N*-terminus. IS1 forms a long surface loop and allows Gln84 to directly coordinate the Ca1 ion in the unautoprocessed form. Hence, Tk-subtilisin nearly completes folding before autoprocessing. The structure of MIP-Tk-subtilisin is nearly identical to that of S324A-subtilisin (Fig. 4.5D,E). S324A-subtilisin is refolded in the presence of Ca^{2+} ions and the absence of Tkpro. This result also suggests that Tk-subtilisin is capable of folding into its native structure without the assistance of Tkpro.

The Ca7 ion is missing in the unautoprocessed form, whereas the other six Ca^{2+} ions are observed in all three forms. The missing Ca^{2+} ion is removed from the Ca7 site when the unautoprocessed form is dialyzed against a Ca^{2+} -free buffer. The Ca7 site is stable in the autoprocessed form and mature form such that the Ca^{2+} ion is bound to these structures even upon dialysis. The Ca7 ion is observed in the unautoprocessed form when the crystal is soaked with 10 mM CaCl_2 solution (Tanaka et al., 2007b). These results suggest that the Ca7 ion weakly binds to the unautoprocessed form, but this binding site is stabilized upon autoprocessing.

4.3.3 Requirement of Ca^{2+} -Binding Loop for Folding

The structure of Pro-Tk-subtilisin greatly changes upon binding to Ca^{2+} ions. Pro-Tk-subtilisin is folded into an inactive form with a molten globule-like structure, which is highly susceptible to proteolysis, in the absence of Ca^{2+} ions, whereas it is folded into its stable native structure in the presence of Ca^{2+} ions. Tk-subtilisin binds seven Ca^{2+} ions. Of those, four Ca^{2+} ions (Ca2–Ca5) continuously bind to a single surface loop, termed the Ca^{2+} binding loop, which mostly consists of Tk-subtilisin's unique insertion sequence (IS2, Pro207–Asp226). This Ca^{2+} binding loop contains a $\text{Dx}[DN]xDG$ motif, which is recognized as the Ca^{2+} binding motif in a variety of Ca^{2+} binding proteins (Rigden et al., 2011). Removal of the Ca^{2+} binding loop from Tk-subtilisin completely

abolishes its ability to fold into its native structure. The loop-deleted mutant (Δ loop-Pro-S324A) is trapped into a molten globule-like structure even in the presence of Ca^{2+} ions. Similarly, the mutation that removes either the Ca2 or Ca3 site substantially reduces the refolding rate of Pro-S324A. We kinetically analyzed the refolding of Pro-S324A and its mutants, $\Delta\text{Ca2-Pro-S324A}$ and $\Delta\text{Ca3-Pro-S324A}$, which lack the Ca2 and Ca3 ion, respectively, by initiating and terminating the refolding reaction by the addition of CaCl_2 and ethylenediaminetetraacetic acid (EDTA), respectively, with appropriate intervals, and determining the amount of the correctly folded protein by SDS-PAGE (Fig. 4.6) (Takeuchi et al., 2009). The rate constant for refolding the protein in the presence of 10 mM CaCl_2 at 30°C is 1.3 min⁻¹ for Pro-S324A, 0.020 min⁻¹ for $\Delta\text{Ca2-Pro-S324A}$, and 0.019 min⁻¹ for $\Delta\text{Ca3-Pro-S324A}$. Our crystallographic analysis revealed that the overall structures of $\Delta\text{Ca2-Pro-S324A}$ and $\Delta\text{Ca3-Pro-S324A}$ are nearly identical to that of Pro-S324A, except that these structures lack the Ca2 and Ca3 ion, respectively. Also, the thermal stability of these proteins was not reduced compared with that of Pro-S324A. These results suggest that the Ca2 and Ca3 ions are responsible for the Ca^{2+} -dependent folding of Tk-subtilisin, but not for the stability gained upon folding. Likewise, the mutation that removes the Ca4 or Ca5 site also greatly reduces the refolding rate of Pro-S324A without seriously affecting its thermal stability, indicating that the Ca4 and Ca5 ions also contribute to the folding of Tk-subtilisin, although the crystal structure of these mutants remains to be determined (Y. Takeuchi, unpublished data).

The Ca^{2+} binding loop is inserted between the sixth α -helix of the mature domain ($\alpha 6\text{m-helix}$), and the fifth β -strand of the mature domain ($\beta 5\text{m-strand}$), which together compose the central $\alpha\beta\alpha$ substructure along with the seventh α -helix of the mature domain ($\alpha 7\text{m-helix}$), as shown in Fig. 4.5G. This loop is rich in aspartate and probably unstructured in the absence of Ca^{2+} ions, because of extensive negative charge repulsions. The Ca^{2+} binding loop is only folded into its correct structure when Ca^{2+} ions bind to the loop, which permits a proper arrangement of the $\alpha\beta\alpha$ substructure. Bryan et al. (1992, 1995) proposed that the formation of the $\alpha\beta\alpha$ substructure is crucial for the folding of bacterial subtilisins. Subsequently, the Ca^{2+} binding loop is probably required to initiate the folding of Tk-subtilisin by stabilizing the $\alpha\beta\alpha$ substructure, and most likely acts as an intramolecular chaperone for Tk-subtilisin. These results suggest that Tk-subtilisin has two intramolecular chaperones, its unique Ca^{2+} binding loop and its cognate propeptide (Tkpro). The propeptides of extracellular proteases, such as bacterial subtilisins, assist folding by reducing the high kinetic barrier between unfolded and folded states, which ensures proteolytic stability and allows these proteases to function in harsh environments (Jaswal et al., 2002). The kinetic barrier of folding may be much higher for Tk-subtilisin than that of mesophilic proteases, because *T. kodakarensis* grows in an extremely hot environment. Hence, Tk-subtilisin might require not only the chaperone function of Tkpro, but also the folding of its Ca^{2+} binding loop to overcome this high kinetic barrier.

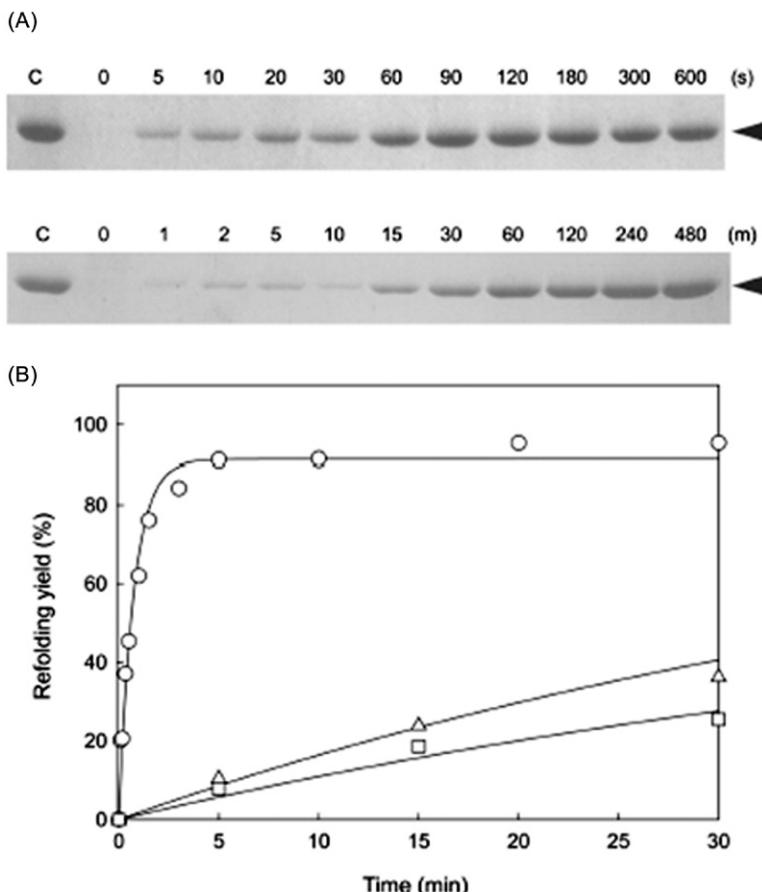


FIGURE 4.6 Refolding rates of Pro-S324A and its derivatives. (A) SDS-PAGE of refolded proteins. Pro-S324A (*top*) and Δ Ca2-Pro-S324A (*bottom*) in a Ca^{2+} - free form were denatured using 6 M GdnHCl, diluted 100-fold with 50 mM Tris-HCl (pH 8.0) containing 10 mM CaCl_2 and 1 mM DTT, and then incubated at 30°C for refolding. At appropriate intervals (shown at the top of the gel), incorrectly folded proteins were digested with chymotrypsin, and refolded proteins were analyzed with 12% SDS-PAGE. (B) Refolding curves. The refolding yields of Pro-S324A (*open circle*), Δ Ca2-Pro-S324A (*open triangle*), and Δ Ca3-Pro-S324A (*open square*) are shown as a function of incubation time. The refolding yields were calculated by estimating the amount of correctly refolded protein from the intensity of the band visualized with CBB staining following SDS-PAGE. Lines represent the optimal fit to the data.

4.3.4 Ca^{2+} Ion Requirements for Hyperstability

The other three Ca^{2+} ions (Ca1, Ca6, and Ca7) bind to the different surface loops, which are located far from one another. The Ca1 site is conserved among bacterial subtilisins and other subtilases. This site in bacterial subtilisin is essential

for protein stability (Bryan et al., 1992; Pantoliano et al., 1989; Voordouw et al., 1976). According to the crystal structure, the Ca1 ion is relatively buried inside the protein molecule, and therefore the dissociation of the Ca^{2+} ion from the Ca1 site is very slow (Bryan et al., 1992), resulting in a high affinity Ca^{2+} binding site. We constructed Pro-Tk-subtilisin and Pro-S324A derivatives lacking the Ca1, Ca6, and Ca7 ions by removing the binding loop or by mutating the aspartate that directly coordinates the Ca^{2+} ions, to examine the role of these three Ca^{2+} ions in Tk-subtilisin. These Pro-Tk-subtilisin derivatives ($\Delta\text{Ca1-Pro-Tk-subtilisin}$, $\Delta\text{Ca6-Pro-Tk-subtilisin}$, and $\Delta\text{Ca7-Pro-Tk-subtilisin}$) were used for maturation analysis, and the Pro-S324A derivatives ($\Delta\text{Ca1-Pro-S324A}$, $\Delta\text{Ca6-Pro-S324A}$, and $\Delta\text{Ca7-Pro-S324A}$) were used for analyses of stability and structure.

Differential scanning calorimetry analysis showed that the removal of the Ca1, Ca6, or Ca7 ions reduces Pro-S324A's thermal stability T_m value by 26.6°C, 11.7°C, or 4.0°C, respectively. The crystal structure of $\Delta\text{Ca6-Pro-S324A}$ revealed that the overall structure is nearly identical to that of Pro-S324A except that the surface loop containing the Ca6 site is disordered owing to the absence of the Ca^{2+} ion. Although the crystal structure of $\Delta\text{Ca1-Pro-S324A}$ and $\Delta\text{Ca7-Pro-S324A}$ was not determined, the secondary structure features of these mutants are indistinguishable from that of Pro-S324A in CD spectroscopy. Additionally, the refolding rate of these mutants was not reduced compared with Pro-S324A. These results suggest that the Ca1, Ca6, and Ca7 ions stabilize the structure of Tk-subtilisin, and especially that the Ca1 ion is critical for stability. The Pro-Tk-subtilisin derivatives lacking these Ca^{2+} ions are autoprocessed in the presence of Ca^{2+} ions, but the subsequent degradation of Tkpro does not occur for $\Delta\text{Ca1-Pro-Tk-subtilisin}$, while the other two mutants are effectively matured with rates comparable to that of Pro-Tk-subtilisin (Fig. 4.7). The active form of $\Delta\text{Ca1-Tk-subtilisin}$, which is obtained by refolding the mature domain alone in the presence of Ca^{2+} ions, exhibits approximately 50% of the activity of Tk-subtilisin at 60°C, and less than 5% at 80°C (Uehara et al., 2012a). The reduced activity of $\Delta\text{Ca1-Tk-subtilisin}$ is probably insufficient for the degradation of Tkpro. Hence, the Ca1 ion is most likely crucial for Tk-subtilisin activity at high temperature, and thereby required for maturation. $\Delta\text{Ca6-Tk-subtilisin}$ and $\Delta\text{Ca7-Tk-subtilisin}$ exhibit activity comparable with that of Tk-subtilisin, but the optimum temperature for proteolytic activity is decreased by 10°C. The Ca6 site is not conserved among bacterial subtilisins, but is found in several subtilases, such as a mesophilic subtilisin, sphericase (Sph) from *Bacillus sphaericus* (Almog et al., 2003), and psychrophilic subtilisin S41 from Antarctic *Bacillus TA41* (Almog, 2009). Because removal of the Ca6 ion increases the flexibility of the binding loop in the $\Delta\text{Ca6-Pro-S324A}$ structure, the Ca6 site may be important in protecting the surface loop from self-degradation. The Ca7 ion is observed in the autoprocessed form and the mature form, but missing in the unautoprocessed form. This autoprocessing, as described above, likely increases Ca^{2+} ion affinity at this site. Removal of the Ca7 ion destabilizes Pro-S324A by only 4°C,

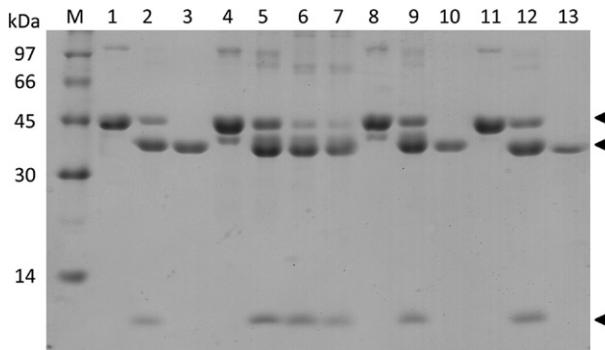


FIGURE 4.7 Maturation of Pro-Tk-subtilisin Ca^{2+} -deleted mutants. Pro-Tk-subtilisin (lanes 1–3), $\Delta\text{Ca}1$ -Pro-Tk-subtilisin (lanes 4–7), $\Delta\text{Ca}6$ -Pro-Tk-subtilisin (lanes 8–10), and $\Delta\text{Ca}7$ -Pro-Tk-subtilisin (lanes 11–13) (0.3 μM each) were incubated in 1 mL of 50 mM CAPS-NaOH (pH 9.5) containing 10 mM CaCl_2 and 1 mM DTT at 80°C for 2 min. Lanes 2, 5, 9, and 12 were incubated for 30 min; lanes 3, 6, 10, and 13 for 60 min; lane 7 was precipitated by the addition of 120 μL of TCA, and subjected to 12% Tricine-SDS-PAGE. Lanes 1, 4, 8, and 11 contained the sample without exposure to the buffer containing Ca^{2+} ions. Lane M contained the low-molecular-weight markers (GE Healthcare). Proteins were stained with CBB. The *arrowheads* indicate Pro-Tk-subtilisin or its derivative, Tk-subtilisin or its derivative, and Tkpro from top to bottom, respectively.

probably because it weakly binds to the unautoprocessed form. Destabilization by removal of the Ca7 ion appears to be more serious in the mature form than in the unautoprocessed form, such that the half-life of $\Delta\text{Ca}7$ -Tk-subtilisin at 95°C is decreased fourfold compared with that of Tk-subtilisin. Interestingly, the N-terminal region of the mature domain (Leu75–Thr80) is disordered when the Ca7 ion is binding to the unautoprocessed form. Gln81 changes the conformation so much that it can stabilize the Ca7 site upon autoprocessing. Therefore, it is tempting to speculate whether the Ca7 ion promotes the autoprocessing reaction by shifting the equilibrium between the unautoprocessed form and the autoprocessed form. However, $\Delta\text{Ca}7$ -Pro-Tk-subtilisin is autoprocessed at a similar rate as Pro-Tk-subtilisin (Uehara et al., 2012a). Thus, the Ca7 ion must contribute to stabilization of the autoprocessed form and the mature form but is likely not responsible for autoprocessing efficiency.

4.3.5 Role of Tkpro

Unlike bacterial subtilisins, Tkpro is not required for the folding of Tk-subtilisin. The crystal structure of the active site mutant of Tk-subtilisin (S324A-subtilisin), which is refolded in the presence of Ca^{2+} ions and the absence of Tkpro, is essentially the same as that of the mature form (Fig. 4.5E). In contrast, the refolding rate of S324A-subtilisin is greatly increased in the presence of Tkpro. Tkpro increases the refolding rate of S324A-subtilisin by more than 100-fold in the presence of 1 mM CaCl_2 , while it is increased 10-fold in the presence of

10 mM CaCl₂, suggesting that the chaperone function of Tkpro is dependent on Ca²⁺ ion concentration, and more predominant at lower concentrations (Tanaka et al., 2009). Hence, Tkpro probably acts as an intramolecular chaperone in an auxiliary manner.

Tkpro interacts with Tk-subtilisin predominantly at three regions, according to the crystal structure of the Tkpro and S324A-subtilisin complex (Tanaka et al., 2007b). These regions are: the C-terminal extended region (His64–Leu69), which binds to the active site cleft in a substrate-like manner; Glu61 and Asp63, which interact with the N-termini of two surface helices (α 6m and α 7m) of the $\alpha\beta\alpha$ substructure by hydrogen bonds; and the hydrophobic loop containing three hydrophobic residues (Phe33, Leu35, and Ile36) between the second and third β -strand of Tkpro (β 2p and β 3p strands), which interact with Glu201 by hydrogen bonds. Glu201 is located in the α 6m-helix. Furthermore, three homologous acidic amino acids are highly conserved among bacterial subtilisins and form similar hydrogen bonds. Either the truncation of the Tkpro C-terminal extended region, or the combination of this truncation plus the double mutations substituting Glu61 and Asp63 to alanine, reduces the chaperone function and binding ability of folded S324A-subtilisin, but does not abolish them. This suggests that the interactions at these two regions are not essential for the chaperone function of Tkpro. However, these interactions do seem to accelerate propeptide-catalyzed folding by promoting binding to a folding intermediate with a native-like structure. In contrast, the mutation at Glu201 almost fully abolishes the chaperone function of Tkpro, but nevertheless Tkpro retains significant binding ability to E201/S324A-subtilisin with a native structure. These results suggest that the hydrophobic loop of Tkpro, which interacts with Glu201 through hydrogen bonds, is required to initiate propeptide-catalyzed folding by promoting binding to a folding intermediate with a nonnative structure. Because the hydrophobic loop is tightly packed with two surface helices (α 6m and α 7m), it seems likely that Glu201-mediated interactions promote an association between the two helices and the subsequent formation of the central $\alpha\beta\alpha$ substructure. Hydrophobic effects with Tkpro may also further stabilize these structures. Therefore, Glu201-mediated interactions might be the first folding step promoted by Tkpro (Tanaka et al., 2009). The glutamate residue corresponding to Glu201 of Tk-subtilisin is well conserved in members of the subtilisin and proteinase K families, although it is occasionally replaced by glutamine, aspartate, or asparagine. Likewise, the hydrophobic loop and two acidic residues (Glu61 and Asp63) are located in the conserved motif of the propeptide (N2 motif). These results suggest that the folding of these subtilases is catalyzed by their cognate propeptides through similar mechanisms. In fact, the propeptide of aqualysin, a member of the proteinase K family, can function as a chaperone for subtilisin (Marie-Claire et al., 2001). Hence, the interactions required for the propeptide-catalyzed folding of Tk-subtilisin might be shared with all members of the subtilisin and proteinase K families.

Tkpro remains bound to the mature domain after autoprocessing and forms an inactive complex. The C-terminal four residues of Tkpro (Ala66–Leu69) are located in the Tk-subtilisin substrate binding pockets (S1–S4 subsites), according to the crystal structure. Therefore, Tkpro competitively inhibits Tk-subtilisin activity when added *in trans* (Pulido et al., 2006, 2007a,b; Uehara et al., 2013a). Our inhibition progress curves of mature Tk-subtilisin by Tkpro revealed a hyperbolic pattern, indicating that Tkpro acts as a slow binding inhibitor, similar to the propeptides of bacterial subtilisins. The inhibition potency of Tkpro is higher than that of bacterial propeptides, such that the concentration of propeptide required for complete inhibition is ~50 nM for Tkpro and 0.5–5 μM for bacterial propeptides at low or middle temperatures. Tkpro is well structured even in an isolated form, while bacterial propeptides are intrinsically unstructured proteins (Hu et al., 1996; Huang et al., 1997; Wang et al., 1995, 1998). Because of its defined structure, Tkpro is effectively degraded by Tk-subtilisin only at high temperatures ($\geq 60^{\circ}\text{C}$), where the stability and binding affinity of Tkpro decrease, and the activity of Tk-subtilisin simultaneously increases. Tk-subtilisin can be refolded in the absence of Tkpro. However, the yield of mature Tk-subtilisin when refolding Tk-subtilisin in the presence of Ca^{2+} ions and the absence of Tkpro is considerably lower than with Tkpro ($\leq 5\%$), because the mature Tk-subtilisin molecules that refold earlier attack the other molecules destined to refold later. Hence, Tkpro is required not only for promoting refolding, but also for increasing the yield of mature Tk-subtilisin by protecting the precursor from autodegradation.

4.3.6 Role of the Insertion Sequences

Tk-subtilisin has three insertion sequences (IS1, IS2, and IS3). All three form long surface loops on the structure. IS2 contains four Ca^{2+} binding sites, and comprises the unique Ca^{2+} binding loop required for folding. In contrast, the other two loops do not bind Ca^{2+} ions. Our crystal structure of the unautoprocessed form revealed that IS1 forms an N-terminal extension loop and allows Gln84 to directly coordinate the Ca1 ion before autoprocessing. This loop is nearly disordered in the autoprocessed form, and most of it (Gly70–Gly78) is cleaved away in the mature form (Tanaka et al., 2007b). This suggests that IS1 specifically stabilizes the structure of the unautoprocessed form by promoting the formation of the Ca1 site. We constructed IS1-deleted mutants (ΔIS1 -Pro-Tk-subtilisin, ΔIS1 -Pro-S324A, and ΔIS1 -Pro-S324C), and characterized the resultant proteins. As described previously for Pro-S324A and Pro-S324C, ΔIS1 -Pro-S324A and ΔIS1 -Pro-S324C represent the unautoprocessed form and autoprocessed form of ΔIS1 -Pro-Tk-subtilisin, respectively. Thermal denaturation analyses showed that ΔIS1 -Pro-S324A is less stable than ΔIS1 -Pro-S324C by a 26.3°C lower T_m value, whereas the thermal stability of Pro-S324A is comparable with that of Pro-S324C (Uehara et al., 2012b). 8-Anilinonaphthalene-1-sulfonic acid

fluorescence spectra demonstrated that the Δ IS1-Pro-S324A structure is not fully folded, and that the interior hydrophobic region is partially exposed. These results suggest that the covalent bond between Leu69 and Ala83 in Δ IS1-Pro-S324A causes a strain, which disrupts the structure of the N-terminal region of the mature domain. Gln84 cannot coordinate Ca^{2+} ions in this structure, because of the large distance between its active site and the Ca1 site. The N-terminus is released from steric strain by autoprocessing, and moves to its original position where Gln84 can coordinate the Ca1 ion. Hence, IS1 is required for the formation of the Ca1 site prior to autoprocessing.

Δ IS1-Pro-Tk-subtilisin is matured without decreasing the yield of mature protein, but the autoprocessing rate is significantly less than that of Pro-Tk-subtilisin. The structure around the scissile peptide bond between Tkpro and the mature domain may change owing to strain caused by the connection between Ala83 and Leu69 in the Δ IS1-Pro-Tk-subtilisin unautoprocessed form. This structural change may be responsible for the slow autoprocessing and maturation of Δ IS1-Pro-Tk-subtilisin. We note that the source organism, *T. kodakarensis*, optimally grows at 85°C (Atomi et al., 2004), yet Pro-Tk-subtilisin might not fold without IS1 at this temperature because of the instability of the unautoprocessed form. In fact, Δ IS1-Pro-Tk-subtilisin cannot mature at 80°C. Hence, IS1 is required for the maturation of Pro-Tk-subtilisin in its native environment, because it not only stabilizes the structure of the unautoprocessed form but also promotes maturation.

Bacterial subtilisins also have the Ca1 site. The crystal structure of the unautoprocessed form has not yet been determined because of instability. We constructed a stable unautoprocessed form of pro-subtilisin E by inserting IS1 between the propeptide and the mature domain (IS1-pro-S221A-subtilisin E) and determined its crystal structure (Uehara et al., 2013b). In this structure, IS1 forms a surface loop and the Ca1 ion is bound to the protein. This result suggests that the N-terminal extension sequence stabilizes the unautoprocessed form of subtilisins containing a Ca1 site by supporting the formation of the Ca1 site. As described above, IS1 increases the autoprocessing rate of Pro-Tk-subtilisin. Likewise, IS1-pro-S221C-subtilisin E is autoprocessed more rapidly than pro-S221C-subtilisin E. When we overproduce pelB-pro-subtilisin E (a pelB leader attached to the pro-subtilisin E N-terminus) in *E. coli*, it accumulates in inclusion bodies, whereas pelB-IS1-pro-subtilisin E does not. IS1-pro-subtilisin E may rapidly fold and mature in the cells. Li et al. (1994) has reported that subtilisin E activity is cytotoxic to host cells. The rapid activation of IS1-pro-subtilisin E may cause serious damage to the cells. This may be the reason why pro-subtilisin E and its homologs do not have insertion sequences corresponding to IS1. In contrast, Pro-Tk-subtilisin requires IS1 to mature at the high temperatures where the source organism grows. The maturation of Pro-Tk-subtilisin is regulated by Ca^{2+} ions and is initiated when Pro-Tk-subtilisin is secreted into the external medium where Ca^{2+} ions are abundant. Therefore, the stabilization of the unautoprocessed form of Pro-Tk-subtilisin by IS1 may not cause serious damage to *T. kodakarensis* cells.

[Henrich et al. \(2005\)](#) has proposed, on the basis of structural models, that the unautoprocessed form of pro-furin, which has a short six residue insertion sequence between its propeptide and mature domain, assumes an incompletely folded structure, in which the Ca1 site is not formed. Meanwhile, that of other pro-protein convertases (pro-PCs) with a long 12 residues insertion sequence assumes a fully folded structure, in which the Ca1 site is formed. These results suggest that the maturation rates of the pro-PCs are controlled by the presence or absence of a long insertion sequence between the propeptide and mature domain.

The role of IS3 (Gly346–Ser358) remains unclear. IS3 is not responsible for binding any Ca^{2+} ions. However, this insertion sequence seems likely to be important for the architecture of the mature domain. The IS3-deleted mutant (ΔIS3 -Pro-Tk-subtilisin) is not autoprocessed, but the structure is significantly changed in the presence of Ca^{2+} ions (R. Uehara, unpublished data). This result suggests that ΔIS3 -Pro-Tk-subtilisin folding is initiated by the binding of Ca^{2+} ions, but not completed. Furthermore, L349A-Pro-Tk-subtilisin and D356A-Pro-Tk-subtilisin, in which Leu349 and Asp356, conserved among subtilases from hyperthermophilic archaeon, are replaced with alanine, respectively, exhibit maturation deficiencies at 80°C. The crystal structure of these active site mutants (Pro-S324A/L349A and Pro-S324A/D356A, PDB code: 3WIU and 3WIV) are nearly identical to that of Pro-S324A, suggesting that these residues in IS3 are not important for the native structure, but are important for folding.

Database searches indicate that, in addition to Tk-subtilisin, only subtilases from hyperthermophilic archaea, such as *Thermococcus onnurineus* NA1 (NCBI YP_002308296), *Pyrococcus* sp. NA2 (NCBI YP_004424756), *Ferroglobus placidus* DSM 10642 (NCBI YP_003436500), *Pyrolobus fumarii* 1A (NCBI YP_004781243), and *Aeropyrum pernix* K1 (NCBI NP_147093), contain these insertion sequences. Among these hyperthermostable proteases, the IS2 and IS3 sequences are highly conserved, while IS1 sequence similarities are poor. This may be because IS1 functions as an extension loop without specific interactions with other residues. Although the insertion sequences of the other subtilases have not been characterized, [Catara et al. \(2003\)](#) reported that the enzymatic activity of *A. pernix* K1 subtilase (pernisine) is greatly reduced by EDTA or ethylene glycol tetraacetic acid treatment. Thus, Ca^{2+} -dependent folding may be a common feature among these hyperthermophilic subtilisins. Our studies on the Tk-subtilisin insertion sequences suggest that the acquisition of these insertion sequences is a strategy that hyperthermophilic subtilases have evolved to adapt to the extremely high temperature environment in which they function.

4.3.7 Cold-Adapted Maturation Through Tkpro Engineering

Tk-subtilisin is effectively matured only at temperatures $\geq 80^\circ\text{C}$, because Tkpro forms a stable, inactive complex with Tk-subtilisin and is barely degraded at mild temperatures. *E. coli* HB101 transformed by a plasmid containing the entire

Prepro-Tk-subtilisin gene do not form halos, that is, clear zones around colonies, at $\leq 70^{\circ}\text{C}$, but do form halos at 80°C on casein-LB-plates. We screened for cold-adapted Pro-Tk-subtilisin mutants, which exhibited the halo-forming activity at $\leq 70^{\circ}\text{C}$, upon random mutagenesis in the propeptide region (Ala1–Leu69). A single Gly56→Ser mutation was identified that greatly accelerated the maturation of Pro-Tk-subtilisin, using this method. The mutant protein (G56S-Pro-Tk-subtilisin) matured within 1 h at 60°C without reducing the mature form yield, whereas Pro-Tk-subtilisin does not complete maturation within 3 h at this temperature. Tkpro containing the G56S mutation (G56S-Tkpro) was overproduced, purified, and characterized, to examine why this mutation accelerates the maturation of Pro-Tk-subtilisin. G56S-Tkpro is unstructured in an isolated form, and its stability and inhibitory potency are greatly decreased. In contrast, G56S-Tkpro retains the ability to bind Tk-subtilisin and is folded into a compact structure in the G56S-Tkpro:S324A-subtilisin complex crystal structure, even though the hydrophobic core of G56S-Tkpro is partially exposed (Pulido et al., 2007b). Gly56 is in the hydrophobic core of Tkpro and assumes a right-handed conformation. When serine is substituted into this position, it assumes a left-handed conformation and leads to a conformational change of the other hydrophobic residues in the core region. The relationship between the stability and the inhibitory potency of the propeptide has been reported for bacterial subtilisins by several researchers (Huang et al., 1997; Kojima et al., 2001; Wang et al., 1995, 1998). These propeptides are almost fully disordered in their isolated form, but adopt significant secondary structure in the presence of 10% glycerol. The association constant of the interaction between subtilisin E and its propeptide is increased 10-fold in the presence of 10% glycerol, indicating that the propeptide folded in an isolated form is a more potent inhibitor of subtilisins. Pro-Tk-subtilisin presumably matures at temperatures $\geq 80^{\circ}\text{C}$ in its native environment, and the independent folding of Tkpro is probably required to strongly bind Tk-subtilisin at high temperatures. We also introduced a Phe17→His mutation in Tkpro to examine whether a nonpolar-to-polar amino acid substitution at a different position in the hydrophobic core would accelerate the maturation of Pro-Tk-subtilisin in a similar manner. The maturation rate of the Pro-Tk-subtilisin Phe17→His mutant (F17H-Pro-Tk-subtilisin) greatly increased compared with that of Pro-Tk-subtilisin, such that F17H-Pro-Tk-subtilisin effectively matures even at temperatures as low as 40°C . This mutation nearly abolishes Tkpro secondary structure in its isolated form, and increases its sensitivity to chymotryptic digestion (Yuzaki et al., 2013). These results suggest that destabilization of the Tkpro hydrophobic core disrupts the structure in its isolated form, and thereby increases the maturation rate of Pro-Tk-subtilisin owing to a rapid degradation of Tkpro. In contrast, these mutations do not seriously affect the refolding rate of Pro-Tk-subtilisin, nor the yield of mature Tk-subtilisin. Tkpro is covalently linked to the mature domain until it is auto-processed. Hence, the mutant propeptides might be folded by interactions with Tk-subtilisin and become fully functional when they act as an intramolecular

chaperone. In fact, F17H-Tkpro and G56S-Tkpro are mostly folded in the crystal structures of Pro-F17H/S324A and G56S-Tkpro:S324A-subtilisin complex, respectively. We propose that destabilization of the Tkpro hydrophobic core by a single mutation is an effective way to promote the degradation of Tkpro, the rate-determining step of maturation, and thereby accelerate the maturation of Pro-Tk-subtilisin without seriously affecting the yield of mature protein.

The third mutation for cold-adapted maturation was found in the Tkpro C-terminus. This Leu69→Pro mutation increases the Pro-Tk-subtilisin maturation rate as much as the G56S mutation does (Fig. 4.8). Unlike the other two mutants, L69P-Tkpro is fully folded in its isolated form and retains stability to proteolysis by other proteases even at high temperatures. Nevertheless, L69P-Tkpro is rapidly degraded by Tk-subtilisin. A kinetic binding analysis showed that Tkpro and L69P-Tkpro bind to Tk-subtilisin at similar rates, but that L69P-Tkpro dissociates faster than Tkpro. In addition, the inhibitory potency of L69P-Tkpro is reduced, especially at high temperatures, although L69P-Tkpro retains its secondary structure at these temperatures. These results suggest that the L69P mutation accelerates maturation by reducing binding ability to Tk-subtilisin. The Tkpro C-terminal extended region binds to the active site cleft of Tk-subtilisin, and Ala66–Leu69 are located in the Tk-subtilisin substrate binding pockets (S1–S4 subsites). The crystal structure of the L69P-Tkpro:S324A-subtilisin complex revealed that the conformation of the L69P-Tkpro C-terminal region is shifted away from the substrate binding pockets compared with that of Tkpro (Uehara et al., 2013a). This conformational change is probably caused by the restricted conformation of the C-terminal proline residue (Pro69). The proline cyclic side chain limits the backbone dihedral φ angle to a small range, and therefore proline acts as a structural disruptor in secondary structure elements. The extended Tkpro C-terminal region assumes a β -strand

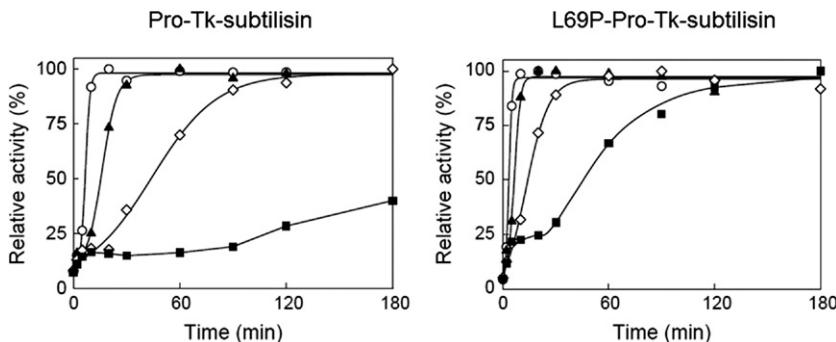


FIGURE 4.8 Accelerated maturation of L69P-Pro-Tk-subtilisin. Pro-Tk-subtilisin and L69P-Pro-Tk-subtilisin were incubated at 50°C (filled square), 60°C (open diamond), 70°C (filled triangle), and 80°C (open circle) for maturation. After incubation at these temperatures, an aliquot was withdrawn and enzymatic activity was determined at 20°C using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as a substrate.

conformation, which forms an antiparallel β -sheet with another β -strand of the mature domain. The hydrogen bonds formed between the C-terminal region and the mature domain are mostly disrupted in the structure of L69P-Tkpro:S324A-subtilisin, indicating that the C-terminal proline reduces interactions between Tkpro and Tk-subtilisin. This mutation does not seriously affect the chaperone function of Tkpro, because the Glu201-mediated interactions, which are essential for the chaperone function of Tkpro, are completely preserved in this structure. We emphasize, however, that the accelerated maturation mechanism of the L69P mutation is different than those of the F17H and G56S mutations. This mutation does not affect structure and stability but does reduce binding ability. Therefore, introducing mutations in the Tkpro C-terminal region may be effective for altering inhibitory potency without affecting stability and chaperone function, and thereby modulating Pro-Tk-subtilisin's maturation rate.

4.3.8 Degradation of PrP^{Sc} by Tk-Subtilisin

Prion diseases are fatal neurodegenerative disorders that include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome, fatal familial insomnia, and kuru in humans, bovine spongiform encephalopathy in cattle, and scrapie in sheep (Prusiner, 1998; Wissmann, 2004). These diseases are associated with an abnormal isoform of the prion protein rich with β -sheets (PrP^{Sc}), which is converted from the normal form containing an α -helix rich conformation (PrP^C). The pathogenic PrP^{Sc} propagates by promoting the conversion of cellular PrP^C to the abnormal form by some unknown mechanism. Therefore, decontamination of PrP^{Sc} attached to medical instruments is essential to prevent secondary infection. However, PrP^{Sc} is highly resistant to standard medical instrument sterilization and decontamination methods. The World Health Organization infection control guidelines recommend autoclaving or strong chemical treatment using a high concentration of sodium hydroxide or sodium hypochlorite to completely inactivate PrP^{Sc} contaminated reusable instruments. Although effective for removing infectivity, these procedures are not applicable to various delicate medical devices. Hence, PrP^{Sc} decontamination methods of sufficient potency and safety have been eagerly anticipated.

Tk-subtilisin is highly active and stable at wide temperature and pH ranges. It exhibits its highest activity toward protein substrates at 90°C and a mildly alkaline pH. In contrast, subtilisin E exhibits its highest activity at 60°C. The maximum specific activity of Tk-subtilisin is approximately sevenfold higher than that of subtilisin E (Pulido et al., 2006). Furthermore, Tk-subtilisin retains almost 100% activity even after 1 h of incubation in the presence of 5% SDS, 8 M urea, and 6 M guanidine hydrochloride (GdnHCl), whereas most commercial proteases are immediately denatured under these conditions. Because of its hyperstability and high activity, Tk-subtilisin is expected to degrade proteolysis-resistant proteins such as PrP^{Sc} under severe physical and chemical conditions where most other proteases would be denatured. We tested whether

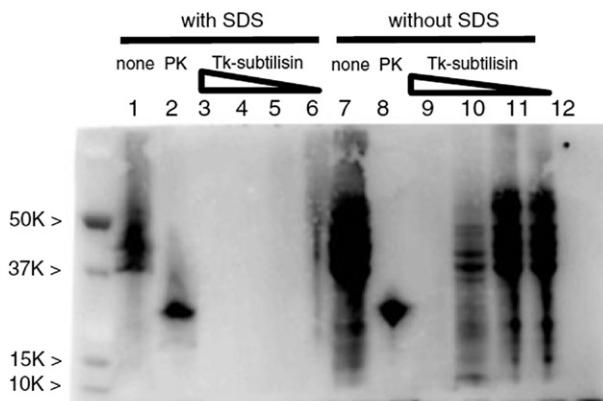


FIGURE 4.9 Proteolysis of PrP^{Sc} in the brain homogenate of a sporadic CJD patient. A sporadic CJD brain homogenate was digested with proteinase K (PK) and Tk-subtilisin in the presence and absence of SDS. Lane 1 and 7 contained no enzyme. Lanes 2 and 8 contained 14 µg (16.7 units) of proteinase K. Lanes 3–6 and 9–12 contained 9100, 910, 91, and 9.1 µU Tk-subtilisin, respectively.

Tk-subtilisin can be applied to PrP^{Sc} decontamination. Brain homogenates of terminally diseased mice infected with the Candler scrapie strain were treated with Tk-subtilisin at various concentrations. Western blot analysis of the proteolysis products showed that Tk-subtilisin is capable of degrading PrP^{Sc} to an undetectable level (Koga et al., 2014). Tk-subtilisin's PrP^{Sc} decontamination activity is much higher than that of proteinase K, a versatile commercial protease; 90 mU of Tk-subtilisin degrades PrP^{Sc} in brain homogenates from sporadic CJD patients more effectively than 16,700 mU of Proteinase K (Fig. 4.9). Tk-subtilisin activity is further increased by the presence of SDS and other industrial surfactants. These results suggest that Tk-subtilisin should be a powerful tool for reducing prion infectivity, and could be applied in many different conditions. Bacterial subtilisins have been used as detergent additives, which improve washing ability. Tk-subtilisin should be useful not only for standard detergents, but also for special medical detergents used for PrP^{Sc} decontamination.

4.3.9 Tk-Subtilisin Pulse Proteolysis Experiments

Determining protein stability and folding kinetics are essential procedures for studying protein structure and energetics. Optical spectroscopy, such as CD and fluorescence, has been conventionally used to characterize the chemical- and thermal-unfolding/refolding properties of proteins, to evaluate ΔG values of stability, and to determine k_f and k_u values for folding reactions. The pulse proteolysis method has recently been developed for taking thermodynamic measurements of protein stability/folding, and for the determination of folding/unfolding pathways without biophysical instrumentation (Okada et al.,

2012; Park and Marqsee 2005). Pulse proteolysis is designed to digest only the unfolded regions of a mixture of folded and unfolded proteins using a protease in a very short incubation time. After proteolysis, the fraction of folded protein versus digested product is determined by SDS-PAGE. The method is simple and can be applied to high-throughput systems or for the measurement of an unpurified protein's stability/folding. However, chemically and thermally stable proteases are required, because the proteolysis reactions are often performed in conditions that would unfold most proteins.

Tk-subtilisin retains high stability and activity in high temperatures as well as in the presence of chemical denaturants. We examined the unfolding pathway of ribonuclease H2 from *T. kodakarensis* (Tk-RNase H2) using pulse proteolysis with Tk-subtilisin (Okada et al., 2012). Tk-subtilisin retains activity in highly concentrated GdnHCl; Tk-RNase H2 unfolds in this situation. The Tk-subtilisin pulse proteolysis degradation products of Tk-RNase H2 during its unfolding were detected with tricine-SDS-PAGE (Fig. 4.10). The intact Tk-RNase H2 band, and several cleavage products were observed. The intact band was reduced by more than half, and the presence of bands representing 20 and 22 kDa fragments developed at early time points. However, the amount of intact protein, and of the 20 and 22 kDa fragments, was less at this early incubation time of 0.5 min, than it was at intermediate incubation times of 2–16 min. These bands gradually disappeared, and then a 9 kDa light chain fragment appeared (fragment 9) over time. At 120 min, only the faint bands of the intact protein and of fragment 9 were observed. Pulse proteolysis using Tk-subtilisin

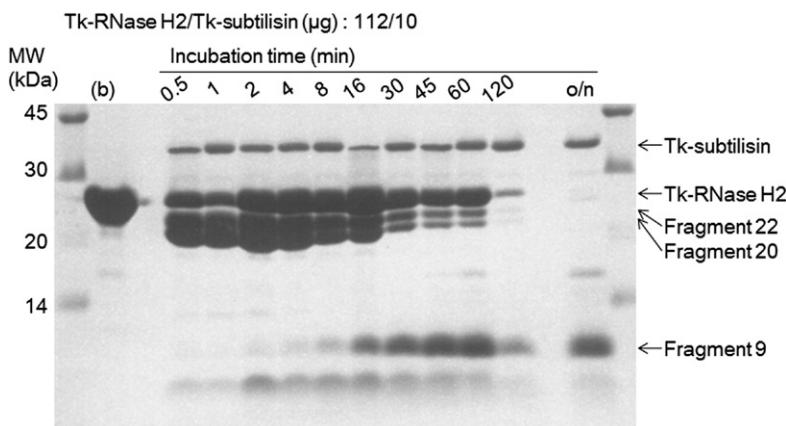


FIGURE 4.10 Tk-subtilisin pulse proteolysis in the kinetic unfolding of Tk-RNase H2. Lane b represents Tk-RNase H2 (112 µg). Tk-RNase H2 was unfolded by adding 4 M GdnHCl. At each time point (0.5–120 min and overnight), the sample was dispensed into tubes, and proteolysis was performed by adding Tk-subtilisin (10 µg) and incubating for 45 s. Proteolysis was quenched by adding 10% TCA, and the products were quantified using tricine-SDS-PAGE. Bands corresponding to Tk-subtilisin, Tk-RNase H2, and the cleavage products are indicated.

enabled us to detect the unfolding intermediates of a hyperthermophilic protein. This is exceptional. The intermediates were further examined and identified by N-terminal sequencing, mass spectrometry, and protein engineering. We found that the Tk-RNase H2 native state (N-state) changes to an I_A-state, which is digested by Tk-subtilisin in the early stage of unfolding. The I_A-state shifts to two intermediate forms, the I_B-state and I_C-state, and the I_B-state is digested by Tk-subtilisin in the C-terminal region, but the I_C-state is a Tk-subtilisin-resistant form. These states gradually unfold through the I_D-state. These results show that pulse proteolysis by the super-stable protease, Tk-subtilisin, is a suitable strategy and an effective tool for analyzing the intermediate structure of proteins.

4.4 TK-SP

4.4.1 Maturation of Pro-Tk-SP

Pro-Tk-SP is a subtilisin-like serine protease coded in the gene Tk-1689 (NCBI YP_184102), of *T. kodakarensis*. The primary structures of Pro-Tk-SP (Ala1–Gly640) and its derivatives are schematically shown in Fig. 4.11. Asp147, His180, and Ser359 form the catalytic triad. When Pro-Tk-SP (68 kDa) is overproduced in *E. coli*, 55 and 44 kDa proteins accumulate and show proteolytic activity in zymographic analysis, although the production levels of these proteins are too low to be detected by Coomassie brilliant blue (CBB) staining of the gel following SDS-PAGE (Fig. 4.12). In contrast, the active site mutant

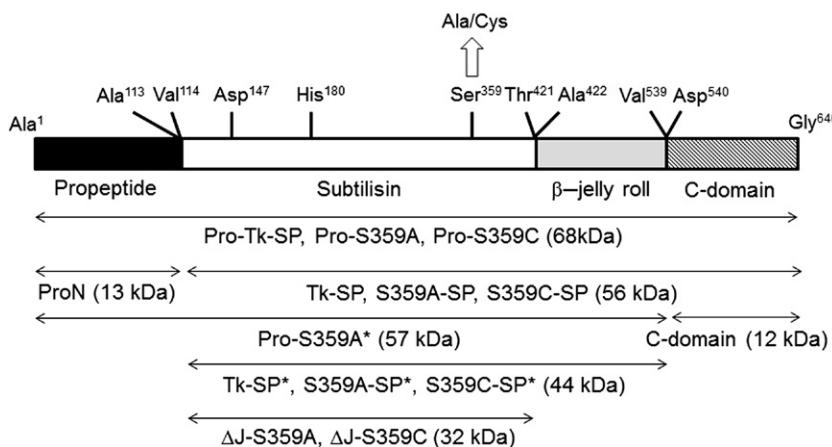


FIGURE 4.11 Schematic representation of the primary structure of Pro-Tk-SP and its derivatives. The dark box represents the propeptide domain (proN); the open box represents the subtilisin domain; the gray box represents the β-jelly roll domain; and the hatched box represents the C-domain. The locations of the three active site residues, Asp147, His180, and Ser359 (alanine and cysteine for the active site mutants), and the N- and C-terminal residues of each domain are shown. Each region and the terminology associated with the recombinant proteins are shown with their molecular masses.

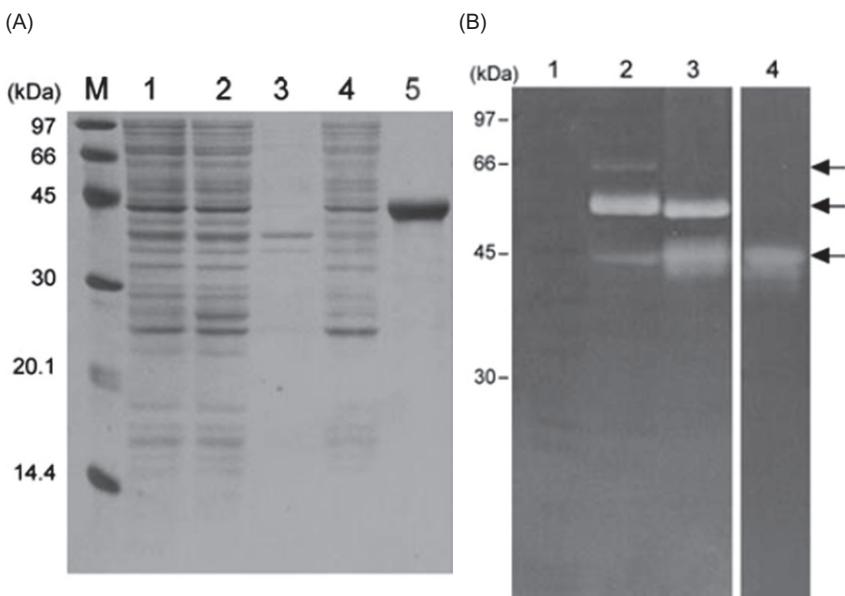


FIGURE 4.12 Overproduction and purification of Pro-Tk-SP. (A) *E. coli* BL21-Codonplus (DE3)-RIL cells transformed by a pET25b derivative designed for the overproduction of Pro-Tk-SP were subjected to 15% SDS-PAGE. The gel was stained with CBB following electrophoresis. Lane M: low-molecular-weight marker; lane 1: whole cell extract without IPTG induction; lane 2: whole cell extract with IPTG induction (1 mM); lane 3: insoluble fraction after sonication lysis of the cells; lane 4: soluble fractions after sonication; lane 5: purified Tk-SP protein (4 µg). (B) Activity staining of gel. The gel contained 0.1% gelatin and SDS. After electrophoresis, the gel was washed with 2.5% (v/v) Triton X-100, incubated in 50 mM Tris-HCl (pH 9.0) at 80°C for 2 h and stained with CBB. Lanes 1, 2, and 3 are the same samples as those loaded onto lanes 1, 2, and 4 in panel A, respectively; lane 4 is the purified Tk-SP protein (1 µg). The arrows indicate the position of the 65, 55, and 44 kDa proteins from top to bottom.

Pro-S359A, in which the catalytic serine (Ser359) is replaced with alanine, accumulates in a soluble form as a 65 kDa protein. These results suggest that Pro-Tk-SP, like bacterial subtilisins, start maturation in *E. coli* cells, and that its proteolytic activity prevents further protein production owing to serious cytotoxicity. The 55 and 44 kDa proteins cannot be separated using normal purification procedures, such as column chromatography. However, the 44 kDa protein fraction gradually increases during purification and eventually becomes the only protein fraction, as the 55 kDa fraction decreases, upon heat treatment at 80°C. Therefore, Pro-Tk-SP (68 kDa) must be autoprocessed into the 44 kDa protein through the 55 kDa protein, and this autoprocessing reaction is promoted at high temperatures.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry revealed that the 44 kDa protein is a Pro-Tk-SP derivative (Val114–Val539) lacking the N-terminal (Ala1–Ala113) and C-terminal (Asp540–Gly640)

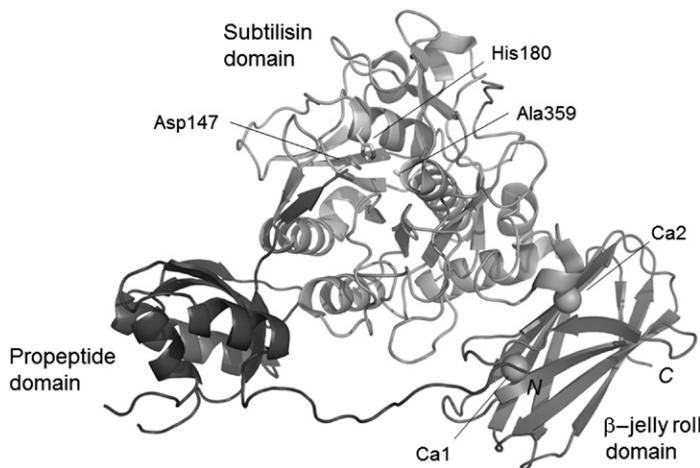


FIGURE 4.13 Crystal structure of Pro-S359A*. The propeptide, subtilisin, and β -jelly roll domain are colored black, gray, and dark gray, respectively. The two active site residues and Ala359, which was substituted for the catalytic serine residue, are indicated with stick models. Two Ca^{2+} ions (Ca1 and Ca2) are shown as gray spheres. N and C represent the N- and C-terminus, respectively.

regions (Foophow et al., 2010a). The 55 kDa protein was identified to be the intermediate (Val114–Gly640) lacking the N-terminal region (Ala1–Ala113). These results suggest that Pro-Tk-SP is matured through the autoprocessing of both the N-terminal and C-terminal domains, and that the first autoprocessing event occurs at the N-termini. The 55 kDa protein, 44 kDa protein, N-terminal domain, and C-terminal domain are designated the Tk-SP, Tk-SP*, ProN, and C-domains, respectively, as shown in Fig. 4.11. Because Tk-SP is correctly refolded into its native structure in the absence of Ca^{2+} ions and it exhibits significant activity in gel assays, Tk-SP requires neither propeptide nor Ca^{2+} ions for folding.

4.4.2 Crystal Structure of Pro-S359A*

We successfully determined the crystal structure of Pro-S359A* at 2.0 Å resolution. The structure consists of the propeptide domain (proN, Lys4–Ala113), the subtilisin domain (Val114–Thr421), and the β -jelly roll domain (Ala422–Pro522), as shown in Fig. 4.13. The structure of Pro-S359A* is similar to that of Pro-S324A (unautoprocessed form of Pro-Tk-subtilisin), but Pro-S359A* has an additional helix (α 1p) and a long extension loop, which extends to the β -jelly roll domain across the subtilisin domain, at the N-terminus. The subtilisin domain lacks four α -helices (α 1m– α 4m of Pro-S324A) and Ca^{2+} ions. The root mean square deviation values between Pro-S359A* and Pro-S324A is 1.5 Å for both the propeptide and subtilisin domains.

The β -jelly roll domain is not observed in the Pro-S324A structure. It is composed of nine β -strands and contains two Ca^{2+} ions. The Ca1 site of Pro-S359A* is located in the β -jelly roll domain, while the Ca2 site is located at the interface between the subtilisin and β -jelly roll domains. These Ca^{2+} binding sites are relatively conserved in the β -jelly roll-like domains of subtilisin-like alkaline serine protease Kp-43 from *Bacillus* sp. (Nonaka et al., 2004), kexin-like proteases (Henrich et al., 2003; Holyoak et al., 2003; Kobayashi et al., 2009), and tomato subtilase 3 (Ottmann et al., 2009). However, the amino acid sequence similarities of the β -jelly roll domains between Pro-S359A* and these proteases are poor, and the site corresponding to the Ca2 site of Pro-S359A* is not located at the interface between the subtilisin and β -jelly roll-like domains of these proteases owing to the different arrangement of the β -jelly roll-like domain relative to the subtilisin domain.

We constructed an S359A-SP* derivative lacking the β -jelly roll domain ($\Delta\text{J-S359A-SP}^*$) to examine whether the β -jelly roll domain is important for the stability of Tk-SP. The far- and near-UV CD spectra of $\Delta\text{J-S359A-SP}^*$ are similar to those of S359A-SP*. Thermal denaturation curves of these proteins were measured by monitoring the change in CD values at 222 nm as the temperature increases, in the presence of either 10 mM CaCl_2 or EDTA. The midpoints of the transition of these thermal denaturation curves, their T_m s, were 88.3 ± 0.86 , 58.8 ± 0.93 , 58.9 ± 1.3 , and $58.4 \pm 0.93^\circ\text{C}$ for S359A-SP*^{Ca}, S359A-SP*^{EDTA}, $\Delta\text{J-S359A-SP}^{\ast\text{Ca}}$, and $\Delta\text{J-S359A-SP}^{\ast\text{EDTA}}$, respectively (Foophow et al., 2010b). The T_m value of $\Delta\text{J-S359A-SP}^{\ast\text{Ca}}$, which was measured in the presence of 10 mM CaCl_2 , is lower than that of S359A-SP*^{Ca} by 29.4°C , whereas those of S359A-SP*^{EDTA} and $\Delta\text{J-S359A-SP}^{\ast\text{EDTA}}$ are similar, suggesting that the β -jelly roll domain contributes to the stabilization of Tk-SP by binding with Ca^{2+} ions.

4.4.3 Role of proN

The N-terminal propeptide domain (proN), similar to the propeptides of other subtilases, binds to the subtilisin domain in a substrate/product-like manner (Fig. 4.13). This suggests that proN inhibits the activity of Tk-SP as a competitive inhibitor. In fact, proN inhibits the activity of Tk-SP* when added *in trans* although the progress curve for the inhibition does not show a clear hyperbolic pattern (M. Yamanouchi, unpublished data). ProN is not required for the folding of these proteins, because Tk-SP and Tk-SP* exhibit activity in gel assays. However, the possibility that proN accelerates the folding rate of Tk-SP, as well as Tkpro, cannot be excluded. The critical interactions of the Glu201 residue for propeptide-catalyzed folding observed in Tk-subtilisin are conserved in the crystal structure of Pro-S359A*, for the most part, indicating that proN may function as an intramolecular chaperone. Further study will be required to completely understand the chaperone function of proN.

4.4.4 Role of the C-Domain

Attempts to obtain S359A-SP and Pro-S359A crystals containing the C-domain have remained elusive, and therefore the structure of the C-domain is unknown. When Pro-Tk-SP is overproduced in *E. coli* and purified, the C-domain is cleaved by Tk-SP during the purification procedures. We constructed the active site mutant of Tk-SP, S359C-SP, to examine the autoprocessing of the C-domain in more detail. The activity of S359C-SP was, similar to S324C-subtilisin, substantially reduced. When S359C-SP was incubated at 80°C in the absence of Ca²⁺, the C-domain was autoprocessed, whereas the autoprocessing reaction was not observed in the presence of 10 mM CaCl₂ (Sinsereekul et al., 2011). Similarly, the isolated C-domain, which was produced in His-tagged form using an *E. coli* expression system, was susceptible to proteolytic degradation by Tk-SP in the absence of Ca²⁺, but was resistant in the presence of Ca²⁺. Therefore, the Ca²⁺-bound form is most likely stable and resistant to autoprocessing owing to a conformational change of the C-domain induced by Ca²⁺ binding. In fact, the far-UV CD spectra of the His-tagged C-domain indicated increased secondary structure in the presence of Ca²⁺. Tk-SP's source organism, *T. kodakarensis*, was isolated from sediments and seawater samples from a solfatara at a wharf of Kodakara Island (Kagoshima, Japan) (Morikawa et al., 1994). Therefore, Pro-Tk-SP must mature in its native growth environment where Ca²⁺ ions are enriched (approximately 10 mM in seawater). We propose that Tk-SP is the mature form, rather than Tk-SP*, because the C-domain is unlikely autoprocessed under the natural conditions.

Thermal denaturation T_m curves of S359C-SP and S359C-SP* show that S359C-SP is more stable than S359C-SP* by 7.5°C in the presence of Ca²⁺, and 25.9°C in the absence of Ca²⁺. These results suggest that the C-domain contributes to the stabilization of Tk-SP by Ca²⁺ binding, although why the C-domain contributes more to the stabilization of Tk-SP in the absence of Ca²⁺ than in the presence of Ca²⁺ remains to be elucidated.

4.4.5 PrP^{Sc} Degradation by Tk-SP

Tk-SP is a highly thermostable enzyme with a half-life of 100 min at 100°C and exhibits its highest activity at 100°C. It is also resistant to treatment with chemical denaturants, detergents, and chelating agents. Therefore, Tk-SP is, like Tk-subtilisin, a promising candidate as a novel detergent enzyme. We tested whether Tk-SP can degrade PrP^{Sc} in scrapie-infected mouse brain homogenates using a combination of chemical treatments (Hirata et al., 2013). Western blot analysis revealed that PrP^{Sc} is completely degraded by Tk-SP in both the absence and presence of 1% SDS (Fig. 4.14). These results suggest that Tk-SP has potential application as a detergent additive for decreasing the infectivity of PrP^{Sc}. Further quantitative assessment of both Tk-SP and Tk-subtilisin for decontaminating

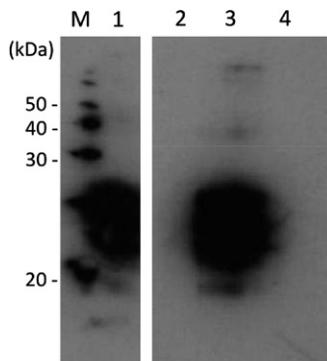


FIGURE 4.14 Western blot analysis of PrP^{Sc} Tk-SP digests. 1% Mouse brain homogenate was subjected to digestion at 100°C for 1 h with buffer only (lane 1), 0.02 mg/mL (0.4 mM) Tk-SP (lane 2), 1% SDS (lane 3), or 0.02 mg/mL (0.4 mM) Tk-SP plus 1% SDS (lane 4).

PrP^{Sc} will be required, because the minimum amount of protease for complete PrP^{Sc} degradation in various individual conditions has not been determined.

4.5 CONCLUDING REMARKS

Our studies have revealed the unique maturation and stabilization mechanisms of two hyperthermophilic proteases. Tk-subtilisin has a Ca^{2+} -dependent maturation mechanism characterized by its unique Ca^{2+} binding loop, which acts as an intramolecular chaperone, and also by its Ca^{2+} -dependent stabilization mechanism. Tk-SP requires neither a propeptide, nor Ca^{2+} ions for its maturation and the stabilization mechanisms, and is characterized by a β -jelly roll domain bound to Ca^{2+} ions. These mechanisms have evolved as successful strategies in hyperthermophilic proteases to adapt to high temperature environments.

Tk-subtilisin and Tk-SP both exhibit superior stability against heat, detergents, and denaturants. Thus, they are potentially applicable to industrial and medical technologies for degrading persistent proteins under harsh conditions where most other proteins would be denatured. In fact, both proteases effectively degrade infectious prion proteins (PrP^{Sc}) from human and mouse brain homogenates in combination with SDS. Our results indicate the great potential of these proteases as versatile detergent enzymes, not only for household use, but also for the decontamination of infectious materials on medical instruments.

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ABBREVIATIONS

- CBB** coomassie brilliant blue
CD circular dichroism
EDTA ethylenediaminetetraacetic acid
GdnHCl guanidine hydrochloride
PAGE polyacrylamide gel electrophoresis
SDS sodium dodecyl sulfate
TCA trichloroacetic acid

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Chapter 5

Enzymes from Basidiomycetes— Peculiar and Efficient Tools for Biotechnology

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5.1 INTRODUCTION

Fungi are the only group of organisms that occupy a kingdom all to themselves, the Kingdom Fungi. At least 100,000 different species of fungi have been identified and recent estimates based on high-throughput sequencing methods suggest that as many as 5.1 million fungal species exist (Blackwekk, 2011). The Ascomycotina, called “sac fungi” (ascomycetes), with over 60,000 described species, and the Basidiomycotina, called “club fungi” (basidiomycetes), with over 30,000 described species are the largest groups of known fungi. Basidiomycetes are called “club fungi” because their spores are attached to a club-shaped structure named *basidium* (pl. *basidia*). Basidiomycetous fungi include edible and medicinal mushrooms, pathogens for plants and animals, symbionts and endophytes in lichens, plant root mycorrhizas, leaves and needles, and saprotrophs.

5.2 BROWN AND WHITE ROT FUNGI

Basidiomycetes and ascomycetes play a crucial role in the balance of ecosystems. They are the major decomposers of lignocellulosic material in several ecosystems and play an essential role in the cycling of carbon and other nutrients. A wide variety of wood types and trees in different stages of decomposition can be colonized by these fungi. The wood-decaying fungi have specific preferences for certain host species and stages of wood decay. In addition, those wood-decaying fungi that are plant pathogens are adapted to survive against the

plant defenses with particular abilities to defeat antifungal substances such as phenolics, tannins, and alkaloids (Maciel et al., 2012).

Wood is a carbon-rich substrate with low contents of nitrogen and other essential nutrients. Lignocellulose, the main component of wood, is a complex mixture of polymers, which include mainly cellulose, hemicellulose, and lignin (Fig. 5.1).

Lignocellulose is the major renewable organic matter in nature. It has been estimated that there is an annual worldwide production of 10–50 billion tons of dry lignocellulose, accounting for about half of the global biomass yield. However, lignocellulose is recalcitrant to degradation. The recalcitrance of lignocellulose, which confers protection against microbial attack and enzymatic action, derives mainly from lignin, an irregular and nonrepeating polymer. Its biosynthesis is the result of oxidative polymerization of several phenyl-propanoid precursors, such as coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol. Polymerization occurs at random by various carbon–carbon and ether

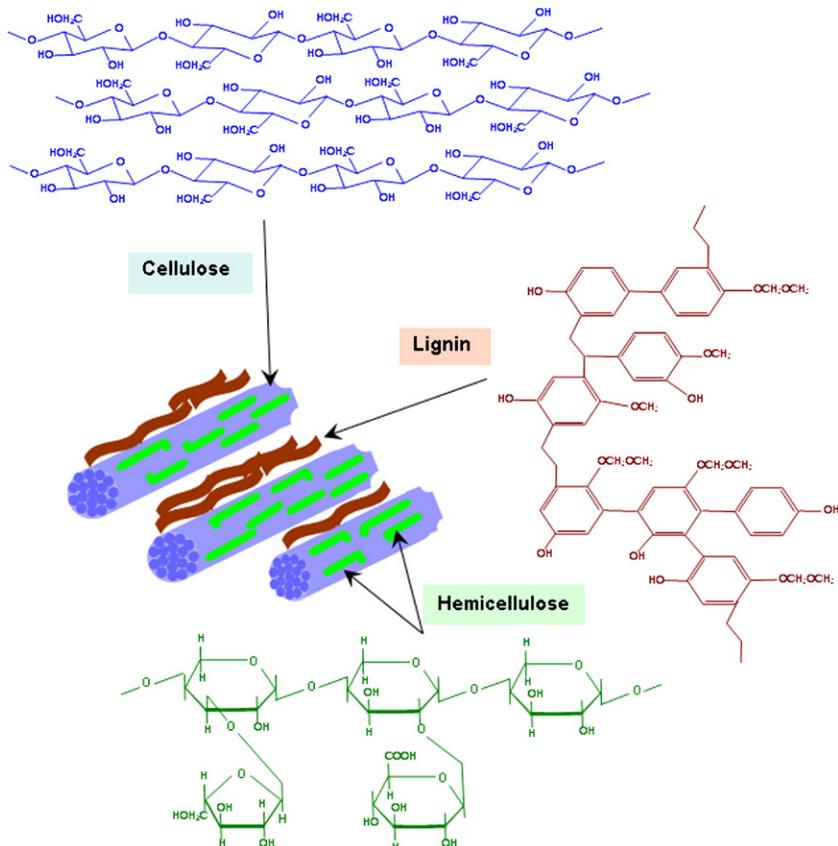


FIGURE 5.1 Main components of lignocellulosic material.

bonds resulting in an irregular structure, impossible to be hydrolyzed under natural conditions. The proportions and location of these polymers (cellulose, hemicelluloses, and lignin) are quite variable among plant groups or species. In order to use woody materials as substrates and to get access to its limited nutrients, the species of wood-decaying fungi have developed distinct mechanisms of growth as well as metabolic and enzymatic abilities, which are also dependent on environmental conditions such as temperature, humidity, and availability of food resources. Based on the ability to degrade lignin along or not with cellulose and hemicellulose, wood decay has traditionally been divided into white rot and brown rot, mainly performed by basidiomycetes, and soft rot, mainly brought about by ascomycetes.

Many of the brown rot fungi produce bracket-shaped fruiting bodies on the trunks of dead trees, but the characteristic feature of these fungi is that the decaying wood is brown and shows brick-like cracking—a result of the uneven pattern of decay, causing the wood to split along lines of weakness. The term “brown rot” refers to the characteristic color of the decayed wood, because most of the cellulose and hemicelluloses are degraded, leaving the lignin more or less intact as a brown, chemically modified framework (Fig. 5.2A).

The term white rot is related to the bleached (white) appearance frequently observed on wood attacked by these fungi (Fig. 5.2B). A common characteristic among white rot fungi is the ability to degrade all main components of the plant cell wall: cellulose, hemicelluloses, and lignin. The decomposition of lignocelluloses is achieved by a series of enzymatic (hydrolases and oxidoreductases) and nonenzymatic mechanisms and the production of ligninolytic enzymes is typical of this group of fungi.

Less than 10% of the wood-rotting basidiomycetes species are brown rots, but they are prevalent in nature. Typical species of brown rot fungi are *Gloeophyllum trabeum*, *Serpula lacrymans*, *Coniophora puteana* (known as the “cellar fungus”), *Schizophyllum commune*, *Postia placenta*, and *Fomes fomentarius* (Fig. 5.3).

The brown rot fungi can degrade cellulose and hemicelluloses, but they can only modify lignin, which remains as a polymeric residue in the decaying wood (Arantes and Goodell, 2014). The main lignin modification carried out by brown rot fungi is a demethylation reaction. The majority of the brown rot

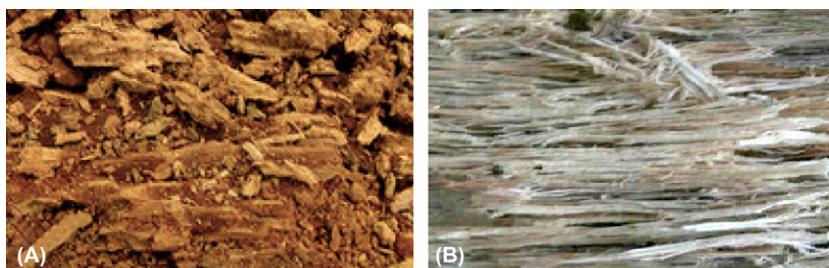


FIGURE 5.2 Wood decay caused by brown rot fungi (A) and white rot fungi (B).

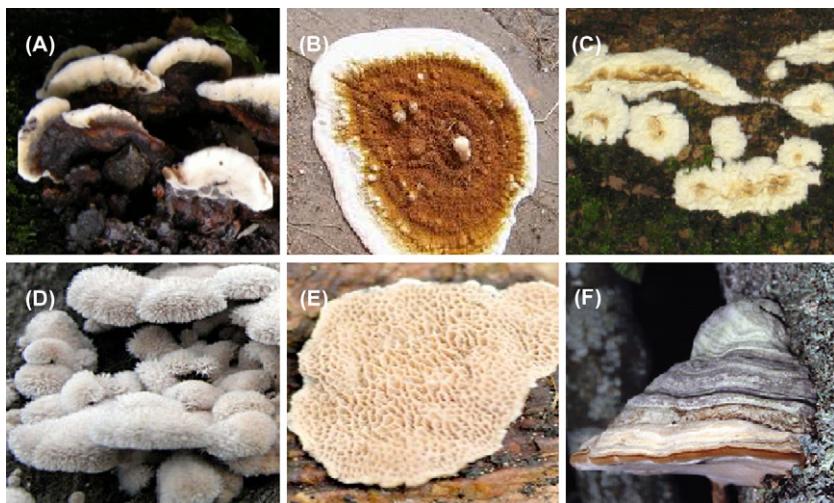


FIGURE 5.3 Brown rot basidiomycetes. (A) *Gloeophyllum trabeum*; (B) *Serpula lacrymans*; (C) *Coniophora puteana*; (D) *Schizophyllum commune*; (E) *Postia placenta*; (F) *Fomes fomentarius*.

basidiomycetes have long been thought to lack the processive cellulases, especially exocellulases. This makes the generation of hydroxyl radicals through Fenton-based reactions ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$), which depolymerize polysaccharides, highly important. However, brown rot fungi such as *Fomitopsis palustris* (Yoon et al., 2007) and *G. trabeum* (Cohen et al., 2005) produce endoglucanase and are capable of degrading microcrystalline cellulose. A study of the genome sequence from *P. placenta* revealed the absence of exocellulases and abundance of genes involved in reactive oxygen species formation (Martinez et al., 2009), reinforcing the idea that brown rot fungi can depolymerize cellulose via a combination of oxidative reactions and endocellulases (Cohen et al., 2005).

White rot fungi are a diverse and abundant group classified into the Agaricomycetes class. These fungi can be found colonizing either living trees (eg, heart rot) or dead wood (eg, logs, stumps) from temperate to tropical climates, presenting a variety of morphologies such as caps, brackets, or resupinaceous (corticioid) basidiomes. There are about 10,000 species of white rot fungi, with varying capacities to degrade lignin, cellulose, and hemicelluloses. However, only a few dozen have been properly studied. Most commonly studied species of white rot fungi are subdivided into six families: Phanerochaetaceae (eg, *Phanerochaete chrysosporium*), Polyporaceae (eg, *Trametes versicolor* and *Pycnoporus sanguineus*), Marasmiaceae (eg, *Lentinula edodes*), Pleurotaceae (oyster mushrooms such as *Pleurotus ostreatus* and *Pleurotus pulmonarius*), Hymenochaetaceae (eg, *Inonotus hispidus* and *Phellinus igniarius*), Ganodermataceae (e.g., *Ganoderma lucidum* and *Ganoderma applanatum*), and Meruliaceae (eg, *Bjerkandera adusta*, *Irpex lacteus*, and *Phlebia radiate*) (Fig. 5.4).



FIGURE 5.4 White rot basidiomycetes. (A) *Phanerochaete chrysosporium*; (B) *Trametes versicolor*; (C) *Lentinula edodes*; (D) *Pleurotus ostreatus*; (E) *Pleurotus pulmonarius*; (F) *Inonotus hispidus*; (G) *Phellinus igniarius*; (H) *Ganoderma lucidum*; (I) *Ganoderma applanatum*; (J) *Bjerkandera adusta*; (K) *Irpex lacteus*; (L) *Phlebia radiata*.

In nature, there are several microorganisms able to produce hydrolases (cellulases and hemicellulases) capable of hydrolyzing to a full extent all polysaccharide components of wood (cellulose and hemicelluloses) into monosaccharides. However, when these polysaccharides are complexed with lignin, they are resistant to enzymic hydrolytic breakdown. Therefore, lignin appears to inhibit hydrolytic activity. This is one reason, among others, why research on biotransformation of lignocellulose has taken a long time to develop. The degradation of lignin is a critical step for efficient carbon cycling. Thus, white rot fungi, as organisms capable of completely metabolizing lignin, are an essential part of forest ecosystems.

The patterns and rates of wood and woody material degradation vary among white rot fungi. Usually, the degradation systems can be divided in two subtypes: (1) those producing oxidative cleavage of lignin and structural polysaccharides at similar rates, leading to progressive erosion and thinning of wood cell walls, often referred to as simultaneous degraders and (2) those capable of removing lignin in advance of cellulose and hemicelluloses which are called selective delignifiers. The first subtype is considered the normal or the most common process of wood degradation and is used by the majority of white rot fungi (Skyba et al., 2013). In some cases, a slight preference for the removal of lignin in advance of carbohydrates may occur, but extensive loss of carbohydrates usually appears simultaneously or immediately after lignin removal. In selective delignification, the white rot fungi preferentially remove lignin from wood, causing moderate losses of hemicelluloses and leaving cellulose practically intact or only slightly degraded. Selective decayed wood presents white pockets consisting entirely of cellulose. Although rare, selective delignification can occur under natural conditions. *G. applanatum* has been associated with the formation of “palo podrido” wood in the evergreen rainforests of Southern Chile, a highly delignified type of wood where cellulose remains intact while hemicellulose and lignin are degraded (Dill and Kraepelin, 1986). *Ceriporiopsis subvermispora*, *Phlebia* spp., and *Physisporinus rivulosus* are some examples of white rot fungi that selectively attack lignin, while *T. versicolor*, *P. chrysosporium*, and *I. lacteus* simultaneously degrade all cell wall components (Maciel et al., 2012). The white rot fungi capable of preferentially removing lignin from lignocellulosic materials have received increasing attention for their applicability in industrial processes, such as biopulping and bioethanol production, as well as in the formulation of cellulose-enriched products in animal feed (Koutrotsios et al., 2014). However, the application of white rot fungi in selective delignification may depend on strain, cultivation methods, and lignocellulosic material composition (Salmones et al., 2005), whereas much variation exists in the ability of certain fungi to cause selective delignification and/or simultaneous rot.

The firstly determined genomes of the white rot fungus *P. chrysosporium* (Martinez et al., 2004) and the brown rot fungus *P. placenta* (Martinez et al., 2009) revealed a gene complement consistent with their respective modes of wood decay. Recently, the analysis of more than 30 genomes, however, has led to the view that

the ability of basidiomycetes to degrade wood should be reclassified based on a continuum, rather than on only two groups (Riley et al., 2014). The argument in favor of a new classification of wood decay is based on the fact that some white rot species, such as *Botryobasidium botryosum* and *Jaapia argillacea*, lack the peroxidases involved in lignin degradation, thus resembling brown rot fungi, but they do possess the cellulose-degrading apparatus typical of white rot fungi.

5.3 ISOLATION AND LABORATORY MAINTENANCE OF WOOD ROT BASIDIOMYCETES

Over several decades the basidiomycetes have been explored as biofactories of novel bioactive substances with great potential for biotechnological applications. In fact, basidiomycetes represent a reservoir for discovering new compounds, such as antibiotics, enzymes, antioxidants, immunomodulators, and anticancer and antiparasitic compounds, for use in the pharmaceutical and agrochemical industries (Erjavec et al., 2012). For this reason, there has been a growing interest in screening for new species and strains. A classical strategy is to collect basidioma or mycelia of white rot fungi in forests, dead trees, and lignocellulosic crop residues showing signs of attack by fungi. Samples of basidioma or mycelia are aseptically transferred onto potato dextrose agar or malt extract agar and subcultured until pure mycelia are obtained. Identification is based on morphological, physiological, biochemical, and genetic characteristics of the basidioma, hyphae, and spores. Molecular biology techniques are also being used in the identification of new isolates. Advancement in molecular methods has permitted a more rational study of the phylogenetic relationships within the various microorganisms. Noncoding internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA seem to be one of the most frequently used analytical tools (Prewitt et al., 2008).

There are several options for the maintenance of brown and white rot basidiomycetes in the laboratory. Continuous growth methods of preservation, in which the fungi are grown on agar (eg, malt extract agar, potato dextrose agar, and yeast extract agar), are typically used for short-term storage. For long-term storage, preservation in distilled water (called the Castelani method) and anhydrous silica gel are some of the most indicated methods. All are considered as low cost methods but none of them is considered permanent. Lyophilization and liquid nitrogen refrigeration (cryopreservation) are expensive methods, but are considered permanent.

5.4 BASIDIOMYCETES AS PRODUCERS OF ENZYMES INVOLVED IN DEGRADATION OF LIGNOCELLULOSE BIOMASS

As stated above, the lignocellulosic biomass consists mainly of three types of polymers, that is, lignin, cellulose, and hemicellulose, interlinked in a heteromatrix.

Consequently, its complete degradation requires the synergistic action of a large number of oxidative, hydrolytic, and nonhydrolytic enzymes. A panoramic view of enzymes involved in the complex processes of lignocellulose degradation will be presented here. Those enzymes typically found in basidiomycetes with potential biotechnological applications will be described in more detail.

5.4.1 Enzymes Involved in the Degradation of Cellulose and Hemicelluloses

The classical model for degradation of cellulose into glucose involves the cooperative action of endocellulases (EC 3.2.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91) glucano-hydrolases (EC 3.2.1.74), and beta-glucosidases (EC 3.2.1.21). Endocellulases hydrolyze internal glycosidic linkages in a random fashion, which results in a rapid decrease in polymer length and a gradual increase in the reducing sugar concentration. Exocellulases hydrolyze cellulose chains by removing mainly cellobiose either from the reducing or the nonreducing ends, which leads to a rapid release of reducing sugars but little change in polymer length. Endocellulases and exocellulases act synergistically on cellulose to produce cello-oligosaccharides and cellobiose, which are then cleaved by beta-glucosidase to glucose.

More recently, it has been shown that some fungi, including *Hypocrea jecorina*, also produce a class of oxidative enzymes, known as polysaccharide monooxygenases, which directly cleave cellulose chains through an oxidative mechanism, and appear to act synergistically with the traditional hydrolytic enzymes (Harris et al., 2010).

Cellulose is particularly resistant to degradation and requires several different enzymatic modes of attack to be effectively degraded. In cellulose fibers, crystalline and amorphous regions alternate. The amorphous regions are formed by cellulose chains with weaker organization, being more accessible to enzymatic attack. On the other hand, the crystalline regions are very cohesive, with a rigid structure formed by the parallel juxtaposition of linear chains, which results in the formation of intermolecular hydrogen bonds. This characteristic contributes to the insolubility and low reactivity of cellulose.

Hydrolysis of hemicelluloses involves enzymes such as glycoside hydrolases, carbohydrate esterases, polysaccharide lyases, endohemicellulases, and others. The concerted action hydrolyzes glycosidic bonds, ester bonds, and removes the substituent chains or side chains. These include endo-1,4- β -xyylanase (EC 3.2.1.8), β -xylosidase (EC 3.2.1.37), β -mannanase (EC 3.2.1.78), β -mannosidase, (EC 3.2.1.25), α -glucuronidase (EC 3.2.1.1), α -L-arabinofuranosidase (EC 3.2.1.55), acetyl xylan esterase (EC 3.1.1.72), *p*-coumaric and ferulic acid esterases (EC 3.1.1.1), and feruloyl esterases (EC 3.1.1.73). The last one can specifically cleave the ester bond between ferulic acid and arabinose, acting synergistically with cellulases, xylanases, and other hemicellulases in the saccharification of lignocellulose.

Taking into consideration the catalytic mechanisms and enzymatic specificities, the carbohydrate active enzymes are divided into families and subfamilies and compiled in the knowledge-based CAZy database (<http://www.cazy.org>) together with information about enzymes with auxiliary activities (Kues, 2015; Levasseur et al., 2013).

Filamentous fungi are considered good sources of hydrolytic enzymes which find application in various fields from process industries to diagnostic laboratories. Traditionally, enzymes from basidiomycetes involved in the degradation of plant cell wall polysaccharides have received less attention than those of soft rot ascomycetes and deuteromycetes. *Trichoderma reesei* (anamorph of *H. jecorina*) is the best known mesophilic soft-rot fungus producer of cellulases and hemicellulases (He et al., 2014; Martinez et al., 2008; Schuster and Schmoll, 2010). It is widely employed to produce enzymes for applications in the pulp and paper, food, feed, and textile industries and, currently, with increasing importance, in biorefining (Kuhad et al., 2011; Seiboth et al., 2011). The genera *Aspergillus*, *Fusarium*, *Humicola*, *Rhizopus*, *Alternaria*, *Monilia*, *Mucor*, and *Penicillium* are also considered great producers of cellulases and hemicellulases, including xylanases and pectinases (Juturu and Wu, 2013; Polizeli et al., 2005). The genera are also explored as producers of amylases and proteases (Payne et al., 2015). These enzymes have been commercially available for more than 30 years, and represent a target for both academic as well as industrial research. Basic and applied studies on cellulolytic and xylanolytic enzymes have demonstrated their biotechnological potential in various fields including food, animal feed, brewing, wine making, biomass refining, textile, pulp and paper industries, as well as in agriculture and laundry.

The brown rot fungi, *C. puteana*, *Lanzites trabeum*, *Poria placenta*, *Tyromyces palustris*, *F. palustris*, and *Piptoporus betulinus*, and the white fungi, *P. chrysosporium*, *Sporotrichum thermophile*, *T. versicolor*, *Agaricus arvensis*, *P. ostreatus*, and *Phlebia gigantea*, are among those most studied concerning their cellulase and xylanase enzymatic complexes (Cohen et al., 2005; Daniel, 2014; Kuhad et al., 2011; Valásková and Baldrian, 2006; Yoon et al., 2007; Zilly et al., 2012).

Considering that the brown rot and the white rot basidiomycetes are able to completely degrade the lignocellulosic materials, it is reasonable to suppose that they are able to produce at least some of the hydrolytic enzymes involved in the degradation of the polymers cellulose and hemicellulose (cellulase and xylanase complexes). Again, the most studied species in this respect is *P. chrysosporium*. The number of extracellular hydrolytic enzymes described in *P. chrysosporium* is elevated and includes several proteases, amylases, xylanases, and other carbohydrases (Dey et al., 1991; Ishida et al., 2007). However, the hydrolases of white rot basidiomycetes are not yet commercially explored. This low interest in the basidiomycete hydrolytic enzymes is easy to understand. Firstly, the soft rot ascomycetes and deuteromycetes can be easily cultured to produce hydrolytic enzymes with high productivity and low cost. Secondly,

when compared with the ascomycete and deuteromycete hydrolytic enzymes, the white rot basidiomycete hydrolytic enzymes do not possess the desired special characteristics, such as thermal stability, tolerance to high temperatures, and stability over a large range of pH values. Also, they do not retain their activity under severe reaction conditions such as the presence of metals and organic solvents (Nigam, 2013).

5.4.2 Enzymes Involved in Lignin Degradation

Lignin degradation by white rot basidiomycetes involves a set of enzymes called lignin modifying enzymes (LMEs). Most LMEs are secreted as multiple isoforms by many different species of white rot fungi under varying conditions. The set of LMEs comprises a phenoloxidase, laccase (Lcc, EC 1.10.3.2), and three peroxidases (high oxidation potential class II peroxidases), lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), and a versatile peroxidase (VP, 1.11.1.16). Laccase, which was firstly described over 128-years ago, is one of the oldest known enzymes. LiP and MnP were initially discovered in *P. chrysosporium*. VP was also added to the group of LMEs and was originally discovered in a strain of *Pleurotus eryngii* (Salvachúa et al., 2013).

White rot fungi usually secrete one or more LMEs in different combinations. Distribution of white rot fungi into groups according to their enzymatic systems has been undertaken. This general classification is based on the capacity of different fungi to produce one or a combination of peroxidases and laccase. Generically, white rot fungi can be distributed into four groups, according to their ability to produce laccases and peroxidases (LiP, MnP, and VP): (1) laccase and MnP and LiP (*T. versicolor*, *B. adusta*), (2) laccase and at least one of the peroxidases (*L. edodes*, *P. eryngii*, *C. subvermispora*), (3) only laccase (*S. commune*), (4) only peroxidases (*P. chrysosporium*). The most frequently observed LMEs among the white rot basidiomycetes are laccases and MnP, and the least observed are LiP and VP (Maciel et al., 2012).

The absence or nondetection of these enzymes in some white rot fungi, the sequencing of white rot fungal genomes, and the discovery of new enzymes can lead to the establishment of different groups. For example, the white rot fungus *C. subvermispora* delignifies lignocellulose with high selectivity, but up to few years ago it appeared to lack the specialized peroxidases, termed LiPs and VPs, that are generally thought important for ligninolysis (Lobos et al., 1994). The recently sequenced *C. subvermispora* genome was screened for genes that encode peroxidases with a potential ligninolytic role (Fernandez-Fueyo et al., 2012). Among 26 peroxidase genes, 2 newly discovered *C. subvermispora* peroxidases are functionally competent LiPs, phylogenetically and catalytically intermediate between classical LiPs and VPs. These results offer new insights into selective lignin degradation by *C. subvermispora*.

In addition to the peroxidases and laccases, fungi produce other accessory process enzymes, unable to degrade lignin on their own, but necessary to complete

the process of lignin and/or xenobiotic degradation: aryl-alcohol dehydrogenase (AAD, EC 1.1.1.90), glyoxal oxidase (GLOX, EC 1.2.3.5), quinone reductase (QR, EC 1.1.5.1), cellobiose dehydrogenase (CDH, EC 1.199.18), superoxide dismutase (SOD, EC 1.15.1.1), glucose 1-oxidase (GOX, EC 1.1.3.4), pyranose 2-oxidase (P2Ox, EC 1.1.3.4), and methanol oxidase (EC 1.1.3.13). These are mostly oxidases generating H₂O₂, dehydrogenases of lignin, and many xenobiotics (Maciel et al., 2012).

Cytochrome P450 monooxygenases (CYPs, EC 1.14.14.1) are also significant components involved in the degradation of lignin and chemically-associated xenobiotics (Coelho-Moreira et al., 2013a; Ning and Wang, 2012). These unspecific monooxygenases are intracellular heme-thiolate-containing oxidoreductases acting on a wide range of substrates in stereoselective and regioselective manners under consumption of O₂. Activated by a reduced heme iron, these enzymes add one atom of molecular oxygen to a substrate, usually by a hydroxylation reaction. A series of other reactions can occur including epoxidation, sulfoxidation, and dealkylation (Kues, 2015).

Recent additions to the enzymatic systems of white rot fungi include dye-decolorizing peroxidases (DyP), involved in the oxidation of synthetic high redox-potential dyes and nonphenolic lignin model compounds (Liers et al., 2010) and aromatic peroxygenases (APO) that catalyze diverse oxygen transfer reactions which can result in the cleavage of ethers (Hofrichter et al., 2010).

5.5 PRODUCTION OF LIGNINOLYTIC ENZYMES BY BASIDIOMYCETES: SCREENING AND PRODUCTION IN LABORATORY SCALE

The potential application of ligninolytic enzymes in biotechnology has stimulated investigations on selecting promising enzyme producers and finding convenient substrates to obtain large amounts of low-cost enzymes. Firstly, screening of fungal species and their variants is important for selecting suitable lignin-modifying enzyme producing organisms. For this reason one usually relies on the use of inexpensive, rapid, and sensitive testing methods. The screening strategy must aim to identify fungal strains and enzymes that will work under industrial conditions. Discovery of novel ligninolytic enzymes with different substrate specificities and improved stabilities is important for industrial applications. Fungi that produce ligninolytic enzymes have been screened either by visual detection of ligninolytic enzymes on solid media containing colored indicator compounds or, alternatively, by enzyme activity measurements in samples obtained from liquid cultivations. The use of colored indicators is generally simpler as no sample handling and measurement are required. As ligninolytic enzymes oxidize various types of substrates, several different compounds have been used as indicators of laccase production. The traditional screening reagents tannic acid and gallic acid (Bavendam reaction) have mainly been replaced by synthetic phenolic reagents, such as guaiacol, or by the

anthraquinonic dye Remazol Brilliant Blue R (RBBR) and the polymeric dye Poly R-478. These dyes are decolorized by ligninolytic fungi and the production of enzymes is observed as a colorless halo around the microbial growth. With guaiacol, a positive reaction is indicated by the formation of a reddish brown halo, while with tannic acid and gallic acid, the positive reaction is seen as a dark-brown colored zone (Bazanella et al., 2013; Ryu et al., 2003).

In general, the production of industrial enzymes on a large scale has been carried out using well-established submerged systems where the fungi are grown in a fully liquid system, which has the advantage of allowing control over process parameters, such as pH, temperature, and aeration. Submerged cultures of several white rot basidiomycetes have been conducted in both Erlenmeyer flasks, and bioreactors with capacity of 5 L or more (Janusz et al., 2007). The production of ligninolytic enzymes by the white rot basidiomycetes depends on the concentration of bioavailable nitrogen and carbon. For several fungal species, such as *P. chrysosporium* producing LiP and MnP under nutrient-limited conditions, ligninolytic enzyme activities are suppressed by high nitrogen concentrations in the medium. For other species such as *P. ostreatus* and *Trametes trogii*, high amounts of laccase and MnP are produced in the presence of high concentrations of nutrient nitrogen, while in *Dichomitus squalens* cultures, MnP is secreted under both high- and low-nitrogen conditions (Janusz et al., 2013). Overproduction of ligninolytic enzymes can be obtained by the addition of inducers. The list of inducers is large and includes oxalic acid, veratryl alcohol, 2,6-dimethoxyphenol, 2,5-xylidine, ferulic acid, vanillin, ethanol, copper, manganese, among others (Piscitelli et al., 2013; Souza et al., 2004).

Cultivation of white rot fungi in solid state systems appears as a promising technology for production of ligninolytic enzymes on large scale. Solid state fermentation (SSF) is defined as the cultivation process in which microorganisms grow on solid materials without the presence of free liquid. Solid state cultivation using different agro-industrial residues appears to be the most adequate technique for culturing basidiomycetes. The solid state fermentations reproduce the natural living conditions of these microorganisms, leading to the production of enzymes with high productivity and low cost. The selection of an adequate support for performing solid state cultivation is very important since the success of the process depends on it.

Selection of agro-industrial residues for utilization in SSF depends on physical parameters, such as particle size, moisture level, intra-particle spacing, and nutrient composition within the substrate. Wheat bran is the most commonly used substrate for the cultivation of white rot fungi. However, the list of possibilities is very large and includes several lignocellulolytic wastes such as sugar cane bagasse, corn cob, wheat straw, oat straw, rice straw, and food processing wastes, such as banana, kiwi fruit, and orange wastes, cassava bagasse, sugar beet pulp/husk, oil cakes, apple pomace, grape juice, grape seed, coffee husk, coir pith, and others (Couto 2008; Holker et al. 2004; Zilly et al., 2012). Even so, it is worth searching for new substrates, especially if they are available in

large amounts, allowing the growth of white rot fungi without further supplementation, and to facilitate obtaining valuable products.

After successful production, the desired enzymes must be separated and purified. This final step is commonly known as downstream processing or bioseparation, which can account for up to 60% of the total production costs, excluding the cost of the purchased raw materials. The downstream processing includes methods such as extraction, concentration, purification, and stabilization.

5.6 GENERAL CHARACTERISTICS OF THE MAIN LIGNINOLYTIC ENZYMES WITH POTENTIAL BIOTECHNOLOGICAL APPLICATIONS

A general description of the mechanisms and functions of the main ligninolytic enzymes is presented here.

5.6.1 Laccases

Laccases are copper oxidases that catalyze the one-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates with a concomitant reduction of O₂ to H₂O (Fig. 5.5).

The enzyme is widely distributed in higher sand fungi and has been found also in insects and bacteria. The majority of laccases characterized so far have been derived from white rot fungi, which are efficient lignin degraders. Many fungi contain several laccase-encoding genes, but their biological roles are not well understood. *Agaricus bisporus*, *Botrytis cinerea*, *B. adusta*, *Coprinus cinereus*, *G. lucidum*, *P. radiae*, *P. ostreatus*, *P. pulmonarius*, *Rigidoporus lignosus*, and *T. versicolor* are examples of basidiomycetes that produce laccases (Manavalana et al., 2013; Viswanath et al., 2014). Fungal laccases are mainly extracellular glycoproteins with molecular weights between 60 and 80 kDa, and acid pI values of 3–6 (Lundell et al., 2010). Fungal laccases occur often as isozymes with monomeric or dimeric protein structures, all showing a similar architecture consisting of three sequentially arranged domains of a β-barrel type structure (Fig. 5.6).

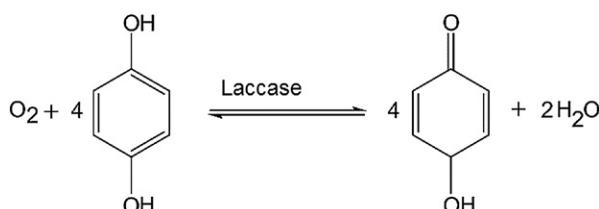
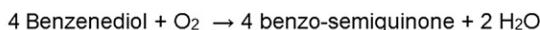


FIGURE 5.5 General reaction catalyzed by laccase.

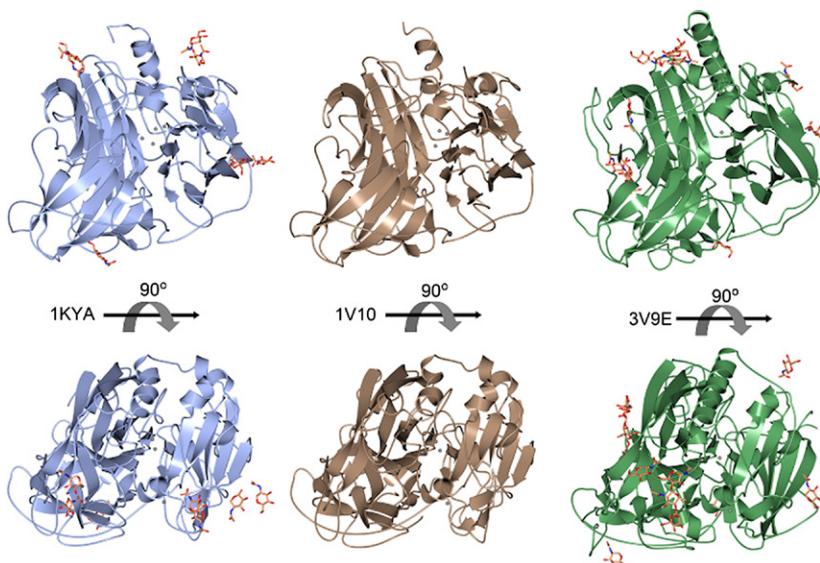


FIGURE 5.6 Ribbon models of laccases from *Trametes versicolor* (pdid: 1KYA); *Rigidoporus lignosus* (pdid: 1V10); and *Botrytis aclada* (pdid: 3V9E) showing a similar fold motif. Crystallographic data evidence carbohydrates (coral color) at glycosylation binding sites and copper ions (gray spheres) at redox centers.

Laccase attacks the phenolic subunits of lignin, leading to C α oxidation, C α -C β cleavage, and aryl-alkyl cleavage. This oxidation results in an oxygen-centered free radical, which can then be converted into quinone by a second enzyme-catalyzed reaction. The quinone and the free radicals can then undergo polymerization (Christopher et al., 2014; Kunamneni et al., 2008). Most monomeric laccase molecules contain four copper atoms in their structure that can be classified into three groups using UV/visible and electron paramagnetic resonance (EPR) spectroscopy. The type I copper (T1) is responsible for the intense blue color of the enzymes (with an absorption peak at 600 nm) and is EPR-detectable. The type II copper (T2) is colorless but is EPR-detectable, and the type III copper (T3) consists of a pair of copper atoms that give a weak absorbance near the UV spectrum but no EPR signal. The T2 and T3 copper sites are located close to each other and form a trinuclear center that is involved in the catalytic mechanism of the enzyme. Based on the type T1–T3 copper properties, laccases can be categorized into enzymes with high (0.6–0.8 V) or low (0.4–0.6 V) redox potential (Christopher et al., 2014). The catalytic efficiency of laccases depends on their redox potential, which explains the interest in laccases with high redox potential, such as the laccases from *T. versicolor*, *Pycnoporus coccineus*, and *P. sanguineus* (Morozova et al., 2007; Uzan et al., 2010).

Due to their specificity for phenolic subunits in lignin, restricted access to lignin in the fiber wall, and low redox potential, laccases have a limited potential

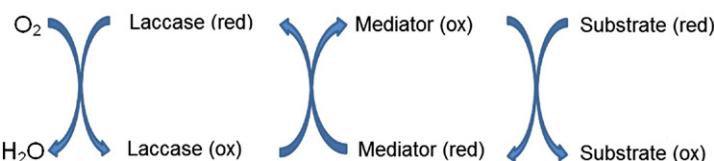


FIGURE 5.7 Schematic representation of laccase-catalyzed cycles for substrate oxidation in the presence of a chemical mediator.

to oxidize lignin and be used for biotechnological applications. Laccases possess relatively low redox potentials ($\leq 0.8\text{ V}$) compared to ligninolytic peroxidases ($> 1\text{ V}$). Thus, their action would be restricted to the oxidation of the phenolic lignin moiety (<20% of lignin polymers). Nonphenolic substrates, having redox potentials above 1.3 V , cannot be directly oxidized by laccases. Nevertheless, this limitation has been overcome through nature mimicking, that is, by using redox mediators in the so-called laccase-mediator system (LMS). These small natural and synthetic low molecular weight compounds with higher redox potential than laccase itself ($> 0.9\text{ V}$), called mediators, may be used to oxidize the nonphenolic part of lignin (Bibi et al., 2011; Camarero et al., 2004; Christopher et al., 2014; Johannes and Majcherczyk, 2000; Poppins-Levlin et al., 1999). In the last few years, the discovery of new and efficient synthetic mediators has extended laccase catalysis towards several xenobiotic substrates (Camarero et al., 2005; Can   and Camarero, 2010). A mediator is continuously oxidized by laccase and subsequently reduced by the substrate. The mediator acts as a carrier of electrons between the enzyme and the substrate (Fig. 5.7).

Phenolic products generated during lignin degradation by white rot fungi, fungal metabolites such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), violuric acid (VLA), 1-hydroxybenzotriazole (HBT), acetosyringone, syringaldehyde, *p*-coumaric acid, vanillin, and 4-hydroxybenzoic acid have been considered as potential mediators. Fig. 5.8 shows the chemical structures of some largely used natural and synthetic mediators.

The applicability and effectiveness of the laccase-mediator system (LMS) depends on the choice of proper mediators (Jeon et al., 2008). It should be noted that mediators, in the same way as enzymes, are also substrate selective. The mediator HBT, for example, efficiently improves the laccase-induced transformation of some dyes, such as Reactive Black 5, Bismarck Brown R, and Lanaset Grey G, but it is not effective in the decoloration of Remazol Brilliant Blue R (Da  ssi et al., 2014). The ideal mediator should be nontoxic, of low-cost, and efficient, with stable oxidized and reduced forms (Morozova et al., 2007) and should be able to maintain a continuous cyclic redox conversion (Christopher et al., 2014). The utilization of synthetic mediators, such as ABTS and HBT, in industrial processes is additionally hindered by their high cost, toxicity, and recycling-associated problems (Can   and Camarero, 2010). Low-cost and environmentally benign mediators are still highly needed to facilitate

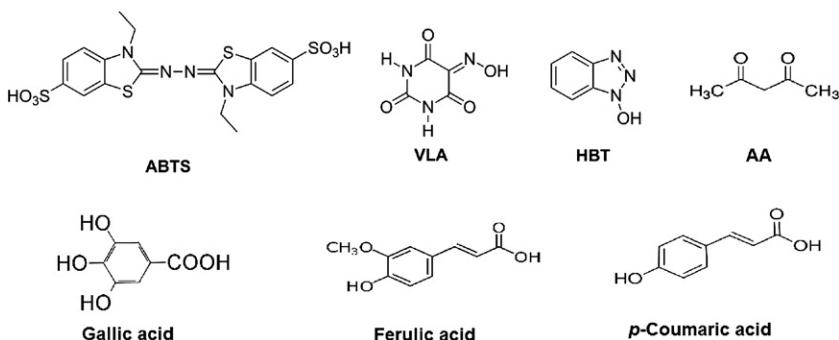


FIGURE 5.8 Chemical structures of natural and synthetic laccase mediators. *ABTS*: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); *VLA*, violuric acid; *HBT*, 1-hydroxybenzotriazole; *AA*, acetylacetone.

the application of laccases in various biotechnological processes in wastewater treatment. Acetylacetone (2,4-pentanedione), denoted as AA in Fig. 5.7, is an inexpensive small molecular diketone which presents low toxicity (Ballantyne and Cawley, 2001). It has been demonstrated that AA could act as a mediator for laccase from *P. coccineus* and *Myceliophthora thermophila* to initiate free radical polymerization of acrylamide (Hollmann et al., 2008). AA was also a highly effective mediator in the *T. versicolor* laccase-induced grafting copolymerization of acrylamide and chitosan, as well as in the decolorization of the dye malachite green (Yang et al., 2015).

5.6.2 Peroxidases

Three peroxidases involved in lignin degradation are produced by white rot fungi. LiP is characterized by its ability to oxidize high redox-potential aromatic compounds (including veratryl alcohol), whereas MnP requires Mn^{2+} to complete the catalytic cycle and forms Mn^{3+} chelates acting as diffusing oxidizers. Lignin peroxidase and manganese peroxidase were first described as true ligninases because of their high redox potential. The third peroxidase, VP is able to oxidize Mn^{2+} as well as nonphenolic aromatic compounds, phenols, and dyes (Martinez, 2002).

The lignin-modifying peroxidases (LiP, MnP, and VP), belonging to class II of fungal heme peroxidases, the so-called LiPs, catalyze the oxidation of various nonphenolic aromatic compounds as well as phenolic aromatic compounds such as veratryl alcohol, which is a metabolite produced by *P. chrysosporium*. Under ligninolytic conditions veratryl alcohol can also act as a mediator (Fig. 5.9).

MnP is the most common lignin-modifying peroxidase produced by almost all wood-colonizing basidiomycetes causing white-rot. Multiple forms of this glycosylated heme protein with molecular weights normally from 40 to 50 kDa are secreted by ligninolytic fungi into their microenvironment (Martínez et al.,

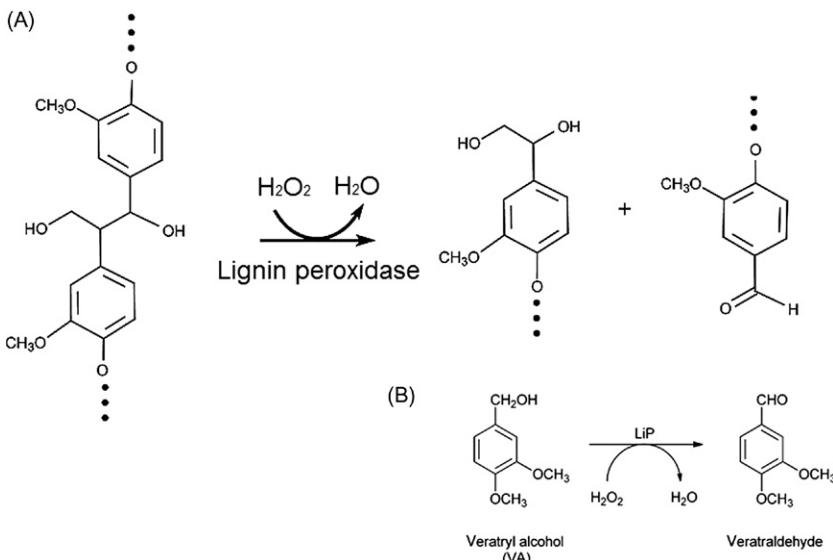


FIGURE 5.9 General reaction catalyzed by lignin peroxidase. (A) cleavage of C-C of lignin; (B) oxidation of veratryl alcohol is generally used to estimate the lignin peroxidase activity.

1996). MnP preferentially oxidizes Mn^{2+} , always present in woods and soils, into the highly reactive Mn^{3+} , which is stabilized by fungal chelators such as oxalic acid. Since Mn^{3+} is unstable in aqueous media, MnP releases it as a Mn^{3+} -carboxylic acid chelate. There are a variety of carboxylic acid chelators including oxalate, malonate, tartrate, and lactate, however oxalate is the most common. Chelated Mn^{3+} , in turn, acts as a low-molecular weight, diffusible redox-mediator that attacks phenolic lignin structures resulting in the formation of unstable free radicals that tend to disintegrate spontaneously. The enzyme has extraordinary potential to oxidize a range of different phenolic and nonphenolic complex compounds (Asgher and Iqbal, 2011). MnP is capable of oxidizing and depolymerizing natural and synthetic lignins as well as entire lignocelluloses (milled straw or wood, pulp) in cell-free systems (in vitro). In vitro depolymerization is enhanced in the presence of cooxidants such as thiols (eg, glutathione) or unsaturated fatty acids and their derivatives (eg, Tween 80).

Classical LiP producers are *B. adusta*, *P. chrysosporium*, *Trametes cervina*, and *T. versicolor*. The occurrence of MnP is higher than LiP. Typical MnP producers are *C. subvermispora*, *D. squalens*, *I. lacteus*, *L. edodes*, *P. chrysosporium*, *P. ostreatus*, *P. pulmonarius*, and *T. versicolor*.

The existence of a VP, a peroxidase with ability to oxidize both Mn^{2+} and aromatic compounds, was first reported in *P. eryngii* (Martinez et al., 1996). Subsequently, other VPs were isolated from *P. pulmonarius*, *P. ostreatus*, *B. adusta*, and *Bjerkandera* sp. (Salsachua et al., 2013). These peroxidases as MnP are able to oxidize Mn^{2+} to Mn^{3+} and to oxidize veratryl alcohol and

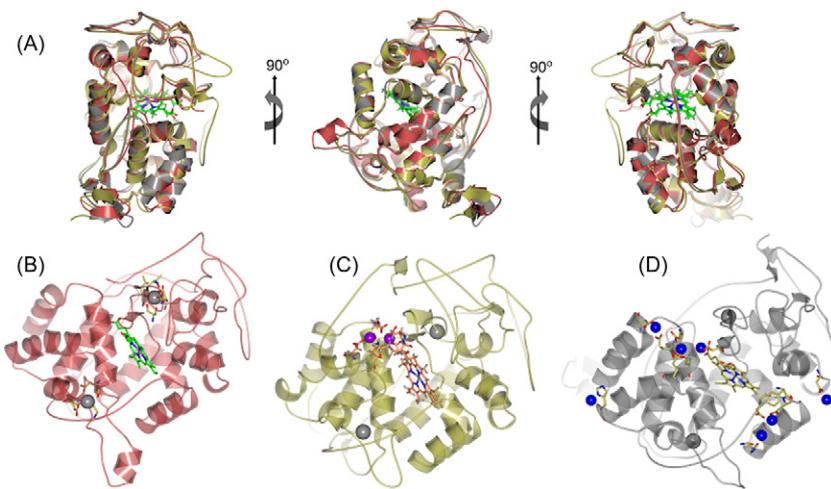


FIGURE 5.10 Ribbon models of peroxidases from *Trametes cervina* (vine color, pdbid: 3Q3U); *Ceriporiopsis subvermispora* (gold color, pdbid: 4CZO); and *Pleurotus eryngii* (gray color, pdbid: 3FKG). (A) The overlay fit of these three enzymes evidences a similar fold motif detaching a heme group (green) common in this protein family (haem peroxidase, pfam id: PF00141). (B) A lignin peroxidase from *T. cervina* detaching two calcium ions (gray spheres) bonded at their sites (yellow sticks). (C) Manganese peroxidase from *C. subvermispora* detaching two manganese ions (violet spheres) bonded at their sites (orange sticks). (D) Versatile peroxidase from *P. eryngii* detaching zinc ions (blue spheres) bonded at their sites (gold sticks). In the case of lignin peroxidase and versatile peroxidase, the heme group is in close contact (within 4.0 Å) with manganese or zinc ions.

p-dimethoxybenzene to veratraldehyde and *p*-benzoquinone, respectively, as reported for lignin peroxidase.

The catalytic cycle of three lignin peroxidases, consisting of the resting peroxidase and compounds I (two-electron oxidized form) and II (one-electron oxidized form), is common to other peroxidases. The unique catalytic properties of ligninolytic peroxidases are provided by the heme environment, conferring high redox potential to the *oxo*-ferryl complex ($\geq 1.0\text{ V}$) and by the existence of specific binding sites (and mechanisms) for oxidation of their characteristic substrates (Anastasi et al., 2013). These include nonphenolic aromatics in the case of LiP, manganous iron in the case of MnP, and both types of compounds in the case of the VP (Martinez et al., 2005). Similar heme environments in the above three peroxidases are located at the central region of the protein (Fig. 5.10).

5.7 INDUSTRIAL AND BIOTECHNOLOGICAL APPLICATIONS OF LIGNINOLYTIC ENZYMES FROM BASIDIOMYCETES

A considerable number of reviews detailing the numerous applications of ligninolytic enzymes have been published during the last few years. Ligninolytic enzymes are applied or present potential application in biofuel production,

TABLE 5.1 Recent Reviews of Industrial and Biotechnological Applications of Ligninolytic Enzymes

Enzyme(s) and application fields	References
Addressing Ligninolytic Enzymes	
Bioethanol industry	Placido and Capareda (2015)
Bioremediation/biodegradation of synthetic dyes	Bazanella et al. (2013)
Pretreatment of recalcitrant lignocellulosic biomass for biofuel production and other industrial and environmental applications such as paper industry, textile industry, wastewater treatment, and degradation of herbicides	Abdel-Hamid et al. (2013)
Polycyclic aromatic hydrocarbon (PAHs) degradation by fungi belonging to different ecophysiological groups (white-rot and litter-decomposing fungi) under submerged cultivation and during mycoremediation of PAH-contaminated soils. The possible functions of ligninolytic enzymes of these fungi in PAH degradation are discussed	Pozdnyakova (2012)
Application of ligninolytic enzymes in several fields such as chemical, fuel, food, agricultural, paper, textile, and cosmetic. Degradation of various xenobiotic compounds and dyes is also discussed	Maciel et al. (2010)
Degradation of persistent organic pollutants in waste waters, emphasizing the utilization of immobilized enzymes, especially laccases	Kues (2015)
Degradation of herbicides by white rot fungi, emphasizing ligninolytic enzymes and cytochrome P450	Coelho-Moreira et al. (2013b)
The role of mushrooms in mycoremediation, emphasizing their capability of biodegradation, bioaccumulation, and bioconversion	Kulshreshtha et al. (2014)
Descriptive information on the several enzymes from various microorganisms, including ligninolytic enzymes, involved in the biodegradation of a wide range of pollutants, applications, and suggestions of how to overcome the limitations of their efficient use	Karigar and Rao (2011)
Obtaining new bioproducts through enzymatic conversion of vegetal biomass, including biofuel, vanillin, and gallic acid	Pothiraj et al. (2006)
Discussion about mixed enzyme systems (ligninolytic and hydrolytic) for delignification of lignocellulosic biomass.	Woolridge (2014)

(Continued)

TABLE 5.1 Recent Reviews of Industrial and Biotechnological Applications of Ligninolytic Enzymes (*Continued*)

Enzyme(s) and application fields	References
Importance of biological pretreatment using microorganisms and their enzymes for selective delignification of lignocellulosic residues for the obtainment of biofuels	Dashtban et al. (2009)
Addressing Laccases	
Use of laccase in several fields including pulp and paper industry, textile industry, food industry, pharmaceutical and cosmetic industries, organic synthesis, biofuel cells, and biosensing	Piscitelli et al. (2013)
Use of immobilized laccase and tyrosinase for various applications, including synthetic and analytical purposes, bioremediation, wastewater treatment, and must and wine stabilization	Duran et al. (2002)
Application of laccases in the decolorization of dyes, detoxification of industrial effluents, wastewater treatment, paper and pulp industries, xenobiotic degradation, bioremediation, and in biosensors. The review also compares several techniques such as micropatterning, self-assembled monolayer, and layer-by-layer techniques, which immobilize laccase and preserve their enzymatic activity	Viswanath et al. (2014)
Application of laccases in pulp and paper industry	Virk et al. (2012)
Patents on applications of laccases within different industrial fields, including pulp and paper industry, textile industry, food industry, bioremediation, organic synthesis, pharmaceutical industry, and nanobiotechnology	Kunamneni et al. (2008)
Application of enzymes in food industry, pulp and paper industry, textile industry, nanobiotechnology, soil bioremediation, synthetic chemistry, and cosmetics	Rodrigues-Couto and Toca-Herrera (2006)
Laccase-mediator systems and their application in areas such as bioremediation and lignocellulose biorefineries	Can� and Camarero (2010)
Recent progress in lignin degradation with laccase-mediator systems	Christopher et al. (2014)
Application of enzymes in bioremediation: degradation of xenobiotics, decolorization of dyes, effluent treatment, among others	Viswanath et al. (2014)

(Continued)

TABLE 5.1 Recent Reviews of Industrial and Biotechnological Applications of Ligninolytic Enzymes (*Continued*)

Enzyme(s) and application fields	References
The mechanism of operation of laccase-mediator systems (LMSs) in xenobiotic degradation mediated by true redox mediators and laccase enhancing agents is considered. Structural formulae of most common laccase mediators and compounds that can be used as agents enhancing the enzyme operation are presented. Examples of LMS application in biotechnology are described	Morozova et al. (2007)
Use of laccase in the environmental area, emphasizing detoxification and bioremediation of polluted wastewaters and soils	Strong and Clauss (2011)
Addressing Peroxidases in General Including Lignin Peroxidases and Manganese Peroxidase	
Application of peroxidases in general. The application of lignin peroxidase and manganese peroxidase in biopulping and biobleaching is emphasized in addition to the degradation of synthetic azo dyes	Hamid and Rehman (2009)
Addressing Manganese Peroxidase	
Describes different approaches to prove lignin decomposition <i>in vitro</i> and lists, in addition, other recalcitrant substances oxidizable by manganese peroxidase	Hofrichter (2002)

bioremediation of several xenobiotics, detoxification of wastewater, organic synthesis, food industry, pharmaceutical and cosmetic industries, among others ([Table 5.1](#)).

Among the ligninolytic enzymes, laccases seem to be the most suitable to be used on a large scale due to the existence of a considerable number of microbial producers, ease of production in both submerged and solid state cultivation, broad substrate specificity, and their ability to use atmospheric oxygen as an electron donor compared to the H_2O_2 requirement of the peroxidases. These facts explain the higher number of research and review articles about laccase when compared to those about peroxidases. Below, a brief description of some important industrial and biotechnological applications of ligninolytic enzymes is presented.

5.7.1 Application of Ligninolytic Enzymes in Delignification of Vegetal Biomass and Biological Detoxification for Biofuel Production

The increasing demand for energy and the depletion of the fossil fuel reserves urge us to find large quantities of alternative precursors for the petrol-based chemical industry and transportation sectors. Cellulosic biomass, derived from nonfood sources, such as trees and grasses, is being explored as a feedstock for cellulosic ethanol production. As mentioned at the beginning of this chapter, the use of lignocellulosic residues to produce bioethanol is hindered by the presence of lignin. The highly recalcitrant structure of lignin makes the enzymatic and chemical degradation highly problematic. For this reason, the previous degradation of lignin is a prerequisite for saccharification of polysaccharides in biomasses. Pretreatments are required to remove or to modify lignin into a lignocellulosic fiber structure in order to facilitate the access of the hydrolytic enzymes to the polysaccharides. Ideally, these pretreatments should predominantly modify lignin without causing major breaks in the structural carbohydrates, making the latter available for fermentation processes. Mild pretreatments, avoiding the generation of waste and pollutants, are desirable. Several chemical and physicochemical pretreatment processes, such as acid pretreatment, alkaline pretreatment, steam explosion, and ammonia fiber explosion, have been used to enhance the enzymatic hydrolysis of lignocellulose (Agbor et al., 2011). However, these processes usually require high temperatures and pressures, resulting in high costs and undesirable products. In this respect, biological pretreatments present interesting alternatives. The biological pretreatment of lignocellulosic residues can be done using ligninolytic fungi (Castoldi et al., 2014; Singh et al., 2008; Salvachúa et al., 2011) or their ligninolytic enzymes. LMS, peroxidases, or mixtures of two or three ligninolytic enzymes have been largely used in biological delignification of lignocellulose (Caná and Camarero, 2010; Gutiérrez et al., 2012; Martínez et al., 2005; Placido and Capareda, 2015; Rico et al., 2014). Ligninolytic enzymes, particularly LMS, can be useful in the detoxification of vegetal biomass after conventional pretreatments such as steam explosion for removing toxic phenolics (Jonsson et al., 2013; Placido and Capareda, 2015). Several soluble phenolics derived from lignin such as vanillin, syringaldehyde, *trans*-cinnamic acid, and hydroxybenzoic acid inhibit cellulases, and consequently reduce the efficiency of saccharification of biomass (Ximenes et al., 2010).

5.7.2 Application of Ligninolytic Enzymes in the Degradation of Xenobiotic Compounds

White rot fungi and their ligninolytic enzymes have been demonstrated to be capable of transforming and/or degrading a wide range of xenobiotic compounds including aromatic amines, a wide number of phenolic compounds

including chlorophenols, secondary aliphatic polyalcohols, polycyclic aromatic hydrocarbons (PAHs), herbicides, and pesticides among others (Ahn et al., 2002; Jegannathana and Nielsen, 2013). Two mechanisms or systems have been proposed. The first consists of transformation in the extracellular space and it involves lignin-degrading enzymes. This powerful capability of white rot fungi resides in the fact that many pollutants have structural similarities to lignin and because ligninolytic enzymes are nonspecific, that is, they can also act on the pollutant molecules. Furthermore, the transformation of some compounds can be enhanced by the use of mediators, which can extend the reactivity of enzymes towards the substrates. In recent years, the capability of white rot fungi and their enzymes to biodegrade several xenobiotics and recalcitrant pollutants has generated considerable research interest in the area of industrial/environmental microbiology. As a consequence, a considerable number of reviews detailing the numerous characteristics and applications of ligninolytic enzymes have been published (Table 5.1). The participation of extracellular enzymes in the transformation of several xenobiotics by white rot fungi has been conclusively demonstrated by studies performed with purified enzymes.

A second system of white rot fungi involved in xenobiotic transformation is an intracellular enzymatic mechanism, represented mainly by cytochrome P450. Purification of fungal cytochrome P450, in order to obtain conclusive data, has been accomplished in only a few studies, due to the difficulties in maintaining the activation of the enzymes during microsome preparation. Hence, most conclusions were drawn from the results of indirect experiments consisting of the addition of specific cytochrome P450 inhibitors to the culture medium, such as piperonyl butoxide and 1-aminobenzotriazole (Coelho-Moreira et al., 2013a,b; Ning and Wang, 2012). Direct evidence is also available. Some experiments were carried out with the microsomal fraction isolated from *P. ostreatus* (Jauregui et al., 2003). In this work, the investigators found that the microsomes transformed the pesticides in vitro in a NADPH-dependent reaction.

5.7.3 Application of Ligninolytic Enzymes in the Degradation of Textile Dyes

More than 0.7 million tons of dyes and pigments are produced annually worldwide presenting more than 10,000 different chemical structures (Young and Yu, 1997). Several of these dyes are very conducive to light, temperature, and microbial attack, making them recalcitrant compounds. Dyes can obstruct the passage of sunlight through water resources, reducing photosynthesis by aquatic plants coupled to consequent decreases of the concentration of dissolved oxygen and diminution of the biodegradation of organic matter. Presently the removal of color from the colored effluents is conducted using physical and chemical methods such as adsorption, precipitation, coagulation–flocculation, oxidation, filtration, and photodegradation, which present advantages and disadvantages (Ferreira-Leitão et al., 2007; Robinson et al., 2001). Many works have reported

dye degradation by the use of ligninolytic enzymes. Different dye classes, such as heterocyclic, polymeric, triphenymethane, azo, indigo, anthraquinones, and phthalocyanin, were degraded by ligninolytic enzymes (Neifar et al., 2011; Zilly et al., 2011; Zilly et al., 2002). Although most studies on dye decolorization are carried out using spectrophotometric analysis, this technique is limited as an analytical tool since it allows only the evaluation of the chemical modifications that occur in the chromophore groups. However, more recently, new methodologies such as liquid chromatography-mass spectrometry (LC-MS), ¹³C-nuclear magnetic resonance (¹³C-NMR), high performance liquid chromatography systems equipped with a diode array detector (HPLC-DAD), liquid chromatography-mass spectrometry-electrospray ionization (LC-ESI-MS), and EPR have been introduced to analyze the decomposition of dyes by ligninolytic enzymes (Baratto et al., 2015; Hsu et al., 2012; Michniewicz et al., 2008; Murugesan et al., 2009; Zhao and Hardin 2007; Zille et al., 2005). These methodologies allow not only the identification of the metabolite products, but they also propose catalytical transformation mechanisms. Peroxidases are applied in the degradation of dyes, but free and immobilized laccases associated or not to natural and synthetic mediators are the most common ligninolytic enzymes used in these studies.

5.7.4 Application of Ligninolytic Enzymes in Pulp and Paper Industry

The manufacture of pulp, paper, and paper products ranks among the world's largest industries. The traditional pulp and paper production process is based on chemicals and mechanical processing, which consumes large amounts of raw materials, water, and energy and creates considerable pressure on the environment (Rosenfeld and Feng, 2011). A range of enzyme applications in the pulp and paper industry, including ligninolytic enzymes, can be useful for reducing the environmental impacts caused by this important economic activity (Jegannathan and Nielsen, 2013). Laccase can be used in the pulp and paper industry in a number of ways, including lignin degradation, deinking, pitch control, grafting on fibers to improve properties, and pulp and paper mill effluent detoxification (Virk et al., 2012). Lignozym-process, which refers to the laccase mediator system employing mediators such as ABTS and HBT, can remove lignin from pulp. This improves the brightness of pulp, thereby making the paper "white" (Call and Mucke, 1997). The combination of laccase-mediators with cellulases and hemicellulases has also been considered for deinking to produce pulps with improved physical and optical properties. The combination of hydrolytic and oxidative enzymes has been described as highly efficient with lower environmental impacts (Woolridge, 2014).

Lignin peroxidases are useful in bleaching of pulp (Bajpai et al., 2006; Sigoillot et al., 2005) and both lignin peroxidase and manganese peroxidase have been shown to be efficient in the decolorization of kraft pulp mill effluents (Ferrer et al., 1991; Moreira et al., 2003).

5.8 CONCLUDING REMARKS

Basidiomycetes represent a reservoir of important bioactives. In this chapter, efforts were made to present a general panorama of the enzymes involved in the capability of these fungi to degrade vegetal biomass as well as their industrial and biotechnological applications. Basidiomycete enzymes involved in the degradation of plant cell wall polysaccharides have been receiving less attention than those of soft rot ascomycetes and deuteromycetes. However, their ligninolytic systems (peroxidases and laccases) have great biotechnological importance. Ligninolytic enzymes have been used especially in the so called white biotechnology, where vegetal biomass can be useful as an alternative to fossil resources for the production of chemicals such as biofuels and biopolymers. Ligninolytic enzymes have also several environmental applications; thanks to their nonspecificity, these enzymes are able to degrade several xenobiotics and recalcitrant pollutants including pesticides, herbicides, and textile dyes. Additionally, ligninolytic enzymes present applications in the food, medical, pharmaceutical, cosmetic, and nanotechnological areas. After the description of so many enzymes, the present trend is to characterize synergism as a real possibility of enhancing efficiency. The final goal is to use highly efficient enzymatic cocktails for industrial purposes.

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Chapter 6

Microbial Production and Molecular Engineering of Industrial Enzymes: Challenges and Strategies

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6.1 INTRODUCTION

Enzymes are widely used in the biopharmaceutical, agricultural, chemical, and food production industries. The products of these industries also are important in the fields of medicine, diagnostics, food, nutrition, detergents, textiles, leather, paper, pulp, and plastics (Sanchez and Demain, 2012). A high-level expression system depends on many factors, including cell growth characteristics, posttranslational modifications, biological activity of the protein of interest, regulatory issues, and so on (Fig. 6.1). To select a proper expression system requires consideration of the cost in the design process and other economic factors (Datar et al., 1993; Makrides, 1996). Recombinant enzymes have been expressed in *Escherichia coli*, bacilli and lactic acid bacteria, filamentous fungi, and yeasts. Bacterial expression systems have high efficiency due to their growing ability and high density on substrates and adaptation of cloning vectors as well as well-characterized genetics (Terpe, 2006). Recombinant DNA technology has derived strategies for protein production. Among large numbers of industrial microorganisms, *E. coli* is known as the most common host for recombinant protein production (Terpe, 2006; Chou, 2007).

Enzyme overproduction by recombinant expression systems was difficult to achieve because of multiplicity and physiological impacts. In addition, the mechanistic link between the stress response and the deteriorated culture performance of hosts is needed. Most genetic strategies for cell physiology are

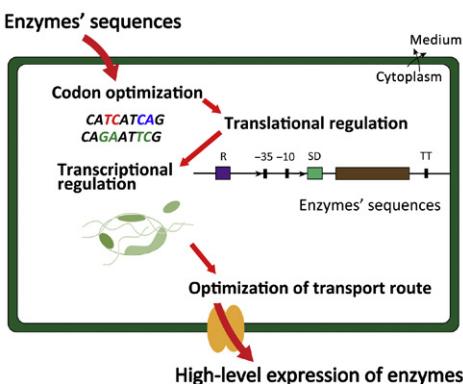


FIGURE 6.1 Strategies to achieve high-level expression of recombinant proteins in hosts.

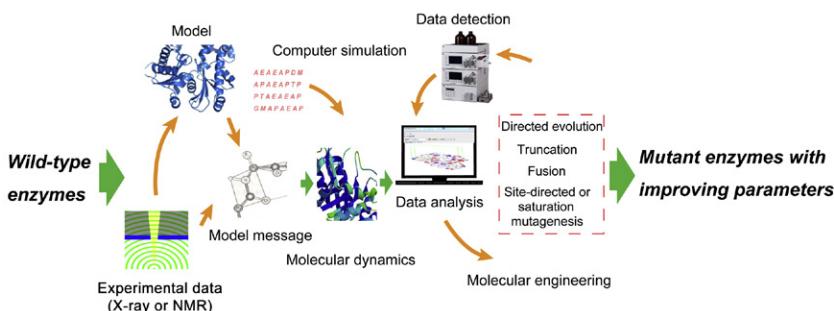


FIGURE 6.2 Molecular engineering strategies to enhance the performance of enzymes.

limited to single-gene manipulation and appear to be target protein-dependent. Furthermore, how to monitor cell physiology during cultivation is another problem.

New enzymes for industrial processes are often found by a metagenomic approach because some microorganisms from extreme environments cannot be cultivated in the laboratory (Martinez-Martinez et al., 2013; Zheng et al., 2013). Some methods such as immobilization and embedding could improve the application properties of enzymes to some extent, but these techniques only work on natural enzymes. The characteristics of natural enzymes need to be optimized. Therefore, molecular engineering methods such as directed evolution, site-directed mutagenesis, terminal fusion, and truncation have been developed (Fig. 6.2). These methods could improve catalytic performance by changing the structure of certain proteins (Hida et al., 2007).

This chapter integrates extensively published literature on gene expression in microbial hosts, and summarizes efficient expression systems and useful experimental approaches for protein overproduction. This review also discusses

the developing trends in molecular engineering to optimize characterization of enzymes.

6.2 STRATEGIES FOR ACHIEVING HIGH-LEVEL EXPRESSION OF INDUSTRIAL ENZYMES IN MICROORGANISMS

6.2.1 Strategies for High-Level Expression of Microbial Enzymes in *E. coli*

The regulation of recombinant protein expression is complex, consisting of interacting elements. An expression plasmid is important for the expression of a particular recombinant protein. The essential elements of an *E. coli* expression vector include promoter, ribosome-binding site, transcription terminator, and copy number.

6.2.1.1 High-Level Expression of Enzymes by Transcriptional Regulation in *E. coli*

High-level protein synthesis needs a strong promoter and tight regulation (Guzman et al., 1995). The most widely used promoters for large-scale protein production are thermal or chemical inducers (Chao et al., 2004). In prokaryotes, transcription termination is affected by rho-dependent transcription termination and rho-independent termination (Makrides, 1996). Efficient transcription terminators are indispensable elements since they serve a crucial function. The transcription terminators can enhance mRNA stability and increase the protein production level (Newbury et al., 1987). It is believed that the antitermination elements are useful for heterologous genes expression in *E. coli*. The transcriptional antitermination region from the *E. coli rrnB* rRNA operon has been used in the expression vector pSE420, which utilizes the *trc* promoter (Brosius, 1992). Tightly regulated promoters could enable the design of many of ingenious and highly repressible expression systems. Several methods employed to study the tight regulation of promoters have provided vital tools for the gene expression armamentarium.

6.2.1.2 High-Level Expression of Enzymes by Translational Regulation in *E. coli*

The protein synthesis initiation factors are difficult to decipher. In *E. coli*, AUG is the preferred codon by two- or threefold, while GUG is slightly better than UUG (Ringquist et al., 1992). The secondary structure at the translation initiation region of mRNA is important for the efficiency of gene expression. SD and other sequences in the mRNA are vital for translation. Translation was enhanced upon interaction with bases 485–466 of the 16S rRNA (Olins and Rangwala, 1989). The mRNA stability also plays an essential role in maintaining cellular levels of a given protein in *E. coli* (Sprengart et al., 1996).

6.2.1.3 Enhancement of the Expression of Enzymes by Different Protein Formations in *E. coli*

Inclusion bodies are important cytoplasmic aggregates. Several experimental methods such as lower temperatures and molecular chaperones have been used to reduce inclusion body formation and improve protein folding.

The oxidative environment of the periplasm plays an important role in facilitating the proper folding of proteins. The signal peptide cleavage during translocation to the periplasm in vivo is more likely to yield the authentic N-terminus of the target protein. Strategies to improve the translocation of periplasmic proteins include supplying components involved in protein transport and processing, overproduction of the signal peptidase I (Zhang et al., 1997), coexpression of the *prlA4* and *secE* genes (Pérez-Pérez et al., 1994), deletion of the twin-arginine translocation motif (Uthandi et al., 2012), type III secretion chaperone (Brinkworth et al., 2011), mutations in *secY* (Brinkworth et al., 2011), and addition of endoplasmic reticulum or Golgi retention sequences (Zhan et al., 1998).

The secretion of enzymes into culture media is preferred owing to several advantages, such as simplifying downstream processing, high expression level, simpler purification, improving protein folding, and level of proteolysis (Tesfai et al., 2012). However, *E. coli* secretes few proteins and the manipulation of various transportation ways to promote foreign protein secretion is an important task. Limited leakage of the outer membrane was induced to improve protein secretion. Several strategies such as signal peptide mutation and the synergistic use of ethylenediaminetetraacetic acid and lysozyme have been reported (Ismail et al., 2011; Liu et al., 2012).

6.2.1.4 Improving Enzyme Production Yield by Fusion Proteins or Molecular Chaperones in *E. coli*

Several fusion proteins have been used to improve enzyme production. The increase in protein yield is due to improved folding, efficient mRNA translation, and so on. Besides, the expression of poorly expressed heterologous genes was improved by translational fusion with fragments of the *trpE* gene (Makoff et al., 1989).

Molecular chaperones can assist protein folding under normal and stress conditions (Hartl, 1996). They could provide higher yields of correctly folded, biologically active proteins for some enzymes that are difficult to produce in *E. coli*. The combination of molecular chaperones and target proteins from the same species is another strategy. It has been successfully used in the production of soluble gp37 by coexpression with two bacteriophage T4-encoded chaperones in a two-vector system in *E. coli* (Bartual et al., 2010).

6.2.1.5 High-Level Expression of Enzymes by Codon Optimization in *E. coli*

Heterologous protein produced in *E. coli* may be diminished by biased codons. Expression of genes in *E. coli* shows a nonrandom usage of synonymous codons.

The rare tRNA competition may also affect the expression of host genes or elicit an adversely stringent response (Burgess-Brown et al., 2008). The arginine codons AGA and AGG are particularly rare in *E. coli*, and they have been shown to lead to mistranslational errors and lower protein expression (Calderone et al., 1996). Codon optimization could improve the fidelity of transformation in order to improve the expression of enzymes (Hutterer et al., 2012). The codon bias can be mitigated by targeted mutagenesis to change rare to more commonly used codons or by coexpressing genes that encode the tRNA. Synthesis of complete genes could be used to obtain codon-optimized and mRNA secondary structure-free sequences. Synthetic DNA has been used successfully to express many genes (Gustafsson et al., 2004; Kim and Lee, 2006). In addition, the expression of genes with rare codons could be rescued by coexpression of rare cognate tRNA genes (Kleber-Janke and Becker, 2000).

6.2.1.6 Fermentation Optimization of Enzyme Production in *E. coli*

Enzyme production in *E. coli* can be infinitely enhanced by high-density culture systems. The methods include batch, fed-batch, and continuous cultures (Kleist et al., 2003; Patnaik et al., 2008). The composition of the growth medium must be optimized because it significantly affects both the cell metabolism and enzyme production (Kumar and Shimizu, 2011). Nutrient composition and fermentation variables such as temperature, pH, and other parameters affect the production levels of enzymes (Fang et al., 2011). However, high-cell-density culture is affected by several factors such as limited availability of dissolved oxygen at a high cell density and high carbon dioxide levels. The decreased growth rates would enhance the formation of acetate and reduce mixing efficiency. The accumulation of acetate is a problem in the production of recombinant proteins in high-cell-density cultures. Nutrient consumption rate and CO₂ production rate are also important factors affecting the enzyme expression level (Kumar and Shimizu, 2011).

6.2.2 High-Level Expression of Microbial Enzymes in *Bacillus*

As well as *E. coli*, Gram-positive *Bacillus* species are popular organisms for recombinant enzyme production. *Bacillus* strains have a high protein secretion ability and can export proteins directly into the extracellular medium, unlike *E. coli*. In addition, few enzymes expressed in the *Bacillus* strains are isomerized (Westers et al., 2004).

The ability to directly secrete proteins into the medium makes *Bacillus subtilis* an interesting system. By comparing different *B. subtilis* expression systems, the subtilin-regulated gene expression (SURE) system was found to be the most efficient expression system, and was suitable for expression of membrane protein complexes (Vavrova et al., 2010). Several recombinant enzymes have been expressed in the *B. subtilis* expression system. Multiple regulators can affect *B. subtilis* secretion machinery and its posttranscriptional functions.

The secretion of heterologous proteins can be enhanced by engineering components involved in the late stages of secretion. The promoter system is also an important factor for expression.

Bacillus megaterium is a well-studied prokaryote used for heterologous protein production. This expression host has several advantages, such as low protease activity, ability to grow on a wide variety of substrates, and structural stability of plasmids. Proteins like sugar-modifying enzymes and hydrolases have been successfully expressed in *B. megaterium* (Gamer et al., 2007). In *B. megaterium* enzyme production processes, the *exo*-enzyme genes are repressed during exponential growth by transition-state regulators.

In the *Bacillus brevis* expression system, enzymes are secreted directly into the culture medium, and are accumulated at high levels. The secreted proteins are generally correctly folded, soluble, and biologically active. Since *B. brevis* has a very low level of extracellular protease activity, the secreted proteins are stable and not significantly degraded. The secretion system of *B. brevis* is enhanced by the use of fungal protein disulfide isomerase.

6.2.3 High-Level Expression of Microbial Enzymes in Lactic Acid Bacteria

The lactic acid bacteria are promising potential hosts for expression of recombinant enzymes. They are safer expression hosts for recombinant production of food and medical proteins, since they do not have endotoxins in their membranes. Modified methods for recombinant protein production, such as strain selection, vector optimization, and secretion systems engineering, have been developed.

Lactobacillus lactis is promising for expression of some recombinant proteins. The *L. lactis* MG 1363 strain was engineered as a recombinant live beta-galactosidase delivery system using food-grade protein-expression techniques and selected probiotics as vehicles. The most widely used inducible expression system in *L. lactis* is a nisin-inducible controlled gene system, which could afford tightly controlled expression and relatively high protein yield (Morello et al., 2008). Secretion is also used to improve recombinant enzymes production in *L. lactis*.

6.2.4 High-Level Expression of Microbial Enzymes in Yeasts

Yeast expression systems have many advantages such as genetic manipulation, rapid growth, and ability of performing eukaryotic posttranslational modifications (eg, glycosylation). The hosts mainly include *Saccharomyces cerevisiae*, *Pichia pastoris*, *Arxula adeninivorans*, *Hansenula polymorpha*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*. Several methods have been used to optimize the transformation system and efficient expression in yeast hosts in order to improve production of recombinant enzymes.

6.2.4.1 High-Level Expression of Microbial Enzymes in *P. pastoris*

P. pastoris is considered an excellent host for the production of enzymes. *P. pastoris* expression strains, including the commonly used auxotrophic mutants GS115 and protease-deficient strains (eg, SMD 1165), are derived from the wild-type strain NRRL-Y 11430. *P. pastoris* has three phenotypes related to methanol utilization, including Mut⁺, Mut^s, and Mut⁻. This expression system has many advantages, especially its excellent protein processing mechanism, including posttranslational modifications inside the cell, protein folding, signal peptide cleavage, and the ability to secrete its products into the medium with normal function.

The *P. pastoris* expression system includes the signal sequence, the ability of proteolytic cleavage, a suitable vector, and a codon-optimized gene. Gateway-compatible vectors, that is, pBGP1-DEST and pPICZ alpha-DEST, have been developed for secretory protein production in recombinant *P. pastoris*. In addition, the choice of the inducer and the induction mechanism highly affect the yield of enzyme production. For high-level expression of recombinant enzymes in high-cell-density fermentation of *P. pastoris*, pH was maintained by ammonium hydroxide and different combinations of glycerol/methanol were supplied as carbon source. The use of glycerol as cosubstrate is one of the most common strategies. However, the excess glycerol in the medium represses the *AOX1* promoter in recombinant enzyme production (Wang et al., 2009). Sorbitol as a cosubstrate was adopted as an alternative to the glycerol/methanol cofeeding strategy. High oxygen transfer rates are necessary in the methanol-utilization pathway to prevent the side reaction oxidizing methanol to formaldehyde. High agitation and oxygen-enriched air can be used to minimize the transfer resistance and keep the dissolved oxygen around 20–30%. The yield is also affected by enzyme degradation by host-specific proteases. To solve this problem, several methods have been adopted, such as control of cultivation conditions, addition of protease inhibitors, and different medium compositions. Although the optimum temperature for the growth is 30°C, a lower induction temperature was found to improve recombinant protein production.

6.2.4.2 High-Level Expression of Microbial Enzymes in *S. cerevisiae*

S. cerevisiae is a safe strain, producing recombinant enzymes which could be applied in the food and drug industries. The most popular enzyme expressed in *S. cerevisiae* is urate oxidase (Demain and Vaishnav, 2009). Various enzymes have been successfully expressed in *S. cerevisiae*, such as esterase (Lopez-Lopez et al., 2010). Several strategies have been employed to enhance the expression yield of proteins, such as translational fusion partners. The secretory pathway of *S. cerevisiae* could be improved by manipulation of the unfolded protein response pathway.

6.2.4.3 High-Level Expression of Microbial Enzymes in Other Yeast Hosts

Several enzymes have been produced in *H. polymorpha*, including catalase. During the final hours of the fermentation, enzyme production in *H. polymorpha* could be enhanced by a mixture of glycerol and methanol. [Kottmeier et al. \(2010\)](#) found that the metabolic flux toward protein production could be diverted by secondary substrate-limited batch fermentation and the yield of green fluorescent protein was increased by 1.9-fold. *Y. lipolytica* has the advantage of secreting high-molecular-weight enzymes via cotranslational translocation. *Kluyveromyces lactis* has been used for producing recombinant enzymes (eg, galactosidase). *S. pombe* is also an attractive host for recombinant enzyme expression.

6.2.5 High-Level Expression of Microbial Enzymes in Filamentous Fungi

Filamentous fungi are extraordinary hosts for the overexpression of recombinant enzymes due to their superior capacities of hyperproducing and secreting proteins. The industrial strains include *Aspergillus*, *Penicillium*, *Trichoderma*, and *Rhizopus* species. Among them, *Aspergillus* and *Trichoderma* species produce and secrete recombinant enzymes at very high levels.

6.2.5.1 High-Level Expression of Microbial Enzymes in Aspergillus Species

Aspergillus species are vital industrial filamentous fungi employed for both homologous and heterologous enzyme production. *Aspergillus oryzae* and *Aspergillus niger* are on the generally recognized as safe (GRAS) list of the Food and Drug Administration (FDA) in the United States. *Aspergillus fumigatus* and *Aspergillus nidulans* produce toxins, so they are not used for the production of recombinant enzymes.

A. niger is a promising host for the expression of recombinant enzymes. Some homologous and heterologous enzymes were overexpressed in *A. niger* by using a standardized expression cassette. Protein engineering was used to study the features that influence protein production and secretion. Protein sequence features were explored in the production of overexpressed extracellular proteins by *A. oryzae*. Tyrosine and asparagine compositions were found to have a positive impact on high-level production of recombinant enzymes ([van den Berg et al., 2012](#)).

A. oryzae is a particularly good host for producing recombinant proteins with its demonstrated capacities to hyperproduce and secrete enzyme proteins. Proteolytic degradation by secreted protease into the culture medium is an important issue that remains to be solved for heterologous protein production by filamentous fungi.

6.2.5.2 High-Level Expression of Microbial Enzymes in Trichoderma Species

The *Trichoderma* spp. include *Trichoderma atroviride*, *Trichoderma reesei*, and *Trichoderma viride*. *T. reesei* has a remarkable ability to secrete proteins and represents a promising host to help replace gasoline with cellulose-derived ethanol. Several methods have been used to obtain a more efficient *T. reesei* expression system, including a strong promoter and RNAi-mediated gene silencing (Qin et al., 2012; Zou et al., 2012).

6.2.5.3 High-Level Expression of Microbial Enzymes in Other Filamentous Fungi Species

The white rot fungi *Penicillium* and *Rhizopus* spp. have the potential for production of recombinant enzymes. Two novel lignin peroxidase genes isolated by RT-PCR and RACE-PCR were transformed into the uracil auxotrophic mutant UV-64 of the white rot fungus *Phanerochaete sordida* YK-624, then expressed successfully and secreted in an active form (Sugiura et al., 2009). In addition, carbon sources can affect the secretion of enzymes in *Penicillium* species. Acetylated xylan and sugar beet pulp strongly regulate the enzymes secreted by *Penicillium purpurogenum* (Navarrete et al., 2012). *Rhizopus oryzae* has also been used in the production of enzymes (eg, glucoamylase and lipase). Three plasmid vectors have been created to drive the expression of heterologous proteins, including the pyruvate decarboxylase (*pdcA*), phosphoglycerate kinase (*pgkI*), and the glucoamylase A (*amyA*) promoter (Mertens et al., 2006).

6.3 MOLECULAR ENGINEERING STRATEGIES

6.3.1 Directed Evolution

Directed evolution is the most efficient and practical means of modifying enzymes to improve catalytic performance. This technology combines random mutagenesis via the error-prone polymerase chain reaction (ep-PCR), DNA shuffling, staggered extension process (StEP), and appropriate high-throughput screening or selection methods. Mutants with desirable properties such as enhanced enzymatic activity, improved environmental durability, and even novel catalytic activities, can be obtained by these strategies. Directed evolution and rational design can further improve the properties of enzymes.

The architecture of protein domains was proposed to have evolved via the combinatorial assembly or exchange of preexisting polypeptide segments. The recombined modular units may be simple secondary structural elements or larger fragments of the subdomain. This process can result from exon shuffling, nonhomologous recombination or alternative splicing and can be simulated by selecting folded proteins from combinatorial libraries of shuffled secondary structure elements (Urvoas et al., 2012). Despite the fact that directed evolution is a practical and efficient method for improving the properties of enzymes, a

trade-off between the targeted property and other essential properties often hinders the efficiency of this method.

6.3.2 Site-Directed Mutagenesis

Site-directed mutagenesis is an invaluable tool to modify genes and study the structural and functional properties of a protein, based on the structure, function, catalytic mechanism, and catalytic residues of enzymes. Site-directed mutagenesis includes single and combinational mutations. It is usually analyzed by bioinformatic methods. Single site-directed mutagenesis and multiple mutations have been used to expedite and simplify methods for mutagenesis ([Hsieh and Vaisvila, 2013](#)). The properties of enzymes can be improved markedly by the combination of site-directed mutagenesis with other methods. For instance, the yield of maltose-binding protein-fused Hepl from recombinant *E. coli* was significantly improved (30.6% increase) by a thermostabilization strategy combining site-directed mutagenesis and calcium ion addition ([Chen et al., 2013](#)).

6.3.3 Saturation Mutagenesis

Site-directed saturation mutagenesis is a unique method for rapid laboratory evolution of proteins. Each amino acid of a protein is replaced with each of the other 19 naturally occurring amino acids. Hotspot sites of enzymes are performed by saturation mutagenesis and variants show improved thermostability or catalytic efficiency. A new method called combinatorial coevolving-site saturation mutagenesis has been suggested by [Wang et al. \(2012\)](#), in which the functionally correlated variation sites of proteins are chosen as the hotspot sites for mutant libraries. This method enhanced the thermostability of wild-type α -amylase from *B. subtilis* CN7 by 8°C ([Wang et al., 2012](#)).

A challenge is how to maximize the quality of mutant libraries and the degree of catalyst improvement. Libraries with higher efficiency require less screening effort. Iterative saturation mutagenesis (ISM) first randomizes appropriate sites in protein comprising through formation of focused libraries. ISM involves (1) randomized mutation of one or more sites, (2) screening of the initial mutant libraries for properties of catalytic efficiency, stereoselectivity, or thermal robustness, (3) use of the best hit in a given library as a template for other sites, and (4) continuation of the optimization process until the expected improvement is achieved ([Gumulya et al., 2012](#)). The standard employed to choose proper randomization sites is different according to the catalytic properties. Stereoselectivity and substrate scope are determined by the combinatorial active-site saturation test ([Reetz and Carballera, 2007](#)). Because only small mutant libraries in the range of 100–3000 transformants are required, the screening effort is minimized. ISM has proven more efficient than all previous systematic efforts using ep-PCR with various mutation rates, saturation mutagenesis at hot spots, or DNA shuffling ([Reetz et al., 2010](#)).

6.3.4 Truncation

Some domains of enzyme proteins are unnecessary for enzyme activity. Thus, random or directed truncation including site-directed truncation and random truncation, has been used to change the enzyme properties, through which truncated enzymes or a truncation library can be obtained. After truncation, the endo-dextranase mutant TM-NCG Δ from *Streptococcus* mutant ATCC 25175 exhibited hydrolytic activity on 0.4% dextran T2000 that was similar to that of SmDex90, and displayed 1.4-fold and 2-fold increased activity on 0.05% dextran T2000 and T10, respectively (Kim et al., 2011).

6.3.5 Fusion

Recently, chimeric enzymes have been constructed with improved thermostability, catalytic activity, substrate specificity, or product selectivity. Most chimeric enzymes are made by fusing the catalytic domain and binding the substrate domain from different enzymes. For instance, carbohydrate-active enzymes often contain a catalytic module and a carbohydrate-binding module (CBM), connected by a flexible linker. A CBM is defined as a contiguous amino acid sequence from a carbohydrate-active enzyme that folds as a separate domain and shows carbohydrate-binding capability (Christiansen et al., 2009). Based on amino acid similarities, there are 54 defined families of CBMs, which display substantial variation in ligand specificity. CBMs are often fused with other enzymes to create new chimeric enzymes. In addition, other functional genes or oligopeptides can be used to construct new chimeric enzymes with multiple activities. For instance, the fusion alkaline α -amylase containing peptide AEAEAKAKAEAKAK exhibited improved catalytic efficiency, alkaline stability, thermal stability, and oxidative stability as compared to the wild-type (Yang et al., 2013a,b).

6.4 CONCLUDING REMARKS

Microbial proteins can be expressed in cell cultures of recombinant bacteria, yeasts, or filamentous fungi. The *E. coli* expression system continues to dominate the bacterial expression systems. Yeasts can grow to high densities rapidly, and the expression level can be regulated by simple medium manipulation. *Bacillus* systems are high secretors and useful host strains, and are preferred for the homologous expression of recombinant enzymes (eg, proteases and amylases). Filamentous fungi became attractive hosts for recombinant DNA technology due to high secretion levels of bioactive enzymes after posttranslation. The application of lactic acid bacteria needs to be further explored, although they are safe and highly effective expression systems. The challenges to meet are mainly in terms of quantity, quality, and cost-effectiveness. An insight into the proteome is necessary for systems-level understanding. It is possible to obtain cost-efficient expression systems with better vectors and promoters by fermentation

techniques and current advances in order to meet the increasing demand for enzyme production.

The reported protein engineering strategies mainly include directed evolution, site-directed mutagenesis, saturation mutagenesis, terminal fusion, and truncation. To select an engineering strategy for a protein, we can use directed evolution, terminal fusion, or truncation based on analysis of gene sequence if the structural information is not given, or use site-directed mutagenesis or other methods after obtaining structure information from experimental data. Molecular design of bioinformatics is widely applied in the optimization of catalytic performance due to rapid developments of technologies. In the future development of rational synthesis, more synthetic enzymes that include three characteristics will be produced: (1) optimum properties, (2) a great distance in sequence space from the amino acid sequence of the most highly homologous enzymes, and (3) combinations of rational design with directed evolution.

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Chapter 7

Metagenomics and the Search for Industrial Enzymes

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7.1 INTRODUCTION

As per published reports there will be a 28% increase in the next 15 years in the world's population (OECD, 2006; Sawaya and Arundel, 2010; Zúñiga et al., 2014) and we will surpass the maximum rate of oil extraction (peak oil) (Jackson and Smith, 2014; Timmis et al., 2014) (Fig. 7.1). This increase will require a significant effort in relation to the implementation of biotechnology in industrial sectors, of which it currently makes up 7% and is estimated to increase to circa 40% by 2030 (Sawaya and Arundel, 2010; Zúñiga et al., 2014) (Fig. 7.2). This change will entail an increase in the annual demand for new enzymes, which currently is growing almost 7% per year and is expected to be close to 10% in 2030 (Fig. 7.3). These data demonstrate that enzymes and the biotechnological and renewable solutions obtained from them will have an important impact in the near future. The issue to address at this point is that the general introduction of enzymatic synthesis into the chemical industry is much slower than previously thought (Bornscheuer et al., 2012; Timmis et al., 2014), despite its increasing prevalence: first, because the number of enzymes available is very limited; and second, because the enzymes available are usually not capable of promoting the desired chemical reactions at the wished extend. Moreover, the process of producing *de novo* an enzyme capable of catalyzing such reactions is slow and expensive. The reason for this limitation is that no efforts have been made in the search and characterization of truly promiscuous and versatile enzymes for the systematic and organized completion of several chemical reactions (Hult and Berglund, 2007; Rashamuse et al., 2009; Beloqui et al., 2010; Alcaide et al., 2013; Drepper et al., 2014; Martínez-Martínez et al., 2014; Pandya et al., 2014). With these factors in mind, from where should these enzymes be obtained? Below we provide answers to this important question relevant for future advances in biotechnology.

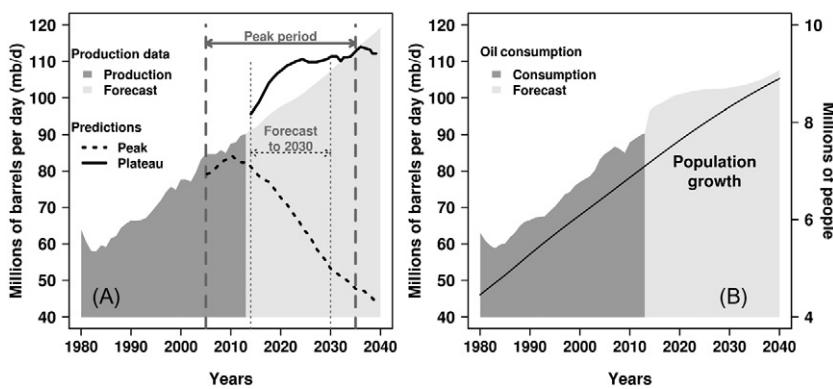


FIGURE 7.1 Past, present, and forecast predictions of the oil consumption and population growth (A) and oil extraction (B). In Panel A, evolution and forecast production data (gray color graph) are shown on the left axis, whereas two-types of predictions (solid and dashed lines) are shown on the right axis. In Panel B, population growth (right axis) is represented by a solid line, while oil consumption (left axis) is shown in the gray color graph. Data have been adapted from Jackson and Smith (2014) and the IEA Releases Oil Market Report (<http://www.iea.org>).

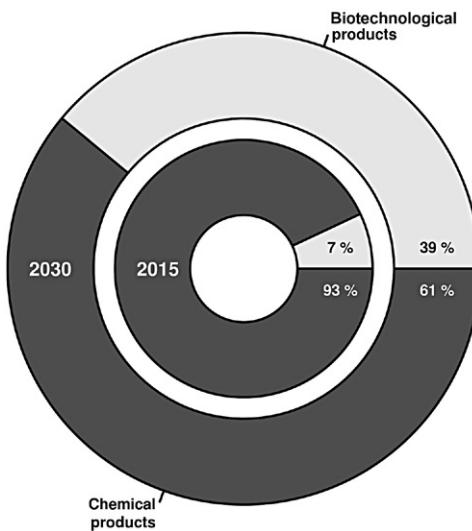


FIGURE 7.2 Implementation of biotechnology in industrial sectors. Actual and forecast predictions by 2030 are indicated. Data have been adapted from Sawaya and Arundel (2010), Zúñiga et al. (2014), and the World Enzymes to 2017 Report (www.mrmarketresearch.com/world-enzymes-to-2017-market-report.html). Note, that industrial biotechnology sectors such as fermentations and biofuels are not considered.

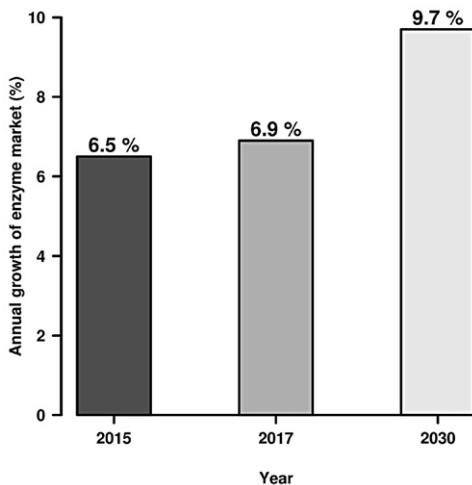


FIGURE 7.3 Evolution of the annual demand of enzymes in the market. Actual and forecast previsions by 2030 are indicated. Data have been adapted from [Sawaya and Arundel \(2010\)](#), [Zúñiga et al. \(2014\)](#), and the [World Enzymes to 2017 Report](#) (www.rnrmarketresearch.com/world-enzymes-to-2017-market-report.html).

7.2 THE DILEMMA BETWEEN KNOWN, ENGINEERED, OR NOVEL ENZYMES

A first approach to identify enzymes for chemical reactions of interest would be to use commercial biocatalysts previously extracted, isolated, or purified directly from biological sources, mainly microorganisms (Song et al., 2005; Esteban-Torres et al., 2014). In fact, from the wide range of sources of commercial enzymes being used industrially, 88% are from microorganisms and the remaining are divided between animal (8%) and plant (4%) sources (Gurung et al., 2013). However, they are very limited, and advances in molecular biology, biochemistry, and microbiology have allowed for other more efficient options, that are detailed below.

A second approach would be to improve the known biocatalysts (Höhne and Bornscheuer, 2014). Previous findings in the literature on both the theoretical and experimental level have demonstrated that it is possible to design enzymes that do not exist in nature or that exhibit very different properties compared to those that currently exist. Thus, it is possible to design enzymes with great increases in activity and specificity towards a great number of molecules (Reetz et al., 2010; Brustad and Arnold, 2011; Alcaide et al., 2013; Nobili et al., 2013; Liu et al., 2014; Sheng et al., 2014). These enzymes have sequences or modifications produced through the use of recombinant techniques. Such mutations can cause structural changes that produce a new enzyme containing an active site specific for the reaction and substrate for which the screening method

was designed. In this process, known as protein engineering, new and/or better enzymes can be generated (Brustad and Arnold, 2011; Mate and Alcalde, 2015). Based on these results, the process is iteratively repeated under desired conditions to produce the variants that fulfill the desired requirements. The technical advances associated with systems known as high-throughput screening provide a competitive advantage. A literature review related to the application of these methods in protein engineering studies reveals that the production of mutant libraries is relatively easy and has limited risk. Additionally, they have success rates, meaning the rates of identifying an improved mutant within a mutant library compared to the native enzyme, ranging between 1:140 and 1:125,000. However, while published studies indicate the possibility of creating new enzymes, the introduction of such enzymes to the industry is very limited (Woodley, 2013). Moreover, it would be expensive to start a protein engineering experiment for each one of the known enzymes and reactions.

A third option would be to identify new biocatalysts (Fernández-Arrojo et al., 2010). Unlike protein engineering, this alternative has the capacity to access truly novel proteins. However, given that there are so many enzymes in nature, how can we search for the most appropriate enzymes for each process of interest? Furthermore, how can it be known which enzymes will have the most promising properties to help them reach industry? These two last questions are currently very difficult to address. However, it is known that microbial diversity is and will be the greatest source of enzymes. Indeed, life in ecosystems constantly evolves, giving rise to great biodiversity. Although the actual scope of microbial biodiversity is unknown, recent studies have revealed that only approximately 11,000 Bacteria and Archaea species have been classified so far, and each year, at least 600 new species are described (Kyrpides et al., 2014; Yarza et al., 2014). Recent estimates have indicated that almost 1000 years would be needed to characterize the approximately 30 million species remaining, 25% of them in seas and oceans (Mora et al., 2011; Yarza et al., 2014). These numbers provide a reference not only for the widely unexplored potential in our planet and the microbial communities inhabiting it but also for their potential in terms of new genes and activities (Kyrpides et al., 2014). Such activities are largely unknown, although their potential for future economic development has been widely recognized (Jemli et al., 2014; Timmis et al., 2014).

7.3 METAGENOMICS AND ITS APPLICATION TO ENZYME RESEARCH

It has been known since the beginning of the last century that most microorganisms cannot be cultured (Puspita et al., 2012); therefore, their enzymes cannot be accessed using conventional methods (Niehaus et al., 2011). As a response to this need, in 1998, the term “metagenomics” was introduced as the application of genomics to the study of microbial communities and enzymes directly extracted from the environment without the need for culturing (Handelsman

et al., 1998). Metagenomics is analogous to genomics, where the genome is not derived from a single organism but, rather, from a whole community. Using this technique, the search for new enzymes can be performed via either massive searches through direct sequencing of environmental genomic material (Mende et al., 2012) or through selection of interesting activities in clone libraries created from environmental DNA (Niehaus et al., 2011; Chistoserdova, 2014; Alcaide et al., 2015).

Metagenomics is a culture-independent technique that makes it theoretically possible to study any type of sample and offers us the possibility of studying DNA from an entire organismal community. In the last 25 years, there has been much talk about the fact that with metagenomic techniques, new enzymes can be accessed with no limit. However, the reality is quite different. Thus, to date, only about 2100 different locations worldwide have been explored using these techniques, 60% of which correspond to marine samples, and new enzymes have been identified in only approximately 11% of them. It is noteworthy that an exhaustive review of the databases and bibliographic records revealed that from these limited number of explored sites about 6100 clones containing active enzymes or purified enzymes have been directly extracted (Fig. 7.4).

These findings demonstrate that only a small fraction of the environment has been tracked at the enzyme level. There is an additional problem: sequencing costs have been reduced by three orders of magnitude. Thus, while 25 years ago,

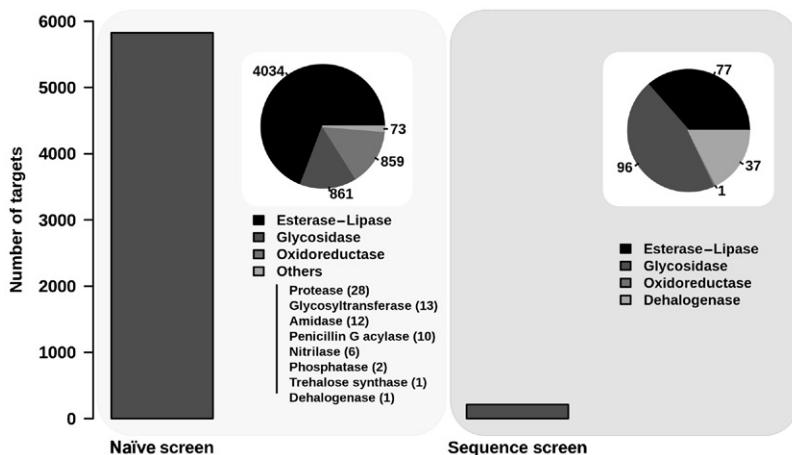


FIGURE 7.4 A survey of the total number of targets (clones and/or single enzymes and/or sequences encoding enzymes) identified by metagenomic studies. The figure is based on studies that were published over the last two decades using naïve (left) and sequence-based (right) screen protocols. The databases used to provide such estimations were SCOPUS, PubMed, WOK, IMG of the US Department of Energy Joint Genome Institute (<http://www.jgi.doe.gov/>), and UniProtKB/Swiss-Prot. The distribution of selected targets as per enzyme type is shown per each of the two screening methods.

450 enzymes out of 1000 had been properly annotated and/or characterized, currently this proportion approaches 0 (Anton et al., 2013), and only 3 out of 100,000 are commonly characterized (Fig. 7.5). It is so inexpensive to sequence DNA either from environmental samples or from pure cultures that databases are full of sequences coding for enzymes no one has characterized (Box 7.1).

Clearly, we must make more effort regarding the search, production, and development of new enzymes if we desire new economic growth (Sawaya and Arundel, 2010; Zúniga et al., 2014). Current estimations have shown that it takes at least 11–18 years for the discovery of a marketable biotech-based drug. However, currently, at least 7 years are required to discover and use a new enzyme from an environmental sample, and it is predicted that this process will be competitive only if it is reduced to 3 years. This reduction will require progress in several areas, including improvements in sampling and selection of

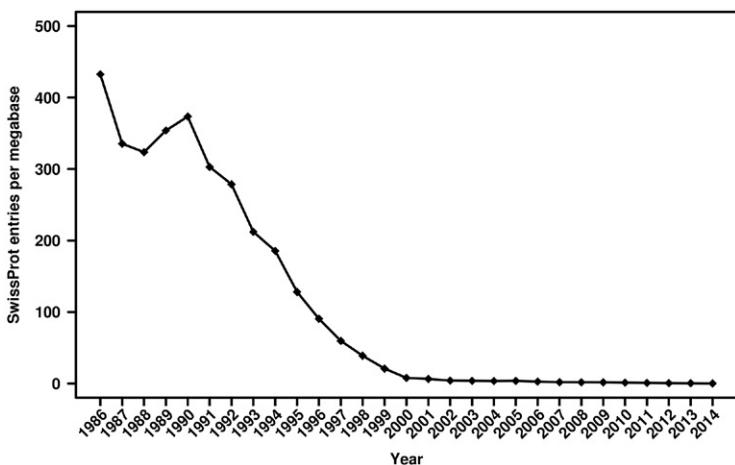


FIGURE 7.5 Evolution in the number of sequences encoding proteins/enzymes being properly annotated and/or characterized. The databases used to provide such estimations were SCOPUS, PubMed, WOK, IMG/M of the US Department of Energy Joint Genome Institute (<http://www.jgi.doe.gov/>), and UniProtKB/Swiss-Prot.

BOX 7.1 Gap Between Enzyme Prediction and Characterization

The revolution in the high throughput DNA sequencing technologies has resulted in a significant reduction of the sequencing costs and thus led to an explosion of in silico data production and dramatic expansion of the databases (Mende et al., 2012). In contrast to that, the pipelines for enzyme isolation and characterization operate at much lower rates and throughputs (Chistoserdova, 2014), opening the gap between the numbers of microbial enzymes predicted in silico and those experimentally characterized in the lab and used at the industrial scale.

interesting activities, expression systems for environmental DNA and the genes it contains, sequencing techniques, and characterization and data integration systems, that will be discussed later. Above all, improvement is required in the process of integration of the new enzymes in biocatalysis and biotransformations. This is currently very rare as integrated research covering all aspects of the entire process ranging from biocatalysts identification and/or optimization to establishment of an industrial process has been neglected by international research. As an example, many research centers do have extensive collections of enzymes and microorganisms and/or sequence information about them, but they have not been tested in production processes of interest. Conversely, many research centers do have ready-to-test immobilization techniques and production processes but do not have access to new biocatalysts. Improvements in such integrated investigations will increase the success rate because, currently, to produce 1 enzyme at an industrial scale, at least 100,000 environmental enzymes must be characterized. This limitation exists because enzymes normally cannot fulfill industrial criteria, such as activity under adverse conditions that are very different from natural conditions, high selectivity, and high reaction rate (Singh, 2010; Martínez-Martínez et al., 2013).

It is also important to identify the difficulties in finding marketable environmental enzymes. The biggest difficulty is the long period required for experimental validation of the enzymatic activities, resulting in a delayed entrance into the market and high costs. Only a few enzymes from available collections of environmental enzymes described in the literature (<5%) fulfill the required criteria for the industrial scale (Niehaus et al., 2011; Martínez-Martínez et al., 2013). The low promiscuity (or low number of substrates capable of being efficiently transformed), low selectivity, and low tolerance to reaction conditions, for example, temperature, are concrete and recurring problems. Other problems are low activity at low/high pH and the low tolerance to high salt concentrations and solvents (Blomberg et al., 2013; Turner and Truppo, 2013; Vergne-Vaxelaire et al., 2013). Despite these factors, the demand for new enzymes to meet industrial criteria is very high (Esteban-Torres et al., 2014; Pandya et al., 2014). The expression and development stages of the process constitute the most costly steps in terms of time and money. In fact, in many cases, these stages constitute more than 90–95% of the overall costs of a biotechnological development process. Clearly, future research windows should be based on the hypothesis that “to produce new molecules,” it is crucial to identify and incorporate new enzymes in the market at reasonable time and cost. As mentioned above, it is agreed that a 3-year process is currently a suitable limit for such developments and for a new enzyme to be competitive against the existing ones.

Altogether, current enzyme collections are limited, and we need to access new enzyme sources. Metagenomics offers this opportunity, but only a few enzymes isolated using these techniques have been thoroughly characterized at the functional and structural levels (for details see Martínez-Martínez et al., 2013; Alcaide et al., 2015). Finally, if we wish to produce new biotechnological

advances and to reduce the time required to introduce new enzymes to the market, we must make efforts in the deep characterization of new enzymes.

7.4 SUCCESS STORIES OF NAÏVE AND DIRECT SEQUENCING SCREENS FOR NEW ENZYMES

Based on bibliographic records, the establishment of enzyme collections from environmental genomes is no longer a problem. However, while the production of clone libraries represents a limited risk, according to literature data from the last 25 years, the low incidence of positive clones is problematic. As a technique, it is possible to access, with no advanced technical resources, a mean of 44 active clones per clone library of approximately 53,000 clones. The incidence of positive clones, that is, the number of positive clones compared to the total clones screened, oscillates between 1:11 and 1:193,200. Nonetheless, current searches based on clonal screening make it possible to find hundreds of enzymes using a single substrate in a timeframe from a day to a couple of months. Culturing or preenrichment under selective conditions can significantly accelerate the selection of interesting enzymes acting on particular substrates or molecules (Jacquiod et al., 2013; Verastegui et al., 2014).

The incidence (or frequency at which a positive clone appears in a clone library) depends on the type of enzyme or activity to be selected, the abundance of genes coding for such an activity in the microbial genomes present in the sample analyzed, and their activity levels. Thus, clones containing enzymes with low activity cannot be identified in the time usually required for colorimetric assays, which varies in the range of seconds to months. For example, in the search for esterase/lipase activity with tributyrin (1% vol/vol), the typical assay time varies from 12 h to 8 days (Jeon et al., 2011); for glycosidases, from a minute scale to 30 days (Vester et al., 2014); for proteases, from 12 h to 4 weeks using skim milk (Niehaus et al., 2011); for phytases, up to 5 days using phytate (Tan et al., 2014); and for mono- and dioxygenases from 2 h (using styrene/NADPH) to 3 days (for the production of pigments such as indigo) (Singh, 2010).

At the enzyme level, considering the six most studied activities at the metagenomic level in the literature, some activities are more common than others, that is, they can be found in higher proportions compared to others in clone libraries. The following order is observed in average incidence rates: acylases (1 positive clone for every 333 clones [1:333]), phosphatases (1:2843), oxidoreductases (1:3370), proteases (1:9388), esterases and lipases (1:17,320), and glycosidases (1:31,190). Another factor affecting the efficacy of screenings is the substrate to be used. Thus, in a recent study, the incidence varied from 1:188 to 1:3937 and 1:15,625 when using 1% (vol/vol) of tributyrin, tricaprylin, and triolein, respectively, when screening the same clone library for esterase/lipase activity (Glogauer et al., 2011). This variation suggests that enzymes with lipase activity are much less abundant (in this study, 83-fold) than those

with esterase activity. Among the most used substrates and methods for the screening of esterase and lipase activities, those based on the use of pH indicators result in a higher incidence of positive clones (1:29), followed by methods using polylactic acid (1:13,334), tributyrin (1:15,478), α -naphthyl acetate (1:19,925), polyethylene terephthalate (1:21,400), triolein/olive oil and rhodamine B (1:22,061), Tween-20 (1:26,496), methyl and ethyl ferulate (1:26,496), 5-bromo-4-chloro-3-indolyl phosphate caprylate (X-caprylate) (1:50,000), and tricaprylin (1:68,279), just to mention the most common substrates and types of activities widely studied. Data reported in the literature have also demonstrated that in many cases, it is better to use wide-spectrum model substrates capable of being transformed by most described enzymes being sought, exposing the chosen clones to selective screenings with the molecule or substrate of interest. This strategy has been tested successfully for the search of clones with specific hydrolytic activities against esters of (S)-ketoprofen (Yoon et al., 2007) or pyrethroids (Li et al., 2008), first selected using screenings with α -naphthol or X-caprylate, respectively.

Besides the selection assays and cloning and expression systems described previously, bioinformatic tools allow a preselection of enzymes that can be further studied in detail (Kube et al., 2013; Nyssönen et al., 2013; Schallmey et al., 2014). The metagenomics boom has been possible due to the emergence of high-throughput technologies for low-cost DNA sequencing (Akondi and Lakshmi, 2013). In this regard, the onset of the Human Genome Project in 1990 started a technological revolution. The development of these techniques has allowed the sequencing of both single and environmental genomes. For example, the GOLD database (*The Genomes On Line Database*; <http://www.genomesonline.org/>) has 58,285 records of ongoing sequencing projects, of which 6620 have been completed. In direct sequencing, a group of nucleotide triplets, known as an open reading frame (ORF), is obtained. Each one of these triplets encodes an amino acid located between a start codon formed by three nucleotides (ATG) and a termination codon, which usually has one of these nucleotide combinations TAA, TAG, or TGA. From the set of sequences obtained in any study, approximately 2–5% of the ORFs in an entire genome code for interesting enzymes (Guazzaroni et al., 2015). This calculation means that there are few enzymes with different interesting activities compared to the total number of genes coding for enzymes in microbial genomes. One of the biggest limitations of methods for the search of sequences coding for enzymes is that the *in silico* functional predictions are based on homology. Currently, bioinformatic and sequence-based modeling tools are being developed that will allow researchers to identify sequences coding for interesting enzymes in data obtained from massive sequencing efforts (Anton et al., 2013).

The sequences obtained using massive sequencing methods can be filtered according to their similarities with databases of general sequences (UniProt, NCBI NR) and databases with conserved domain sequences (Pfam and *Common Domains Database* [CDD]). They can also be filtered using specific

databases containing sequences and biochemical information for certain groups of enzymes, such as the *Carbohydrate-Active Enzyme* database (Cantarel et al., 2009; Tasse et al., 2010), which is used to identify glycosidases, and a database for identifying laccases (accessible since 2011) (Sirim et al., 2011), peroxidases (Fawal et al., 2013), oxidoreductases (Duarte et al., 2014), and the lipase engineering database (Fang et al., 2014; <http://www.led.uni-stuttgart.de/>). High-performance bioinformatic methods have been successfully used for searches or screens within sequences accessible in databases or generated via massive sequencing of genes coding for epoxide hydrolases and haloalkane dehalogenases (Barth et al., 2004). Prediction tools such as antiSMASH have been recently developed for the search of genes coding for enzymes for the synthesis of secondary or bioactive metabolites, such as lactones, bacteriocins, siderophores, ectoine, and polyketides (Blin et al., 2013, 2014). Schallmey et al. (2014) have also used conserved motifs to identify 37 new halohydrin dehalogenases, which were very promiscuous after experimental verification, when public databases were examined. It is important to mention that in a second stage of analysis, for each selected sequence, it is possible to obtain general information, such as the molecular mass and pK, and information regarding the existence of conserved domains or structures that are specific for the desired activity (Marsh et al., 2012).

A comparison between the numbers of sequences encoding enzymes of industrial interest identified so far revealed that the naïve screens with appropriated substrates are by far the methods that have provided the biggest collection of active clones or enzymes (Box 7.2) in the bibliography, followed to a much lower extent (c. 27-fold) by the massive sequencing screen methods (Fig. 7.4).

BOX 7.2 Functional Metagenomic as Major Source of Characterized Enzymes

There has been much talk about the fact that low-cost DNA sequencing technologies will be the major source of environmental microbial enzymes. They may substitute naïve screen methods where the type of vectors, hosts, and availability of chemicals for screenings are limiting the success (Vester et al., 2015). However, one should consider the low proportion of genes present in metasequences encoding for industrial enzymes compared to the total number of genes in microbial metagenomes, and the yet-problematic assignations of activities to hypothetical sequences. This required extensive sequencing efforts to avoid obtaining partial sequences encoding enzymes of interest (Cantarel et al., 2009; Tasse et al., 2010) and the design of advanced bioinformatics. Having said that, bibliographic records revealed that naïve screening is actually the major source of characterized enzymes (Fig. 7.4). However, both methods should be performed in parallel. For example, the prescreening of clone libraries by naïve screens followed by the pyrosequencing of the inserts led to a 106-fold increase in the success rate of identifying genes encoding enzymes of interest as compared to direct sequencing approaches (Tasse et al., 2010).

7.5 SUCCESS STORIES FOR INTRODUCING ENVIRONMENTAL ENZYMES INTO THE MARKET

Funding agencies, worldwide companies, and laboratories have adopted a number of actions, and there are ongoing research activities to decrease this time-frame and the very expensive and time-consuming biocatalysts optimization phase while increasing the efficiency of the processes. However, there are very few cases in which a new environmental biocatalyst has been brought to a process in recent times (Fernández-Arrojo et al., 2010). In fact, only a few metagenomics-based enzyme products have been patented and placed in the market. Having said that, industrial enzymes will have to be novel and not found in the patented literature, since this is the only chance for new enzymes to make an impact “beyond the state of the art.” In this sense several metagenomic enzymes have been patented, for example, nitrile hydratases (EP2369009A3), soil-metagenome-derived gene *wes* (WO2013125808A1), cow rumen-derived esterases (EP04015920.4), cellulases (EP04015680.4), laccases (GB01P006EP), and an esterase from uncultured microorganisms able to degrade terephthalate esters, an important component of bioplastics (WO 2007017181). It is important to note that the enzyme discovered is not only key when patenting, but also its application: the use of a new enzyme for the same industrial application would infringe the “inventive” requirement for any new IP application, and for this reason finding new applications for new enzymes is also desired. Having said that, targets in common metagenomic investigations are enzymes that are predominantly used in biocatalysis and industrial sectors (ie, food, laundry, biofuels), such as nitrilases, transaminases, ketoreductases, glycosyl hydrolases, and lipases/esterases (Fernández-Arrojo et al., 2010; Bayer et al., 2011; Gong et al., 2013; Vergne-Vaxelaire et al., 2013), due to their remarkable market potential. Fig. 7.4 shows that within them, lipases/esterases and glycosidases are the most selected enzyme targets for metagenomic investigations whatever the screen method used (naïve or sequence-based screens). Other enzymatic activities which are redundant in bibliography are summarized in Fig. 7.4.

7.6 ENZYME SEARCH: LIMITATIONS OF METAGENOMICS AND SOLUTIONS

There are several problems limiting the use of metagenomics for the identification of new enzymatic activities, and new developments are being performed to reduce the time required from the identification of an enzyme to its application in technological developments (Jemli et al., 2014). The biggest technological challenges are: (1) the low number of genes present in metagenomic DNA coding for enzymes with activities of interest (Guazzaroni et al., 2015), (2) the low proportion of enzymes selected under conditions required in industrial processes (Martínez-Martínez et al., 2013), (3) the lack of relevant industrial substrates for functional screenings (Fernández-Arrojo et al., 2010), (4) the low

efficacy of screening methods for rare activities (Alcaide et al., 2013), (5) the low performance of enzymes isolated using these techniques under nonnatural conditions (Fernández-Arrojo et al., 2010), (6) the high number of identified enzymes that are inactive after expression in *Escherichia coli* (Loeschke et al., 2013), (7) the lack of reliable bioinformatic sources for the analysis of massive sequencing data (Nyssönen et al., 2013), and (8) the lack of reliable systems to predict enzymatic activities in sequences coding hypothetical proteins (Anton et al., 2013; Bastard et al., 2014).

To overcome such problems, in the recent years, work has focused on several solutions including: (1) a more selective screening plan for enzymatic activities from extensive clone libraries and for the selection of genes coding for interesting enzymes (Yoon et al., 2007), (2) a preenrichment under conditions similar to those required by processes or biotransformations (Jacquiod et al., 2013; Verastegui et al., 2014), (3) identification of genes and proteins being expressed in selective enrichments under conditions and substrates of interest—this will select enzymes with higher specific activities towards substrates of industrial interest (Akeroyd et al., 2013; Chang et al., 2013), (4) prioritizing the selection of enzymes with multiple activities, wide substrate specificity ranges, and high stability in a wide range of conditions (Alcaide et al., 2013) to allow the use of the same enzyme in several processes and under different conditions, (5) on demand de novo synthesis of small molecules or substrates with similar functions as those required in industrial scales to be used in screenings or selective enrichments (Lim et al., 2013), (6) on demand development of vectors and hosts for the screening and expression of genes coding for interesting activities (Loeschke et al., 2013; Terrón-González et al., 2013; Liebl et al., 2014), (7) *in silico* or experimental design using protein engineering for new enzymes identified by metagenomic techniques to produce more favorable biotechnological variants (Alcaide et al., 2013), (8) development of a computational workflow for the identification of sequences encoding enzymes based on structure-based function prediction (Watson et al., 2007), active sites modeling, and clustering (de Melo-Minardi et al., 2010) through the integration of customized bioinformatic methods, and (9) development of techniques known as “*Unknown BLAST*” that allow the mapping or search of orthologs to enzymes with unknown activities (Anton et al., 2013).

7.7 CONCLUDING REMARKS

Currently, there has been a substantial increase in the number of enzymes obtained and characterized using metagenomic techniques (Martínez-Martínez et al., 2013). While the results indicate a high potential for interesting reactions, we must consider that in a biocatalysis process, as in any chemical reaction in general, the solvent or reaction medium is often as important as the substrate’s structure and the enzymatic mode of action. Therefore, this variable adds an important factor of complexity when designing screening protocols for, and biotransformation processes with, new enzymes.

It is well known that biotechnological developments are easier when starting from commercially available enzymes, whereas these are much more complex, expensive, and time-consuming when a new enzyme must be identified and produced. Therefore, the chance of using metagenomic techniques in industrial processes can be mostly afforded by large industrial corporations with proportionate technical capacity, whereas for most SMEs and small labs such a possibility is limited. This is especially noticeable when the sales volumes of the compounds to be produced with the new enzymes are low and commercial margins are very tight. Thus, only when the identification and production of new enzymes become cost-efficient in terms of time and money, may SME and small labs compete with large corporations.

Therefore, in the future, basic research in biocatalysis should be focused on the systematic and organized resolution of chemical reactions with industrial applications via the use of newly discovered enzymes. Special focus should also be paid to those reactions with high application potential but that are currently framed in poorly developed chemical reactions (Kourist et al., 2007; Liu et al., 2014; Martínez-Martínez et al., 2014). Regarding this point, the addition of a catalogue accessible to companies and academic groups (Timmis et al., 2014) where the general properties of such enzymes are summarized would be especially interesting and should be completed to attract funding or to start collaborations in the field of biocatalytic development. Second, assays of enzymatic capacities in the presence of organic and nonaqueous media should be performed to increase the catalytic capacities of the enzymes (Khan and Jithesh, 2012; López-López et al., 2014). Moreover, a detailed study of the effects of the immobilization processes on the enzymatic activities of the different enzymes should be performed (Bommarius and Payne, 2013). This study should be independent of the evident improvements they show in terms of being recovered at the end of the processes and being used again and the improvements of their resistance to environmental effects and solvents. Only through the study of these and other variables can we acknowledge the potential of new enzymes for future biotransformations of basic and applied interests.

Finally, we would like to mention that in the study of biocatalytic processes, it is of paramount importance to have a deep knowledge of the mechanism of a particular chemical reaction to understand how the enzyme modifies the transition energy states undergone through the catalytic process to reduce the amount of energy required for the chemical reaction to take place. This study, which requires biochemical characterization and the crystallization of the enzyme to determine its structure, especially the active site, and a noticeable calculation capacity, must be performed specifically for each new marketable environmental enzyme. The new structures elucidated can open up future studies along these lines in which the use of molecular dynamics techniques, quantum mechanics, and other analysis systems (Martín-Garcia et al., 2013; Mendieta-Moreno et al., 2014) could be of interest, along with tools for directed and random mutagenesis. Altogether, this may produce optimized biocatalysts from wild-type environmental variants.

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Chapter 8

The Pocket Manual of Directed Evolution: Tips and Tricks

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8.1 INTRODUCTION

Directed molecular evolution is a powerful engineering strategy to obtain enzymes that are tailor-made for specific biotechnological processes, ranging from industrial and environmental applications to drug discovery. Similarly, such studies offer insights into protein structure-function relationships and natural protein evolution (Bloom and Arnold, 2009; Tracewell and Arnold, 2009; Bornscheuer et al., 2012; Goldsmith and Tawfik, 2012). This methodology recreates the Darwinian principles of mutation, recombination, and selection in the laboratory, but collapsing the evolutionary timeline from thousands of years to months or even days. For more than two decades now, members of the six enzyme classes have been engineered by directed evolution (Jackel and Hilvert, 2010; Cobb et al., 2013; Martínez and Schwaneberg, 2013). Indeed, the limits of directed evolution are now being pushed beyond those imposed by nature, as proven by enzymes evolved to catalyze chemical reactions that have no known natural counterparts (Renata et al., 2015) or those that act in nonnatural environments (Zumarraga et al., 2007; Lehmann et al., 2012; Mate et al., 2013a,b; Alvizo et al., 2014).

In recent years, excellent protocols for library creation and screening have been published in comprehensive directed evolution handbooks (Brakmann and Johnson, 2002; Arnold and Georgiou, 2003a,b; Brakmann and Schwienhorst, 2004; Gillan et al., 2014). Thus, the purpose of this pocket manual on directed evolution is to provide a quick but wide glance at the most common techniques and methods used in protein evolution, as well as to mention new research trends in this groundbreaking field of biotechnology. Throughout this guide,

you will find our own personal views on how to perform directed evolution in the most efficient manner. We will pay special attention to the evolutionary methods developed by laboratories elsewhere, while offering tips and tricks to overcome bottlenecks during the step of library construction and exploration, all of which can help carry out more productive directed evolution studies.

The first section of the guide deals with the variety of techniques used to construct mutant libraries in order to generate DNA diversity. The second section discusses the use of computational tools in laboratory evolution, while the third part details the most representative host organisms employed for directed evolution. The fourth section describes the selection and screening technologies currently used to evaluate mutant libraries, and finally, the last section highlights the new trends in this thrilling research field ([Fig. 8.1](#)).

8.2 METHODS TO GENERATE DNA DIVERSITY

All directed evolution experiments involve the creation of mutant libraries using one or more parental DNA sequences encoding the enzyme/s of interest as the starting point. The target gene(s) is subjected to random mutation and/or recombination, although the fidelity with which these *in vitro* processes mimic nature remains unknown. While the mutagenesis process can be performed with mutagenic strains, the undesired introduction of mutations in regions outside of the gene frontiers (eg, promoters, terminators, selection markers) compromise the quality of the mutant libraries, discouraging their use ([Wong et al., 2006a](#)). Accordingly, most of the *in vitro* or *in vivo* methods used to generate DNA diversity only target the gene under study.

The success of a directed evolution experiment not only depends on the quality of the gene library but also, on the availability of an efficient cloning system. For years, cloning strategies have relied on restriction enzymes to create sticky ends in target sequences, digesting the cloning vector with the same enzymes and then ligating the fragments using DNA ligases. In directed evolution experiments, poor ligation efficiency, incomplete digestion, or the lack of unique restriction sites can hamper standard cloning and diminish transformation efficiencies. To overcome these drawbacks, a series of commercial kits has been released that are based on the use of homologous sequences followed by *in vitro* or *in vivo* recombination, overlapping PCR or the use of megaprimer ([Tee and Wong, 2013](#)), as well as the joining of multiple overlapping DNA fragments in a single-tube reaction ([Gibson et al., 2009, 2010](#)).

8.2.1 Mutagenic Methods

8.2.1.1 Random Mutagenesis

Irrespective of the cloning strategy chosen for your directed evolution experiment, there are two key issues to be considered when incorporating random mutations into the target DNA sequence: the quantity and the quality of the

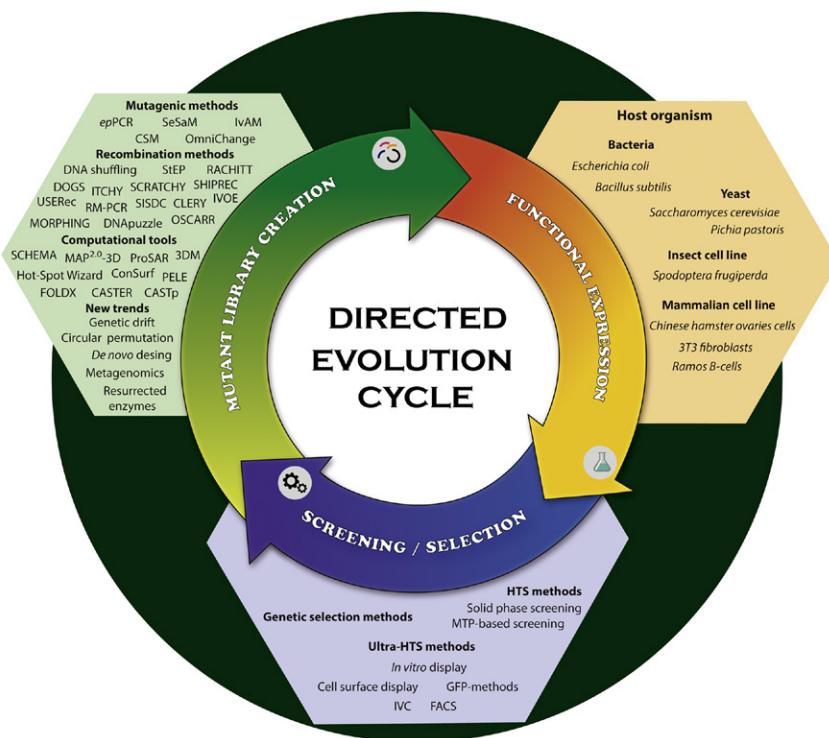


FIGURE 8.1 The process of directed evolution. The methods employed to generate diversity are highlighted in green, the host organisms for directed evolution are shown in orange, and the screening and selection methods are shown in blue: *epPCR*, error-prone PCR; *SeSaM*, Sequence Saturation Mutagenesis; *IvAM*, *In vivo* Assembly of Mutant libraries; *CSM*, Combinatorial Saturation Mutagenesis; *StEP*, Staggered Extension Process; *RACHITT*, RAndom CHimeragenesIs on Transient Templates; *DOGS*, Degenerate Oligonucleotide Gene Shuffling; *ITCHY*, Incremental Truncation for the Creation of HYbrid enzymes; *SCRATCHY*, *ITCHY*+DNA Shuffling; *SHIPREC*, Sequence Homology-Independent Protein REcombination; *USERec*, *USER* friendly DNA Recombination; *RM-PCR*, Random Multi-recombinant PCR; *SISDC*, Sequence-Independent Site-Directed Chimeragenesis; *CLERY*: Combinatorial Libraries Enhanced by Recombination in Yeast; *IVOE*, *In vivo* Overlap Extension; *MORPHING*, Mutagenic Organized Recombination Process by Homologous *In vivo* Grouping; *OSCARR*, One-pot, Simple methodology for CAssette Randomisation and Recombination; *MAP*, Mutagenesis Assistant Program; *PROSAR*, Protein Sequence Activity Relationship; *3DM*, 3-Dimensional Matching; *ConSurf*, Conservation Surface-mapping; *PELE*, Protein Energy Landscape Exploration; *FoldX*, Force field; *CASTER*, Combinatorial Active-site Saturation Test aid software; *CASTp*, Computed Atlas of Surface Topography of proteins; *HTS*, High-Throughput Screening; *MTP*, MicroTiter Plate; *GFP*, Green Fluorescent Protein; *IVC*, *In vitro* Compartmentalization; *FACS*, Fluorescence Assisted Cell Sorting.

mutations. On the one hand, the quantity (referred to as mutational load or frequency) reflects the number of mutations per kilobase pair. Although the sequence of each protein has different tolerance to mutations, the mutational load suitable for directed evolution studies generally ranges from 2 to 7 nucleotide

substitutions per gene (Wong et al., 2006a). A good trick to estimate the mutational load is to construct small mutant libraries with different mutational loads (one or two 96-well plates), evaluating the mutational landscape by counting the number of clones with less than 10% of the parental activity. Typically, a landscape in which 35–50% of the total clones screened have less than 10% of the parental activity is suitable for directed evolution campaigns, although this figure varies in function of the target protein and its activity (Arnold and Georgiou, 2003b). Conversely, the quality of the mutations is determined by the mutational bias, which is strictly dependent on the mutagenic method. For instance, mutagenesis based on DNA polymerases is strongly biased towards a higher rate of transitions (T_s ; change from purine to purine or pyrimidine to pyrimidine) over transversions (T_v ; change from purine to pyrimidine or vice versa). Some mutagenic methods (see below) have been developed to equilibrate the T_s/T_v ratio or to make them independent of the DNA polymerase bias. As a general tip, we suggest combining different polymerases by alternating between them in consecutive generations of evolution.

Leaving aside the expensive synthetic chemistry-based methods for mutagenesis and the use of nonreliable mutator strains, PCR-based methods (also known as error-prone PCR or *ep*PCR methods) are the most commonly employed for random mutagenesis. They are based on the use of low fidelity DNA polymerases and chemicals that interfere with the activity of the DNA polymerase. The DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (*Taq* DNA polymerase) is frequently used in these experiments due to its high error rate, which is linked to the lack of 3'→5' proofreading exonuclease activity. Moreover, the mutational loads in *Taq* libraries can be altered by the addition of MnCl₂, the use of unbalanced dNTP concentrations and the reduction in the concentration of gene template. The GeneMorph II random mutagenesis kit is a valuable alternative to the standard *Taq* libraries as it minimizes the mutational bias by using the Mutazyme II, which is a blend of the Mutazyme I DNA polymerase and a *Taq* polymerase mutant (Stratagene, 2004). Alternative methods to produce a less biased mutational spectrum include SeSaM and IvAM. Sequence Saturation Mutagenesis (SeSaM) is a protocol to modify each nucleotide position of the target gene independent of polymerase bias (Wong et al., 2004). It is a process based on the random introduction of α-phosphorothioate nucleotides that are ultimately replaced by universal bases. The mutational spectrum is dependent on the concentration of α-phosphorothioate nucleotides, the combination of two or more of them, and the preference of the universal base for different base-pairings. Among other examples, the utility of SeSaM has been proven in directed evolution studies on phytases (Shivange et al., 2012), proteases (Li et al., 2012; Martinez et al., 2013), and DNA polymerases (Kardashliev et al., 2014). In vivo Assembly of Mutant libraries with different mutational spectra (IvAM) involves the recombination of two or more mutagenic libraries created by different *ep*PCR-based methods in *Saccharomyces cerevisiae* (Zumárraga et al., 2008). The libraries can be mixed in different proportions to adjust the mutational bias and their mutagenic loads. Thus, IvAM

represents a good alternative when the host organism is *S. cerevisiae*, this yeast having been used comprehensively during the evolution of ligninases (Maté et al., 2010; Garcia-Ruiz et al., 2012; Molina-Espeja et al., 2014).

8.2.1.2 Saturation Mutagenesis

Since *epPCR* methods are not useful to introduce consecutive mutations (ie, they only produce a single nucleotide substitution per codon), on average we can only access 5.6 residues of the 20 amino acids in the protein alphabet. Moreover, the degeneracy of the genetic code (with four letters, there are 64 possible codons: 61 codons for the 20 amino acids plus 3 stop codons), along with the mutational bias of DNA polymerases, generate amino acid changes of similar chemical nature. A recent study demonstrated that in low load *epPCR* libraries, around 45% of the library explored is wasted due to: (1) the presence of the parental type (33%), (2) mutants with identical substitutions (9%), and (3) mutational errors (3%) (Zhao et al., 2014). All these shortcomings can be alleviated by using saturation mutagenesis (SM) methods, whereby single codons are replaced by all the codons that will generate the 20 naturally occurring amino acids (Georgescu et al., 2003; Martínez and Schwaneberg, 2013). SM methods can be used as a fine-tuning step on residues of particular interest, such as those revealed by conventional *epPCR*. As SM methods involve the rational selection of the site/s to be mutated and screened, they are considered to be so-called semirational approaches (Chica et al., 2005; Lutz, 2010). In fact, the residues subjected to SM are typically identified by molecular modeling assisted by the resolution of protein crystal structures, together with a range of computational tools (see Section 8.2).

When several codons are simultaneously subjected to SM the process is referred as to combinatorial saturation mutagenesis (CSM), which is often a useful approach to unmask optimal interactions and synergies between residues. Iterative saturation mutagenesis (ISM) is a CSM approach performed in consecutive cycles (Reetz and Carballera, 2007). In ISM, several positions are targeted for SM in the initial and in independent rounds, where the best hit of the offspring is then chosen as the parental type for a new ISM cycle, which also includes the second best hit. The process is repeated for as many rounds as necessary until the desired trait is obtained. More recently, Omnichange, was described as a versatile method for multiple SM (Dennis et al., 2011), a protocol that permits up to five independent codons to be saturated simultaneously. Unlike ISM, this strategy does not require a minimum distance to be maintained between the mutated codons while permitting a combinatorial assembly of the positions subjected to mutagenesis in a simple manner (ie, it is a restriction enzyme-free method).

In CSM experiments, it is very important to pay attention to the size of the library to be screened. For example, when using NNK or NNS degenerate primers (N = A, T, C or G; K = G or T; S = G or C; codon coverage 32; coded amino acids 20), exploration of three positions simultaneously implies screening

libraries of approximately 100,000 clones. Unless ultrahigh-throughput assays are available, we strongly suggest using NDT primers (N = A, T, C or G; and D = A, G, or T) for two or more positions (Currin et al., 2014). These degenerate oligos provide a good variety of polar, nonpolar, aliphatic, aromatic, negative and positive charged residues, notably reducing the demand for screening (eg, three positions can be easily handled in libraries of only 5184 clones for a coverage of 95%). Indeed, one good option is to construct couple-NDT libraries for the rapid analysis of synergies between two positions with minimal experimental effort (432 clones per library). Another clever trick involves a mixture of three primers (NDT, VHG (where V = no T, H = no G) and nondegenerate TGG = tryptophan) that allows a degeneracy of 22 unique codons for the 20 natural amino acids to be created. With this approach, codon redundancy and screening effort are decreased (eg, to saturate two positions with NNK primers, 3068 clones must be screened while using the three primer trick only 1450 clones are needed for a library coverage of 95%) (Kille et al., 2013).

8.2.2 DNA Recombination Methods

DNA recombination is fundamental to harness the selected mutant offspring from *epPCR* libraries or to create a complete set of chimeric proteins from distant parental types. Since the invention of in vitro DNA shuffling by Stemmer in 1994, many DNA recombination protocols have been reported, fostering the rapid growth of directed enzyme evolution. Here, we will only refer to the most common methods and to some protocols developed in our laboratory that are supported by the machinery of *S. cerevisiae*.

8.2.2.1 In Vitro Methods

8.2.2.1.1 Homology-Dependent Recombination Methods

Due to their extreme simplicity in terms of structural requirements and the ease of library construction, homology-dependent recombination methods are the most frequently employed protocols for switching two or more templates (eg, DNA shuffling (Stemmer, 1994); StEP (Zhao et al., 1998); RACHITT (Coco et al., 2001); DOGS (Gibbs et al., 2001)).

1. *In vitro DNA shuffling*: this method allows the recombination of parental genes with only 63% sequence identity (Stemmer, 1994). It takes advantage of the random fragmentation of parental genes by DNase I and their reassembly in a primer-free PCR reaction. DNA shuffling is commonly used to recombine highly distant parental types (DNA family shuffling) or to complement other in vitro recombination protocols (see below).
2. *StEP (Staggered Extension Process)*: this method involves iterative cycles of denaturing and priming/extension from two or more parental genes in the absence of primers (Zhao et al., 1998). In all PCR cycles, each amplified fragment will anneal to a different parental template due to sequence

complementarity, and it will continue the extension process giving rise to a full collection of chimeric products. It is useful to recombine the best mutant offspring from a round of directed evolution, as well as to mix different genes from the same family with sequence identities above 80%.

8.2.2.1.2 Homology-Independent Recombination Methods

Distinct homology-independent recombination methods have emerged to fill the gap when attempting to recombine structural related enzymes but with weak DNA identity (eg, ITCHY (Ostermeier et al., 1999); SCRATCHY (Lutz et al., 2001a); SHIPREC (Sieber et al., 2001); USERec (Villiers et al., 2010); RM-PCR (Tsuij et al., 2001); SISDC (Hiraga and Arnold, 2003)).

- 1. ITCHY (Incremental Truncation for the Creation of HYbrid enzymes):** ITCHY allows chimeric libraries to be designed between two distant parental types (even those that have a sequence identity below 50%). Firstly, the two parental types are cloned in tandem (ie, in a single vector), followed by the vector linearization between them. Subsequently, exonuclease III from *Escherichia coli* incrementally truncates one parental gene from its 3' end and the other from its 5' terminus, which is followed by intramolecular ligation of random truncated fragments to yield a full set of chimeric products (Ostermeier et al., 1999). New versions of this method include thio-ITCHY, which uses α -phosphorothioate dNTPs to avoid inaccuracies during time-dependent exonuclease III digestion (Lutz et al., 2001b).
- 2. SHIPREC (Sequence Homology-Independent Protein RECombination):** Like ITCHY, SHIPREC is employed to design chimeras irrespective of their relative sequence identity (Sieber et al., 2001). In this case, a fusion gene is created that is comprised of the two parental types by linking the C- and N-termini with a small linker containing a unique restriction site. This fusion construct can then be fragmented randomly by DNase I and S1 nuclease, yielding a library of blunt end-fusion constructs. Upon circularizing the library by intramolecular blunt-end ligation and by introducing a cleavage step using the restriction site, a library enriched in chimeric genes is produced that can be cloned into a suitable expression vector.

8.2.2.2 In Vivo Methods

Since its discovery in 1981, the homologous recombination in *S. cerevisiae* has generated great interest among cell and molecular biologists (Orr-Weaver et al., 1981), and it has since then become the model of choice to study homologous recombination. It is well known that homologous sequences of 40 to 50 base pairs are sufficient to drive in vivo splicing of DNA fragments, one to another (Oldenburg et al., 1997). This principle can be applied to the in vivo cloning of mutant libraries together with a linearized plasmid, as well as to perform DNA shuffling of parental genes (Gonzalez-Perez et al., 2012). Homologous recombination in vivo is independent of cleavage sites since it neither requires

restriction enzymes nor in vitro ligation. The sequence generated after recombination is similar to that obtained by in vitro methods but without the disruptions due to the insertion of restriction sites or linker sequences. All the protocols described in this section are based on the high frequency of homologous recombination in *S. cerevisiae*. As an important tip for the methods described below, the design of overlapping overhangs to favor the in vivo splicing and recombination between fragments is crucial for rapid and efficient mutant library creation.

1. *In vivo DNA shuffling*: Full parental genes (unfragmented) with DNA sequence identities as low as approximately 50% can be in vivo shuffled at the same time that they are cloned into a linearized plasmid with overhangs complementary to the ends of target genes. This simple method is extremely useful when evolving eukaryotic genes in yeast, permitting the recombination of several parental types and their cloning in one-pot (Cherry et al., 1999). In our experience, mutations at a distance of ≥ 20 residues from one another can be shuffled by the yeast's in vivo gap repair mechanism. We have used this method as the main recombination protocol in the evolution of different ligninolytic genes (Garcia-Ruiz et al., 2014; Alcalde, 2015).
2. *CLERY (Combinational Libraries Enhanced by Recombination in Yeast)*: CLERY combines in vitro and in vivo DNA shuffling in a single round of evolution. This method was validated for the creation of chimeric human cytochrome P450 with 74% nucleotide sequence identity (Abecassis et al., 2000), and more recently, to design chimeric laccases with only 51% nucleotide sequence identity (Pardo et al., 2012).
3. *Mutagenic StEP + in vivo DNA shuffling*: We recently modified the StEP method to introduce point mutations during amplification (mutagenic StEP). The chimeric library obtained in this way was subjected to further recombination through in vivo shuffling in order to design thermostable versatile peroxidases (Garcia-Ruiz et al., 2010, 2012) and blood tolerant laccases (Mate et al., 2013a,b). Both CLERY and this method are useful when trying to increase the number of crossover events in chimeric libraries.
4. *IVOE (In Vivo Overlap Extension)*: This is a fast and reliable method that mimics classic sequence overlap extension (SOE, also known as double PCR). By transforming the PCR fragments containing homologous overhangs along with the linearized plasmid into yeast, several amplification and ligation steps are avoided, while the proof-reading apparatus of *S. cerevisiae* prevents the inclusion of undesired mutations. This approach can be efficiently used for site-directed mutagenesis (including insertion and deletion mutagenesis), CSM, site-directed recombination, and gene assembly (Alcalde, 2010). IVOE has been exploited exhaustively in our laboratory for the directed evolution of ligninases (Garcia-Ruiz et al., 2014; Alcalde, 2015; Mate and Alcalde, 2015).
5. *MORPHING (Mutagenic Organized Recombination Process by Homologous IN vivo Grouping)*: We designed MORPHING as a random domain

mutagenesis method to focus mutational loads and recombination events on specific protein segments while the remaining protein regions keep unaltered (Gonzalez-Perez et al., 2014a). Mutant segments as short as 30 amino acids are subjected to *ep*PCR while the remaining fragments are amplified with high-fidelity polymerases. The DNA fragments are flanked with homologous overhangs at their 5' and 3' ends, and they are spliced in vivo along with the linearized vector in a one-pot step. MORPHING has been used to improve the oxidative stability of a versatile peroxidase (Gonzalez-Perez et al., 2014b), to enhance secretion of an unspecific peroxygenase (Molina-Espeja et al., 2014) and to find consensus-ancestor mutations in aryl alcohol oxidases (Viña-Gonzalez et al., 2015).

6. *DNA puzzle*: This method was developed recently in our lab to recover putative beneficial mutations that are lost through the evolution process (Gonzalez-Perez et al., 2014b). With DNA puzzle, mutations discarded by regular recombination approaches are relocated in independent sequence blocks that can be amplified and recombined with each other to identify epistatic effects in new mutational environments.

As a final reflection, the combination of random mutagenesis and DNA recombination methods with rational and semirational approaches has proved to be effective in directed evolution experiments (Fig. 8.2). Such strategies not only compensate the mutational bias of each method but they also permit sequence diversity to be enriched, increasing the fraction of functional enzymes in the mutant library. As a rule of thumb, a thorough analysis of each round of evolution gives clues and directions about which approaches should be followed in successive rounds. Given that there are very few chances to find beneficial mutations in a directed evolution experiment (generally less than 0.5% of random mutations are beneficial (Bloom and Arnold, 2009)), it is much easier to damage an enzyme than to improve it. Therefore, the subtle incorporation of mutations and their recombination in consecutive generations, analyzing the synergies and interactions between the mutations carried by the offspring of interest, is our preferred approach.

8.3 COMPUTATIONAL TOOLS

Combining computational algorithms with directed evolution allows CSM, DNA recombination or *ep*PCR methods to be performed in a more effective way, leading to biocatalysts with strong biotechnological potential while reducing notably the demands on screening. Indeed, the courtship between *in silico* computational approaches and directed evolution reduces the time to construct smart mutant libraries. In recent years, different computational methods have emerged that take advantage of the thousands of sequences and structures deposited in gene and protein databases (Verma et al., 2012a,b). Comprehensive reviews of these computational tools have been recently published

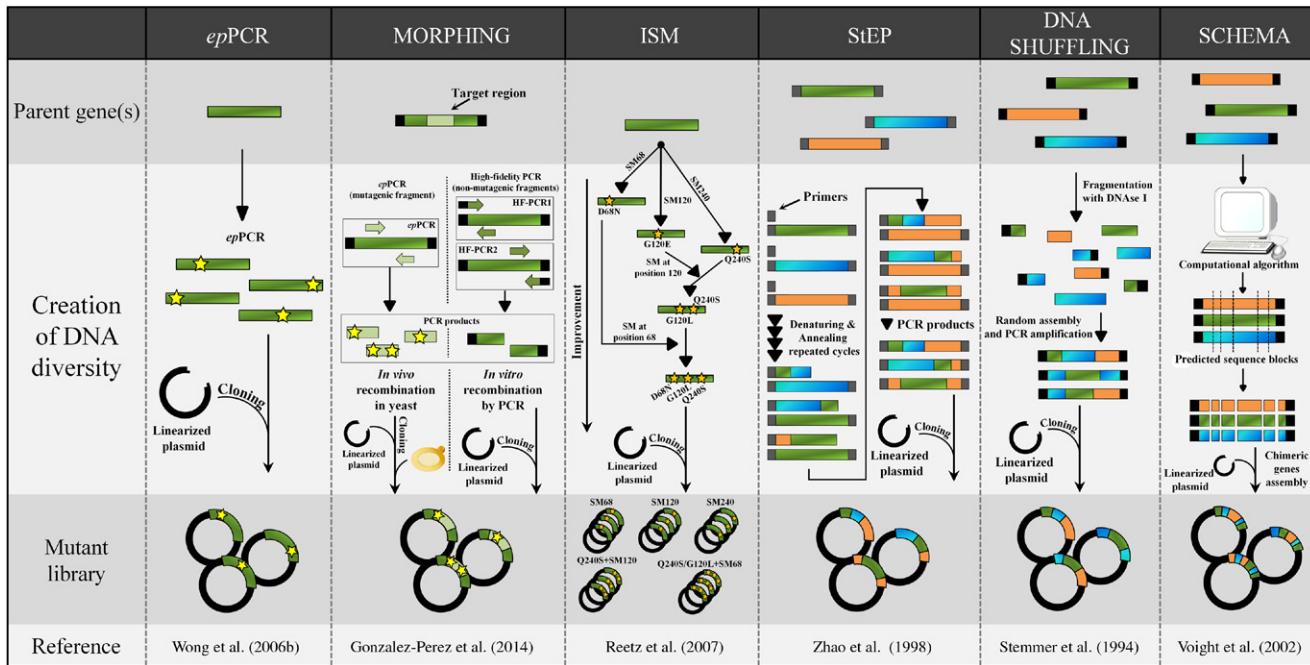


FIGURE 8.2 Some representative methods used to mutagenize/recombine target gene(s). *ep*PCR: error-prone PCR; MORPHING: Mutagenic Organized Recombination by Homologous *In vivo* Grouping; ISM: Iterative Saturation Mutagenesis; StEP: Staggered Extension Process.

(Damborsky and Brezovsky, 2014; Sebestova et al., 2014), so here we will simply mention a few representative examples of the most common computational algorithms used to date.

- 1. SCHEMA:** This computational method allows libraries to be designed by recombination of several homologous sequences (with as little as 55 % identity) while maximizing the number of folded proteins (Voigt et al., 2002). SCHEMA predicts the fragments that must be inherited in the same parent, thereby allowing the computational selection of blocks from which novel proteins (chimeras) can be assembled. The selection of crossover locations is based on a metric for disruption, defined as the number of interactions of a hybrid protein that are broken when a given pattern of fragments is inherited from each of the parents (ie, the pairs of residues within 4.5 Å of each other that are not present as the same pair in any of the parents—defined as SCHEMA energy $\langle E \rangle$). This method requires a multiple sequence alignment of the parental sequences, a PDB file, and a UNIX-based computer. While SCHEMA can be combined with the RASSP algorithm for sequence elements that are contiguous along the polypeptide chain (Endelman et al., 2004), the swapping of elements that are not contiguous in primary sequence can be performed by using the NCR algorithm in combination with SCHEMA (Smith et al., 2013).
- 2. MAP (*Mutagenesis Assistant Program*):** MAP compares the different random mutagenesis methods available and their consequences in terms of mutational bias at the level of amino acid substitution (Wong et al., 2006b). MAP is used to predict the quality of the mutant library based on the *epPCR* method chosen and the nucleotide composition of the target gene. It works at three levels of information using three distinct indicators: (1) the protein structure indicator provides data about the likelihood of introducing stop codons and residues that destabilize the helical structure (Pro, Gly), (2) the amino acid diversity indicator shows the proportion of variants with preserved amino acids (ie, amino acids that do not change with a single nucleotide substitution—silent mutations—and the average number of amino acid substitutions after a single nucleotide change, and (3) the chemical diversity indicator provides the chemical diversity generated in terms of amino acid substitution (aliphatic, aromatic, neutral, charged). The new version of the server (MAP^{2.0} 3D) permits further mapping of the sequence indicators onto the target protein’s 3D structure, correlating the pattern of amino acid substitution with structural information regarding local interactions solvent accessibility, residual motility, and secondary elements (Verma et al., 2012a,b, 2014). To work, MAP^{2.0} 3D requires only the target gene sequence and a PDB file (or a homology model) of the protein.
- 3. HOT-SPOT WIZARD:** This algorithm predicts hot-spot residues for CSM to modify activities and stability, precluding catalytic determinants from mutagenesis (Pavelka et al., 2009). For each individual amino acid, the tool

gives information about the degree of mutability (and possible amino acid substitution at that position), functional and structural significance, and similar residues in related enzymes. The server integrates data from several bioinformatics databases allowing structural and evolutionary previews to be performed. The requirements to run HotSpot Wizard are minimal, only the PDB file of the target protein.

4. **3DM:** The 3DM protein superfamily platform is a commercial database that is useful to explore sequence-function relationships ([Kuipers et al., 2010](#)). By superimposing protein family structures from different sources (including mutations reported in the literature), a structure sequence alignment provides valuable information about conserved residues and more mutable amino acids to create CSM libraries.
5. **ProSAR (Protein Sequence Activity Relationship):** This computational platform unmasks beneficial mutations from experimental mutant libraries (even from mutants with reduced function) to be incorporated into new iterative rounds of directed evolution. ProSAR follows the same principles that rule QSAR (an algorithm used for drug design) but applied to protein engineering to sort out beneficial, neutral, and detrimental mutations from a given mutant library in order to find new epistatic effects within a given protein scaffold ([Fox et al., 2007](#)).

8.4 FUNCTIONAL EXPRESSION SYSTEMS

The choice of a suitable host to functionally express a target enzyme is critical in directed evolution. The main organisms used include bacteria (*E. coli* and *Bacillus* sp.), yeast (*S. cerevisiae*, *Pichia pastoris*), and to a much lesser extent mammalian and insect cell lines. Gene expression in heterologous systems may imply differences in codon usage, chaperones, and posttranslational modifications, all of which must be taken into account before starting a directed evolution process. Depending on the host, the directed evolution timeline can vary notably due to the different growth rates of the organisms. For example, the doubling time for a prokaryotic host like *E. coli* is between 0.25–0.33 hours, whereas it takes from 2 to 3 days in the insect cell line *Spodoptera frugiperda* Sf9 ([Pourmir and Johannes, 2012](#)). For all these reasons, the bacteria *E. coli* and the brewing yeast *S. cerevisiae* are by far the most common hosts for directed evolution of prokaryotic and eukaryotic genes, respectively.

Since its discovery in 1886 by Theodor Escherich, *E. coli* has been widely studied and hence, dozens of standardized protocols exist for its genetic manipulation. Due to its ease of handling and biological attributes (fast growth, high transformation efficiency), *E. coli* leads the list of host organisms used in directed evolution. However, in vitro library creation is often complicated and more significantly, *E. coli* lacks essential posttranslational modification and specific chaperones needed when expressing eukaryotic genes. Thereby, many attempts to produce eukaryotic proteins in *E. coli* result in the accumulation of

proteins in inclusion bodies and/or misfolding ([Khow and Suntrarachun, 2012](#)). In such cases, the organism of choice is *S. cerevisiae* as it offers similar processing machinery to higher eukaryotic cells. Moreover, although *S. cerevisiae* has a slower growth rate (with a doubling time of 1.25–2 hours) and its transformation efficiency is approximately 2 orders of magnitude lower than that of *E. coli* (10^7 – 10^8 and 10^8 – 10^{10} transformants per μg of DNA, respectively), it does possess a high frequency of homologous DNA recombination with proof-reading activity that is very convenient to create mutant libraries, as described above ([Gonzalez-Perez et al., 2012](#)). Another attractive advantage of this host is that it can secrete heterologous proteins to the culture broth, avoiding tedious lysis steps and cumbersome interactions during screening assays. However, what is probably the main pitfall of using *S. cerevisiae* relates with its poor level of secretion when compared to other industrial hosts (eg, *Aspergillus oryzae*, *P. pastoris*). Unfortunately, the latter are far from ideal for directed evolution due to the lack of episomal vectors, their low transformation efficiencies and complex plasmid recovery. Thus, using tandem-expression systems (ie, *S. cerevisiae* for evolution and *P. pastoris* or *A. oryzae* for overproduction) can help to circumvent these hurdles, as long as the evolved enzyme properties are maintained in the production host ([Mate et al., 2013b; Molina-Espeja et al., 2015](#)).

8.5 MUTANT LIBRARY EXPLORATION

Along with the creation of genetic diversity and the functional expression of mutant libraries, the success of directed evolution experiments depends on the development of reliable and robust methods to explore the desired enzyme property ([Arnold and Georgiou, 2003b; Martínez and Schwaneberg, 2013](#)). Ideally, such methods must be robust, reliable, sensitive, and reproducible. The techniques available to explore mutant libraries can be classified as genetic selection methods, or as high-throughput and ultrahigh-throughput screening assays ([Table 8.1](#)).

8.5.1 Genetic Selection Methods

Genetic selection methods allow very large libraries ($\sim 10^9$ clones per round) to be explored and they do not require special instrumentation. They are specifically designed to detect enzyme features linked to the survival of the host ([Leemhuis et al., 2009](#)) and therefore, these selection methods are mainly limited to explore detoxifying enzymes or enzymes associated to host metabolism ([Boersma et al., 2008; Reetz et al., 2008](#)). For this reason they are complicated to use when attempting to improve nonnatural functions, such as activity on nonnatural substrates or in harsh environments (eg, high temperature, extreme pH, or in the presence of nonconventional media like organic cosolvents and ionic liquids).

TABLE 8.1 Selection and Screening Methods Used in Directed Evolution

Approach	Library size	Advantages	Limitations	References
Selection	$\sim 10^9$	Yields desirable variants. No special instrumentation required	Only possible for detoxifying enzymes or enzymes that synthesize essential nutrients for cell growth and survival	Leemhuis et al. (2009)
Agar plate-based screening	$\sim 10^5$	Easy manipulation. Useful as prescreening method	Low sensitivity. Substrate must be supplemented as part of the growth medium	Tobias and Joern (2003)
Microtiter plate screening	$\sim 10^3\text{--}10^4$	Availability of a wide range of analytical tools and robotic automation	Relatively low screening capacity	Salazar and Sun (2003)
Cell surface display	$\sim 10^9$	Large libraries	Fluorescence detection	Lane and Seelig (2014)
Liposome display	$\sim 10^7$	Rapid and efficient evolution of various proteins, including pore-forming proteins, transporters and receptors	Fluorescence detection	Fujii et al. (2013) and Fujii et al. (2014)
Megavalent bead display	$\sim 10^9$	Up to 10^6 copies of the DNA and the protein or peptide of interest are displayed on the beads	Fluorescence detection	Diamante et al. (2013)
Cell as microreactor	$\sim 10^9$	Large libraries. No compartmentalization needed. Femtoliter scale (not much reagent needed)	Product should stay in the cell. Differences in catalytic performance depending on the metabolic state of cells	Martínez and Schwaneberg (2013)
GFP and its variants as reporters	$\sim 10^7\text{--}10^8$	No need of fluorescent substrate	Desired function or enzyme activity must be linked to GFP expression	Yang and Withers (2009)

Ligand-mediated eGFP expression system (LiMEx)	$\sim 10^7$	No need of fluorescent substrate. Allows the detection of subtle differences in intracellular concentrations of metabolites	Desired function or enzyme activity must be linked to GFP expression	Cheng et al. (2015)
Cell-in-droplet screening	$\sim 10^9$	Large libraries. Femtoliter scale (not much reagent needed)	Substrates and products that do not cross the oil-phase barrier of the droplets are needed. Differences in catalytic performance depending on the metabolic state of cells	van Rossum et al. (2013)
Cellular High-Throughput Encapsulation Solubilization and Screening (CHESS)	$\sim 10^8$	Applicable for directed evolution of both soluble and membrane proteins	Proteins lower than 70kDa can diffuse out of the capsules. Capsules are not resistant to highly basic solutions. A fluorescent read-out of protein integrity is required for FACS	Scott and Plückthum (2013) and Yong and Scott (2015)
Fur-shell technology	$\sim 10^7$	Adaptable to other hydrolytic enzymes that use substrates in which β -D-glucose is released upon hydrolysis	Differences in catalytic performance depending on the metabolic state of cells	Pitzler et al. (2014)
In vitro compartmentalization (IVC)	$\sim 10^{10}$	Large libraries. No cloning and transformation steps. Femtoliter scale (not much reagent needed)	Substrates and products that do not cross the oil-phase barrier of the droplets are needed	Griffiths and Tawfik (2006)
Phage-assisted continuous evolution (PACE)	n.r.	Dozens of rounds of evolution in one day with minimal researcher intervention. It does not require the manufacture of specialized components	The gene encoding the protein target of evolution must be linked to pIII production in <i>E. coli</i>	Esveld et al. (2011) and Dickinson et al. (2014)
<i>n.r.:</i> not reported.				

8.5.2 High-Throughput Screening (HTS) Assays

Although with a weaker capacity for exploration than selection methods, HTS assays allow enzyme function to be uncoupled from host survival. These methods are generally based on changes in absorbance or fluorescence emission, such that small improvements derived from single amino acid substitutions can be detected. HTS assays can be performed in solid or liquid phase.

1. *Solid phase screening assays:* Agar plate-based screening involves visual changes after substrate conversion (ie, qualitative measurements in terms of color, fluorescence, or halo formation around the colonies), changes that can be detected with the naked eye or by digital image analysis ([Tobias and Joern, 2003](#)). The main advantages of these solid phase assays are their relatively easy of handling and medium throughput capacity (~ 10^5 clones per round) ([Leemhuis et al., 2009](#)). Nevertheless, they are often limited by: (1) their poor sensitivity since soluble products diffuse away from the colony and only very active mutants can be detected and (2) interference with cell growth provoked by the chromogenic or fluorogenic substrates that are added as supplements to the agar medium (although the use of transfer membranes can help alleviate this problem ([Aharoni et al., 2005](#))).
2. *Liquid phase screening assays:* Known as microtiter plate (MTP) based screening methods, are the most common format used. They offer a low to medium screening capacity (~ 10^3 – 10^4 clones per round) but they permit the individual analysis of each component of the mutant library and their comparison with the parental type ([Leemhuis et al., 2009](#)). Typically, single colonies from a mutant library are inoculated into 96-well plates (master plates), grown until stationary phase (biomass production), and replicated into a second 96-well plate for enzyme expression. All the protein variants are then extracted, either by cell lysis (intracellular enzymes) or by removing the biomass (extracellular enzymes), and screened in microtiter plate format to quantify the changes in absorbance or fluorescence ([van Rossum et al., 2013](#)).

The optimization and validation of MTP-based screening methods is typically performed by evaluating the linearity of the assay and the coefficient of variance (CV). In practice, CV values below 15% are acceptable for directed evolution experiments. It is also important to adjust the conditions of the assay so that the activity of the wild-type/parental enzyme is close to the detection limit of the assay (never below). The use of special equipment helps to achieve good CV values (humidity shakers, colony pickers, liquid handlers, pipetting robots, etc.), avoiding the abundant generation of false positives. If available, solid-phase assays can be used for prescreening before the selected clones are further subjected to MTP-based screening. In MTP assays, it is wise to include a full column in each plate with the parental type (internal standard) in order to estimate the improvements achieved for each individual mutant in each single plate.

8.5.3 Ultrahigh-Throughput Screening Assays

Next in the throughput scale are the fluorescence-activated cell sorting (FACS)-based screening methods. FACS cytometers are complex instruments that can analyze the fluorescent characteristics of individual “particles” (eg, cells, liposomes, beads, emulsion droplets, or polymer shells) at extremely high event rates ($\sim 10^7$ per hour), isolating those with the strongest signal for the desired trait (Yang and Withers, 2009; Pitzler et al., 2014). The main bottleneck in the use of these systems is that they are not generally applicable since they require linking the genotype (a nucleic acid that can be replicated) and phenotype (a functional feature like binding or catalytic activity) in an ultrahigh-throughput context (Griffiths and Tawfik, 2006). In other words, active enzyme mutants should only label the particle in which they are produced. This can be attempted through the accumulation of a fluorescent product in the particle, permitting the identification and recovery of the active variants, although this is not always an easy task (Martínez and Schwaneberg, 2013). FACS-based screening techniques can be classified into five distinct categories according to the methods used to link the genotype and phenotype: (1) cell surface display (or *in vivo* display) of active enzymes, (2) *in vitro* display, (3) green fluorescent protein (GFP) methods, (4) entrapment of the product inside the cells, and (5) *in vitro* compartmentalization.

- 1. Cell surface display:** This involves the attachment of heterologous enzymes to the surface of bacteria (bacterial surface display (van Bloois et al., 2011)), yeast (yeast surface display (Boder and Wittrup, 1997)) or phages (phage surface display (Fernandez-Gacio et al., 2003)). Cell display offers ultrahigh-throughput capacity of up to 10^9 clones per round and allows direct contact between the enzyme and the added substrate, without requiring diffusion through the cell membrane. However, this accessibility is at the same time a major drawback as the product is also freely diffusible.
- 2. In vitro display:** This method is based on the synthesis of the target protein for evolution through an *in vitro* translation system so that the genotype-phenotype linkage is achieved using molecules like ribosomes (ribosome display), puromycin (mRNA display), and the DNA replication initiator protein RepA (CIS display). More recently, novel *in vitro* display approaches have been reported that include liposome and megavalent bead display. Liposome display enables membrane proteins to be engineered, and it relies on their display on liposome membranes through the transcription and translation of a single DNA molecule using an encapsulated cell-free translation system (Fujii et al., 2014). Megavalent bead surface display allows to tailor-made protein binders by laboratory evolution. In this method, the gene that encodes the protein of interest is attached to a bead by strong noncovalent interactions and the corresponding protein is displayed via a covalent thioether bond on the DNA (Diamante et al., 2013).

3. *Green fluorescent protein (GFP) methods:* Due to their autofluorescent properties, GFP and its variants are ideal candidates for single-cell fluorescence analysis and, therefore, they can be used in flow cytometry screening ([Yang and Withers, 2009](#)). The main advantages of GFP-based screening methods are their very high-throughput ($\sim 10^7$ – 10^8 clones per round) and the lack of expensive fluorescent substrates. Nonetheless, the enzymatic function or activity of interest must be linked to GFP expression, a requirement that cannot always be met by all protein systems.
4. *Entrapment of the product inside the cells:* An alternative to cell surface display strategies and GFP-based screening methods are *in vivo* FACS-based screening approaches in which the fluorescent product is trapped within the cell ([Yang and Withers, 2009](#)). This approach, also known as a cell microreactor, must overcome the challenge of designing substrates that are accessible to the enzyme inside the cell and ensuring that the diffusible fluorescent products stay within the cell. This can be readily achieved if the enzyme catalyzes reactions in which the size, polarity, or other chemical properties of the substrate are modified, resulting in the retention of the product within the cell. The drawback of substrate/product entrapment in the cell has been overcome by developing cell microencapsulation techniques that imitate natural cell compartmentalization eg, including the target enzyme in microemulsion droplets along with the substrate and products ([Griffiths and Tawfik, 2006](#)). The main advantages of the cell-in-droplet approach are its ultrahigh-throughput capacity ($\sim 10^9$ clones per round) and the reduced volume of the microemulsions (typically ~ 5 femtoliters), such that little substrate is needed ([Aharoni et al., 2005](#)). Very recently, novel screening platforms using polymer encapsulated cells have been developed like: (1) the cellular high-throughput encapsulation, solubilization and screening (CHESS) method to allow the encapsulation of libraries of approximately 10^8 mutants in a polymer to form cell-like microcapsules ([Yong and Scott, 2015](#)) and (2) the polymer shell (fur-shell) method that relies on fluorescent hydrogel formation around *E. coli* cells ([Pitzler et al., 2014](#)).
5. *In vitro compartmentalization (IVC):* Microencapsulation can be performed in the absence of cells, directly producing the enzyme from the DNA library by *in vitro* protein translation. Such IVC offers the advantage of generating a large number of droplets per emulsion volume unit ($> 10^{10}$ in 1 mL of emulsion), coupled to the ease of preparing emulsions and their high stability to changes in temperature, pH, and salt concentrations ([Aharoni et al., 2005](#)). Additionally, IVC prevents the gene diversity loss derived from the cloning and transformation steps by *in vitro* protein translation of the DNA used to generate the mutant library; moreover, it reduces experimental time by skipping the cell growth steps associated with cloning, transformation, and protein production ([Martínez and Schwaneberg, 2013](#)).

For all the strategies described above, a single round of directed evolution involves mutation, gene expression, screening, or selection, all of which

typically requires weeks with frequent researcher involvement. In order to speed up the overall process, the phage-assisted continuous evolution (PACE) platform was recently developed, which enables extremely rapid evolution of biomolecules with minimal human intervention (Esveld et al., 2011). The PACE system is based on a fixed-volume vessel (a “lagoon”) where a continuously replicating phage carries an evolving gene of interest. Phage-free host *E. coli* cells are constantly supplied to the lagoon and as the infection ability of phage is linked to the function of the target gene, phage-encoding inactive mutants produce non-infectious host cells that are diluted out of the lagoon, permitting hundreds of rounds of evolution to be carried out in a few days.

In summary, there are a variety of screening and selection techniques currently available for laboratory evolution. The choice of one method or another depends on the convergence of several factors, which include the nature of the enzyme target, the feature to be engineered, and the facilities that are available in a given laboratory. Current MTP-based screening methods are those most often used in directed evolution, thanks to their easy set-up and the wide availability of compatible instruments. Although the number of sophisticated ultra-HTS platforms has grown significantly in recent years, there is still a long way to go before they can be implemented as a routine approach to screen mutant libraries.

8.6 FORTHCOMING TRENDS IN DIRECTED EVOLUTION

In this last section we will briefly discuss some emergent methods and new strategies in directed enzyme evolution.

- 1. Neutral genetic drift:** Neutral drift is an evolutionary approach that emulates neutral natural evolution, whereby many different genotypes can lead to the same phenotype. Neutral drift in the laboratory is based on the accumulation of neutral mutations by iterative cycles of *epPCR* and screening to maintain the protein's native function (Peisajovich and Tawfik, 2007). Libraries created by this technique are characterized by their high diversity and quality, that is, they are enriched in folded and active variants. This contrasts with conventional directed evolution where the library is mostly comprised of inactive variants due to the accumulation of deleterious mutations, especially when using high mutational loads (Gupta and Tawfik, 2008).

Repeated rounds of random mutation and purifying selection generate highly polymorphic networks, which if expanded sufficiently can generate different genotypes with a similar functional phenotype (ie, natural activity), while unmasking hidden properties in terms of stability and substrate promiscuity (Bloom et al., 2007a,b; Bershtain and Tawfik, 2008; Tokuriki et al., 2008; Romero and Arnold, 2009; Tokuriki and Tawfik, 2009). Neutral drift can be utilized when searching for activity on novel substrates to which the parental protein displayed weak (latent activity) or no activity. Furthermore, it can be applied to further enhance an activity that appears to have reached

an activity plateau (ie, after several rounds of directed evolution the improvements in activity are barely detectable (Tokuriki et al., 2012)). Neutral drift is also useful to increase stability, even by drifting the protein network closer to the consensus/ancestral sequence (Bershtain and Tawfik, 2008). In addition, it can be used prior to the start of an evolution campaign to enhance stability or the promiscuous activity of the parental type.

The critical point in neutral drift is to determine the selection cut-off which is dependent on the final objective of the experiment. When searching for high stability variants, suggestions include the use of low mutational loads while strictly purging the library to retain a high level of native activity (over 75% of the parental activity). On the other hand, when pursuing latent activities, the advice is to use higher mutational loads and milder cut-offs to generate a very diverse population (over 30% of the parental activity). If the cut-off is set too high, the library will become too uniform and promiscuity will be very difficult to achieve (Kaltenbach and Tokuriki, 2014) (Fig. 8.3).

2. *Circular permutation:* In nature, this consists in the relocation of the N- and C-termini of a given protein, which possibly commences through the tandem duplication of a precursor gene. The evolutionary advantages of circular permutation in nature are not fully understood, yet in the laboratory circular permutation is providing a new twist to directed evolution experiments as

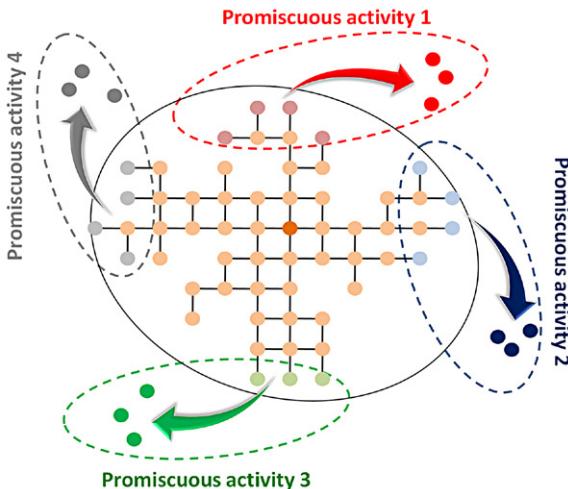


FIGURE 8.3 Combination of neutral drift and adaptive evolution to pursue novel functions. After employing neutral drift on the wild-type protein (represented as the small circle in *dark orange*) and expanding the neutral polymorphic network (enclosed in the large *black circle*), latent promiscuous activities are detected and can be defined by the sequences in the intersecting regions (*small circles in light orange*). Those sequences, although retaining some or all native activity (depending on the threshold selected), represent ideal starting points for adaptive evolution towards promiscuous activity 1, 2, 3, or 4 (arrows and *small circles in red, dark blue, green, and grey*, respectively).

it can modify activities, specificities and stabilities (Yu and Lutz, 2011). Through in vitro circular permutation, the amino acid composition of the target enzyme is not altered but rather, the structural reorganization of the whole protein simply gives rise to new topologies with unexpected functions. Circular permutation protocols include: (1) the covalent connection of the native N- and C-termini of the target gene with the help of a short peptide linker, (2) random DNase I cleavage of a peptide bond in the primary sequence, and (3) screening of the circular permutation library. Among the most important issues to consider when creating circular permutation libraries are: (1) the peptide linker length and composition, (2) that the native C- and N-terminus of the protein lie within 20 Å of each other (as is the case for most natural proteins), and (3) the inclusion of start and stop codons preceding the cloning site in the linearized vector.

3. *De novo enzyme design:* In recent years, the development of quantum mechanics/molecular mechanics (QM/MM), molecular dynamics and Monte Carlo algorithms has permitted their implementation in directed evolution and in rational studies to evaluate protein–substrate interactions, catalytic determinants, and stability (Kiss et al., 2013; Carvalho et al., 2014). Taking advantage of computational simulations, the engineering of *de novo* enzymes has generated significant scientific interest as it allows artificial biocatalysts to be tailor-made using algorithms like ROSETTA or ORBIT, mimicking enzyme function (Golynskiy and Seelig, 2010; Kries et al., 2013). The process involves four essential steps: (1) selection of the target reaction, including the catalytic mechanism and the functional groups involved, (2) modeling of the most stable structure of the rate limiting transition state (theozyme), (3) docking of the theozyme into a palette of protein scaffolds from PDB, and (4) production of the *de novo* enzyme in a heterologous host. Nonetheless, these types of enzymes show often poor catalytic rates, an inconvenience that can subsequently be surpassed by directed evolution (Blomberg et al., 2013).
4. *Metagenomics:* The exploitation of the vast genetic reservoir of bacteria and fungi from extreme environments can be useful to isolate more robust genes/enzymes. As such, metagenomics offers a great potential for industrial biocatalysis due to its access to the limits of natural biological and molecular diversity (Lorenz and Jürgen, 2005; Chistoserdova, 2014). Indeed, the combination of metagenomics and directed evolution can aid the pursuit of more potent biocatalysts for numerous applications where processing conditions are severe. Verenium, the successor of Diversa, is a biotech company whose old slogan “*evolve the best from nature*” anticipated this approach almost two decades ago. Nevertheless, there are only a few examples of academic groups that apply directed evolution to enzymes that have emerged from metagenomic environments, among them: a metagenome-derived thermostable lipase from a hot spring engineered to enhance its catalytic efficiency (Kumar et al., 2013); an alkaline laccase from mangrove soil to decolorize textile dyes (Liu et al., 2011); and a metagenome-derived epoxide hydrolase

from a microbial biofilter community to improve enantioselectivity (Kotik et al., 2013). Even though enzymes from metagenomics sources are ideal starting points for directed evolution due to their implicit stability, the lack of suitable hosts for library construction and exploration (especially in the case of fungi) has precluded faster development. Hence, the identification and engineering of suitable heterologous hosts is being actively pursued to enhance the efficiency of metagenomic gene expression and directed evolution (Yang and Ding, 2014).

5. *Resurrection of ancestral proteins:* The increasing growth of sequence databases, DNA synthesis techniques, and phylogenetic evolutionary methods are helping us to understand the origin of the proteins on our planet and of life as we know it (Benner et al., 2007). Indeed, paleoenzymology allows us to travel back in time to recover ancestral proteins at the molecular level of organisms that are already extinct. This approach is very valuable to create ancestral and ancestral-like proteins whose activities and stabilities extend beyond those of their extant counterparts (Gaucher et al., 2008; Arnaud, 2013). While enzyme reconstruction and resurrection has been used over the years to discern the principles behind natural protein evolution, its combination with directed evolution could lead to a new generation of biocatalysts. We can already find solid examples of highly thermostable and promiscuous resurrected Precambrian enzymes in the literature, such as thioredoxins and β -lactamases (Perez-Jimenez et al., 2011; Rissó et al., 2013), many of which could be now evolved *in vitro* to further exploit their rediscovered traits. Our laboratory is devoted to evolve different resurrected enzymes with strong tolerance to high mutational loads (due to the mutational robustness linked to their stability) and wide substrate promiscuity. Indeed, the combined directed evolution of modern and ancestral enzymes will provide a deeper understanding of protein evolution while driving the engineering of superior biocatalysts.

8.7 CONCLUDING REMARKS

Directed evolution has opened several avenues for protein engineers and molecular biologists that only existed in our dreams some years ago. The rapid progress in synthetic biology and metabolic engineering has led to a new way of making biocatalysts, which has had a strong impact on each of the four colors in biotechnology (white, industrial; green, agricultural; red, biopharmaceuticals; and blue, marine biotechnologies (Alcalde, 2015)).

Still, there are several challenges to be overcome regarding directed evolution: (1) the need to implement sophisticated ultra-high-throughput screening assays as routine and universal tools for library exploration, (2) the restricted DNA diversity covering the full spectrum of the protein alphabet, which may be possibly dealt with new random codon mutagenesis strategies, and (3) the demand for more reliable computational algorithms to reduce the resources required for screening. In the near future, solutions to these problems will accelerate the

design of enzymes, metabolic pathways, or even whole microorganisms that can be used in the production of chemicals, biofuels, medicines, and foodstuffs.

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ABBREVIATIONS

CLERY Combinatorial Libraries Enhanced by Recombination in Yeast
CHESS Cellular High-throughput Encapsulation, Solubilization and Screening
CSM Combinatorial Saturation Mutagenesis
CV Coefficient of Variance
GFP Green Fluorescent Protein
epPCR Error prone PCR
FACS Fluorescence-Activated Cell Sorting
GFP Green Fluorescent Protein
HTS High-Throughput Screening
IvAM In Vivo Assembly of Mutant libraries with different mutation spectra
IVC In Vitro Compartmentalization
IVOE In Vivo Overlap Extension
ISM Iterative Saturation Mutagenesis
ITCHY Incremental Truncation for the Creation of HYbrid enzymes
MAP Mutagenesis Assistant Program
MORPHING Mutagenic Organized Recombination Process by Homologous IN vivo Grouping
MTP MicroTiter Plate
PCR Polymerase Chain Reaction
PACE Phage-Assisted Continuous Evolution
ProSAR Protein Sequence Activity Relationship
QM/MM Quantum Mechanics/Molecular Mechanics
SeSaM Sequence Saturation Mutagenesis
SHIPREC Sequence Homology-Independent Protein RECombination
SM Saturation Mutagenesis
SOE Sequence Overlap Extension
StEP Staggered Extension Process
Ts Transition
Tv Transversion

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Chapter 9

Insights into the Structure and Molecular Mechanisms of β -Lactam Synthesizing Enzymes in Fungi

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9.1 INTRODUCTION

Following the discovery of penicillins and cephalosporins in fungi, and later of cephamycins in some actinobacteria, there was a great interest in finding novel molecules containing the β -lactam ring. This class of secondary metabolites, in addition to the classical β -lactam antibiotics (penicillin, cephalosporin C, and cephamycins), include the cephobacins, monobactams, olivanic acid, thienamycin, clavulanic acid, the antifungal clavams, and the plant toxin tabtoxin (Liras and Martín, 2009), among others. Of these, β -lactams, penicillins, cephalosporins, and clavulanic acid have contributed greatly to combat bacterial infections and still are among the most important selling drugs (Demain, 2014).

In nature, the ability to produce β -lactam antibiotics is distributed among fungi and Gram-positive (and some Gram-negative) bacteria, although the genetic information required is frequently restricted to a few species within a genus, or even to certain strains within a particular species (Laich et al., 2002; Kim et al., 2003; Houbraken et al., 2011). The distribution of β -lactam producing microorganisms in nature has been reviewed (Aharonowitz et al., 1992) and its possible transmission by horizontal gene transfer has been proposed (Landan et al., 1990; Peñalva et al., 1990; Liras et al., 1998).

The biosynthesis of metabolites containing the β -lactam ring is an important source of information on novel enzymes with interesting molecular mechanisms of catalysis. Particularly well known are the enzymes involved in penicillin biosynthesis and the early steps of cephalosporin biosynthesis. These are

excellent models to understand the biochemistry of related secondary metabolites. However, some of the enzymes involved in cephalosporin biosynthesis are not so well known (eg, the two-component isopenicillin N epimerization system).

This chapter is focused on the molecular characterization of the enzymes involved in penicillin biosynthesis and the early steps of cephalosporin formation, in order to gain insight into their molecular mechanisms.

9.1.1 Penicillin and Cephalosporin Biosynthesis: A Brief Overview

Penicillins and cephalosporins are nonribosomal peptide antibiotics containing the β -lactam ring formed by cyclization of a linear tripeptide. The biosynthesis of these antibiotics starts with the condensation of the amino acids L- α -aminoadipic acid, L-cysteine, and L-valine to form the tripeptide δ -(L- α -aminoadipyl)-L-cysteinyl-D-valine (ACV). Activation of the three amino acids and the condensation reactions are catalyzed by a nonribosomal peptide synthetase (NRPS) named ACV synthetase (ACVS). In a second step, the ACV tripeptide is cyclized to isopenicillin N (IPN) by the enzyme isopenicillin N synthase (IPNS), an iron-dependent oxidase. In *Penicillium chrysogenum* NRRL1951 (syn. *Penicillium rubens*), the IPN is converted to hydrophobic penicillins, such as benzylpenicillin or phenoxyethylpenicillin, by the isopenicillin N acyltransferase (IAT), an enzyme with amidase, acyltransferase, and transacylase activities. In *Acremonium chrysogenum* and in actinomycetes, IPN is later isomerized to penicillin N, a step required for the last cephalosporin reactions to occur. The epimerization reaction in filamentous fungi is still poorly understood (Ullan et al., 2002; Martín et al., 2004), whereas in *Streptomyces clavuligerus* and *Amycolatopsis lactamdurans*, it is mediated by the pyridoxal phosphate-dependent isopenicillin N epimerase (Usui and Yu, 1989; Laiz et al., 1990). The penicillin N is then converted by a ring expanding oxygenase, which uses 2-oxoglutarate as cosubstrate, to deacetoxycephalosporin C (DAOC) and deacetylcephalosporin C (DAC), and finally transformed to cephalosporin C by acetylation via the DAC acetyltransferase (Brakhage, 1998; Martín et al., 2012a).

9.1.2 Genes Involved in Penicillin Biosynthesis

The central enzymes of the penicillin biosynthetic pathway in *P. chrysogenum* are encoded by a set of three genes linked together in a cluster (the *pen* cluster) in chromosome I of *P. chrysogenum* or in chromosome VI of *Aspergillus nidulans* (Montenegro et al., 1992). The core of the penicillin biosynthetic pathway is encoded by three genes, *pcbAB* (11.5 kbp, encoding the ACVS), *pcbC* (1.0 kbp, encoding the IPNS), and *penDE* (1.2 kbp, encoding the IAT).

The *P. chrysogenum* *pcbAB* gene consists of a very large open reading frame (11,376 bp). It lacks introns and encodes a protein of 3791 amino acids (Díez

et al., 1990). The orthologous gene of *A. chrysogenum* is very similar (54.9% identity) to that of *Penicillium* (Gutiérrez et al., 1992). The *pcbAB* gene is linked to the *pcbC* and *penDE* genes, which are clustered together with other nonessential ORFs in a 56.8 kb DNA region amplified in tandem repeats in high-penicillin producing strains (Fierro et al., 1995; 2006; van den Berg et al., 2007). In wild-type *P. chrysogenum*, the penicillin (*pen*) gene cluster is present in a single copy. The number of copies of this amplifiable unit can be changed by recombination (Fierro et al., 1995) and even removed completely, resulting in a nonproducer strain (Fierro et al., 1995, 1996; Harris et al., 2009).

In *A. chrysogenum*, the first two genes of the pathway (*pcbAB* and *pcbC*) are linked to the genes *cefD1* and *cefD2*, encoding the two components of the epimerization system, and to two MFS transporter-encoding genes for cell membrane and organelle (peroxisome) transport systems. The two late genes of the pathway, *cefEF* and *cefG*, encoding, respectively, the ring expandase (DAOC synthase/hydroxylase) and the DAC acetyltransferase form a separate gene cluster located on a different chromosome (Gutiérrez et al., 1992; Martín et al., 2012a).

The basic molecular genetics of penicillin and cephalosporin biosynthesis is well known (Aharonowitz et al., 1992; Martín, 2000a; Brakhage et al., 2004; Liras and Demain, 2009; Liras and Martín, 2009) and is not detailed here.

9.2 ACV SYNTHETASE

ACV synthetase is one of the first known fungal NRPSs and serves as a model of more complex peptide synthetases (Zhang and Demain, 1992; Aharonowitz et al., 1993; Byford et al., 1997). NRPSs are multimodular proteins involved in the biosynthesis of thousands of microbial and plant secondary metabolites (Marahiel et al., 1997). The large size of the NRPSs and their multiple enzyme activities has made difficult the characterization of their catalytic sites. The in vitro activities of these enzymes are usually very poor (van Liempt et al., 1989; Theilgaard et al., 1997) because they may lose their native structure or suffer alterations, for example, dissociation of NRPS-ancillary proteins that improve substrate affinity in vivo (Walsh et al., 2001; Baltz, 2011). However, molecular genetic approaches allow their characterization in vivo (Wu et al., 2012).

In the cells, ACV can be found either in a reduced monomeric form or as an oxidized dimer (bis-ACV) (Theilgaard and Nielsen, 1999). In the presence of oxygen, bis-ACV is the predominant form released to the culture broth (López-Nieto et al., 1985; García-Estrada et al., 2007). Intracellularly, the ACV is kept in the reduced form by the thioredoxin and thioredoxin reductase system (Cohen et al., 1994) as only the reduced monomer is used as substrate for the IPN synthase (Aharonowitz et al., 1992; Theilgaard and Nielsen, 1999). The bis-ACV secreted to the culture broth appears to be a waste product of the β -lactam pathway since is not reintroduced into the producer cells (García-Estrada et al., 2007) and does not serve as inducer in a quorum-sensing mechanism (Martín

et al., 2011). Its secretion reflects the existence of limiting steps in the middle and late parts of the penicillin or cephalosporin pathways (eg, the *A. chrysogenum* N2 strain; Shirafuji et al., 1979; Ramos et al., 1986) but the mechanism by which the ACV tripeptide is secreted is still unknown.

9.2.1 The ACV Assembly Line

As indicated above, ACV is synthesized by condensation of three amino acids: L- α -amino adipic acid, which is an intermediate in the lysine biosynthetic pathway, in fungi L-cysteine, and L-valine (Zhang and Demain, 1992; Byford et al., 1997; Martín, 2000b). Amino acid condensation is catalyzed by the non-ribosomal multidomain ACV synthetase (Zhang and Demain, 1992; Kallow et al., 2000). The ACVS is a large cytosolic protein with a molecular mass of about 415 kDa that is not located in peroxisomes, unlike the IAT (see below). However, the recent finding of a vacuolar membrane transporter that affects ACV biosynthesis may imply that ACVS is a cytosolic protein associated with vacuoles (Fernández-Aguado et al., 2013), as proposed also for the more complex cyclosporin synthetase (Hopper et al., 2001).

The ACVS contains three different modules, each of approximately 1000 amino acids, and is able to catalyze multiple reactions, including substrate amino acid activation by adenylation, translocation to the phosphopantetheine arm, peptide bond formation, epimerization, and tripeptide release by an integrated thioesterase (Aharonowitz et al., 1993; Byford et al., 1997; Martin, 2000b). The domains of each NRPSs module are partially conserved giving a reiterated structure (Zucher and Keller, 1997). Each ACVS module contains adenylate-forming (designated A for activation), aminoacyl (or peptidyl) carrier PCP (also abbreviated T, for thiolation) and condensation (C) domains (Marahiel et al., 1997). In the third module, there is an epimerization domain (E). These domains are arranged in the characteristic order ATC ATC ATE. The A domain is involved in ATP binding and amino acyl-adenylate formation, and determines substrate specificity (Rausch et al., 2005; Röttig et al., 2011; Prieto et al., 2012). The aminoacyl/peptidyl carrier domain contains a conserved serine residue that binds a thiol-containing phosphopantetheine cofactor through its phosphate group (Baldwin et al., 1991). Phosphopantetheine is added to the apo-ACVS by the enzyme 4'-phosphopantetheine transferase (PPTase), encoded by the *pptA* gene (Keszenman-Pereyra et al., 2003; Márquez-Fernández et al., 2007; García-Estrada et al., 2008a). The C domain of ACVSs is involved in the condensation of two amino acids activated on adjacent modules and catalyzes the peptide elongation reaction (Crècy-Lagard et al., 1995).

At the end of the third module, there is an epimerase (E) domain (365 amino acids), which was proposed to catalyze the conversion of the precursor amino acid L-valine into D-valine to form the tripeptide LLD-ACV (Díez et al., 1990; Gutiérrez et al., 1991; Stachelhaus and Marahiel 1995). Several conserved motifs (E1–E7) have been found within this domain in the ACVSs from

different fungi, including *P. chrysogenum*, *A. chrysogenum*, *A. nidulans* (Kallow et al., 2000), *Aspergillus oryzae*, *Kallychoma tethys* (Kim et al., 2003), and in the actinobacteria *A. lactamdurans* and *S. clavuligerus* (Coque et al., 1991; Martín, 2000b). In all these microorganisms, the consensus sequence for the E4 motif (EGHGREGRE) is fully conserved as well as in the epimerization domains of other NRPSs (Martín, 2000b).

9.2.2 The Cleavage Function of the Integrated Thioesterase Domain

Next to the epimerase domain and integrated in the C-terminal region of the ACVS, there is a thioesterase domain (designated TE in Fig. 9.1) of about 230 amino acids. Within the thioesterase region, the fungal and bacterial ACVSs contain a motif (GXSXG) which is homologous to the amino acid sequence present in the active center of the oleyl-ACP thioesterase I of vertebrate fatty acid synthetases (Díez et al., 1990; Theilgaard et al., 1997). This domain was proposed to catalyze the hydrolysis of the thioester bond between the nascent ACV tripeptide and the enzyme-bound phosphopantetheine.

The cleavage function of the integrated thioesterase domain has been clearly established by directed in vitro mutation of the serine residue at the thioesterase

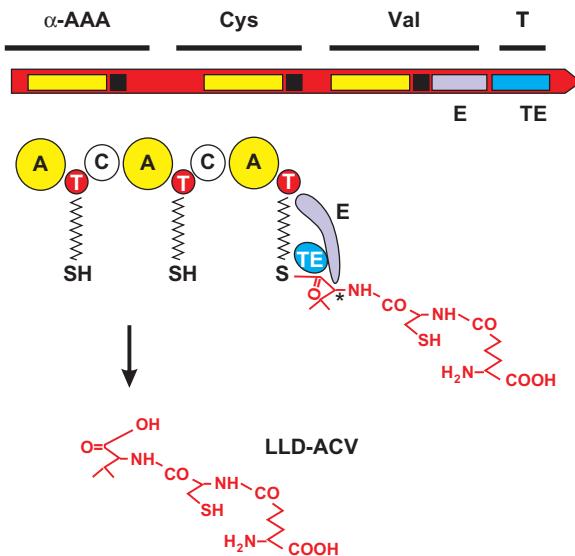


FIGURE 9.1 Scheme of the modules and domains of the ACV synthetase showing the interaction of the epimerization domain (E) with the nascent peptide while it is attached to the pantetheine of the third module, and with the thioesterase (TE) domain (see text for details). A, activation; T, thiolation (peptidyl carrier); C, condensation domains. The C-2 carbon atom of valine that is epimerized to the D-configuration is indicated by an asterisk.

active center (Kallow et al., 2000; Wu et al., 2012) and by deletion of the entire TE domain (Wu et al., 2012). In both studies, mutants in the thioesterase domain were unable to produce significant amounts of penicillin. When the thioesterase domain of *A. nidulans* ACV synthetase was modified, the resulting mutant showed more than a 95% decrease in penicillin production, but unexpectedly, some ACV was released, part of which had the LLL configuration (Kallow et al., 2000). These results suggest that there is an interaction between the epimerase and thioesterase domains which are adjacent in the C-terminal regions of the ACVSs.

9.2.3 The Quality Control (Proofreading) Role of the Thioesterase Domain

Growing evidence indicates that the TE domain of NRPSs exerts quality control of the nascent peptide. The study of mutants altered in the E4 motif of the epimerase domain allowed us to understand the possible role of the integrated thioesterase domain in tripeptide quality control. Indeed, when the EGHGRE amino acid sequence was mutated maintaining the two glycine residues (to keep the protein folding) and the mutant gene was introduced in *P. chrysogenum* Δ pen (strain *npe10*, unable to produce ACV and penicillin because it lacks the entire penicillin gene cluster), the mutant ACVS was unable to synthesize ACV in vivo, in contrast to transformants in which the nonmutated wild-type *pcbAB* gene was introduced in the same host strain. The control transformant synthesizes normal amounts of LLD-ACV as shown by HPLC. The LLD-configuration of the tripeptide was confirmed by its in vivo conversion to IPN (and to benzylpenicillin) (Wu et al., 2012). The inability of the mutant at the E4 site of the epimerase to release the tripeptide, despite the presence of the functional thioesterase domain at the carboxy terminal end of ACVS, indicates that the thioesterase has a selective activity releasing only the tripeptide with the correct configuration LLD. In summary, when L-valine cannot be epimerized to the D-configuration, the LLD-ACV is not released and the enzyme activity is blocked because the peptide assembly line does not proceed.

The same result is obtained in vivo when an engineered *P. chrysogenum* strain Δ *pcbAB* is complemented with either the wild-type or the epimerase E4 *pcbAB* mutant genes. This strain, which lacks the *pcbAB* gene, but contains functional *pcbC* and *penDE* genes, produces penicillin when complemented with the native *pcbAB* gene, but interestingly lacks formation of IPN and benzylpenicillin when the E4 mutant ACVS is introduced. Since the benzylpenicillin assay is very sensitive, these results clearly indicate that the epimerase mutant is unable to form and release the LLD-tripeptide in vivo. The mechanism of tripeptide quality control (proof reading) by the thioesterase must involve interaction of the thioesterase domain with the nascent tripeptide while it is bound to the third module, and probably also with the epimerase domain (Fig. 9.1).

The proposed quality control role of the thioesterase domain of ACVS is strongly supported by the recent finding of a similar mechanism exerted by the C-terminal thioesterase domain of the bacterial NRPSs that catalyze the synthesis of the pentapeptide precursor of nocardicin A (Gaudelli and Townsend, 2014). In this case, in addition to the presence of D-amino acids (eg, D-p-Hydroxyphenyl glycine, D-pHPG), another characteristic feature of the nocardicin precursor peptide is the formation of a β -lactam ring derived from an internal serine residue. The pentapeptide, formed by the combined action of two NRPSs, that is, NocA (3 modules) and NocB (2 modules), is cleaved during biosynthesis by removal of the first two amino acids yielding the tripeptide D-pHPG-L-serine-L-pHPG that is the direct precursor of nocardicins G and A. Gaudelli and Townsend (2014) proved that the integrated TE domain located at the C-terminus of NocB NRPS has a high selectivity for the full-fledged pentapeptide release. Only when the L-pHPG in module 3 of NocA is epimerized to the D-configuration, and the β -lactam ring has been formed, is the TE able to release the pentapeptide from the NocA/NocB complex. If any of these pentapeptide modifications is incomplete, the pentapeptide is retained, linked to the NRPSs. Then, it is rapidly released by the TE when the internal β -lactam ring is formed. This quality control activity (so-called gatekeeper role) allows the cell to be sure that a bioactive product containing the D-amino acids and the β -lactam ring is formed.

9.2.4 ACV Analog Dipeptides and Tripeptides Synthesized by the ACVS in Vitro

Using purified *A. chrysogenum* ACVS, Baldwin and coworkers (Baldwin et al., 1994; Shiau et al., 1995a,b) observed that the enzyme, in the presence of ATP, Mg²⁺, and the substrates valine and cysteine (or cysteine analogs), is able to form, in absence of L- α -amino adipic acid, several cysteine analog-valine dipeptides, all of which contain a C-terminal valine. When ¹⁴C-valine was added to the reactions, the radioactive label was incorporated in the C-terminal valine residue of the dipeptides. The structure of the dipeptide formed was established by MS and NMR analysis after HPLC purification of the reaction products (Shiau and Liu, 2002). The cysteine analogs that are best recognized by the cys-activating module of ACVS are *N*-acetylcysteine, vinylglycine, and *N*-acetylallylglycine. Other cysteine analogs, including *O*-methyl-serine, were also recognized by the ACVS with apparently lower efficiency.

When the above described enzyme reaction mixture was supplemented with α -amino adipic acid, tripeptides containing L- α -amino adipic acid, vinylglycine (or allylglycine) and D- or L-valine were produced with efficiencies similar to that of authentic ACV formation in control reactions containing the three native amino acids (Shiau and Liu, 2002). Those ACV analog tripeptides that lack the thiol group of cysteine cannot form the thiazolidine ring of IPN and probably are not formed in significant amounts in vivo.

Based on the formation of dipeptides containing cysteine (or cysteine analogs) and valine, [Shiau and Liu \(2002\)](#) proposed that the growing peptide chain may start with valine and terminate with α -amino adipic acid, in contrast to the commonly accepted α -amino adipic acid to valine (N to C) orientation ([Fig. 9.1](#)). The Shiau proposal (C to N peptide growth) is incompatible with the previously reported formation of the AC dipeptide in ACVS reactions in vitro ([Adlington et al., 1983; Banko et al., 1986](#)). The most likely interpretation of the formation of “cysteine-analog”-valine dipeptides is that the enzyme in vitro efficiently recognizes vinylglycine, allylglycine, or their *N*-acetyl derivatives at the cysteine-activating site, and these activated compounds, particularly the *N*-acetyl derivatives, mimic the α -amino adipyl-cysteinyl dipeptide and are transferred to the phosphopantetheine molecule of the second module and then translocated to, and condensed with, the L-valine activated at the third module. This dipeptide synthesis may not occur in vivo or perhaps, if synthesized, the dipeptides might not be approved by the quality control function of the C-terminal thioesterase domain. This would imply that the interaction ability of the TE domain with the rest of the ACVS is debilitated during cell disruption and enzyme purification, thus relaxing the strict selectivity for the release of the tripeptide observed in vivo. The same alteration of the proof-reading ability of the TE domain might occur during enzyme purification of the *A. nidulans* ser³⁵⁹ ACVS mutant; when tested in vitro, it releases some LLL-ACV ([Kallow et al., 2000](#)) (see above).

9.3 ISOPENICILLIN N SYNTHASE

The second enzyme of the penicillin and cephalosporin pathway was purified and characterized in the 1980s from extracts of *P. chrysogenum* ([Ramos et al., 1985](#)) and *Cephalosporium acremonium* (syn. *Acremonium chrysogenum*) ([Pang et al., 1984](#)). These early biosynthetic studies have been reviewed ([Aharonowitz et al., 1992; Martín, 1998](#)) and are not discussed here. Studies in the last decade have provided considerable insight into the mechanisms of IPN biosynthesis.

The isopenicillin N synthase, a mononuclear iron-containing nonheme oxidase that may also work as an oxygenase, is related to other nonheme oxygenases. When acting on its natural substrate ACV, this enzyme works as an oxidase consuming a full molecule of O₂ and removing four H atoms from the substrate to form two molecules of H₂O ([Feig and Lippard, 1994](#)). This oxidative reaction results in the closure of the β -lactam and thiazolidine rings, characteristic of penicillins. The IPNS belongs to a group of iron enzymes that bind O₂ to a ferrous iron-containing center and activate O₂ to react with the organic substrate ([Feig and Lippard, 1994; Solomon et al., 2000](#)). These enzymes contain a catalytic center consisting of two histidine residues and an acidic amino acid carboxylate group (the so-called “facial triad”). Most of these enzymes work as oxygenases but two of them, IPNS and the closely related 1-aminocyclopropane 1-carboxylic acid oxidase, exhibit a reaction mechanism different from that of oxygenases and act as oxidases.

The O₂ cleavage mechanism in iron-dependent oxygenases was believed to be a homolytic cleavage, in which the Fe^{II}-hydroperoxide complex formed after binding of O₂, follows a Fenton reaction resulting in the cleavage of the O–O bond and formation of an hydroxyl radical and a Fe^{III}-oxo species. However, in the IPNS, cleavage of the O₂ molecule was found to be heterolytic with an entirely different mechanism, as explained below.

9.3.1 Binding and Lack of Cyclization of the LLL–ACV

During early studies on substrate specificity of IPNS, a large number of possible substrates were tested (Abraham, 1986; Baldwin and Bradley, 1990; Huffman et al., 1992). In those studies on the substrate specificity of IPNS, it was observed that this enzyme is unable to convert the LLL–ACV tripeptide to a penicillin molecule. It was concluded that only the LLD–ACV stereoisomer, the fully modified product of ACV synthetase, served as a substrate of IPNS. However, those studies revealed that the LLL–ACV stereoisomer interferes with the IPNS cyclization activity. Later, Howard-Jones et al. (2005), using crystallographic studies with the LLL-tripeptide analog δ-(L-α-amino adipyl)-L-cysteinyl-L-3,3,3,3',3',3' hexafluorovaline, proved that the LLL-tripeptide binds strongly to the active center of IPNS as does the LLD–ACV stereoisomer but the configuration of the valine apparently prevents the correct cyclization.

9.3.2 The Iron-Containing Active Center

Initial studies of Randall et al. (1993) identified the ferrous active site of IPNS. Using spectroscopic techniques, the coordination of Fe^{II} at the active site with O₂, NO (a dioxygen structural analog) and the ACV substrate was identified, leading to the proposal of a Fe^{II}-ACV-[NO] complex at the active center (Brown et al., 2007). The crystal structure of IPNS complexed with Fe^{II}-ACVS and NO revealed a six coordinate iron center.

9.3.3 The Crystal Structure of IPNS

The first crystal structure of IPNS was provided by Roach et al. (1995) using recombinant *A. nidulans* enzyme complexed with manganese (instead of Fe^{II}) at a resolution of 2.4 Å. These authors reported that (1) the protein has a “jelly-roll”-like structure and (2) the active center is deeply buried inside the protein structure, lined by a set of hydrophobic amino acids. The secondary structure of IPNS consists of 10 α-helices and 16 β-strands. Eight of the β-strands fold into the so-called jelly-roll structure. Several β-strands are combined to give a large sheet on each side of the roll and the active center is buried inside the β-barrel. The C-terminal region of the protein (amino acids 324–331), extending from the last α helix (α10), enters between the two faces of the jelly-roll barrel allowing the glutamine residue conserved in all sequenced IPNSs (Gln³³⁰ in the *P. chrysogenum* amino acid sequence) to bind to the metal in the active center.

The manganese (or iron in the native enzyme) is bound as a six coordinate nucleus to four protein ligands (His^{214} , Asp^{216} , His^{270} , and Gln^{330}) and to two molecules of water (Roach et al., 1995). These authors propose that the substrate ACV and O_2 bind to the coordination sites initially occupied by the two water molecules and to the Gln^{330} .

In further work (Roach et al., 1997), the *A. nidulans* IPNS was crystallized and complexed with ferrous iron and ACV at a resolution of 1.3 Å and a mechanism of ACV cyclization was proposed. These studies suggest that the reaction is initiated by binding of the thiolate of ACV to the Fe^{II} at the active center. Binding of the substrate thiolate to Fe^{II} creates a vacant coordination site in the Fe^{II} center into which the O_2 molecule is bound. According to the proposed model, the cyclization then proceeds by removal of the four H atoms from ACV to form the IPN double ring structure, converting the O_2 into two water molecules. This model was supported by studies using the O_2 structural analog molecule NO and the substrate ACV (Roach et al., 1997). This cyclization model proposes that the IPNS activity participates in the early stages of the reaction to create an Fe^{II} -oxygen species, that then cyclizes the substrate without further direct involvement of the protein ligands in transfer of the H atoms.

In summary, the accumulated evidence indicates that the cyclization step initiates by the formation of a bond between the hydroperoxide in the nucleus complex and the N atom of the amide bond of cys-val at the ACV.

9.3.4 Oxidase and Oxygenase Activities of IPNS

The IPNS may work as an oxidase or as an oxygenase introducing only one of the oxygen atoms of molecular oxygen, when some ACV analogs, such as ACOV (δ -L- α -amino adipyl-L-cysteinyl-D- α -hydroxyisovaleroyl ester), are used as substrate (Brown-Marshall et al., 2010). The oxidase activity corresponds to a mechanism that is unusual among nonheme iron enzymes which act normally as oxygenation enzymes, introducing one oxygen atom in their substrates. The explanation of this particular oxidase behavior of the IPNS has been provided by spectroscopic and crystallographic studies in the last decade (Brown-Marshall et al., 2010).

In a series of articles, Baldwin and coworkers studied the crystal structures of IPNS complexed with Fe^{II} and different substrate analogs, including some tripeptides with substitutions of the valine residue of ACV (Long et al., 2003, 2005; Howard-Jones et al., 2005; Ogle et al., 2001; Ge et al., 2008). One of those substrate analogs was ACOV in which the amide N atom is replaced by an oxygen atom. It is noteworthy that the IPNS in this case behaves as an oxygenase yielding hydroxylated substrate at the β -carbon of cysteine (Baldwin and Bradley, 1990). The ACOV substrate that lacks one of the atoms (the amido N), contributes to heterolytic O_2 cleavage and, therefore, the mechanism of IPNS is modified resulting in a substrate oxygenase rather than in its characteristic oxidase activity. The crystallographic studies allowed a rational explanation of

the oxidase versus oxygenase activities of IPNS (Brown-Marshall et al., 2010). The absence of the NH amide group in ACOV prevents the initial abstraction of H from ACV and changes the Fe^{II}-hydroperoxide type of complex.

9.3.5 Recent Advances on the Cyclization Mechanism

Functional Density Theory studies have contributed to refine the previous ACV cyclization mechanism, proposing that the initial iron (Fe^{III}) atom at the active center abstracts an H atom from the cysteine β-carbon of ACV. This H atom abstraction includes an electron transfer from ACV to the oxygen yielding the known Fe^{II}-hydroperoxide complex and a double bond between the S atom of the L-cysteine and the adjacent carbon atom of cysteine in the ACV. The Fe^{II}-hydroperoxide complex deprotonates either the amide NH (MacFaul et al., 1998; Long et al., 2005; Ge et al., 2008) or the iron bound H₂O molecule (Lundberg and Morokuma, 2007; Lundberg et al., 2009), resulting in the heterolytic cleavage of the O–O bond (Brown-Marshall et al., 2010).

9.4 ACYL-COA LIGASES: A WEALTH OF ACYL-COA LIGASES ACTIVATE PENICILLIN SIDE CHAIN PRECURSORS

In addition to hydrophobic penicillins containing aromatic side chains, *P. chrysogenum* produces a variety of natural penicillins containing saturated (6–8 atom carbon) or unsaturated fatty acids. Indeed, the IAT in vitro is able to accept a variety of acyl-CoA molecules as substrates, including hexanoyl-CoA, hex-3-enoyl-CoA, octanoyl-CoA, among others (Luengo et al., 1986; Aplin et al., 1993). The use of side chain precursors for penicillin biosynthesis requires their activation as thioesters, usually as CoA derivatives, although other thioesters, for example, acyl-glutathione, are also used in vitro by the IAT (Alvarez et al., 1993).

The phenylacetyl-CoA ligase-encoding gene (*phlA*) was first cloned by Lamas-Maceiras et al. (2006). The encoded protein, PhlA (also known as PCL) belongs to a well-known family of acyl-CoA synthetases that use ATP to activate fatty acids and organic acids as acyl-AMP intermediates. The PhlA protein belongs to the subfamily of *p*-coumaroyl-CoA ligases that are widely used in the biosynthesis of plant and microbial secondary metabolites. Detailed analysis of the *P. chrysogenum* genome revealed the presence of at least five genes encoding enzymes of this subfamily in *P. chrysogenum* (Martín et al., 2012b). The PhlA protein contains a canonical PTS1 peroxisomal targeting sequence that supports the biochemical description of a peroxisomal acyl-CoA ligase (Gledhill et al., 2001). Disruption of the *phlA* gene reduced the production of benzylpenicillin by about 50% and, interestingly, the cell extract of the disrupted mutant still contained 60% of phenylacetyl-CoA ligase activity (Lamas-Maceiras et al., 2006), leading to the conclusion that *P. chrysogenum* contains other acyl-CoA ligases involved in penicillin biosynthesis.

Later, Wang et al. (2007) described one of these redundant acyl-CoA ligases, named PhlB as an isozyme involved in the activation of phenylacetic acid. However, Koetsier and coworkers (2009, 2010) purified this second acyl-CoA ligase and concluded that it activates medium and long chain fatty acids but has essentially no activity on phenylacetic acid. This second enzyme activates adipic acid and might be involved in the synthesis of some natural nonaromatic penicillins and in the synthesis of adipyl-7-aminodeacetoxycephalosporin C in engineered *P. chrysogenum* strains. The PhlB protein has also a peroxisomal targeting sequence (Wang et al., 2007; Koetsier et al., 2009) and is proposed to be located in the peroxisomes.

Expression of *phlA* gene is strongly induced by phenylacetic acid (Lamas-Maceiras et al., 2006; Harris et al., 2009) whereas expression of *phlB* is induced by adipic acid (Koetsier et al., 2010).

Recently, a third acyl-CoA ligase-encoding gene, *phlC*, has been cloned from *P. chrysogenum* and the encoded enzyme has been characterized (Yu et al., 2011). This third acyl-CoA ligase is an ortholog of a putative *p*-coumaroyl-CoA ligase of *Aspergillus fumigatus* (73% identical amino acids) whereas it has only 37% and 38% identity with PhlA and PhlB, respectively. The PhlC protein also contains the peroxisomal targeting sequence in its C-terminus (Table 9.1). The recombinant enzyme expressed in *E. coli* showed broad substrate specificity and was able to activate phenylacetic acid, although inefficiently. It was more active on caproic acid (C6) and the aromatic cinnamic and coumaric acids. Although PhlC is able to activate phenylacetic acid, the enzyme turnover was very low, and therefore, the contribution of PhlC to penicillin biosynthesis appears to be very limited (Yu et al., 2011). Unfortunately, no mutants lacking the *phlC* gene have been obtained and therefore, it is impossible to estimate the real contribution of this enzyme to penicillin biosynthesis. Other putative acyl-CoA ligases identified in the *P. chrysogenum* genome by bioinformatic analysis remain to be characterized (Table 9.1). In summary, several acyl-CoA ligases with characteristic substrate specificity are involved in the biosynthesis of different natural penicillins. Some of them, for example, PhlB, may have to be overexpressed to enhance production of adipyl-containing recombinant β -lactams.

9.5 ISOPENICILLIN N ACYLTRANSFERASE

The initial IAT purification studies (Alvarez et al., 1987) revealed that IAT may use either IPN or 6-APA as a substrate in the acylation/transacylation reaction. The activity on 6-APA, that is, phenylacetyl-CoA:6 APA acyltransferase, is very high as compared to the activity on IPN and this led to the initial proposal that there might be two enzymes involved in the conversion of IPN to benzylpenicillin that might require two encoding genes, *penD* and *penE* (Martín et al., 1990). However, we found that a single gene, *penDE* (Barredo et al., 1989; Montenegro et al., 1992), encodes five related enzyme activities in the IAT protein, namely IPN aminohydrolase, IPN acyltransferase, 6-APA acyltransferase, benzylpenicillin (PenG) acylase, and PenG/PenV transacylase (Alvarez et al., 1993).

TABLE 9.1 Phenylacetyl-CoA, Adipyl-CoA, and Related Aryl-CoA Ligases in the Genome of *P. chrysogenum*

Gene	<i>P. chrysogenum</i> gene number	PST1 targeting sequence (C-terminal)	Enzyme	Reference
<i>phlA</i>	Pc22g14900	SKI	Phenylacetate-CoA ligase	Lamas Maceiras et al. (2006)
<i>phlB</i>	Pc22g202700	AKL	Adipyl and fatty acyl-CoA ligase	Wang et al. (2007); Koetsier et al. (2009, 2010)
<i>phlC</i>	Pc13g12270	AKL	Aryl (coumaroyl, phenylacetyl)-CoA ligase	Yu et al. (2011)
<i>ary1</i>	Pc22g24780	AKL	Similar to <i>A. thaliana</i> 4-coumaroyl-CoA ligase	This work
<i>ary2</i>	Pc21g22010	SKL	Similar to <i>A. thaliana</i> 4-coumaroyl-CoA ligase	This work
<i>ary3</i>	Pc06g01160	TKI	Similar to <i>A. thaliana</i> 4-coumaroyl-CoA ligase	This work
<i>ary4</i>	Pc21g20650	ARL	Similar to <i>A. thaliana</i> 4-coumaroyl-CoA ligase	This work
<i>ary5</i>	Pc21g07810	Lacks PTS1	Similar to <i>A. thaliana</i> 4-coumaroyl-CoA ligase	This work

The *ary1* to *ary5* genes found in *P. chrysogenum* genome (van den Berg et al., 2008) have not been studied biochemically.

9.5.1 Posttranslational Maturation of the IAT

The mature IAT, as occurs with other penicillin and cephalosporin acylases (CAs), is a heterodimeric protein consisting of two subunits: α (11 kDa) and β (29 kDa). Both subunits derive from a pro-IAT precursor protein by cleavage of the gly¹⁰²-cys¹⁰³ bond of the precursor protein (Barredo et al., 1989; Aplin et al., 1993; Tobin et al., 1995), although in some analyses, the thr-104 was identified in the N-terminus of the β subunit (Barredo et al., 1989).

Several amino acid residues of the IAT were shown to be important for IAT activity (Tobin et al., 1994, 1995). One of the critical amino acid residues in the IAT is Cys-103, which after cleavage of the IAT precursor (pro-IAT), becomes the N-terminal amino acid of the β subunit. This finding and the similarity with the maturation mechanisms of self-processing penicillin G acylases (PGA), penicillin V acylases (PVA), and CAs of *E. coli* and other Gram-negative bacteria, indicates that the IAT belongs to the Ntn (N-terminal nucleophile) hydrolase family (Brannigan et al. 1995). The *E. coli* PGA has a serine (Ser-264) as the nucleophile and, indeed, it is an autoprocessing enzyme that cleaves itself at the 263–264 peptide bond (Duggleby et al., 1995), the so-called excision site. However, IAT has low overall identity to PGA (10% identity) or PVA (11% identity) indicating that IAT belongs to a different class of Ntn proteins (see below). Although different members of the Ntn protein superfamily do not show extensive amino acid similarity, they all share the same folding structure composed of two layers of β sheets sandwiched between two layers of α -helices ($\alpha\beta\beta\alpha$ structure).

An interesting finding on both the fungal IAT and the bacterial PGA is that the nucleophile residue at the active center is involved in both the self-processing during maturation of the pro-enzyme and the cleavage of the substrate (IPN for IAT, PenG for PGA or PenV for PVA). Hewitt et al. (2000) reported that a slow self-processing PGA variant is also less active in penicillin G hydrolysis. Similarly, we observed that the *A. nidulans* IAT (less active than the *P. chrysogenum* IAT) is a very slow self-processing enzyme (Fernández et al., 2003), presumably due to differences in some amino acid residues of these two fungal enzymes. The amino acids present at the cleavage site of both the *P. chrysogenum* and *A. nidulans* IATs are conserved (Fig. 9.2), but other important residues are different (Montenegro et al., 1992).

The fungal IATs have a cysteine nucleophile whereas other enzymes have a serine or threonine at that position (Murzin et al., 1995; Andreeva et al., 2004). During autoprocessing, the side chain of the cysteine, serine, or threonine nucleophile attacks the carbonyl (CO) group of the preceding peptide bond and cleaves it. Directed mutagenesis of the cys-103 to ser or to ala makes the pro-IAT unprocessable (Tobin et al., 1994; García-Estrada et al., 2008b; Bokhove et al., 2010). The cys103ser mutant has not sufficient nucleophilicity, as compared to cys-103 in the wild type, to attack the peptide bond. The inducer molecule, or cellular condition that activates the enzyme to trigger the nucleophilic

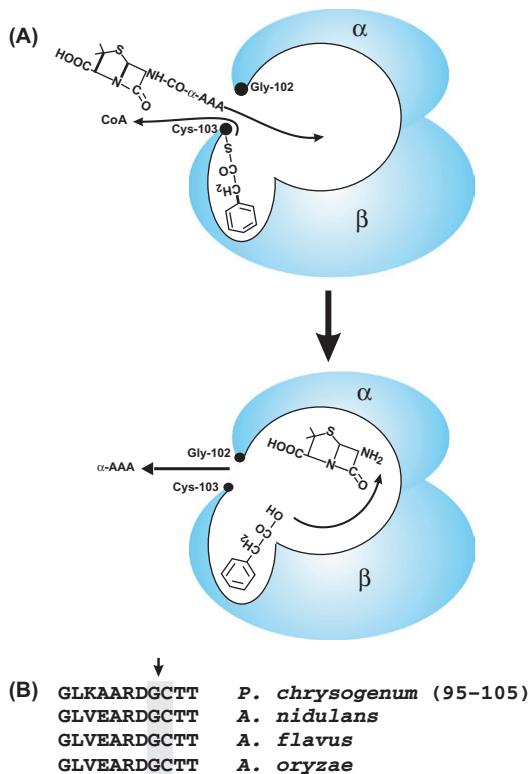


FIGURE 9.2 (A) Model of the reactions occurring in the cavity between the α and β subunits of IAT. Upper drawing: Entry of IPN (with α -AAA as arrowhead) into the cavity and binding of phenylacetic acid to the SH of cys-103 as thioester. Lower drawing: Cleavage of the CO-NH bond between α -AAA and 6-APA by the nucleophile attack and exit of α -amino adipic acid; subsequently, the phenylacetic acid is attached to the 6-APA in the 6-APA binding pocket of the cavity. (B) Comparison of the amino acids forming the lid (residues 95–102 in *P. chrysogenum* enzyme) that closes the entrance to the cavity in *P. chrysogenum* and *A. nidulans*, *Aspergillus flavus*, and *A. oryzae* IATs. The cleavage site G^{102} - C^{103} is indicated by a vertical arrow. Note the small sequence differences between the lids of *Penicillium* and *Aspergillus*.

attack, is unknown but it may be related to its localization in peroxisomes. The higher pH environment of the peroxisomal matrix (above pH 7.0), as compared to the cytosol, and the accumulation of hydrogen peroxide and catalase in these organelles may be relevant to trigger the nucleophile attack on the gly^{102} - cys^{103} peptide bond and, therefore, for the hydrolytic activity on IPN. Using the unprocessable *cys103ser* (*C103S*) variant enzyme, we proved that the unprocessed IAT is perfectly targeted to the peroxisomes and transported into these organelles (Garcia Estrada et al., 2008b), but it is as yet unclear if the wild-type enzyme is cleaved or not in the preperoxisomal traffic of the protein from the ER to the peroxisomes (Roze et al., 2011; Martín et al., 2012b; 2013).

Hengsens et al. (2002) and Bokhove et al. (2010) crystallized the IAT and studied its autoprocessing mechanism. They proposed that the peptide extending from positions 91–02 of the pro-IAT acts as a lid that closes the access of IPN to the substrate binding pocket in the precursor IAT. When the gly¹⁰²-cys¹⁰³ bond is cleaved, the C-terminal peptide of the α -subunit (amino acids 95–102) departs from the α – β heterodimeric structure, opening the substrate binding pocket in the precursor protein. The proposed flexibility of the 95–102 peptides is based on models deduced from electron density maps of the mature IAT and its C103A variant after crystallization. The distance between residue 101 in the C-terminus of the α -subunit and the cys-103 (β subunit) in the mature enzyme was over 25 Å. The gly-102 C-terminal residue of the α subunit does not show up in the electron density maps, indicating that it is not in a rigid permanent position (Bokhove et al., 2010).

9.5.2 The IPN/6-APA/PenG Substrate Binding Pocket

The IAT crystallographic studies have shown that there is a large cavity between the α and β subunits with a narrow entrance in which the nucleophilic cys103 interacts with the IPN substrate (Bokhove et al., 2010). Cleavage of the gly102-cys103 bond and opening of the lid (amino acids 95–102) do not drastically change the structure of the cavity and, therefore, the uncapped narrow entrance to the cavity still poses a limitation for bulky substrates such as benzylpenicillin, or phenoxyethylpenicillin. Based on the data of amino acids lining the cavity, Bokhove et al. (2010) proposed a tetrahedral reaction intermediate. In this model, IPN enters the cavity as an arrow with the α -amino adipic acid chain as the arrowhead establishing bonds through the α -amino and carboxylic groups. Following the nucleophilic attack, cleavage of the amide bond that releases the α -amino adipic moiety takes place. α -Amino adipic acid has to leave the cavity providing room for the entry of the aromatic acyl side chain precursor. These authors propose that the 6-APA formed in the hydrolytic reaction is temporarily stored in a substrate subpocket of the cavity, allowing the released α -amino adipic acid to leave the reaction cavity. The exit of α -amino adipic acid might be a limiting step. This model explains our early observation that the IPN amidohydrolase reaction is more inefficient (and therefore probably rate-limiting in the IAT) than the 6-APA acyltransferase reaction (Alvarez et al., 1993).

9.5.3 A Transient acyl-IAT Intermediate

Alvarez et al. (1987, 1993) observed that the IAT has a thioesterase site (GXS³⁰⁹XG) close to the C-terminus of the β -subunit, that appears to be involved in recognition of the phenylacetyl-CoA (or related acyl-CoA thioesters) and hydrolysis of the thioester bond transferring the phenylacetic acid moiety to a conserved cysteine residue in the IAT.

The serine residue in the GXS³⁰⁹XG sequence is indeed essential for IAT activity (Tobin et al., 1994). Although no enzymatic analysis has been made of the putative thioesterase activity, Aplin et al. (1993) using electrospray protein mass spectrometry concluded that an acyl-IAT intermediate with a mass increase of 116 Da is formed in phenylacetic acid-supplemented culture medium that appears to correspond to a phenylacetyl-IAT transient intermediate. The mass increase was different when the culture was supplemented with phenoxyacetic acid. Similar transient mass increases were observed in in vitro reactions when the enzymatic reaction was carried out in the presence of other acyl-CoAs that correlate with the acyl group mass (Aplin et al., 1993). Bokhove et al. (2010) proposed that the transient acyl-enzyme intermediate is formed at the SH group of cys-103 at the entrance of the substrate pocket. This allows attack of the thioester bond of the acyl enzyme intermediate by the amino group of 6-APA, stored temporarily at the “parking subpocket,” resulting in the formation of the final product of the pathway, benzylpenicillin, which leaves the cavity. When 6-APA, instead of IPN, is used as a substrate in vitro, the 6-APA acyltransferase reaction is facilitated by the absence of α -amino adipic acid. The IAT could be crystallized, complexed with 6-APA that interacts efficiently with amino acids lining the reaction pocket (Bokhove et al., 2010).

9.5.4 Interconversion of Aromatic Penicillins

The penicillin acylase and penicillin acyltransferase activities (interconverting enzymes) described initially by Meesschaert and Eyssen (1981), and shown to be part of the IAT activities, are explained by the action of the amidohydrolase activity on PenG (or PenV) instead of the normal substrate IPN. The released 6-APA would be temporarily stored at the “parking subpocket” until it reacts with a different acyl chain provided by hydrolysis of the second penicillin (eg, PenV) molecule. However, the narrow entrance to the active center may limit the access of bulky penicillins. Alternatively, the aromatic side chain cleavage in vivo may involve an entirely different *P. chrysogenum* acylase that forms 6-APA as product (Whiteman et al., 1990; Whiteman and Abraham, 1996).

9.5.5 The Origin of IAT: An Homologous AT in Many Fungal Genomes

The *penDE* gene, encoding IAT, contains three introns in *P. chrysogenum*, *A. nidulans*, and *A. oryzae*, and its origin is different from that of *pcbAB* and *pcbC*, which lack introns and appear to be of bacterial origin (Landan et al., 1990; Peñalva et al., 1990; Liras et al., 1998). The *penDE* gene is only present in penicillin-producing fungi but not in the cephalosporin producer *A. chrysogenum*. It is closely related to the orthologous *penDE* gene (also named *aat*) in other penicillin-producing fungi and its origin is intriguing.

The IAT belongs to a group of related fungal Ntn amidase/acylases that are encoded by genes designated as *ial* (for IAT-like) that occur in many ascomycetes (García-Estrada et al., 2009). The *ial* gene of *P. chrysogenum* was initially described during analysis of the *P. chrysogenum* genome (van den Berg et al., 2008) and the orthologous gene of *A. nidulans* (named *aatB*) was then identified (Spröte et al., 2008). The IAL protein shares 54% similarity (34% identity) with *P. chrysogenum* IAT and 52% similarity (35% identity) with *A. nidulans* IAT. This similarity is higher than that of bacterial PGA or PVAs (10% identity). IAL has an orthologous gene in most ascomycetes that contains 2 or 3 introns. They encode proteins with similarities ranging from 76% to 81% with proteins of unknown function (members of the IAL subfamily).

The *P. chrysogenum* and *A. nidulans* IAL proteins contain the characteristic Ntn motifs of self-processing enzymes, but lacks the peroxisomal targeting sequence ARL or ANI that occurs in the IAT. Using enzyme purified after expression of *ial* in *E. coli*, it was confirmed that the 40 kDa pro-IAL is processed into two subunits α (about 12 kDa) and β (28 kDa) by self-cleavage, presumably at the G¹⁰⁵-C¹⁰⁶ bond, as occurs with the IAT. The *ial* gene is located away from the penicillin gene cluster, and mutants lacking the *penDE* gene (eg, *npe10 AB-C*) but containing a functional *ial* gene were unable to produce penicillin, indicating that the *ial* gene does not encode a second IAT enzyme. Conversely, mutants deleted in the *ial* gene, but conserving the penicillin gene cluster, produce normal amounts of benzylpenicillin (García-Estrada et al., 2009). Since the IAL protein lacks the peroxisomal targeting signal, an engineered protein carrying the canonical C-terminal ARL amino acids was used in complementation studies. Even when targeted to peroxisomes, the IAL failed to show in vivo IPN-amidolyase acid or IPN acyltransferase activities. Detailed analysis of the protein concluded that the lack of IPN acyltransferase activity in IAL is probably due to the lack of the thioesterase center (at ser-309 in the IAT) that participates in the cleavage of phenylacetyl-CoA and the transfer of phenylacetic acid to form the phenylacetyl-enzyme intermediate in the acyltransferase reaction. Indeed, the *A. nidulans* IAL enzyme (named AatB), that conserves the ser-309 amino acid, shows some penicillin biosynthesis activity (Spröte et al., 2008), even though the protein lacks the canonical peroxisomal targeting signal.

In summary, differences in the protein sequence explain the different activities of IAT and IAL proteins, but the real substrate(s) of IAL in all ascomycetes remains unknown. Although the initial observation of *ial* genes in β -lactam producing fungi led to speculation that the *penDE* and *ial* genes might be related, their low overall identity and the lack of contribution of *ial* to penicillin biosynthesis suggest that the role of IAL in fungal cells is different from that of IAT. However, both genes may derive from a common acyltransferase ancestor gene. Further biochemical characterization of the IAL substrates and reaction products will clarify the relationship between these two enzymes.

9.6 CONCLUDING REMARKS

Considerable progress has been made on our understanding of the structure and function of the main enzymes involved in penicillin formation and the early steps of cephalosporin biosynthesis. Still our knowledge of the structure-activity relationship of the ACVS is very limited and needs to be studied in more detail. The protein–protein interactions between ACVS and IPNS, suggested many years ago, have not been studied. These two proteins are encoded by linked genes in all penicillin-, cephalosporin-, and cephemycin-producing microorganisms. In fungi, both genes *pcbAB* and *pcbC* are expressed from a promoter region as divergent transcripts, which facilitates their coordinate regulation (Brakhage, 1998; Martín 2000a). The availability of advanced proteomic and mass-spectrometry tools should be explored to gain insight into their protein–protein interactions.

In bacterial β -lactam gene clusters, the *pcbAB* and *pcbC* genes are in a tail to head organization and both genes are expressed as a polycistronic transcript from a promoter located upstream of *pcbAB*. The knowledge of the β -lactam biosynthetic enzymes is useful for further improvement of β -lactam production. For instance, the adipyl-CoA ligase activity encoded by *phlB* is useful for the synthesis of adipyl-containing cephalosporins in engineered *P. chrysogenum* strains. Similarly, some members of the Ntn acylases for example, PGA and CA (also named glutarylacylase) are very useful for semisynthetic penicillin and cephalosporin production and may be modified to improve their enzyme kinetics and thermal stability.

Knowledge of the protein structure, substrate specificity, cofactor requirements, and possible modifications of the kinetic parameters by targeted mutation of the encoding genes will facilitate the application of these enzymes for specific substrate conversions in vitro (Barends et al., 2004).

ABBREVIATIONS

- 6-APA** 6-aminopenicillanic acid
- AC dipeptide** δ -(L- α -amino adipyl)-L-cysteinyl dipeptide
- ACOV** δ -(L- α -amino adipyl)-L-cysteinyl-D- α -hydroxyisovaleryl ester
- ACP** Acyl carrier protein
- ACV** δ -(L- α -amino adipyl)-L-cysteinyl-D-valine
- ACVS** ACV synthetase
- DAC** Deacetylcephalosporin C
- DAOC** Deacetoxyccephalosporin C
- D-pHPG** D-*p*-Hydroxyphenyl glycine
- ER** Endoplasmic reticulum
- HPLC** High performance liquid chromatography
- IAL** Isopenicillin N acyltransferase like protein
- IAT** Isopenicillin N acyltransferase
- IPN** Isopenicillin N

- IPNS** IPN synthase
MFS Major facilitator superfamily
NO Nitrogen oxide
NRPS Nonribosomal peptide synthetase
Ntn N-terminal nucleophile
ORF Open reading frame
PCP Peptidyl carrier protein
PGA Penicillin G acylase
PTS1 Peroxisomal targeting sequence 1
PVA Penicillin V acylase
TE Thioesterase

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Chapter 10

The Cellulosome: A Supramolecular Assembly of Microbial Biomass-Degrading Enzymes

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10.1 INTRODUCTION

Plant biomass consisting of lignocellulosic materials is the most abundant organic matter on earth. Its degradation in nature is catalyzed by a myriad of microbial biomass-degrading enzymes including cellulases, hemicellulases, and ligninases. The best-studied cellulase/hemicellulase system is from the fungus *Trichoderma reesei*. *T. reesei* produces various *exo*- and *endo*- β -glucanases, and hemicellulases. These secreted enzymes are free (monomeric) proteins and it has also been established that enzymatic cellulose degradation is facilitated by the synergistic action between *exo*- and *endo*- β -glucanases.

To degrade a crystalline cellulose, the *endo*-enzyme first makes a nick on the cellulose chain, creating reducing and nonreducing ends, respectively. *Exo*-enzymes then remove cellobiose units in a step-wise fashion from the chain ends, ultimately leading to complete degradation of the crystalline cellulose. In this mode of action, the *endo*-enzyme is expected to diffuse away from the site of the nick to vacate the space for the *exo*-enzymes. The enzyme diffusion process appears to be tedious and less efficient than if the *endo*- and *exo*-enzymes would form a complex and work collaboratively on the site of degradation. However, the enzyme, once secreted, can be quickly diluted in the extracellular environment, making complex formation less favorable. Instead, the fungus appears to compensate for the relative inefficiency of monomeric proteins by producing a large quantity of extracellular proteins to ensure synergism among these enzymes.

In the early 1980s, scientists who studied the cellulase system of *Clostridium thermocellum*, a thermophilic and anaerobic bacterium, discovered that it exists mainly in the form of a mysterious large protein aggregate. The aggregate was later termed “the cellulosome” (Lamed et al., 1985). The ensuing research for the next two to three decades, aided by the availability of the genome sequence as well as modern biophysical and bioinformatics tools, has revealed that the cellulosome is a well-organized supramolecular assembly that works as a molecular machine, in a cell-bound or cell-free fashion, for cellulosic biomass degradation. In this chapter, the composition, structure and assembly of the cellulosome of *C. thermocellum* will be described.

10.2 STRUCTURE OF THE CLOSTRIDIUM THERMOCELLUM CELLULOSOME

10.2.1 Model of the Cellulosome Assembly

The structure of the cellulosome had remained elusive for a long time since the protein aggregate was discovered. The first breakthrough came when an anchor–enzyme model, based on biochemical characterization of dissociated cellulosome components, was proposed (Wu, 1987; Wu and Demain, 1988; Wu et al., 1988). In this model, the largest cellulosome component CipA (cellulosome integrating protein A; $M_r = \sim 250\text{kD}$) served as an anchor on the cellulose surface for catalytic subunits (Gerngross et al., 1993). The DNA sequence of CipA later revealed nine repeated modules, which are termed type I cohesins (Bayer et al., 1994). Each type I cohesin serves to bind to a catalytic subunit through its type I dockerin (Bayer et al., 1994), a duplicated sequence of a total molecular mass of about 8 kD, borne mostly at the C-terminal end of the catalytic subunit. A cellulose-binding module (CBM) lies in between the second and third cohesins, which anchors CipA and its tethered catalytic subunits to the cellulose, corroborating with the anchor–enzyme concept (Fig. 10.1).

10.2.2 Scaffoldin Proteins of the Cellulosome

The key scaffoldin, CipA, which binds to catalytic subunits and anchors them on the cellulose surface, also contains a dockerin (type II) at its C-terminal end. The type II dockerin binds to the corresponding type II cohesin born in three cell-surface proteins: (1) SdbA (scaffoldin dockerin binding protein A, containing one type II cohesion) (Leibovitz et al., 1997); (2) Orf2 (open reading frame 2, containing two type II cohesins) (Leibovitz and Beguin, 1996a); and (3) OlpB (outer layer protein B, containing seven type II cohesins) (Lemaire et al., 1995a). Each of these three proteins contains a cell-surface anchor domain composed of three SLH (S-layer homology) modules, through which

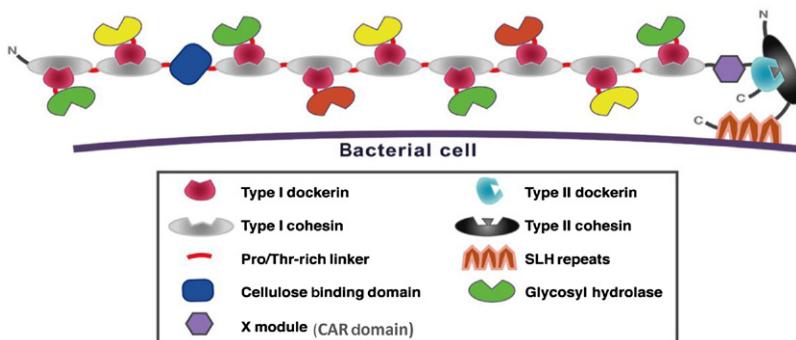


FIGURE 10.1 A schematic diagram of the *Clostridium thermocellum* cellulosome. The type I dockerins mediate attachment of the catalytic subunits to the scaffoldin CipA, which is comprised of nine type I cohesins, a cellulose-binding domain (CBD), a hydrophilic domain of unknown function (X or CAR), and a type II dockerin. The scaffoldin likewise binds through its type II dockerin domain to a type II cohesin-containing protein on the bacterial cell surface that is thought to anchor the complex through a series of three surface-layer homology (SLH) domains (see Fig. 10.2 for details). Figure adapted from Volkman *et al.*, 2004 with permission.

they mediate the attachment of the cellulosome unit to the bacterial cell wall (Fig. 10.2). The fact that OlpB contains seven type II cohesins allowing the attachment of multiple cellulosome units, each consisting of a CipA scaffodin and its nine tethered catalytic subunits, may explain the huge polycellulosomes of 1.5–6 MDa observed by electron microscopy (Felix and Ljungdahl, 1993). On the other hand, heretofore-unknown mechanisms may also be involved in the formation of the huge polycellulosomes.

It is interesting that another scaffold protein, that is, 7CohII (Cthe_0736) contains seven type II cohesins as OlpB but without the type II dockerin. Lacking the dockerin, 7CohII is presumed to exist in a cell-free environment. It therefore appears to be a cell-free version of OlpB. Thus, the cellulosome (and the polycellulosome) is expected to exist in a cell-associated form as well as in a cell-free form. It would be interesting to determine how the bacterial cell regulates the relative abundance of these two forms of the cellulosome and how the ratio affects the overall activity of the cellulolytic system. Besides regulation at the transcriptional level, it is possible that the cell-associated cellulosome can be freed by proteolytic removal of the type II dockerin in a posttranslational fashion. On the other hand, two cell-surface proteins, OlpA (Fujino *et al.*, 1992) and OlpC (Pinheiro *et al.*, 2009), contain a type I cohesin. Thus, it is presumed that each docks a catalytic subunit born with a type I dockerin. OlpA has a cell-surface anchor domain composed of three SLH modules. OlpC, on the other hand, has a different cell wall-binding module. The physiological functions of OlpA and OlpC are not clear. It has been postulated that these two proteins function as a “warehouse” by transiently retaining cellulosomal catalytic subunits before they are assembled into the cellulosome unit.

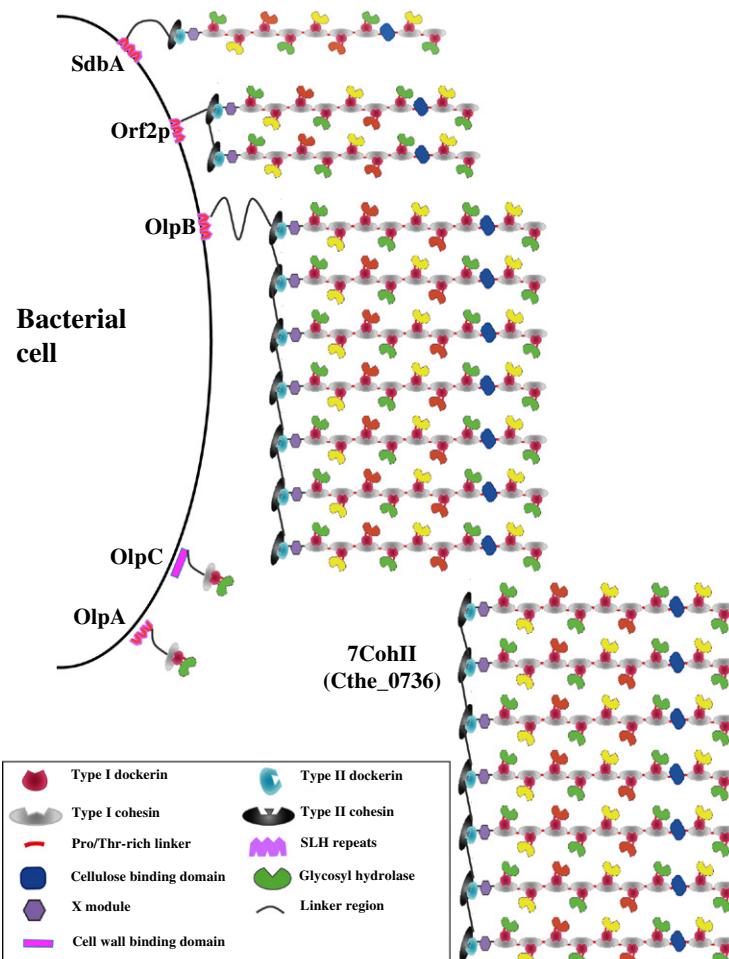


FIGURE 10.2 A schematic drawing depicting cellulosome attachment to the cell surface through the type II dockerin-cohesin interaction with SLH domain-containing cell surface proteins, SdbA, Orf2p, and OlpB. On the other hand, OlpA and OlpC, each of which contains a type I cohesin, is presumed to anchor to an enzyme or protein containing a type I dockerin. 7CohII contains seven type II cohesins but without the type II dockerin. *Figure adapted from (Deman et al., 2005)*.

The important role of CipA in cellulose hydrolysis has been investigated by gene deletion. Deletion of *cipA* from the *C. thermocellum* genome resulted in a 100-fold reduction in cellulose degradation rate, but 80% of the degradation extent was eventually achieved (Olson et al., 2013). The result corroborates with the presumption that the cellulosome organization is the bacterial solution for achieving accelerated cellulosic biomass degradation. Deletion of six type I cohesins of CipA decreased the cellulose hydrolysis rate by 46%, indicating the importance of the length of CipA. Further deletion of the CBM of CipA resulted

TABLE 10.1 Cohesion-Containing Proteins of *Clostridium thermocellum* ATCC 27405

Protein	Function	Gene	Module(s)	References
CipA	Cellulosome scaffoldin	Cthe_3077	2(Coh1)-CBM3a-7 (Coh1)-Car-Doc2	Bomble et al. (2011)
				Olson et al. (2013)
OlpA	Cell-surface anchor	Cthe_3080	Coh1-3(SLH)	Fujino et al. (1992)
OlpC	Cell-surface anchor	Cthe_0452	CAO-Coh1	Pinheiro et al. (2009)
–	Cellulosome anchoring	Cthe_0735	Coh2	
7CohII	Cellulosome anchoring	Cthe_0736	7(Coh2)	
OlpB	Cell-surface anchor	Cthe_3078	7(Coh2)-X-3(SLH)	Lemaire et al. (1995b)
Orf2p	Cell-surface anchor	Cthe_3079	2(Coh2)-3(SLH)	
SdbA	Cell-surface anchor	Cthe_1307	Coh2-3(SLH)	Leibovitz and Beguin (1996b)

in an additional reduction of hydrolysis rate by 89–92% (Hong et al., 2014). These results confirm that the advantage of cellulosomal organization over free enzymes indeed resides in the anchor–enzyme mechanism, in which the anchor (CipA) brings a battery of enzymes to the substrate surface.

The importance of cell association of the cellulosome has also been investigated by gene deletion. Deletion of the type II dockerin of CipA, or of any of the four secondary scaffoldins (OlpB, 7CohII, Orf2p, or SdbA), resulted in a moderate reduction of cellulose hydrolysis rate. Thus, the cell-associated cellulosome appears to be more efficient than the cell-free cellulosome under the experimental conditions. Again, the role of the cell-free cellulosome and the differential regulation of the cell-bound versus cell-free cellulosome await further study.

10.2.3 Catalytic Components of the Cellulosome

Sequence analysis of the draft genome of *C. thermocellum* ATCC 27405 revealed >70 type I dockerin-containing genes and CipA (Demain et al., 2005; Zverlov et al., 2005a). Analysis of the complete genome sequence revealed that the genome encodes eight type I/II cohesin-containing, noncatalytic proteins (Table 10.1) and 73 Doc1-containing proteins, a large number of them being carbohydrate-active enzymes (CAZ) belonging to various glycoside hydrolase (GH) families (Table 10.2).

TABLE 10.2 Type I Dockerin-Containing Cellulosomal Proteins of *Clostridium thermocellum* ATCC 27405

Protein	Function	Gene	Module(s)	References
Cellulase/β-glucanase				
CelO	Cellobiohydrolase	Cthe_2147	CBM3a-GH5-Doc1	Zverlov et al. (2002b)
CelB	Endoglucanase	Cthe_0536	GH5-Doc1	Bomble et al. (2011)
CelG	Endoglucanase	Cthe_2872	GH5-Doc1	Latimer and Ferry (1993)
CelL	Endoglucanase	Cthe_0405	GH5-Doc1	
CelE	Endoglucanase	Cthe_0797	GH5-Doc1-CE2	Montanier et al. (2009)
CelA	Endoglucanase	Cthe_0269	GH8-Doc1	
GH81	β-1,3-Glucanase	Cthe_0660	GH81-Doc1	
CbhA	1,4-β-Cellobiosidase	Cthe_0413	CBM4-Ig-GH9-2X1-CBM3b-Doc1	Bomble et al. (2011)
CelK	1,4-β-Cellobiosidase	Cthe_0412	CBM4-Ig-GH9-Doc1	Kataeva et al. (1999)
CelD	Endoglucanase	Cthe_0825	Ig-GH9-Doc1	Joliff et al. (1986)
GH9	1,4-β-Glucanase	Cthe_2761	GH9-CBM3a-Doc1	
GH9	Glycoside hydrolase	Cthe_0433	GH9-CBM3c-Doc1	
CelN	Endoglucanase	Cthe_0043	GH9-CBM3c-Doc1	Zverlov et al. (2003)
CelR	Endoglucanase	Cthe_0578	GH9-CBM3c-Doc1	Zverlov et al. (2005a)
CelQ	Endoglucanase	Cthe_0625	GH9-CBM3c-Doc1	Arai et al. (2001)
CelF	Endoglucanase	Cthe_0543	GH9-CBM3c-Doc1	
CelW	Endoglucanase	Cthe_0745	GH9-CBM3a-Doc1	

CelV	Endoglucanase	Cthe_2760	GH9-CBM3a-Doc1	
CelU	Endoglucanase	Cthe_2360	GH9-CBM3b,3c-Doc1	
CelP	1,4- β -Glucanase	Cthe_0274	GH9-Doc1	
CelT	1,4- β -Glucanase	Cthe_2812	GH9-Doc1	Kurokawa et al. (2002)
CelJ	Cellulose	Cthe_0624	Ig-GH9-GH44-Doc1-PKD-CBM30	Arai et al. (2003)
CelH	Endoglucanase	Cthe_1472	GH26-GH5-CBM11-Doc1	Carvalho et al. (2004)
CelS	Exoglucanase	Cthe_2089	GH48-Doc1	Bomble et al. (2011)
Cel124A	Endoglucanase	Cthe_0435	Doc1-UN	Brás et al. (2011)

Xylanases

XynD	Xylanase	Cthe_2590	CBM4-GH10-Car-Doc1	
XynC	Xylanase	Cthe_1838	CBM22-GH10-Doc1	Zverlov et al. (2005b)
XynY	Xylanase	Cthe_0912	CBM22-GH10-CBM22-Doc1-CE1	Najmudin et al. (2010)
–	Xylanase	Cthe_3012	GH30-CBM6-Doc1	
XynA/U	Xylanase	Cthe_2972	GH11-CBM4-Doc1-NodB	Hayashi et al. (1999)
XynZ	Xylanase	Cthe_1963	CE1-CBM6-Doc1-GH10	
CE1	1,4- β -Xylanase	Cthe_2194	CE1-CBM6-Doc1	
–	1,4- β -Xylnase	Cthe_2879	Doc1-UN	

(Continued)

TABLE 10.2 Type I Dockerin-Containing Cellulosomal Proteins of *Clostridium thermocellum* ATCC 27405 (Continued)

Protein	Function	Gene	Module(s)	References
GH43	Xylanase	Cthe_2196	GH43-CBM6-Doc1	
GH43	Xylanase	Cthe_1271	GH43-2(CBM6)-Doc1	
GH43	β -Xylosidase	Cthe_0661	GH43-CBM13-Doc1	
Xyl5A	–	Cthe_2193	GH5-CBM6-CBM13-Fn3-CBM62-Doc1	Montanier et al. (2011)
Cse3	Lipase	Cthe_0798	2(CE3)-Doc1	
Hemicellulases				
LicB	Lichenase	Cthe_0211	GH16-Doc1	
ChiA	Chitinase	Cthe_0270	GH18-Doc1	Zverlov et al. (2002a)
ManA	Mannanase	Cthe_2811	CBM35-GH26-Doc1	Halstead et al. (2000)
Man5A	Mannanase	Cthe_0821	GH5-CBM32-Doc1	Mizutani et al. (2012)
GH26	1,4- β -Mannosidase	Cthe_0032	CBM35-GH26-Doc1	
Glycoside hydrolases				
GH53	β -Galactosidase	Cthe_1400	GH53-Doc1	
GH2	Glycoside hydrolase	Cthe_2197	GH2-Ig-CBM6-Doc1	
XghA	Xylogalacturonan hydrolase	Cthe_1398	GH74-CBM2-Doc1	Zverlov et al. (2005b)
Carbohydrate esterases				
G-D-S-L	Lipase	Cthe_3141	Fn3-CE12-Doc1-CBM6-CE12	

Pectinases				
PL1	Pectate lyase	Cthe_2950	PL3-Doc1-CBM6	Chakraborty et al. (2015)
PL9	Pectate lyase	Cthe_2179	PL3-Doc1-CBM35-PL9	Chakraborty et al. (2015)
PL11	Rhamnogalacturonan lyase	Cthe_0246	Doc1-CBM35-RGL11	
–	Pectinesterase	Cthe_2949	CE8-Doc1	
Arabinogalactan hydrolases				
–	α-L-Arabinofuranosidase	Cthe_2139	GH30-GH54-GH43-Doc1	
–	α-L-Arabinofuranosidase	Cthe_0015	GH54-Doc1-GH43	
–	α-L-Arabinofuranosidase	Cthe_2549	UN-Doc1	
–	β-Xylosidase	Cthe_2138	ABFB-GH43-Doc1	
–	α-L-Arabinofuranosidase	Cthe_2038	PGU1-Doc1	
–	Endopolygalacturonase	Cthe_0640	PL3-Doc1	
Proteases and inhibitors				
–	Peptidase S8 subtilisin-like serine protease	Cthe_3136	PepS8-2(Doc1)	
–	Proteinase inhibitor I4, serpin	Cthe_0190	Fn3-Doc1-Serpin	
–	Proteinase inhibitor I4, serpin	Cthe_0191	Fn3-Doc1-Serpin	

(Continued)

TABLE 10.2 Type I Dockerin-Containing Cellulosomal Proteins of *Clostridium thermocellum* ATCC 27405 (Continued)

Protein	Function	Gene	Module(s)	References
Other functions				
NagA	–	Cthe_2195	UN-CBM6-Doc1	
CseP	–	Cthe_0044	CotH-Doc1	Zverlov et al. (2003)
–	–	Cthe_0239	2(LTD)-Fn3-CotH-Doc1	
–	–	Cthe_0109	Doc1	
–	LytTR transcription regulator	Cthe_0258	Doc1-5(RCC1)	
–	–	Cthe_0729	Doc1-UN	
–	–	Cthe_0918	Car-Doc1	
–	–	Cthe_1806	SerH-SLD-Doc1	
–	–	Cthe_1890	Doc1-PDK-UN-LRP5-UN	
–	–	Cthe_2137	Lac-2(CBM35)-Doc1	
–	–	Cthe_2271	2(Doc1)	
–	–	Cthe_3132	SCP-Doc1	
–	–	Cthe_0438	Doc1	

GHs may consist of one or more catalytic modules linked to a CBM(s) and/or another ancillary module(s). The functions of many of these catalytic modules and CBMs have been demonstrated biochemically or inferred from sequence homology to enzymes with known activities (Table 10.2). GHs (Cantarel et al., 2009) and CBMs (Sakon et al., 1997) are classified into more than 120 and 60 sequence-based families, respectively (see CAZy database <http://www.cazy.org/>).

The most prominent catalytic subunit of the cellulosome is the family 48 (Sukharnikov et al., 2012) exoglucanase CelS (Wang et al., 1993). Exoglucanases are crucial for degrading crystalline cellulose. Among the cellulosomal catalytic components, CelS is the only exoglucanase unambiguously characterized (Kruus et al., 1994; Wang et al., 1994). It is one of the most, if not the most, abundantly expressed cellulosomal catalytic subunits. The crucial role of CelS in degrading crystalline cellulose was proven, as deletion of *celS* reduced the cellulose hydrolysis rate by 40% and led to a lower cell yield on crystalline cellulose. The purified cellulosome derived from the *celS*-deletion mutant has a 35% lower specific activity on Avicel, a commercial preparation of crystalline cellulose (Olson et al., 2013).

Some of the type I dockerin-containing proteins display little sequence similarity to enzymes in the CAZy database. One of them, termed Cel124A, was recently shown to be an endoglucanase (Brás et al., 2011) that acts in synergy with CelS. Recently, Chakraborty et al. (Chakraborty et al., 2015) showed that the catalytic domains of two pectinolytic enzymes, PL1 (Cthe_2950; family 1) and PL9 (Cthe_2179; family 9), display endo-pectate lyase activities. Another interesting observation is that the CE2 module of CelE displays divergent catalytic esterase and noncatalytic carbohydrate-binding functions within the same active site (Montanier et al., 2009).

Besides the CBM of CipA, catalytic subunits may have their own CBMs. The CBM of a cellulosomal enzyme adsorbs to a polysaccharide and brings the linked catalytic domain into close and prolonged contact with it to promote its degradation (Shoseyov et al., 2006). However, many cellulosomal enzymes do not have a CBM. For example, CelT lacks a family 3c CBM and an immunoglobulin (Ig)-like domain often found in other family nine enzymes (Kurokawa et al., 2002). They are thus relying on the CBM of CipA for gaining the proximity to the cellulosic substrate.

10.2.4 Noncellulosomal Enzymes

Although this chapter focuses on the cellulosomal enzymes, it is worth noting that *C. thermocellum* also produces free enzymes (Table 10.3) (Raman et al., 2011). Among them, Cell, a processive endoglucanase, is an analog to the cellulosomal enzyme CelR. Likewise, the noncellulosomal exoglucanase CelY is homologous to the principal cellulosomal enzyme CelS (Vazana et al., 2010). Knocking out *celY* in the *celS*-deletion mutant did not significantly affect celulase activity (Olson et al., 2010). However, this should not be surprising as CelY was not highly expressed under the experimental conditions. The roles

TABLE 10.3 Noncellulosomal Glycoside Hydrolases and CBM-Containing Proteins of *Clostridium thermocellum* ATCC 27405

Protein	Presumed function	Gene	Module(s)	References
BglB	β -Glucosidase B	Cthe_1256	GH3-GH3C-Fn3	
Bgl	β -Galactosidase	Cthe_1428	GH1	
GH23	Lytic transglycosylase	Cthe_2744	GH23	
LicA	β -1,3-1,4-Glucanase	Cthe_2809	3(SLH)-GH16-4(CBM4)	Fuchs et al. (2003)
Rsi24C	Antisigma factor	Cthe_1471	GH5	
CelC	Endoglucanase	Cthe_2807	GH5	
BglX	β -Hexosaminidase	Cthe_0322	GH3	
GH13	α -Glucan branching enzyme	Cthe_2191	2(CBM48)-GH13	
GH51	α -L-Arabinofuranosidase	Cthe_2548	GH51	
GH84	Glycosyltransferase 36	Cthe_1221	GH84-2(GH94)	
GH13	α -Amylase	Cthe_0795	CBM34-GH13	
CenC	Carbohydrate-binding protein	Cthe_1257	CBM3-CBM4-vWFA	
Cell	Endoglucanase	Cthe_0040	GH9-CBM3c-CBM3b	Zverlov et al. (2003)
–	Glycosyltransferase	Cthe_0275	GH94	
–	Glycosyltransferase	Cthe_2989	GH94-GDE	
–	Acetyl xylan esterase	Cthe_3063	CE7	
–	Glycoside hydrolase	Cthe_2895	CAO-GH18	
Rsgl4	Antisigma factor	Cthe_0404	Rsgl-CBM3a	
Rsgl6	Antisigma factor	Cthe_2119	Rsgl-GH10	
OrfZ	Cellulose-binding protein	Cthe_0271	CBM3a	
Rsgl1	Antisigma factor	Cthe_0059	CBM3a	
–	Carbohydrate-binding	Cthe_3163	CBM25	
–	Carbohydrate-binding	Cthe_1080	CBM25	
CelY	Exoglucanase	Cthe_0071	GH48-CBMX2-CBM3	

of the noncellulosomal enzymes in the overall cellulolytic process remain to be investigated. It is possible that the bacterium acquires a new enzyme through lateral gene transfer in the form of a free enzyme before adding a dockerin to the enzyme through the evolution process.

10.3 REGULATION OF CELLULOSOMAL GENE EXPRESSION

10.3.1 Sigma Factor—Anti-Sigma Factor

Since crystalline cellulose is water-insoluble, how the bacterial cell senses the existence of the substrate has been an intriguing question. It was postulated that the cell surface bears cellulose sensors, which transmit the signal into the cell similar to the way cell-surface receptors transmit signals in eukaryotic cells. The hypothesis was verified when the sigma factor–anti-sigma factor pairs, each equipped with a CBM, were found to serve as the cell-surface substrate sensor.

Such an anti-sigma factor (RsgI) contains an extracellular CBM, a transmembrane domain, and an intracellular sigma factor-binding domain. RsgI binds to its cognate sigma factor in the absence of the polysaccharide signal. When the polysaccharide becomes available, RsgI binds to it and releases the sigma factor, which in turn activates its own bicistronic operon and the target cellulase-related gene(s).

Six putative bi-cistronic operons, encoding alternative sigma factors and their cognate membrane-associated anti-sigma factors (RsgI), have been identified (Table 10.4, 1–6). Among them, RsgI 1, 3, and 6 have been shown to bind cellulose and/or xylan specifically (Kahel-Raifer et al., 2010). Many SigI target genes were predicted by binding-sequence analysis, including CelS,

TABLE 10.4 Sigma Factors and Anti-Sigma Factors Identified in *Clostridium thermocellum*

σ/Anti-σ	Locus_tag	Sensing domain	References
σI1-RsgI1	Cthe_0058-9	CBM3	Kahel-Raifer et al. (2010)
σI2-RsgI2	Cthe_0268-7	CBM3	Kahel-Raifer et al. (2010)
σI3-RsgI3	Cthe_0315-6	PA14 dyad	Kahel-Raifer et al. (2010)
σI4-RsgI4	Cthe_0403-4	CBM3	Kahel-Raifer et al. (2010)
σI5-RsgI5	Cthe_1272-3	CBM42	Kahel-Raifer et al. (2010)
σI6-RsgI6	Cthe_2120-19	GH10	Kahel-Raifer et al. (2010)
σ24C-RsgI24C	Cthe_1470-1	GH5	Nataf et al. (2010)
σI7-RsgI7	Cthe_2521-2		Rydzak et al. (2012)
σI8-RsgI8	Cthe_2975-4		Kahel-Raifer et al. (2010)

CelA. SdbA, CelR, cellulosome-anchoring protein (Cthe_0735), and GH26 (Cthe_0032) (Nataf et al., 2010). The ability of SigI1 to activate CelS has been verified by in vitro runoff transcription assays. Natal and colleagues also identified the Sig24C- Rsi24C pair (Nataf et al., 2010). In addition, two more sets have been reported, that is, SigI7-RsgI7 (Cthe_2521-2) (Rydzak et al., 2012; Wei et al., 2014) and SigI8-RsgI8 (Cthe_2975-4) (Kahel-Raifer et al., 2010).

10.3.2 Cellulosome Gene Clusters

Clostridium thermocellum cellulosomal genes are scattered throughout the genome, but small clusters are found, sometimes including noncellulosomal genes. A few published studies have demonstrated that members of clusters are coordinately regulated in the form of an operon. Using northern blot hybridization, Fujino et al. (1993) detected bi-cistronic expression of *cipA-olpB* and *orf2-polpA* (Fig. 10.3, cluster 21). Newcomb et al. (2011) showed the presence of a

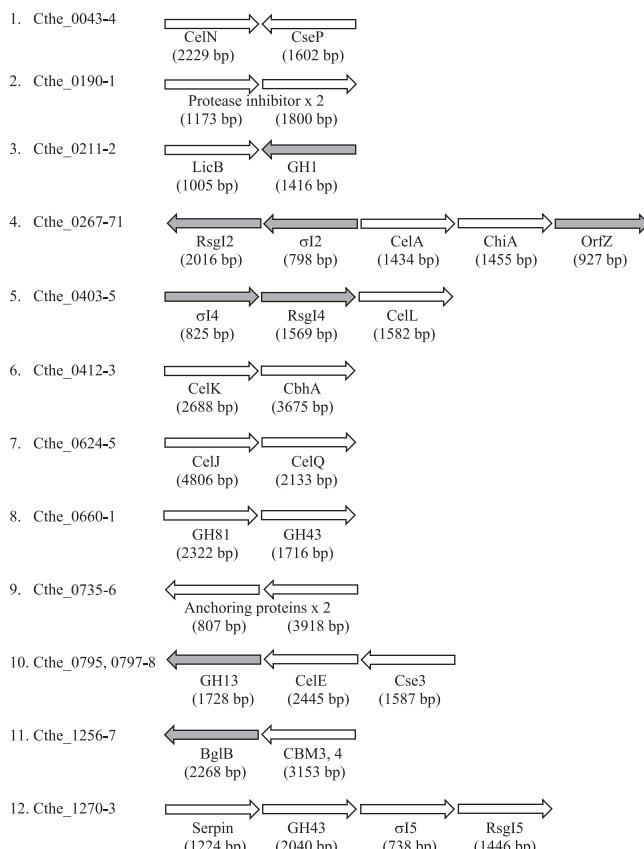


FIGURE 10.3 Cellulosomal (*open arrows*) and noncellulosomal (*filled arrows*) gene clusters identified in the genome of *Clostridium thermocellum* ATCC 27405. Figures are not drawn to scale. *GH*: Glycoside hydrolase gene family.

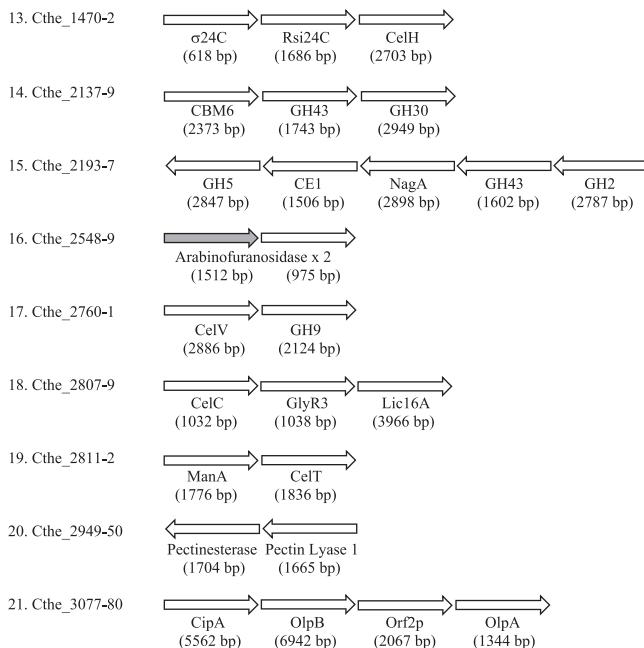


FIGURE 10.3 Continued

celC-glyR3-licA (Fig. 10.3, cluster 18) poly-cistronic messenger by northern blotting and a *manA-celT* bicistronic messenger by RT-PCR mapping. Newcomb and colleagues (Newcomb et al., 2007) showed that GlyR3, a LacI family repressor, negatively regulates the expression of two noncellulosomal enzymes, CelC and LicA. The repression is released when the disaccharide laminaribiose, a hemicellulose degradation product, is present, providing another mechanism of sensing the degradation product of a water-insoluble substrate.

10.3.3 Expression of Cellulosomal Genes

It has been demonstrated that production of the overall cellulolytic activity by *C. thermocellum* is influenced by the carbon source, but it is not clear how many individual genes are subject to carbon source regulation. The study was hampered by the overwhelming large number of genes involved in the degradation process. In recent years, this problem has been overcome by the availability of advanced genomic tools. Gold and Martin (2007) used a metabolic isotope-labeling strategy in combination with nano-liquid chromatography-electrospray ionization mass spectrometry peptide sequencing to detect a total of 41 cellulosomal proteins that were expressed, including 36 type I dockerin-containing proteins. All differential expression data were normalized to the scaffoldin CipA such that protein per cellulosome unit was compared. They also detected four noncellulosomal proteins. Similarly, Raman and colleagues (2009) used ¹⁵N-metabolic

labeling and multidimensional LC-MS/MS to quantify changes in levels of cellosomal proteins normalized to CipA. The cells were grown on a variety of carbon sources, including dilute-acid pretreated switchgrass, amorphous cellulose, crystalline cellulose (Avicel), cellobiose, and combinations of crystalline cellulose with pectin or xylan or both. In all samples, proteomic analysis identified 59 dockerin- and 8 cohesin-containing proteins. In general, the expression of 56–78% of the cellosomal components was distinctly different for substrates cellobiose, amorphous cellulose and pretreated switchgrass, as compared to crystalline cellulose. Raman et al. (2011) used whole-genome microarrays to investigate the temporal expression of *C. thermocellum* mRNA during growth on crystalline cellulose. They found that three sigma factors (σ I1, I2, and I4), and their cognate anti-sigma factors bearing CBM3 domains, were all upregulated in stationary phase. Furthermore, several genes encoding GH9 family cellulases were also upregulated with peak expression in early-to-late stationary phase. Noncellulolytic enzymes, including mannanases, xyloglucanase, pectate lyase, and xylanases, were also increased in expression (Raman et al., 2011). Wilson et al. (2013) reported on 12 highly expressed cellulosome-related genes (including CipA, CelS, Orfz, CelJ, scaffodin 7[Coh2], XynZ, pectinesterase, etc.) during growth on switchgrass or *Populus* by using both DNA microarray and RNA-sequencing (RNA-seq) methods. Wei et al. (2014) used RNA-seq to analyze the transcriptional profile of *C. thermocellum* grown on pretreated yellow poplar (PYP) or cellobiose. They found that 47 cellosomal protein-encoding genes were upregulated, 30 genes were unchanged, and three genes were downregulated during growth on PYP. Taken together, the results from the genomic and proteomic studies validate the expression of the many cellulosome-related genes and prove that they are regulated by the growth substrate. The composition and hence “the quality” of the cellulosome may very accordingly.

10.4 MOLECULAR ASSEMBLY OF THE CELLULOSOME

The key to stably maintain the cellulosome assembly in an extracellular environment lies in the high affinity between the cohesin and dockerin. In fact, the affinity between the two modules has to be close to or exceed that of the antigen–antibody interaction. Thus, identifying molecular interactions contributing to the stable complex formation are important for understanding the molecular assembly of the cellulosome. Many aspects of these interactions have been elucidated, thanks to structural studies involving X-ray crystallography, protein NMR, and other advanced technologies.

10.4.1 Molecular Structures of the Cohesin, Dockerin, and Their Complex

The crystal structures of the second (Shimon et al., 1997) and the seventh (Tavares et al., 1997) cohesins of CipA have been determined. A cohesin consists of three

β sheets, each in turn consisting of three β strands. The β sheets fold into a compact elongated structure with an overall jelly roll-like topology (Yaron et al., 1995). On the other hand, the highly-conserved dockerin domain is characterized by two Ca^{2+} -binding sites with sequence similarity to the EF-hand motif. As the dockerin alone does not seem to crystallize, its structure was determined by 2D protein NMR. The NMR solution structure of the CelS dockerin consists of two Ca^{2+} -binding loop-helix motifs connected by a linker; the E helices entering each loop of the classical EF-hand motif are absent from the dockerin domain. It thus represents a novel fold of calcium-binding protein. Due to its duplicated sequence, the dockerin assumes a symmetrical structure. Each dockerin Ca^{2+} -binding subdomain is stabilized by a cluster of buried hydrophobic side-chains (Lytle et al., 2001). Both Ca^{2+} -binding segments are required for high affinity binding to the cohesin (Lytle and Wu, 1998). Interestingly, the dockerin is one of the very few proteins whose folding is known to be induced by Ca^{2+} (Lytle et al., 2000). In the absence of Ca^{2+} , the protein exists in the form of a random coil. Thus, without Ca^{2+} , the dockerin is “naturally denatured.”

It was not clear how the dockerin, with its symmetric structure, docks to the cohesin, which is not symmetric. The puzzle was resolved after the crystal structure of the cohesin–dockerin complex was determined by coexpressing a cohesin and dockerin genes in *Escherichia coli* to obtain the crystal of the complex. The crystal structure of the complex containing the second cohesion of CipA and the dockerin of XynY, showed that cohesin recognition is predominantly through helix-3 of the dockerin (Carvalho et al., 2007). Thus the dockerin only uses half of its structure to dock to the cohesin. Remarkably, when helix 3 is rendered inactive by site-directed mutation, the dockerin rotates 180 degrees, thus allowing helix 1 to bind to the cohesin. This dual binding mode is likely to allow flexibility in the orientation of the catalytic subunit within the cellulosome assembly. On the other hand, the single binding mode is the mechanism involved in the interaction of cohesins of OlpC and OlpA with the dockerins of Cel124A and Cthe_918, respectively (Brás et al., 2012).

Adams et al. (2005) studied the structure of the C-terminal end of CipA, consisting of the carboxypeptidase regulatory-like (Car) domain and the type II dockerin (X-Doc), using circular dichroism and fluorescence spectroscopy. They found that Ca^{2+} -binding to the dockerin induces the exposure of a hydrophobic surface and homodimerization of the X-Doc molecule. Addition of the SdbA type II cohesin caused dissociation of the X-Doc homodimer and formation of a stable type II Coh–X-Doc complex. The crystal structure of type II cohesin is very similar to that of type I cohesin with nine β strands folded into a jelly-roll topology and a conserved dockerin binding site (Carvalho et al., 2005). The specificities of type I and type II cohesin–dockerin pairs may be due to subtle differences in the topology and a lack of sequence identity in the β strands of the dockerin binding site. Furthermore, Adams et al. (2006) showed that the Car domain stabilizes the type II dockerin structure and enhances type II cohesin recognition.

10.4.2 Molecular Structures of Other CipA Modules

The crystal structure of the CBM3a domain of CipA contains two antiparallel β sheets that stack together to form a β sandwich (Tormo et al., 1996). The interface between the sheets contains two hydrophilic patches, which bind to a Ca^{2+} ion. The flat side of the β sandwich, with conserved aromatic residues in a planar strip, is likely involved in cellulose binding, whereas the function of the other side with a shallow groove is unknown.

The cellulosomal components contain many linker segments ranging from 5–700 amino acids in length, to link various functional modules. There are nine linkers (between adjacent cohesins and CBM3a) in CipA. Yaniv et al. (2012) identified a nine-residue consensus linker sequence as a probe to study the interaction between CBM and linkers. Using isothermal titration calorimetry assays, they showed that the shallow-groove region of the CBM3a of CipA interacts with the linker peptide.

10.4.3 Dynamics of the Cellulosome Structure

The molecular structures of individual domains and the cohesin-dockerin complex provide only a small glimpse of the overall structure of the cellulosome. Recently, the crystal structure of the C-terminal trimodular fragment of CipA (the ninth CohI, connected by a linker to the X-DocII) in complex with the SdbA CohII, was determined (Adams et al., 2010). The trimodular fragment has an elongated topology and forms a dimer with another.

Furthermore, the crystal structure of a “four-module” fragment, consisting of the type-I dockerin module of Cel9D in complex with the above-mentioned trimodular fragment of CipA, has been determined (Currie et al., 2012). The structure displays two rigid domains formed by the cohesin–dockerin complexes, that is, a type I cohesin–dockerin complex and the X module-type-II cohesin–dockerin complex. The result also supported the previously observed homodimerization of CipA scaffoldin. Small angle X-ray scattering analysis of the ternary complex revealed that the intermodular linker of the scaffoldin subunit is highly flexible in solution, allowing the cellulosomal unit to bend in different directions.

Cryo-electron microscopic examination of the three-dimensional structure of a minicellulosome, comprising three consecutive cohesin modules, each bound to one Cel8A cellulase, revealed that the three cellulosomal catalytic domains project outward from CipA and away from each other. Furthermore, the linkers between cohesin modules can extend, allowing dynamic changes in cellulosomal structure from a compact to a relaxed/flexible configuration (García-Alvarez et al., 2011).

It is possible that the cellulosome is assembled randomly (Yaron et al., 1995). However, the shape and modularity of the catalytic subunit may play

a role in the assembly. For example, a model predicts that the multimodular enzyme, CbhA, binds statistically more frequently to the scaffoldin than CelS or Cel5B (Bomble et al., 2011). The enhanced binding is attributed to the flexible nature and multimodularity of this enzyme, providing a longer residence time around the scaffoldin. Furthermore, the initial binding of a given enzyme to CipA may influence the type of enzyme that binds subsequently (Borne et al., 2013). The preferential integration appears to be related to the length of the intercohesin linker.

10.5 CONCLUDING REMARKS

Research on the cellulosome of *C. thermocellum* has provided a window for us to peek into how microbial enzymes can exist in the form of a mysterious extracellular organelle. It sheds light onto a new aspect of how abundant water-insoluble plant biomass is degraded in Nature by such a well-organized protein complex. The design of the cellulosome facilitates many advantages, including: (1) proximity of the enzymes to their substrates, (2) proximity of *endo*- to *exo*-enzymes and cellulases to hemicellulases, and (3) negating the need of enzyme diffusion, thus increasing efficiency. In addition, the cellulosome system enables the bacterium to evolutionarily acquire a new cellulosomal subunit, change its composition by gene regulation, change the ratio of cell-bound and cell-free versions, and change the orientation of the catalytic subunit relative to its position on the scaffoldin. The study has led to new understanding of how enzymes and proteins work and new fundamental knowledge, including calcium-induced protein folding and a new fold of a calcium-binding protein (the dockerin structure). Although, the classical biochemical textbook teaches that a protein functions through primary, secondary, tertiary, and quaternary structures, the cellulosome adds a new dimension to the structure–function relationship by allowing enzymes to “dance,” by rotating, bending, and dimerizing. Despite intensive studies over the last three decades, commencing from the hypothesis of “the anchor–enzyme model,” our understanding of the cellulosome likely only scratches the surface. Future studies will definitely shed more light onto the structure–function relationship of this intriguing biomass-degrading organelle, revealing more fascinating science as well as facilitating biotechnological applications.

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Chapter 11

Microbial Enzymes of Use in Industry

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11.1 INTRODUCTION

Many microbes such as bacteria, actinomycetes, fungi, and yeast extracellularly or intracellularly produce a group of versatile and attractive enzymes with a wide variety of structures and commercial applications. Many microbial enzymes, such as amylases, proteases, pectinases, lipases, xylanases, cellulases, and laccases, are extracellularly produced. Some enzymes such as catalase from *Saccharomyces cerevisiae* and *Aspergillus niger* are intracellular enzymes (Fiedurek and Gromada, 2000; Venkateshwaran et al., 1999). As biocatalytic molecules, microbial enzymes are ecologically effective and highly specific, which can result in the formation of stereo- and regio-chemically defined reaction products with a rate acceleration of 10^5 to 10^8 (Gurung et al., 2013; Koeller and Wong, 2001). Among the industrial enzymes, 50% are made by fungi and yeast, 35% from bacteria, while the remaining 15% are from plants (Saranraj and Naidu, 2014). When compared to animal and plant enzymes, microbial enzymes have several advantages. First, the microbial enzymes are more active and stable than plant and animal enzymes. Through the development of fermentation processes, particularly selected strains are able to produce purified, well-characterized enzymes on a large scale. Second, enzymes produced by microorganisms have high yield and are easy for product modification and optimization owing to the biochemical diversity and susceptibility to gene manipulation. Engineering techniques have been applied to microorganisms in order to improve the production of enzymes and alter the properties of enzymes by protein engineering (Gurung et al., 2013). Third, microbes represent a rich source for the discovery of microbial enzymes by many modern techniques such as

metagenome screening, genome mining, and exploring the diversity of extremophiles (Adrio and Demain, 2014; Zhang and Kim, 2010).

Currently, approximately 200 types of microbial enzymes from 4000 known enzymes are used commercially. However, only about 20 enzymes are produced on a truly industrial scale. The world enzyme demand is satisfied by about 12 major producers and 400 minor suppliers. Nearly 75% of the total enzymes are produced by three top enzyme companies, that is, Denmark-based Novozymes, US-based DuPont (through the May 2011 acquisition of Denmark-based Danisco) and Switzerland-based Roche. The market is highly competitive, has small profit margins and is technologically intensive (Li et al., 2012). With the improved understanding of the microbial recombination, metagenome mining, fermentation processes, and recovery methods, an increasing number of industrial enzymes can be supplied. For example, the recombinant DNA technology can be applied to microorganisms to produce enzymes commercially that could not be produced previously. Approximately 90% of industrial enzymes are recombinant versions (Adrio and Demain, 2014).

The industrial applications for microbial enzymes have grown immensely in recent years. For example, the estimated value of worldwide sales of industrial enzymes for the years 2012, 2013, and 2015 are \$1 million, \$3 billion, and \$3.74 billion, respectively (Deng et al., 2010; Godfrey and West, 1996a,b). Protease sales represent more than 60% of all industrial enzyme sales in the world (Rao et al., 1998) and still constitute the largest product segment in the 2015 global industrial enzymes market. Amylases comprise about 30% of the world's enzyme production. Lipases represent the other major product segment in the market. Geographically, demand for industrial enzymes in matured economies, such as the United States, Western Europe, Japan, and Canada, was relatively stable during the recent times, while the developing economies of Asia-Pacific, Eastern Europe, and Africa and Middle East regions emerged as the fastest growing markets for industrial enzymes. On the basis of the application, commercial applications of enzymes can be divided in nine broad categories including food and feed, detergents, etc. (Sharma et al., 2010). About 150 industrial processes use enzymes or whole microbial cell catalysts (Adrio and Demain, 2014). Food and feed represents the largest segment for industrial enzymes. Detergents constitute the other major segment for industrial enzymes. This chapter covers the classification, resource, production, and applications of biotechnologically and industrially valuable microbial enzymes.

11.2 CLASSIFICATION AND CHEMICAL NATURE OF MICROBIAL ENZYMES

Based on the catalyzed reaction, microbial enzymes can be classified into six types: Oxidoreductases (EC 1, catalyze oxidation/reduction reactions), Transferases (EC 2, transfer a functional group), Hydrolases (EC 3, catalyze

the hydrolysis of various bonds), Lyases (EC 4, cleave various bonds by means other than hydrolysis and oxidation), Isomerases (EC 5, catalyze isomerization changes within a single molecule), and Ligases (EC 6, join two molecules with covalent bonds) (<http://www.chem.qmul.ac.uk/iubmb/enzyme/>). Currently, there are 510 commercial useful microbial enzymes in the metagenomics database (Sharma et al., 2010). Of the industrial enzymes, 75% are hydrolytic (Li et al., 2012).

11.2.1 Amylases

Amylases are a class of enzymes that catalyze the hydrolysis of starch into sugars such as glucose and maltose (Sundaram and Murthy, 2014). Amylases are divided into three sub classes— α - β - γ -amylase according to the type of bond/link they are able to cleave (Fig. 11.1). α -Amylases (EC 3.2.1.1) catalyze the hydrolysis of internal α -1,4-*O*-glycosidic bonds in polysaccharides with the retention of α -anomeric configuration in the products. Most of the α -amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity, and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes. β -Amylases (EC 3.2.1.2) are exohydrolase enzymes that act from the nonreducing end of a polysaccharide chain by hydrolyzing α -1, 4-glucan linkages to yield successive maltose units. Since β -amylases are unable to cleave branched linkages present in branched polysaccharides, such as glycogen or amylopectin, the hydrolysis is incomplete and dextrin units remain. γ -Amylases (EC 3.2.1.3) cleave α (1-6) glycosidic linkages, in addition to cleaving the last α (1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, unlike the other forms of amylase, yielding glucose. α -Amylase is produced by several bacteria, fungi, and genetically modified species of microbes. The most widely used source among the bacterial species are *Bacillus* spp.—*B. amyloliquefaciens* and *B. licheniformis*. Fungal sources of α -amylase are mostly limited to *Aspergillus* species and to only a few species of *Penicillium*, *P. brunneum* being one of them.

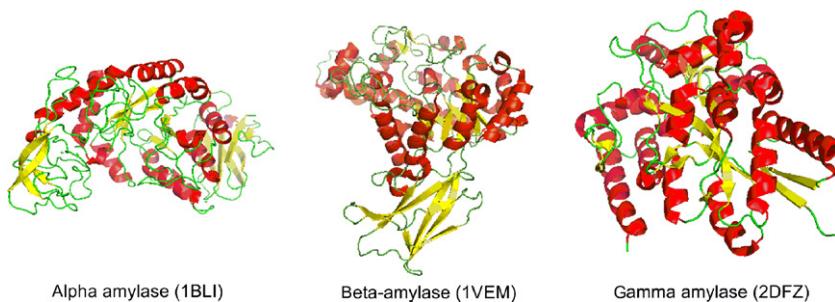


FIGURE 11.1 Structures of selected microbial amylases.

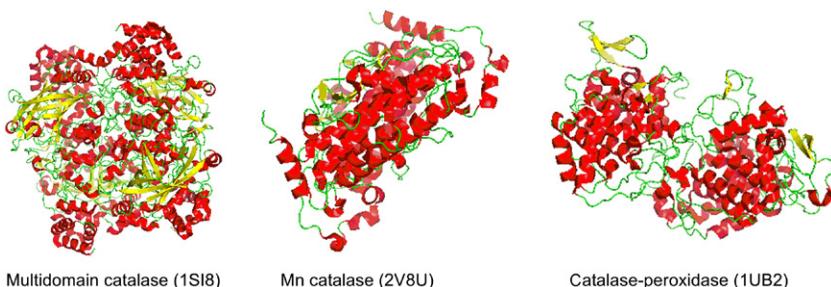


FIGURE 11.2 Structures of selected microbial catalases.

11.2.2 Catalases

Catalases (EC 1.11.1.6) are antioxidant enzymes that catalyze the conversion of hydrogen peroxide to water and molecular oxygen. According to the structure and sequence, catalases can be divided into three classes (Fig. 11.2): monofunctional catalase or typical catalase, catalase-peroxidase, and pseudocatalase or Mn-catalasee (Zhang et al., 2010). Currently, there are at least eight strains that can produce catalases (Zhang et al., 2010): *Penicillium variable*, *A. niger*, *S. cerevisiae*, *Staphylococcus*, *Micrococcus lysodeiktius*, *Thermoascus aurantiacus*, *Bacillus subtilis*, and *Rhizobium radiobacte*. Catalases are used in several industrial applications such as food or textile processing to remove hydrogen peroxide that is used for sterilization or bleaching.

11.2.3 Cellulases

Cellulases are enzymes that hydrolyze β -1,4 linkages in cellulose chains. The catalytic modules of cellulases have been classified into numerous families based on their amino acid sequences and crystal structures (Henrissat, 1991). Cellulases contain noncatalytic carbohydrate-binding modules (CBMs) and/or other functionally known or unknown modules, which may be located at the N- or C-terminus of a catalytic module. In nature, complete cellulose hydrolysis is mediated by a combination of three main types of cellulases (Juturu and Wu, 2014; Kuhad et al., 2011; Sukumaran et al., 2005; Yang et al., 2013) (Fig. 11.3). These are endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and glucosidases (EC 3.2.1.21). Endoglucanases hydrolyze glycosidic bonds at the amorphous regions of the cellulose generating long chain oligomers (nonreducing ends) for the action of exoglucanases or cellobiohydrolases, which cleave the long chain oligosaccharides generated by the action of endoglucanases into short chain oligosaccharides. There are two types of exoglucanases, acting unidirectionally on the long chain oligomers either from the reducing (EC 3.2.1.176) or nonreducing ends (EC 3.2.1.91) liberating cellobiose, which is further hydrolyzed to glucose by β -glucosidases (EC 3.2.1.21) (Juturu and Wu, 2014). Cellulases are inducible

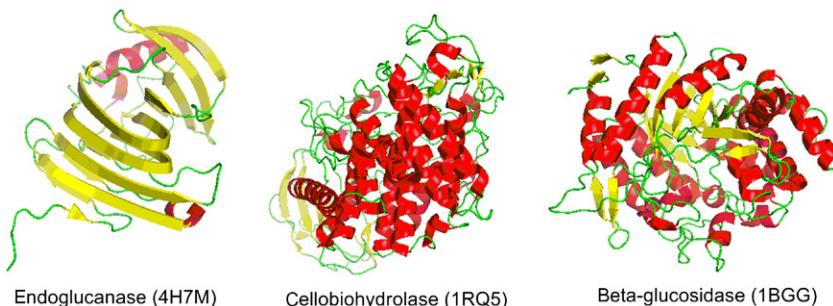


FIGURE 11.3 Structures of selected microbial cellulases.

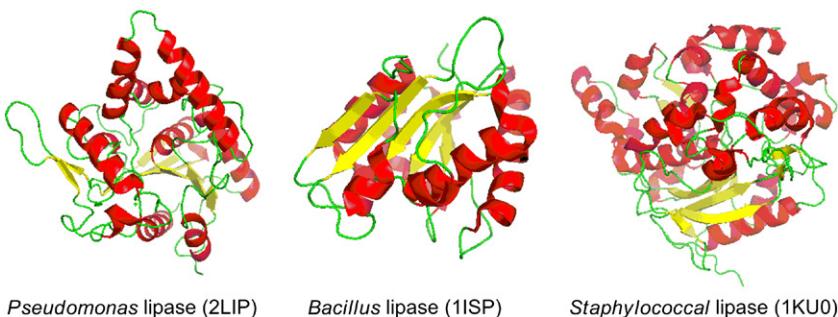


FIGURE 11.4 Structures of selected microbial lipases.

enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials (Ma et al., 2013; Quintanilla et al., 2015). These microorganisms can be aerobic, anaerobic, mesophilic, or thermophilic. Among them, the genera of *Clostridium*, *Cellulomonas*, *Thermomonospora*, *Trichoderma*, and *Aspergillus* are the most extensively studied cellulose producers (Kuhad et al., 2011).

11.2.4 Lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols to glycerol, diacylglycerols, monoglycerols, and free fatty acids (Treichel et al., 2010). Bacterial lipases are classified into eight families (families I–VIII) based on differences in their amino-acid sequences and biological properties (Arpigny and Jaeger, 1999). Family I of true lipases is the most represented one and can be further divided into *Pseudomonas* lipase subfamily, *Bacillus* lipase subfamily, *Staphylococcal* lipase subfamily, etc. (Fig. 11.4). Lipases belong to the class of serine hydrolases. Therefore, lipases do not need any cofactor. Lipases catalyze the hydrolysis of ester bonds at the

interface between an insoluble substrate phase and the aqueous phase where the enzymes remain dissolved. Lipases do not hydrolyze dissolved substrates in the bulk fluid. In nature, lipases have considerable variations in their reaction specificities. From the fatty acid side, some lipases have an affinity for short-chain fatty acids (C2, C4, C6, C8, and C10), some have a preference for unsaturated fatty acids (oleic, linoleic, linolenic, etc.) while many others are nonspecific and randomly split the fatty acids from the triglycerides. Some of the most commercially important lipase-producing fungi belong to the genera *Rhizopus* sp., *Aspergillus*, *Penicillium*, *Geotrichum*, *Mucor*, and *Rhizomucor* (Gupta et al., 2004; Treichel et al., 2010). The main terrestrial species of yeasts that were found to produce lipases are (Treichel et al., 2010): *Candida rugosa*, *Candida tropicalis*, *Candida antarctica*, *Candida cylindracea*, *Candida parapsilopsis*, *Candida deformans*, *Candida curvata*, *Candida valida*, *Yarrowia lipolytica*, *Rhodotorula glutinis*, *Rhodotorula pilimornae*, *Pichia bispora*, *Pichia mexicana*, *Pichia sivicola*, *Pichia xylosa*, *Pichia burtonii*, *Saccharomyces crataegensis*, *Torulaspora globosa*, and *Trichosporon asteroides*. Among bacterial lipases being exploited, those from *Bacillus* exhibit interesting properties that make them potential candidates for biotechnological applications (Gupta et al., 2004; Treichel et al., 2010). *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus stearothermophilus*, and *Bacillus alcalophilus* are the most common lipase producing strains. In addition, *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Burkholderia multivorans*, *Burkholderia cepacia*, and *Staphylococcus caseolyticus* are also reported as bacterial lipase producers (Gupta et al., 2004).

11.2.5 Pectinases

Pectinases are a group of enzymes that catalyze pectic substance degradation through depolymerization (hydrolases and lyases) and deesterification (esterases) reaction (Pedroli et al., 2009). According to the cleavage mode and specificity, pectic enzymes are divided into three major types (Fig. 11.5): pectinesterases (PE), depolymerizing enzymes and cleaving (Tapre and Jain,

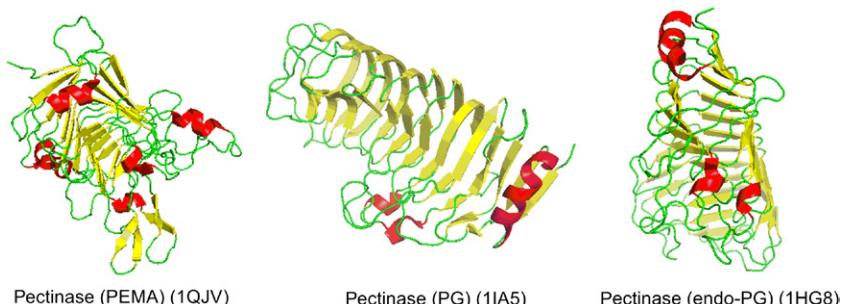


FIGURE 11.5 Structures of selected microbial pectinases.

2014). These types can be further divided into 13 groups, protopectinases, pectin methyl esterases (PME), pectin acetyl esterases (PAE), polymethylgalacturonases (PMG), polygalacturonases (PG), polygalacturonate lyases (PGL), pectin lyases (PL), rhamnogalacturonan rhamnohydrolases, rhamnogalacturonan galacturonohydrolases, rhamnogalacturonan hydrolases, rhamnogalacturonan lyases, rhamnogalacturonan acetylesterases, and xylogalacturonase (Pedrolli et al., 2009). For example, PME or pectinesterase (EC 3.1.1.11) catalyzes deesterification of the methoxyl group of pectin forming pectic acid and methanol. The enzyme acts preferentially on a methyl ester group of galacturonate unit next to a nonesterified galacturonate unit (Kashyap et al., 2001). PAE (EC 3.1.1) hydrolyzes the acetyl ester of pectin forming pectic acid and acetate (Shevchik and Hugouvieux-Cotte-Pattat, 1997). PG catalyzes hydrolysis of α -1,4-glycosidic linkages in polygalacturonic acid producing D-galacturonate. Both groups of hydrolase enzymes (PMG and PG) can act in an endo- or exo- mode. Endo-PG (EC 3.2.1.15) and endo-PMG catalyze random cleavage of substrate, whereas exo-PG (EC 3.2.1.67) and exo-PMG catalyze hydrolytic cleavage at the substrate nonreducing end producing monogalacturonate or digalacturonate in some cases (Kashyap et al., 2001). Among those enzymes, homogalacturonan degrading enzymes are well known (Pedrolli et al., 2009). It has been reported that microbial pectinases account for 25% of the global food enzymes sales and 10% of global industrial enzymes produced (Ceci and Lozano, 1998; Jayani et al., 2005; Saranraj and Naidu, 2014). Pectinase production has been reported from bacteria including actinomycetes, yeast, and fungi (Murad and Azzaz, 2011; Saranraj and Naidu, 2014). However, almost all the commercial preparations of pectinases are produced from fungal sources (Singh et al., 1999). *Aspergillus niger* is the most commonly used fungal species for the industrial production of pectinolytic enzymes (Gummadi and Panda, 2003).

11.2.6 Proteases

Proteases (EC 3:4, 11-19, 20-24, 99) (peptidase or proteinase) constitute a very large and complex group of enzymes that catalyze the hydrolysis of covalent peptide bonds. Proteases can be classified on the basis of pH, substrate specificity, similarity to well characterized enzymes, and the active site amino acid (Ellaiah et al., 2002). Based on the pH optima, they are referred to as acidic, neutral, or alkaline proteases (Rao et al., 1998). On the basis of their site of action on protein substrates, proteases are broadly classified as endo- or exo-enzymes (Rao et al., 1998). They are further categorized as serine proteases, aspartic proteases, cysteine proteases, or metallo proteases-depending on their catalytic mechanism (Jisha et al., 2013) (Fig. 11.6). Microorganisms account for a two-third share of commercial protease production in the enzyme market across the world. Alkaline serine proteases are the most dominant group of proteases produced by bacteria, fungi, yeast, and actinomycetes. Currently,

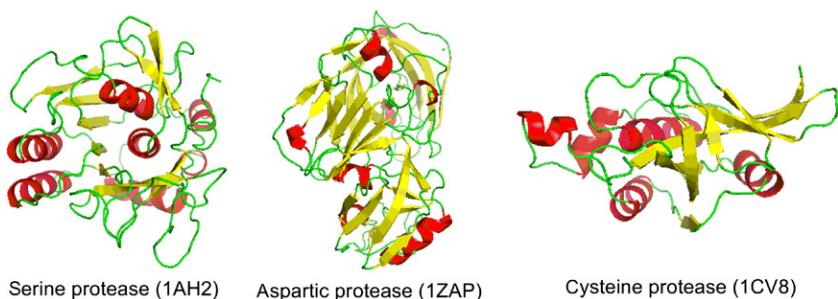


FIGURE 11.6 Structures of selected microbial proteases.

there are at least 29 *Bacillus* species and 17 fungal producers that have been reported to produce alkaline proteases (Jisha et al., 2013). Commercial producers of alkaline proteases include protein engineered *Bacillus licheniformis*, alkalophilic *Bacillus* sp., and *Aspergillus* sp. (Ellaiah et al., 2002).

11.2.7 Xylanases

Xylanases are among the xylanolytic enzyme system that include endoxylanase, β -xylosidase, α -glucuronidase, α -arabinofuranosidase, and acetylxyran esterase (Juturu and Wu, 2012). Xylanases are a group of glycoside hydrolase enzymes that degrade the linear polysaccharide xylan into xylose by catalyzing the hydrolysis of the glycosidic linkage (β -1,4) of xylosides. Xylanases have been classified in at least three ways: based on the molecular weight and isoelectric point (Wong et al., 1988), the crystal structure (Jeffries, 1996) and kinetic properties, or the substrate specificity and product profile (Motta et al., 2013). The favorable system for the classification of xylanases is based on the primary structure and comparison of the catalytic domains (Collins et al., 2005; Henrissat and Coutinho, 2001). According to the CAZy database (<http://www.cazy.org>), xylanases (EC3.2.1.8) are related to glycoside hydrolase (GH) families 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, and 62. Among those, xylanases in GH 10 and 11 are the two families that have been thoroughly studied (Fig. 11.7). GH family 10 is composed of endo-1,4- β -xylanases and endo-1,3- β -xylanases (EC 3.2.1.32) (Motta et al., 2013). Members of this family are also capable of hydrolyzing the aryl β -glycosides of xylobiose and xylotriose at the aglyconic bond. Furthermore, these enzymes are highly active on short xylooligosaccharides, thereby indicating small substrate-binding sites. Family 11 is composed only of xylanases (EC 3.2.1.8), leading to their consideration as “true xylanases,” as they are exclusively active on D-xylose-containing substrates. Among all xylanases, endoxylanases are the most important due to their direct involvement in cleaving the glycosidic bonds and in liberating short xylooligosaccharides (Collins et al., 2005). Although several *Bacillus* species secrete high levels of extracellular xylanase (Beg et al., 2001), filamentous fungi secret

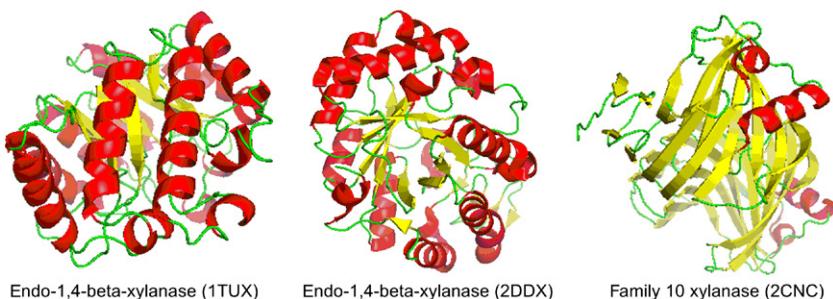


FIGURE 11.7 Structures of selected microbial xylanases.

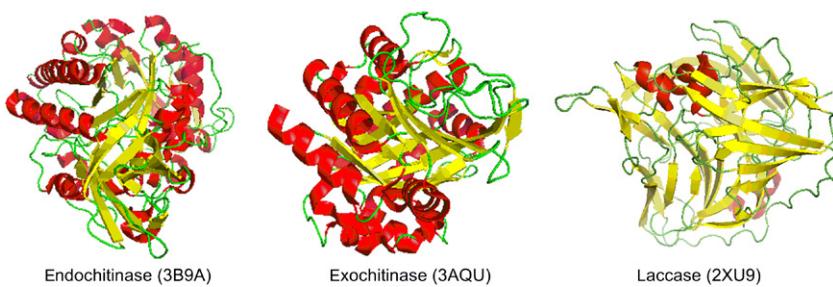


FIGURE 11.8 Structures of selected microbial chitinases and laccase.

high amounts of extracellular xylanase, which are often accompanying celulolytic enzymes—for example as in species of *Trichoderma*, *Penicillium*, and *Aspergillus* (Kohli et al., 2001; Polizeli et al., 2005; Wong and Saddler, 1992).

11.2.8 Other Enzymes

Chitinases have been divided into two main groups: endochitinases (EC 3.2.1.14) and exo-chitinases (Fig. 11.8). The endochitinases randomly split chitin at internal sites, thereby forming the dimer di-acetylchitobiose and soluble low molecular mass multimers of GlcNAc, such as chitotriose and chitotetraose. The exochitinases have been further divided into two subcategories: chitobiosidases (EC 3.2.1.29), which are involved in catalyzing the progressive release of di-acetylchitobiose starting at the nonreducing end of the chitin microfibril, and 1-4- β -glucosaminidases (EC 3.2.1.30), cleaving the oligomeric products of endochitinases and chitobiosidases, thereby generating monomers of *N*-acetylglucosamine (GlcNAc). Chitinases (EC 3.2.1.14) can catalyze the hydrolysis of chitin to its monomer *N*-acetyl-D-glucosamine. Chitinases are widely distributed in bacteria such as *Serratia*, *Chromobacterium*, *Klebsiella*, *Pseudomonas*, *Clostridium*, *Vibrio*, *Arthrobacter*, *Beneckea*, *Aeromonas*, and *Streptomyces*. They are also found in fungi like *Trichoderma*, *Penicillium*,

Lecanicillium, Neurospora, Mucor, Beauveria, Lycoperdon, Aspergillus, Myrothecium, Conidiobolus, Metharhizium, Stachybotrys, and Agaricus (Felse and Panda, 2000; Islam and Datta, 2015; Matsumoto, 2006).

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper oxidases that participate in cross-linking of monomers, degradation of polymers, and ring cleavage of aromatic compounds. These enzymes contain 15–30% carbohydrate and have a molecule mass of 60–90 kDa (Shraddha et al., 2011) (Fig. 11.8). Laccases contain four copper atoms termed Cu T1 (where the reducing substrate binds) and trinuclear copper cluster T2/T3 (electron transfer from type I Cu to the type II Cu and type III Cu trinuclear cluster/reduction of oxygen to water at the trinuclear cluster) (Gianfreda et al., 1999). These four copper ions are classified into three categories: Type 1 (T1), Type 2 (T2), and Type 3 (T3). Laccases carry out one electron oxidation of phenolic and its related compound and reduce oxygen to water. When substrate is oxidized by a laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or nonenzymatic reactions including hydration, disproportionation, and polymerization. Most laccases are extracellularly produced by fungi (Agematu et al., 1993; Brijwani et al., 2010; Chandra and Chowdhary, 2015; Mougin et al., 2003). The production of laccase can also be seen by soil and some freshwater Ascomycetes species (Banerjee and Vohra, 1991; Junghanns et al., 2005; Rodríguez et al., 1996; Scherer and Fischer, 1998). In addition, laccases are also produced by *Gaeumannomyces graminis*, *Magnaporthe grisea*, *Ophiostoma novo-ulmi*, *Marginella*, *Melanocarpus albomyces*, *Monocillium indicum*, *Neurospora crassa*, and *Podospora anserina* (Binz and Canevascini, 1997; Edens et al., 1999; Froehner and Eriksson, 1974; Iyer and Chattoo, 2003; Kiiskinen et al., 2002; Molitoris and Esser, 1970; Palonen et al., 2003; Thakker et al., 1992).

Cytochromes P450 (CYPs) catalyze various types of reactions, such as hydroxylation, epoxidation, alcohol and aldehyde oxidation, *O*-dealkylation, *N*-dealkylation, oxidative dehalogenation, and oxidative C–C bond cleavage (Sakaki, 2012). Among these, regio- and enantioselective hydroxylation by P450 is quite attractive as a bioconversion process. There are 10 classes of CYPs (Kelly and Kelly, 2013). Most bacterial CYPs are class I and driven by ferredoxin and ferredoxin reductases. CYPs have potential applications on bioconversion processes, biosensors, and bioremediation due to their regio- and enantioselective hydroxylation which is difficult for chemical synthesis. *Streptomyces carbophilus* CYP105A3 is a CYP that are successfully applied in industry for bioconversion to produce pravastatin (Watanabe et al., 1995).

11.3 PRODUCTION OF MICROBIAL ENZYMES

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms. It has been widely used for the production of many microbial enzymes (Aehle, 2007). Much work has been

focused on the screening of enzyme-producing microorganisms, physiological optimizations for substrates, carbon source and nitrogen source, pH of the media, and the cultivation temperature during the fermentation process (Ellaiah et al., 2002; Juturu and Wu, 2014; Sundaram and Murthy, 2014).

11.3.1 Fermentation Methods

There are two types of cultivation methods for all microbial enzymes: submerged fermentation (SmF) and solid state fermentation (SSF). Submerged fermentation involves the nurturing of microorganisms in high oxygen concentrated liquid nutrient medium. Viscosity of broth is the major problem associated with the fungal submerged fermentations. When fungal cells grow and a mycelium is produced, this hinders impeller action, due to this limitation occurring in oxygen and mass transfer. SSF is suitable for the production of enzymes by using natural substrates such as agricultural residues because they mimic the conditions under which the fungi grow naturally.

Since SSF involves relatively little liquid when compared with SmF, downstream processing from SSF is theoretically simpler and less expensive. During the past 10 years, a renewed interest in SSF has developed due, in part, to the recognition that many microorganisms, including genetically modified organisms (GMO), may produce their products more effectively by SSF (Singh et al., 2008). SSF has three major advantages: (1) high volumetric productivity, (2) relatively higher concentration of the products, and (3) less effluent generation, requirement for simple fermentation equipment, etc. Moreover, the biosynthesis of microbial enzymes in SmF process is of economic importance because it is strongly affected by catabolic and end product repressions. The amenability of SSF technique to use up to 20–30% substrate, in contrast to the maximum of 5% in SmF process, has been documented (Pamment et al., 1978).

11.3.2 Purification Methods

Enzymes are manufactured in bioreactors for commercial use. These enzymes are in the crude form and have to be purified for further use. The extraction methods are followed by the purification processes. There are mainly three major purification methods for microbial enzymes: (1) based on ionic properties of enzymes, (2) based on the ability to get adsorbed, and (3) based on difference in size of molecules. Special procedures employed for enzyme purification are crystallization, electrophoresis, and chromatography. The main applications of industrial-scale chromatography were the desalination of enzyme solutions by use of highly cross-linked gels such as Sephadex G-25 and batch separations by means of ion exchangers such as DEAE-Sephadex A-50. The stability and hydraulic properties of chromatographic media have been improved so that these techniques are now used on a production scale. Important parameters for the scale-up of chromatographic systems are the height of the column, the

linear flow rate, and the ratio of sample volume to bed volume. Zone spreading interferes with the performance of the column. Factors that contribute to zone spreading are longitudinal diffusion in the column, insufficient equilibration, and inadequate column packing. Longitudinal diffusion can be minimized by using a high flow rate. On the other hand, equilibration between the stationary and the mobile phases is optimal at low flow rates. Because good process economy depends to a large extent on the flow rate, a compromise must be made. In addition, the flow rate is also dependent on particle size; the decisive factor is usually the pressure drop along large columns. Although optimal resolution is obtained only with the smallest particles, the gel must have a particle size that favors a good throughput and reduces processing times. The use of segmented columns prevents a large pressure drop in the column. Above all, the column must be uniformly packed so that the particle-size distribution, porosity, and resistance to flow are the same throughout the column. If this is not done, viscous protein solutions can give an uneven elution profile, which would lead to zone bleeding. The design of the column head is important for uniform distribution of the applied sample. This is generally achieved by symmetrical arrangement of several inlets and perforated inserts for good liquid distribution. The outlet of the column must have minimal volume to prevent back-mixing of the separated components ([Aehle, 2007](#)).

11.4 APPLICATIONS OF MICROBIAL ENZYMES

Microbial enzymes are of great importance in the development of industrial bio-processes. The end use market for industrial enzymes is extremely widespread with numerous industrial applications ([Adrio and Demain, 2014](#)). Over 500 industrial products are being made using different microbial enzymes ([Kumar and Singh, 2013](#)). The demand for industrial enzymes is on a continuous rise driven by a growing need for sustainable solutions.

Microbes are one of the largest and most useful sources of many enzymes ([Demain and Adrio, 2008](#)). A large number of new enzymes have been designed with the input of protein-engineering and metagenomics. Various molecular techniques have also been applied to improve the quality and performance of microbial enzymes for their wider applications in many industries ([Chirumamilla et al., 2001](#); [Nigam, 2013](#)). Many microorganisms including bacteria, actinomycetes, and fungi have been globally studied for the synthesis of economically viable preparations of various enzymes for commercial applications ([Pandey et al., 1999](#)). The special characteristics of enzymes are exploited for their commercial interest and industrial applications ([Table 11.1](#)), which include thermo-tolerance, tolerance to a wide range of pH, and stability of enzyme activity over a harsh reaction conditions.

The majority of currently used industrial enzymes are hydrolytic and they are used for the degradation of various natural substances. Proteases are one of most important classes of enzymes for the detergent and dairy industries.

TABLE 11.1 Microbial Enzymes and Their Applications

Industry	Name of enzymes	Applications	References
Food, dairy and beverage	Protease, lipase, lactase, pectinase, amylase, laccase, amyloglucosidase, phospholipase	Degradation of starch and proteins into sugars, Production of low caloric beer, Fruit juice processing, Cheese production, Glucose production from lactose, Dough stability and Conditioning.	Gurung et al. (2013) and Nigam and Singh (1995)
Detergents	Amylase, cellulase, lipase, protease, mannanase	Remove protein after staining, Cleaning agents, Removing insoluble starch, fats and oils, To increase effectiveness of detergents.	Pandey et al. (2000a) and Wintrode et al. (2000)
Textiles	Amylase, cellulase, pectinase, catalase, protease, peroxidase, keratinase	Fabric finishing in denims, Wool treatment, Degumming of raw silk (biopolishing), Cotton softening.	Liu et al. (2013) and Saha et al. (2009)
Animal feed	Phytase, xylanase	Increase total phosphorus content for growth, Digestibility	Mitidieri et al. (2006) and Tomschy et al. (2000)
Ethanol production	Cellulase, ligninase, mannanase	Formation of ethanol	Jolly (2001)
Paper and pulp	Amylase, lipase, protease, cellulase, hemicellulase, esterase, ligninase, xylanase	Degrade starch to lower viscosity, aiding sizing, deinking, and coating paper. Cellulase and hemicellulase smooth fibers, enhance water drainage, and promote ink removal. Lipases reduce pitch and ligninase remove lignin to soften paper.	Kirk et al. (2002), Kohli et al. (2001), and Polizeli et al. (2005)
Leather	Protease, lipase	Unhearing, bating, depicking	Parameswaran et al. (2013) and Saha et al. (2009)
Pharmaceuticals	Penicillin acylase, peroxidase	Synthesis of semisynthetic antibiotics, Antimicrobials	Neelam et al. (2013) and Roberts et al. (2007)
Molecular biology	DNA ligase, restriction enzymes, polymerase	Manipulate DNA in genetic engineering. DNA restriction and the polymerase chain reaction. Important in forensic science.	Nigam (2013) and Roberts et al. (2010)

Carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent, and baking industries, represent the second largest group ([Underkofler et al., 1958](#)). The fastest growth over the past decade has been seen in the baking and animal feed industries, but growth is also being generated from applications established in a wealth of other industries spanning from organic synthesis to paper and pulp and personal care. The use of enzymes in animal nutrition is important and growing, especially for pig and poultry nutrition.

Enzymes play key roles in numerous biotechnology products and processes that are commonly encountered in the production of food and beverages, detergents, clothing, paper products, transportation fuels, pharmaceuticals, and monitoring devices ([Gurung et al., 2013](#)). As the industrial enzyme market has expanded at a rate of about 10% annually, microbial enzymes have largely replaced the traditional plant and animal enzymes. DNA technology has been used to modify substrate specificity and improve stability properties of enzymes for increasing yields of enzyme-catalyzed reactions. Enzymes can display regional stereospecificity, properties that have been exploited for asymmetric synthesis and racemic resolution. Chiral selectivities of enzymes have been employed to prepare enantiomerically pure pharmaceuticals, agrochemicals, chemical feedstock, and food additives.

11.4.1 Food and Beverage

In the 20th century, enzymes began to be isolated from living cells, which led to their large-scale commercial production and wider application in the food industry. Food and beverage enzymes constitute the largest segment of industrial enzymes with revenues of nearly \$1.2 billion in 2011 which is expected to grow to \$2.0 billion by 2020. Enzymes used in food can be divided into food additives and processing aids. Most food enzymes are considered as processing aids used during the manufacturing process of foodstuffs ([Saha et al., 2009](#)) with only a few used as additives, such as lysozyme and invertase. The applications of different enzymes in the food industry are shown in [Table 11.2](#).

Amylases are the most important enzymes in the industrial starch conversion process. Amylolytic enzymes act on starch and related oligo- and polysaccharides ([Pandey et al., 2000a](#)). The application of these enzymes has been established in starch liquefaction, paper, food, sugar, and pharmaceutical industries. In the food industry, amylolytic enzymes have a large scale of applications, such as the production of glucose syrups, maltose syrup, reduction of viscosity of sugar syrups, to produce clarified fruit juice for longer shelf-life, solubilization of starch in the brewing industry ([Pandey et al., 2000b](#)). The baking industry uses amylases to delay the staling of bread and other baked products.

The major application of proteases in the dairy industry is for the manufacture of cheese. Calf rennin (chymosin) had been preferred in cheese making due to its high specificity, but microbial proteases are also used. Chymosin is

TABLE 11.2 Applications of Enzymes in the Food Industry

Process	Enzyme	Applications	References
Baking	Amylase, protease	Conversion of sugar into ethanol and CO ₂ . To prepare bread	Collar et al. (2000)
Brewing	Amylase, protease	Conversion of sugar into ethanol and CO ₂ . To prepare alcoholic drink	Pandey et al. (2000a)
Corn syrup	Amylase	Preparation of low dextrose equivalent syrups	Kirk et al. (2002)
Cheese making	Rennin, lipases	Milk clotting, Flavor production	Okanishi et al. (1996)
Baby foods	Trypsin	Digestion	Parameswaran et al. (2013)
Coffee	Pectinase	Coffee bean fermentation	Kirk et al. (2002)
Dairy industry	Protease, lactase lactoperoxidase	Preparation of protein hydrolysates, preparation of milk and ice cream, Cold sterilization of milk	Tucker and Woods (1995)
Fruit juices	Glucose oxidase, pectinase	Oxygen removal, clarification of fruit juices	Godfrey and West (1996,b)
Soft drinks	Glucose oxidase	Stabilization	Kirk et al. (2002)
Meat and fish industries	Proteinase	To tenderize meat and solubilize fish products	Saha and Demirjian (2000)

an aspartic acid protease which causes the coagulation of milk. The primary function of these enzymes in cheese making is to hydrolyze the specific peptide bond that generates para-k-casein and macropeptides (Rao et al., 1998). Production of calf rennin (chymosin) in recombinant *A. niger* var. *awamori* amounted to about 1 g/L after nitrosoguanidine mutagenesis and selection for 2-deoxyglucose resistance. Four recombinant proteases have been approved by FDA for cheese production (Bodie et al., 1994; Pariza and Johnson, 2001).

The application of enzymes (proteases, lipases, esterases, lactase, and catalase) in dairy technology is well established. Rennets (rennin) are used for coagulation of milk in the first stage of cheese production. Proteases of various kinds are used for acceleration of cheese ripening, for modification of functional properties and for modification of milk proteins to reduce the allergenic properties of cow milk products for infants. Lipases are used mainly in cheese ripening for development of lipolytic flavors. Lactase is used to hydrolyze lactose to

glucose and galactose as a digestive aid and to improve the solubility and sweetness in various dairy products. Lactose hydrolysis helps these lactose-intolerant people to drink milk and eat various dairy products (Tucker and Woods, 1995). Lactases have also been used in the processing of dairy wastes and as a digestive aid taken by humans in tablet form when consuming dairy products. Recently three novel xylanases thermophilic in nature (XynA,B,C) have been characterized (Du et al., 2013); these were produced by *Humicola* sp. for their potential applications in the brewing industry. This XynA also possessed higher catalytic efficiency and specificity for a range of substrates.

Proteinases, either indigenous (cathepsin) or those obtained from plants and microorganisms, are used in the meat and fish industries to tenderize meat and solubilize fish products. Tenderization of meat can be achieved by keeping the rapidly chilled meat at 1–2°C to allow proteolysis by indigenous enzymes. Enzymes are also used to facilitate separation of hemoglobin from blood proteins and removal of meat from bones. For the preparation of pet food, minced meat or meat by-products are hydrolyzed by proteases to produce a liquid meat digest or slurry with a much lower viscosity (Saha and Demirjian, 2000). Fish protein concentrates are generally prepared by treating ground fish parts with a protease.

In the baking industry, there is an increasing focus on lipolytic enzymes. Recent findings suggest that phospholipases can be used to substitute or supplement traditional emulsifiers, as the enzymes degrade polar wheat lipids to produce emulsifying lipids. Also, research is currently devoted towards the further understanding of bread staling and the mechanisms behind the enzymatic prevention of staling in presence of α -amylases and xylanases (Andreu et al., 1999). Lipases are commonly used in the production of a variety of products, ranging from fruit juices, baked foods, and vegetable fermentations. Fats, oils, and related compounds are the main targets of lipases in food technology. Accurate control of lipase concentration, pH, temperature, and emulsion content is required to maximize the production of flavor and fragrance. The lipase mediation of carbohydrate esters of fatty acids offers a potential market for use as emulsifiers in foods and pharmaceuticals. There are three recombinant fungal lipases currently used in the food industry, one from *Rhizomucor miehi*, one from *Thermomyces lanuginosus*, and another from *Fusarium oxysporum* (Mendez and Salas, 2001).

Enzymes can play important roles in preparing and processing various fruit and vegetable juices, such as apple, orange, grapefruit, pineapple, carrot, lemon etc. Fruits and vegetables are particularly rich in pectic substances. Pectin, a hydrocolloid, has a great affinity for water and can form gels under certain conditions. The addition of pectinases, pectin lyase, pectin esterase, and polygalacturonase, reduces viscosity and improves pressability as the pectin gel collapses (Tucker and Woods, 1995). For complete liquefaction of fruits and vegetable juices, hemicellulases and amylases can be used with pectinases. Flavoprotein glucose oxidase, is used to scavenge oxygen in fruit juice and beverages to

prevent color and taste changes upon storage. Glucose oxidase is produced by various fungi such as *A. niger* and *Penicillium purpurogenum* (Godfrey and West, 1996b).

11.4.2 Detergents

The detergent industry occupies about 30% of the entire industrial enzyme market. The application of enzymes in detergents enhances the detergents ability to remove tough stains and also makes detergent ecofriendly. Constantly, new and improved engineered versions of the “traditional” detergent enzymes, proteases and amylases, are developed. These new second- and third-generation enzymes are optimized to meet the requirements for performance in detergents. Over half of the laundry detergents contain enzymes such as protease, amylase, lipase, and cellulase. These enzymes must be very efficient in laundry detergent environments, work at alkaline pH conditions and high temperatures, be stable in the presence of chelating agents and surfactants, and possess long storage stability. Proteases are the most widely used enzymes in the detergent industry. DNA technology has been used extensively to modify the protein catalysts, primarily for increasing stability properties (Bisgaard-Frantzen et al., 1999; Wintrode et al., 2000). These detergent enzymes (serine proteases) are produced by fermentation of *B. licheniformis*, *B. amyloliquefaciens*, or *Bacillus* sp.

Novo Industri A/S produces and supplies three proteases, Alcalase, from *B. licheniformis*, Esperase, from an alkalophilic strain of a *B. licheniformis*, and Savinase, from an alkalophilic strain of *B. amyloliquefaciens*. GistBrocades produce and supply Maxatase, from *B. licheniformis*. Alcalase and Maxatase are recommended for use at 10–65°C and pH 7–10.5. Savinase and Esperase may be used at up to pH 11 and 12, respectively.

Amylases are the second type of enzymes used in the detergent formulation, and 90% of all liquid detergents contain these enzymes (Mitidieri et al., 2006). These enzymes are used for laundry and automatic dishwashing to clean up residues of starchy foods such as mashed potato, custard, oatmeal, and other small oligosaccharides. The α -amylase supplied for detergent use is Termamyl, the enzyme from *B. licheniformis*, which is also used in the production of glucose syrups. α -Amylase is particularly useful in dishwashing and destarching detergents.

Lipases facilitate the removal of fatty stains, such as lipsticks, frying fats, butter, salad oil, sauces, and tough stains on collars and cuffs. Recently, an alkali-stable fungal cellulose preparation has been introduced for use in washing cotton fabrics. Treatment with these cellulose enzymes removes the small fibers extending from the fabric, without apparently damaging the major fibers, and restores the fabric by improving color brightness and enhancing softness feel. Cellulases are used in textile manufacturing to partially remove dye (indigo) from denim, producing a stone-washed appearance. Bleach-stable enzymes (amylase, protease) are now available for use in automatic dishwashing

detergents. The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry and in household dishwashers. To improve detergency, modern types of heavy duty powder detergents and automatic dishwasher detergents usually contain one or more enzymes, such as protease, amylase, cellulase, and lipase (Ito et al., 1998).

11.4.3 Textiles

Enzymes are being used increasingly in textile processing, mainly in the finishing of fabrics and garments. Some of the more important applications are desizing and jeans finishing. The use of enzymes in the textile industry allows the development of environmentally friendly technologies in fiber processing and strategies to improve the final product quality. The consumption of energy as well as increased awareness of environmental concerns related to the use and disposal of chemicals into landfills, water, or release into the air during chemical processing of textiles are the principal reasons for the application of enzymes in finishing of textile materials (O'Neill et al., 2007).

Enzymes have been used increasingly in the textile industry since the late 1980s. Many of the enzymes developed since the late 1990s are able to replace chemicals used by mills. The first major breakthrough was when enzymes were introduced for stonewashing jeans in 1987. Within a few years, the majority of denim finishing laundries had switched from pumice stones to enzymes.

The main enzymes used in textile industry are hydrolases and oxidoreductases. The group of hydrolases includes amylases, cellulases, proteases, pectinases, lipases and esterases. Amylases were the only enzymes applied in textile processing until the 1980s. These enzymes are still used to remove starch-based sizes from fabrics after weaving. Nowadays, amylases are commercialized and preferred for desizing due to their high efficiency and specificity, completely removing the size without any harmful effects on the fabric (Etters and Annis, 1998). Cellulases have been employed to enzymatically remove fibrils and fuzz fibers, and have also successfully been introduced to the cotton textile industry. Further applications have been found for these enzymes to produce the aged look of denim and other garments. The potential of proteolytic enzymes was assessed for the removal of wool fiber scales, resulting in improved antifelting behaviour. Esterases have been successfully studied for the partial hydrolysis of synthetic fiber surfaces, improving their hydrophilicity and aiding further finishing steps. Besides hydrolytic enzymes, oxidoreductases have also been used as powerful tools in various textile-processing steps. Catalases have been used to remove H₂O₂ after bleaching and to reduce water consumption. In the textile industry lipases are used for the removal of size lubricants, which increases fabrics absorbance ability for improved levelness in dyeing (Raja et al., 2012). In the denim abrasion systems, it is used to lessen the frequency of cracks and streaks.

11.4.4 Animal Feed

Animal feed is the largest cost item in livestock and poultry production, accounting for 60–70% of total expenses. To save on costs, many producers supplement feed with enzyme additives, which enable them to produce more meat or to produce the meat cheaper and faster. Found in all living cells, enzymes catalyze chemical processes that convert nutrients into energy and new tissue. They do this by binding to substrates in the feed and breaking them down into smaller compounds. For example, proteases break down proteins into amino acids, carbohydrases split carbohydrates into simple sugars, and lipases take apart lipids into fatty acids and glycerol.

Animal feed is composed mainly of plant materials, such as cereals, agricultural and grain milling byproducts, and agricultural waste residues. These contain nonstarch polysaccharides, protein, and phytic acid. Monogastric animals generally cannot fully digest and utilize the fiber-rich feedstuffs. Due to the complex nature of the feed materials, starch sequestered by β -glucans and pentosans is also not digestible. Feed enzymes can increase the digestibility of nutrients, leading to greater efficiency in feed utilization (Choct, 2006). Currently, feed enzymes commercially available are phytases, proteases, α -galactosidases, glucanases, xylanases, α -amylases, and polygalacturonases (Selle and Ravindran, 2007). The use of enzymes as feed additives is restricted in many countries by local regulatory authorities (Pariza and Cook, 2010) and applications may therefore vary from country to country.

During recent years focus has been on the utilization of natural phosphorus bound in phytic acid in cereal-based feed for monogastrics. Phytic acid forms complexes with metal ions such as calcium, magnesium, iron, and zinc, thus preventing their assimilation by the animal. Better utilization of total plant phosphorus, of which 85–90% is bound in phytic acid, is only obtained by adding the enzyme phytase to the feed. Microbial phytase liberates part of the bound phosphorus and makes it possible to reduce the amount of supplements (phosphorus, calcium, and other nutrients) added to the animal diet. Phytase in animal feed can alleviate environmental pollution from bound phosphorous in animal waste and development of dietary deficiencies in animals (Lei and Stahl, 2000). The most common source of microbial phytase is *Aspergillus ficuum*.

Protein utilization from vegetables can be enhanced by using microbial proteases. Thus, feed utilization and digestion by animals can be enhanced by adding enzymes to the feed (Lehmann et al., 2000). Various microbial enzymes are now used as feed enhancers and hold the prospect of serving larger roles in animal and poultry production. Commercially-available enzymes can be derived from plants and animals (eg, actininidin from kiwi and rennet from calf stomachs) as well as microorganisms (eg, amylase from *Bacillus* and lactase from *Aspergillus*).

11.4.5 Ethanol Production

In the alcohol industry, the use of enzymes for the production of fermentable sugars from starch is also well established. The process of making ethanol from starch involves the basic steps of preparation of the glucose feedstock, fermentation of glucose to ethanol, and recovery of ethanol. Enzymes have an important role in preparing the feedstock, that is, in converting starch into the fermentable sugar, glucose. Corn kernels contain 60–70% starch, and it is the dominant source (97%) of starch feedstock used for ethanol production.

Over the past decade, there has been an increasing interest in fuel alcohol as a result of increased environmental concern and higher crude oil prices. Therefore, intense efforts are currently being undertaken to develop improved enzymes that can enable the utilization of cheaper and partially utilized substrates such as lignocellulose, to make bioethanol more competitive with fossil fuels (Wheals et al., 1999; Zaldivar et al., 2001).

Two methods are used industrially to process corn for making starch accessible to enzymes in subsequent treatment. In the wet-milling process, corn is steeped in acidic water solutions and the oil, protein, and fiber fractions are successively removed as products leaving the starch fraction. Enzymatic liquefaction and saccharification of the starch fraction are then carried out for the production of glucose. Microbial enzymes have replaced the traditional hydrolytic enzymes formerly supplied by adding malt. Glucose is fermented by the traditional yeast *S. cerevisiae* to ethanol, which can be recovered by distillation. In beverage ethanol processes, the beer may be treated with acetolactate decarboxylase from *Bacillus brevis* or *Lactobacillus* sp. to convert acetolactate into acetoin via nonoxidative decarboxylation. Saccharification and fermentation steps can also be carried out concurrently in a process known as simultaneous saccharification and fermentation. In the United States, most ethanol (over 80%) from corn is produced from corn processed through dry grind facilities because of the lower capital investment required in comparison to that of wet mills. In the typical dry grind process, corn is mechanically milled to coarse flour. Following liquefaction, enzymatic saccharification using glucoamylase and fermentation using the conventional yeast are carried out simultaneously (Taylor et al., 2000, 2001). The addition of protein-splitting enzymes (proteases) releases soluble nitrogen compounds from the fermentation mash and promotes growth of the yeast, decreasing fermentation time. The residue left after fermenting the sugars is known as distiller's grains, which is used as animal feed. Typically, large-scale industrial fermentation processes provide 12–15% (v/v) ethanol with an ethanol yield as high as 95% of theoretical, on the basis of starch feedstock.

11.4.6 Other Applications

In recent years, tremendous research efforts have been made to reduce the amount of chlorine used for bleaching of kraft pulp after the pulping processes.

Environmental regulatory pressures have prompted the pulp and paper industry to adapt new technology to eliminate the presence of various contaminants in the bleaching plant effluents. The main constituents of wood are cellulose, hemicellulose, and lignin. Research in the use of enzymes in pulp manufacture involves the degradation or modification of hemicellulose and lignin without disturbing the cellulose fibers.

Xylanase preparations used for wood processing in the paper industry should be free of cellulose activity. Cellulase-free xylanase preparations have applications to provide brightness to the paper due to their preferential solubilization of xylans in plant materials and selective removal of hemicelluloses from the kraft-pulp. The production of cellulase free extracellular endo-1,4- β -xylanase has been studied at a higher temperature of 50°C and at pH 8.5 employing a strain of *Thermoactinomyces thalophilus* (Kohli et al., 2001). The paper and pulp industry requires a step of separation and degradation of lignin from plant material, where the pretreatment of wood pulp using ligninolytic enzymes is important for a milder and cleaner strategy of lignin removal compared to chemical bleaching. Bleach enhancement of mixed wood pulp has been achieved using coculture strategies, through the combined activity of xylanase and laccase (Dwivedi et al., 2010). The ligninolytic enzyme system is used in biobleaching of craft pulp and in other industries. Fungi are the most potent producers of lignin degrading enzymes. The use of laccase to promote degradation of lignin and bleaching of pulp has attracted considerable interest as a cost-effective replacement for chlorine bleaches. Thermophilic laccase enzyme is of particular use in the pulping industry. Recently, the biophysical characterization of thermophilic laccase isoforms has been reported (Kumar and Srikumar, 2013).

The removal of pitch by chemical pulping or bleaching is not efficient. Pitch is the sticky resinous material in wood. Treatment with lipases has been found to be useful in reducing pitch deposits since lipases hydrolyze the triglycerides in the wood resin to fatty acids and glycerol making the material less viscous. The enzyme does not affect the cellulose quality. Removal of ink is an important part of waste paper processing. Conventional deinking involves pulping of the paper in highly alkaline solution. It has been reported that cellulase enzymes can increase the efficiency of the deinking process. The coating treatment makes the surface of paper smooth and strong to improve the writing quality of the paper. For paper sizing the viscosity of the natural enzyme is too high, and this can be changed by partially degrading the polymer with α -amylases in a batch or continuous processes (van der Maarel et al., 2002). Starch is considered to be the good sizing agent for the finishing of paper, improving the quality and reusability, besides being a good coating for the paper.

In the leather industry, skins are soaked initially to clean them and to allow rehydration. Proteolytic enzymes effectively facilitate the soaking process. Lipases have also been used to dissolve and remove fat. Dehairing is then carried out using alkaline protease, such as subtilisin. Alkaline conditions swell the

hair roots, easing removal of the hair by allowing proteases to selectively attack the protein in the hair follicle. Conventional dehairing processes require harsh chemicals, such as slaked lime and sodium sulfide, which essentially swell the hide and loosen and damage the hair (Godfrey and West, 1996b). Enzyme-based dehairing has led to much lower pollution emissions from tanneries.

Enzymes are used in various analytical methods, both for medical and non-medical purposes. Immobilized enzymes are used as biosensors for the analysis of organic and inorganic compounds in biological fluids. A glucose biosensor consists of a glucose oxidase membrane and an oxygen electrode, while a biosensor for lactate consists of immobilized lactate oxidase and an oxygen electrode. The lactate sensor functions by monitoring the decrease in dissolved oxygen which results from the oxidation of lactate in the presence of lactate oxidase (Saha et al., 2009). The amperometric determination of pyruvate can be carried out with the pyruvate oxidase sensor. A bioelectrochemical system for total cholesterol estimation was developed, based on a double-enzymatic method. In this system, an immobilized enzyme reactor containing cholesterol esterase and cholesterol oxidase is coupled with an amperometric detector system. An amino acid electrode for the determination of total amino acids has also been developed using the enzymes L-glutamate oxidase, L-lysine oxidase, and tyrosinase. Enzyme electrodes are used for continuous control of fermentation processes.

Successful application of enzymatic processes in the chemical industry depends mainly on cost competitiveness with the existing and well-established chemical methods (Tufvesson et al., 2011). However, new scientific developments in genomics, as well as in protein engineering, facilitate the tailoring of enzyme properties to increase that number significantly (Jackel and Hilvert, 2010; Lutz, 2010). An enzymatic conversion was devised to produce the amino acid L-tyrosine. Phenol, pyruvate, pyridoxal phosphate and ammonium chloride are converted to L-tyrosine using a thermostable and chemostable tyrosine phenol lyase obtained from *Symbiobacterium toebii* (Kim do et al., 2007; Sanchez and Demain, 2011).

The numerous biocatalytic routes scaled up for pharmaceutical manufacturing have been recently reviewed (Bornscheuer et al., 2012), showing the competitiveness of enzymes versus traditional chemical processes. Enzymes are useful for preparing beta-lactam antibiotics such as semisynthetic penicillins and cephalosporins (Volpatto et al., 2010). The semisynthetic penicillins have largely replaced natural penicillins and about 85% of penicillins marketed for medicinal use are semisynthetic. 6-Aminopenicillanic acid is obtained by the hydrolysis of the amide bond of the naturally occurring penicillin with the enzyme penicillin amidase, which unlike chemical hydrolysis does not open the β -lactam ring. The most important applications in biocatalysis are the synthesis of complex chiral pharmaceutical intermediates efficiently and economically. Esterases, lipases, proteases, and ketoreductases are widely applied in the preparation of chiral alcohols, carboxylic acids, amines, or epoxides (Zheng and Xu, 2011). Kinetic resolution of racemic amines is a common method used in

the synthesis of chiral amines. Acylation of a primary amine moiety by a lipase is used by BASF for the resolution of chiral primary amines in a multithousand ton scale ([Sheldon, 2008](#)). Atorvastatin, the active ingredient of Lipitor, a cholesterol-lowering drug can be produced enzymatically. The process is based on three enzymatic activities, such as a ketone reductase, a glucose dehydrogenase, and a halohydryl dehalogenase. Several iterative rounds of DNA shuffling for these three enzymes led to a 14-fold reduction in reaction time, a sevenfold increase in substrate loading, a 25-fold reduction in enzyme use, and a 50% improvement in isolated yield ([Ma et al., 2010](#)).

Therapeutic enzymes have a wide variety of specific uses such as oncolytics, thrombolytics, or anticoagulants and as replacements for metabolic deficiencies. Enzymes are being used to treat many diseases like cancer, cardiac problems, cystic fibrosis, dermal ulcers, inflammation, digestive disorders etc. Proteolytic enzymes serve as good anti-inflammatory agents. Collagenase enzyme, which hydrolyzes native collagen and spares hydrolysis of other proteins, has been used in dermal ulcers and burns. Papain has been shown to produce marked reduction of obstetrical inflammation and edema in dental surgery. Deoxyribonuclease is used as a mucolytic agent in patients with chronic bronchitis. Trypsin and chymotrypsin have been successfully used in the treatment of athletic injuries and postoperative hand trauma. Hyaluronidase has hydrolytic activity on chondroitin sulphate and may help in the regeneration of damaged nerve tissue ([Moon et al., 2003](#)). Lysozyme hydrolyzes the chitins and mucopeptides of bacterial cell walls. Hence, it is used as antibacterial agent usually in combination with standard antibiotics. Lysozyme has also been found to have activity against HIV, as the RNase A and urinary RNase U present selectively degrade viral RNA ([Lee-Huang et al., 1999](#)) showing possibilities for the treatment of HIV infection.

Cancer research has some good instances of the use of enzyme therapeutics. Recent studies have proved that arginine-degrading enzyme (PEGylated arginine deaminase) can inhibit human melanoma and hepatocellular carcinomas ([Ensor et al., 2002](#)). Currently, another PEGylated enzyme, Oncaspar1 (pegaspargase), is showing good results for the treatment of children newly diagnosed with acute lymphoblastic leukemia. The further application of enzymes as therapeutic agents in cancer is described by antibody-directed enzyme prodrug therapy (ADEPT). A monoclonal antibody carries an enzyme specific to cancer cells where the enzyme activates a prodrug and destroys cancer cells but not normal cells. This approach is being utilized for the discovery and development of cancer therapeutics based on tumor-targeted enzymes that activate prodrugs. Certain enzymes such as L-asparaginase have been found to be useful in treating cancer. L-asparaginase, by lowering the concentration of asparagine, retards the growth of cancer cells. It has proven particularly useful in treating lymphoblastic leukemia and certain forms of lymphomas.

Genetic engineering basically involves taking the relevant gene from the microorganism that naturally produces a particular enzyme (donor) and inserting

it into another microorganism that will produce the enzyme more efficiently (host). The first step is to cleave the DNA of the donor cell into fragments using restriction enzymes. The DNA fragments with the code for the desired enzyme are then placed, with the help of ligases, in a natural vector called a plasmid that can be transferred to the host bacterium or fungus. In recombinant DNA technology, restriction enzymes recognize specific base sequences in double helical DNA and bring out cleavage of both strands of the duplex in regions of defined sequence. Restriction enzymes cleave foreign DNA molecules. The term *restriction endonuclease* comes from the observation that certain bacteria can block virus infections by specifically destroying the incoming viral DNA ([Adrio and Demain, 2014](#)). Such bacteria are known as restricting hosts, since they restrict the expression of foreign DNA. Certain nicks in duplex DNA can be sealed by an enzyme-DNA ligase which generates a phosphodiester bond between a 5'-phosphoryl group and a directly adjacent 3'-hydroxyl, using either ATP or NAD⁺ as an external energy source.

11.5 FUTURE OF MICROBIAL ENZYMES

Enzymes are some of the most important biomolecules, which have a wide range of applications in industrial as well as biomedical field. Today enzymes are some of the most important molecules that are widely used in every sector, whether that may be dairy, industrial, agriculture, or pharmaceutical fields. The global market for industrial enzymes is estimated at \$3.3 billion in 2010 and is expected to reach \$5 billion by 2020. The market segmentation for various areas of application shows that 34% of the market is for food and animal feed, followed by detergent and cleaners (29%). Paper and pulps share an 11% market while 17% of the market is captured by the textile and leather industries ([Parameswaran et al., 2013](#)).

The ongoing progress and interest in enzymes provide further success in areas of industrial biocatalysis. There is the need for exciting developments in the area of biotransformation and molecular biology. There are many factors influencing the growing interest in biocatalysts, which include enzyme promiscuity, robust computational methods combined with directed evolution, and screening technologies to improve enzyme properties to meet process prospects ([Adrio and Demain, 2014](#)).

Recent advances in genomics, proteomics, efficient expression systems, and emerging recombinant DNA techniques have facilitated the discovery of new microbial enzymes from nature or by creating enzymes with improved catalytic properties. A future trend is to develop more effective systems that use much smaller quantities of chemicals and less energy to attain maximum product yield. Modern biotechnology will lead to the development of enzyme products with improved effects with diverse physiological conditions. Biotechnology offers an increasing potential for the production of goods to meet various human needs. Enzyme technology is a subfield of biotechnology where new processes

had been developed and are still developing to manufacture both bulk and high added value products utilizing enzymes as biocatalysts, in order to meet needs in food, fine chemicals, agricultural, and pharmaceuticals, etc.

Enzymes contribute to more environmentally-adapted clean and green technology due to their biodegradable nature. They can be used in order to develop environmentally friendly alternatives to chemical processes in almost all steps of textile fiber processing (Araujo et al., 2008). Further research is required for the implementation of commercial enzyme-based processes for the biomodification of synthetic and natural fibers. An active field of research is the search for new enzyme-producing microorganisms and enzymes extracted from extremophilic micro-organisms (Schumacher et al., 2001).

Since the late 1990s, enzyme applications have been continuously increasing with highly research and development oriented activity covering various scientific and technological issues. Many enzymes need rigorous research and development to explore commercially through basic research in enzymology and process engineering. It is likely that the functional understanding of different enzyme classes will provide new applications in the future. Multidisciplinary research involving industry is required to develop application-oriented research on enzymes. Over the past 10 years, major advances in DNA technologies and in bioinformatics have provided critical support to the field of biocatalysis. These tools have promoted the discovery of novel enzymes in natural resources and have substantially accelerated the redesign of existing biocatalysts. Next-generation DNA sequencing technology has allowed parallel sequence analysis on a massive scale and at dramatically reduced cost (Born scheuer et al., 2012).

New and exciting enzyme applications are likely to bring benefits in other areas like less harm to the environment, greater efficiency, lower cost, lower energy consumption, and the enhancement of product properties. New enzyme molecules capable of achieving this will be developed through protein engineering and recombinant DNA techniques. Industrial biotechnology has an important role to play in the way modern foods are processed. New ingredients and alternative solutions to current chemical processes will be the challenge for the enzyme industry. When compared with chemical reactions, the more specific and cleaner technologies made possible by enzyme-catalyzed processes will promote the continued trend towards natural processes in the production of food.

11.6 CONCLUDING REMARKS

The enzyme industry is one of the major industries of the world and there exists a great market for enzymes. Enzymes are used in many different industrial products and processes and new areas of applications are constantly being added because of advances in modern biotechnology. Microorganisms provide an impressive amount of catalysts with a wide range of applications across many industries, such as food, animal feed, technical industries, paper, fine chemicals, and pharmaceuticals. The unique properties of enzymes, such as high

specificity, fast action, and biodegradability, allow enzyme-assisted processes in industry to run under milder reaction conditions, with improved yields and a reduction in waste generation. Naturally occurring enzymes are often modified by molecular biology techniques to redesign the enzyme itself in order to fine-tune substrate specificity activity and thermostability. Enzyme technology offers a great potential for many industries to meet challenges in the future with the help of recombinant technology.

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Chapter 12

Significance of Microbial Glucokinases

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12.1 INTRODUCTION

The transferase family (EC 2) includes a group of enzymes capable of transferring phosphorus-containing groups to an alcohol group as an acceptor (EC 2.7.1). Inside this broad group are the glucokinases (Glks). These enzymes are responsible for glucose phosphorylation and the product of this reaction, glucose-6-phosphate (G6P), may follow different fates. In yeast, the role of hexokinases in glucose uptake/oxidation, and in controlling the pentose phosphate pathway and energy metabolism has been demonstrated (Gao and Leary, 2003). Glks (α -glucose-6-phosphotransferases) transfer the group γ -phosphate to the OH group from the C₆ of α -glucose, using various phosphate donors, such as ATP (EC: 2.7.1.1 and EC: 2.7.1.2), ADP (EC: 2.7.1.147), and polyphosphate (PolyP) (EC: 2.7.1.63) (Kyoto Encyclopedia of Genes and Genomes). Glks are present both in prokaryotes and eukaryotes. However, the phosphorylation of glucose in eukaryotes is mostly achieved by ATP-dependent kinases and these enzymes show broad substrate specificity for hexoses, and are called hexokinases (HKs). In contrast to HKs, bacterial Glks usually show high specificity for glucose. Based on their primary structure, Glks/HKs from eukaryotes or prokaryotes are broadly classified into two distinct nonhomologous families: HK and ribokinase (RK) (Kawai et al., 2005). The RK family comprises Glks from euryarchaeota and eukaryotes (mammals). Glks in the RK family show homology in primary and tertiary structures, whereas the HK members only have a few conserved motifs, initially identified in conserved tertiary structures. Glks in the HK family are subgrouped in HK, A and B. The HK group consists entirely of HKs from eukaryotes. Group A is composed by Glks from Gram-negative bacteria, cyanobacteria, and amitochondriate protists. Group B includes HKs from crenarchaeota, Glks from Gram-positive bacteria, some PolyP-Glks, and also

proteins belonging to the ROK family (Repressor ORF Kinase). Based on their primary amino acid structure, another previous classification divided microbial Glks into three distinct families ([Lunin et al., 2004](#)): (1) Glks from archaea, (2) ATP-Glks without the ROK motif, and (3) ATP-Glks belonging to the ROK family.

1. The first family involves ADP-dependent Glks (ADP-Glks) from archaea and higher eukaryotes ([Fig. 12.1](#)). Nowadays, approximately 154 sequences belonging to this family are compiled in Pfam: PF04587 (Protein family database, [Finn et al., 2013](#)). These enzymes are involved in a modified Embden-Meyerhof pathway in archaea requiring ADP as the phosphoryl group donor, instead of ATP ([Hansen et al., 2002; Siebers and Schonheit, 2005](#)). In euryarchaeota, two types of glucose-phosphorylating enzymes have been reported: (1) the ADP-Glks from the hyperthermophilic euryarchaea *Pyrococcus furiosus* ([Kengen et al., 1995; Koga et al., 2000; Tuininga et al., 1999](#)), *Thermococcus litoralis* ([Koga et al., 2000](#)), and *Archaeoglobus fulgidus* strain 7324 ([Labes and Schonheit, 2003](#)) and (2) the ATP-dependent glucose-phosphorylating enzymes.

Also, a bifunctional ADP-Glk/phosphofructokinase has been described in *Methanococcus jannaschii* ([Sakuraba et al., 2002](#)). In the Pfam database, a greater number of ADP-dependent Glks from eukaryotes were reported, followed by archaea and bacteria as can be seen in [Fig. 12.1](#). Likewise, 11 of such Glks were reported in firmicutes, 3 in actinobacteria, 2 in proteobacteria, and only 1 in spirochaetia.

2. The second family (Pfam: PF02685) groups together ATP-Glks that do not contain the ROK motif. The Pfam database currently contains approximately 1500 full or partial protein sequences belonging to this family ([Fig. 12.2](#)). Most members of this family are of bacterial origin, four from eukaryotes and three from archaea. The main members from bacteria belong to Proteobacteria and Cyanobacteria (COG0837).
3. The third family (ATP-Glks belonging to the ROK family) PFAM: PF00480, essentially comprises ATP-Glks from both archaea and bacteria (primarily Gram-positive) with a ROK motif. Even though a vast number of ROK members are from a prokaryotic origin, there are proteins with ROK domains in all branches of life ([Conejo et al., 2010](#)) ([Fig. 12.3](#)). Sugar kinases that are classified as members of the ROK family have been found in many bacterial species, constituting the largest family of bacterial Glks. Approximately 5000 ROK-family proteins have been identified so far, mostly in prokaryotes, but family members are found in all kingdoms of life ([Swiatek et al., 2013](#)). Sugar kinases that are classified as members of the ROK family have been found in many bacterial species and constitute the largest family of bacterial Glks with approximately 3600 members in Pfam. From these members, most are present in firmicutes, followed by proteobacteria and actinobacteria ([Fig. 12.3](#)).

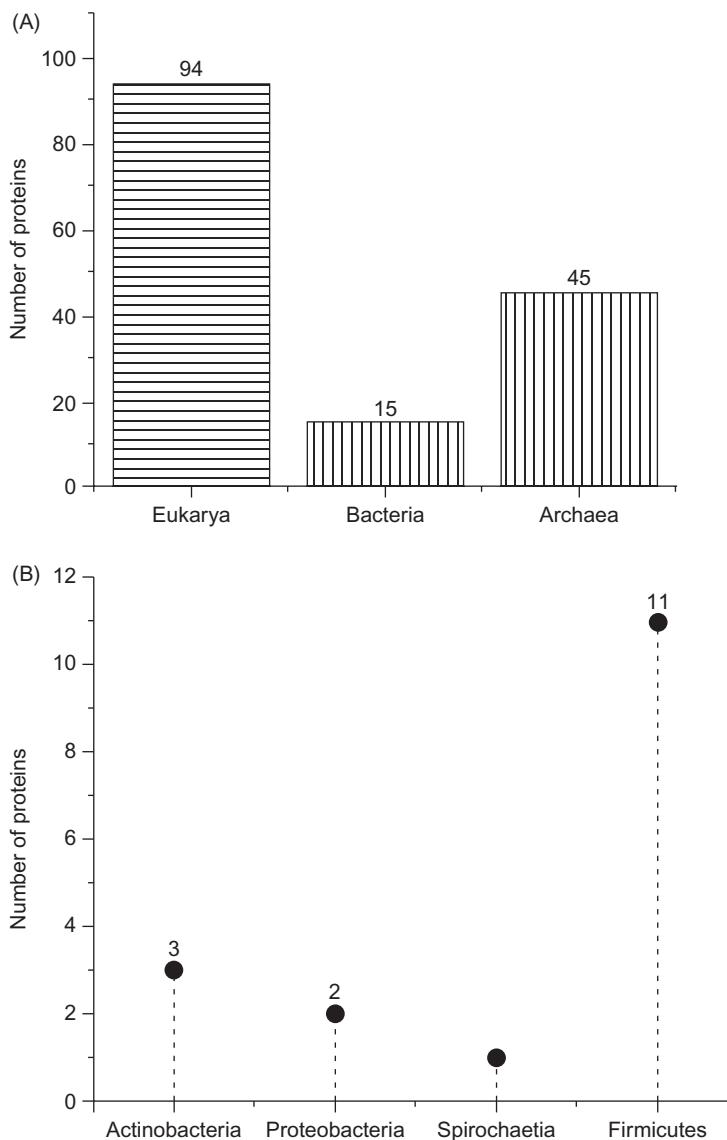


FIGURE 12.1 Distribution of ADP-dependent Glks (ADP-Glks) (Pfam: PF04587) in the three domains of life (Panel A) and bacterial phyla (Panel B). The presence of ADP-Glks were searched in the Pfam database; thus, numbers indicate how many proteins belonging to PF04587 are reported.

This family also includes many Glks that use inorganic PolyP as a phosphate donor (PolyP-Glks). PolyP can be found in organisms that represent species from each domain in nature: Eukarya, Archaea, and Bacteria. Among other functions in prokaryotes, PolyP and its associated enzymes play a crucial role

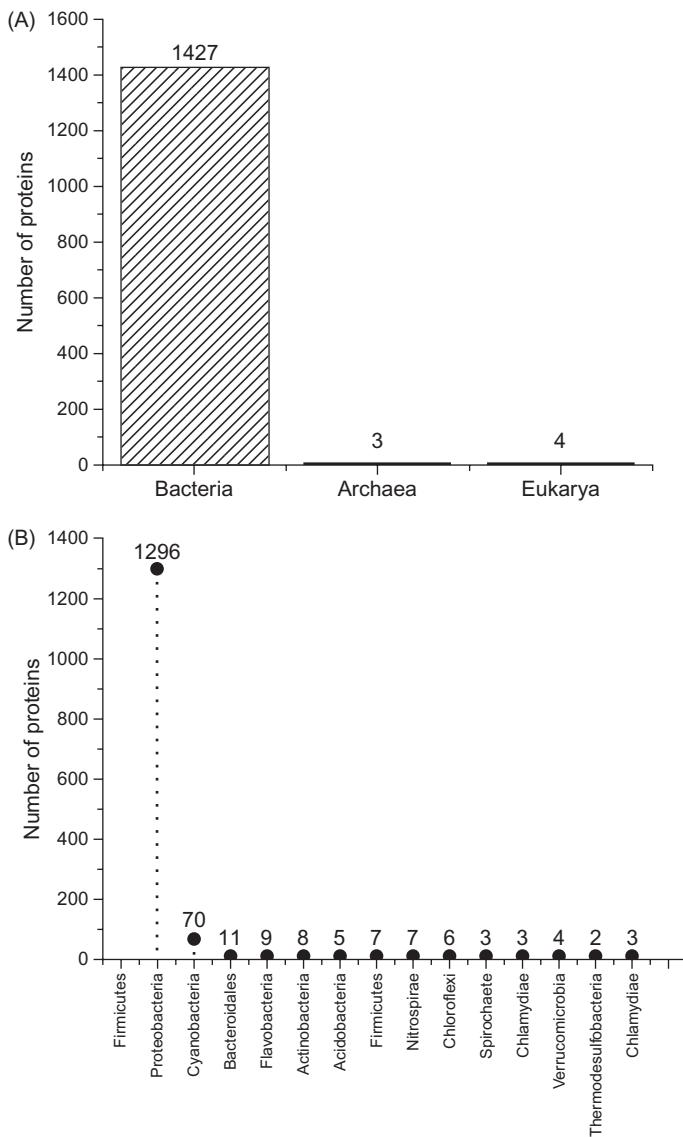


FIGURE 12.2 Distribution of ATP-dependent Glks (ATP-Glks) (Pfam: PF02685) in the three domains of life (Panel A) and in bacterial Phyla (Panel B). The presence of ATP-Glks were searched in the Pfam database; thus, numbers indicate how many proteins belonging to PF02685 are reported.

in basic metabolism and stress responses (Rao et al., 2009, Whitehead et al., 2013). PolyP-Glks were first reported in the actinobacterium *Mycobacterium phlei* (Szymona and Ostrowski, 1964), although nowadays there are many other reports in other species of actinobacteria. This Glk uses PolyP as the phosphoryl donor, as well as ATP. Inorganic PolyP is an energy- and phosphorus-rich

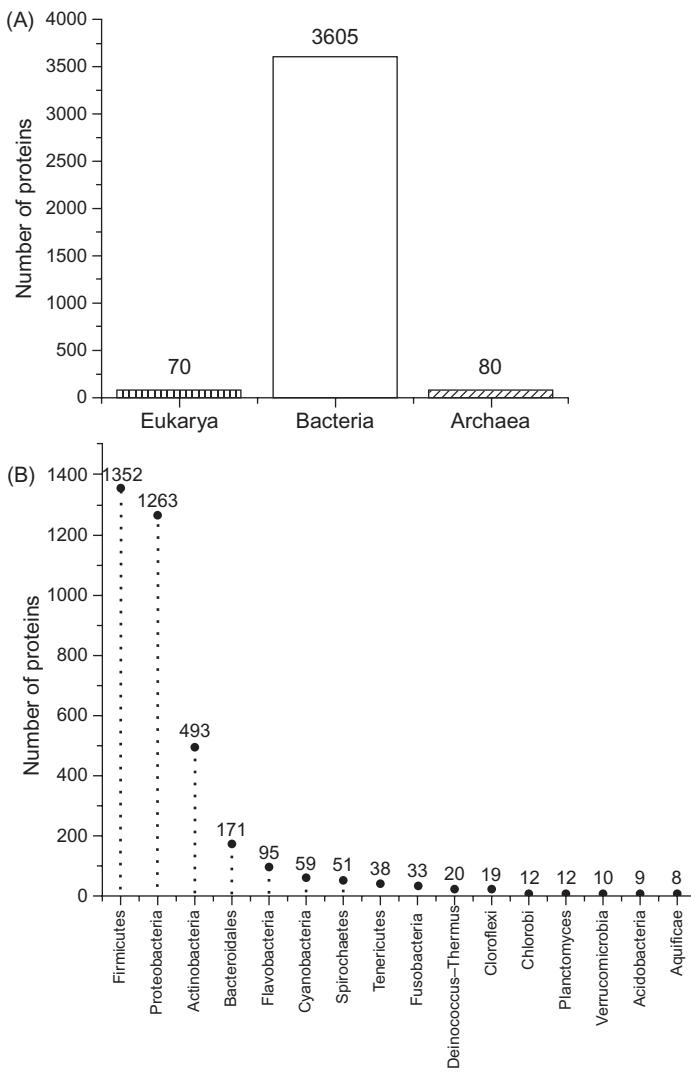


FIGURE 12.3 Distribution of ATP-dependent Glks (ATP-Glks) with the ROK motif (Pfam: PF00480) in the three domains of life (Panel A) and in bacterial Phyla (Panel B). The presence of ATP-Glks were searched in the Pfam database; thus, numbers indicate how many proteins belonging to PF00480 with the ROK motif are reported.

biopolymer that is present in a variety of organisms. The energy contained in the phosphodiester bonds of PolyP is thermodynamically equivalent to the energy obtained from ATP and can be utilized directly or indirectly for the phosphorylation of cellular molecules (Mukai et al., 2004; Rao et al., 2009). Furthermore, two strictly PolyP-Glks that utilize PolyP as the sole phosphoryl donor were reported in *Microlunatus phosphovorus* and *Termobifida fusca* (Tanaka et al.,

2003; Liao et al., 2012). Evolutionarily, PolyP/ATP-Glk and PolyP/ATP-NAD kinases are presumed to be intermediate enzymes between an ancient PolyP specific kinase and a present day ATP specific kinase family (Kawai et al., 2005).

As outlined before, Glks and HKs are widely distributed among practically all living beings. Regarding eukaryotes, mammals have four HK isoenzymes while the yeast *Saccharomyces cerevisiae* contains three enzymes that catalyze the phosphorylation of glucose, that is, HKs1 and 2 (HxK1 and Hxk2) and Glk (GIK1). Nevertheless, HxK2 is the isoenzyme that predominates when this microorganism is grown on glucose (Rodríguez et al., 2001).

12.2 SYNTHESIS, BIOCHEMICAL PROPERTIES, AND REGULATION

In general, Glks from Gram-positive and Gram-negative bacteria have been cloned and expressed in *Escherichia coli* in order to determine their biochemical characteristics. Some of these recombinant Glks and their properties are listed in Table 12.1. Additionally, kinetic properties for different yeast systems have also been summarized (Gao and Leary, 2003). The biochemical properties and substrate specificities of HK isoforms can be used to distinguish between pathogenic and nonpathogenic species. For instance, *Entamoeba histolytica*, responsible for amoebic colitis, is morphologically indistinguishable from non-pathogenic species but due to the HK isoenzyme patterns, it is possible to distinguish the pathogenic from the nonpathogenic species (Kroschewski et al., 2000).

For many years, Glks and other glycolytic enzymes were viewed just as “housekeeping” proteins with the functional purpose of fueling more important and complex biochemical processes (Kim and Dang, 2005). Nevertheless, evidence is emerging to support the unexpected multifunctional roles of glycolytic proteins.

In particular, the Glk from *Streptomyces coelicolor* (Angell et al., 1994; Mahr et al., 2000; van Wezel et al., 2007) and the Hxk2 from *S. cerevisiae* (Rodríguez et al., 2001) have been implicated in glucose regulation and carbon catabolite repression.

In order to understand the Glk/HK functions and signaling properties, it is critical to have insight into its regulation to eventually be able to manipulate them or their metabolic pathways.

In bacteria from the genus *Streptomyces*, like *S. coelicolor*, Glk seems to be constitutively expressed while its kinase activity largely depends on the carbon source present as a result of a possible posttranscriptional mechanism (van Wezel et al., 2007). In *Streptomyces peucetius* var. *caesius*, the ATP and PolyP-Glk activities are both induced by glucose (Ruiz-Villafán et al., 2014). In the yeast *S. cerevisiae*, the principal Glk, HxK2, has long been implicated in the carbon catabolite repression (CCR) process. Initially, this property was attributed to its catalytic activity but now seems to be the result of an interaction

TABLE 12.1 Some Biochemical Characteristics of Glks

Microorganism	Phosphoryl donor	Km (mM)	Vmax for glucose	Source	References
<i>Sporolactobacillus inulinus</i> Y2-8	ATP	ATP 1.03 Glc 4.26	62.3 U/mg	Overexpressed in <i>E. coli</i>	Zheng et al. (2012)
<i>Leptospira interrogans</i>	ATP	ATP 1.011 Glc 0.43	NR	Overexpressed in <i>E. coli</i>	Zhang et al. (2011)
<i>Bacillus subtilis</i>	ATP	ATP 0.77 Glc 0.24	93 U/mg	Overexpressed in <i>E. coli</i>	Skarlatos and Dahl (1998)
<i>Thermus caldophilus</i>	ATP	ATP 0.77 Glc 0.13	196 U/mg	Overexpressed in <i>E. coli</i>	Bae et al. (2005)
<i>Sulfolobus tokodaii</i>	ATP	ATP 0.12 Glc 0.05	67 U/mg	Overexpressed in <i>E. coli</i>	Nishimasu et al. (2007)
<i>Thermotoga maritima</i>	ATP	ATP 0.36 Glc 1	365 U/ mg	Overexpressed in <i>E. coli</i>	Hansen and Schönheit (2003)
<i>Methanococcus jannaschii</i>	ADP	ADP 0.032 Glc 1.6	21.5 U/mg	Overexpressed in <i>E. coli</i>	Sakuraba et al. (2002)
<i>Pyrococcus furiosus</i>	ADP	ADP 0.45 Glc 2.61	1740 U/mg	Overexpressed in <i>E. coli</i>	Verhees et al. (2002)
<i>Corynebacterium glutamicum</i>	ATP/PP	ATP 6 PP 1 Glc 21.1	21.1 U/mg	Overexpressed in <i>E. coli</i>	Lindner et al. (2010)
<i>Streptomyces coelicolor</i>	ATP/PP	PoliP 3.87×10^{-3} Glc 1.24×10^{-2}	NR	Overexpressed in <i>E. coli</i>	Koide et al. (2013)
Yeast	ATP	ATP 0.235	Yeast Sigma		Socorro et al. (2000)
		Glc 2.857			
<i>S. cerevisiae</i>	ATP	ATP 0.21	Self expression		Golbik et al. (2001)
		Glc 0.071			
<i>S. pombe</i>		ATP 0.0886	Self expression		Tsai and Chen (1998)
		Glc 0.1097			

with other regulatory proteins (Pérez et al., 2014). For instance, HxK2 from *S. cerevisiae* could interact with the transcriptional repressor Mig1 and the Snf1 kinase. Hxk2 interacts with Mig1 when the yeast grows in a high glucose concentration, preventing its phosphorylation at serine 311 by Snf1, avoiding Mig1 nuclear export and de-repression of genes subjected to CCR (Ahuatezi et al., 2004, 2007).

12.3 STRUCTURE

The tertiary structure of Glk consists of two domains (a large and a small) (Steitz et al., 1981). Between them, there is a deep cleft, where the substrate's binding site is formed (Fig. 12.4).

The binding of glucose causes movement of the two domains, so that they get close to the cleft. It leads to a change in the conformation of the enzyme, a phenomenon known as induced fit. The domains acquire a closed and open conformation in the presence and absence of glucose, respectively (Fig. 12.5).

The bound glucose molecule is in a chair conformation and adopts the β -anomeric configuration. The glucose molecule participates in an extensive hydrogen bonding network within the active site pocket.

The Glks contain an N-terminal ATP-binding motif denoted by the sequence LXXDXGGTNXRXXXL. The PP-Glk have two putative sequences for PP-binding: (1) TXGTGIGXA and (2) SXXX-W/Y-A (Ruiz-Villafán et al., 2014).

The main amino acids binding the glucose-forming hydrogen bonds are Gly, Asn, Asp, Glu, and His in the conserved ATP-Glk (Fig. 12.6). The large number of hydrogen bonds formed between the enzyme and glucose contribute to the stability of the closed structure. The putative catalytic amino acid that acts as a base in the reaction mechanism of Glk is preserved for: Asp100 *E. coli*, Asp189

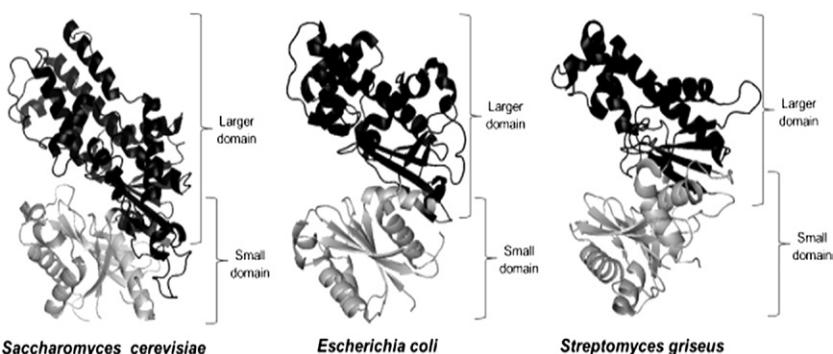


FIGURE 12.4 Ribbon model of the glucokinase monomer. The larger (black) and small domain (gray) for the hexokinase I from *Saccharomyces cerevisiae* (PDB, 3B8A), Glk from *Escherichia coli* (PDB, 1Q18), and ATP-dependent Glk from *Streptomyces griseus* (PDB, 3VGK).

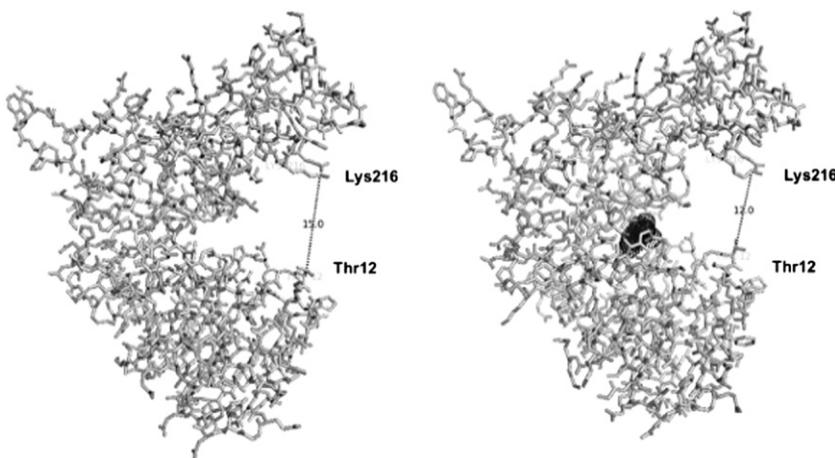


FIGURE 12.5 Comparison of open (left) and closed (right) structure of glucokinase “induced fit” of ATP-dependent Glk from *Streptomyces griseus* (PDB 3VGK apo form and 3VGM glucose complex).

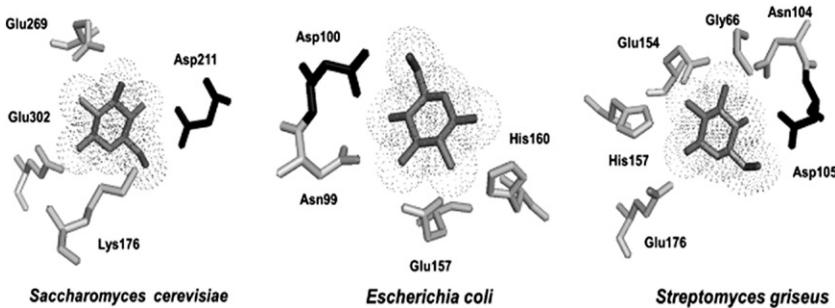


FIGURE 12.6 Structure of the active site region of hexokinase I from *Saccharomyces cerevisiae* (PDB, 3B8A), Glk from *Escherichia coli* (PDB, 1SZ2), and ATP-dependent Glk from *Streptomyces griseus* (PDB, 3VGM). Showing electron density for glucose (dark gray), amino acid residues that link glucose with hydrogen bonds (gray), and the catalytic residue Asp (black).

S. cerevisiae, Asp451 *T. litoralis*, Asp440 *P. furiosus*, Asp443 *Pyrococcus horikoshii*.

12.4 CATALYTIC MECHANISM

Although a conformational change in microbial HKs and Glks can be induced by the presence of glucose, their mechanism can be different. In the case of ADP-dependent Glk enzymes of Archaea such as *T. litoralis*, *P. horikoshii*, and

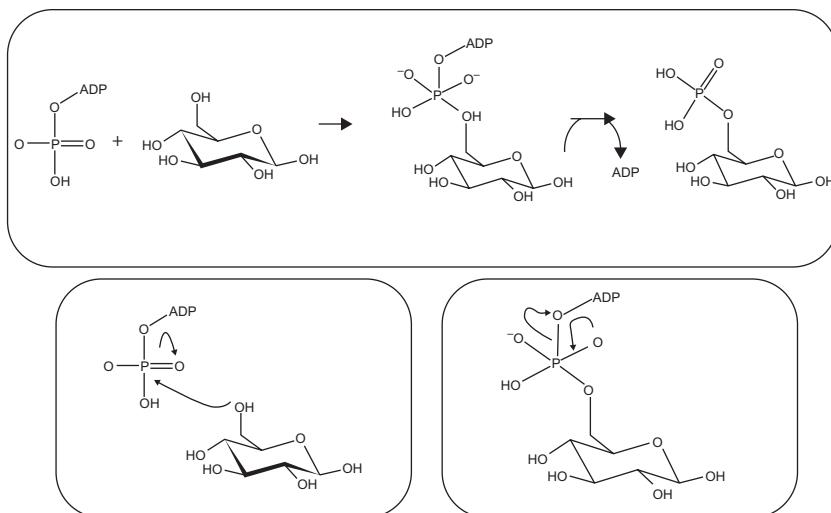


FIGURE 12.7 Mechanism of Glk activity. Simultaneous binding of ATP and glucose to the enzyme provides the proximity for the nucleophilic attack of the 6-OH of glucose on the terminal phosphoryl of ATP. Electron rearrangement leads to the production of glucose-6-phosphate and ADP.

Aeropyrum pernix, a sequential mechanism has been observed, where a ternary complex must be formed before the products are released. In this sequential mechanism, Mg•ADP⁻ is the first substrate to bind the catalytic site and Mg•AMP is the last released product.

The most striking structural aspect about the nucleotide and D-glucose binding to the active site is that both binding events indicate a sequential conformational change. Thus, Mg•ADP⁻ triggers the first structural change (semiclosed conformation), which favors the oncoming of the small domain toward the large domain. This is followed by the entry of D-glucose, which in turn leads to a ternary complex formation and closure of the total domain. This sequential conformational change strongly suggested an induced-fit mechanism (Rivas-Pardo et al., 2013).

The crystal structure of ADP-Glk from *P. furiosus* revealed that on closing of the domain, 17 water molecules were completely confined in the active-site cleft and formed a hydrophilic pocket. This is the only report of ADP-Glk from Archaea whose native structure corresponds to a homodimer (Ito et al., 2003).

Analogous to other bacterial kinases, the ATP chemical mechanism of Glk catalysis in *E. coli* is an S_N2 nucleophilic attack over the glucose O6 atom on the electropositive P atom of the γ-phosphoryl group of ATP (Fig. 12.7). Initial

abstraction of the proton from the CH_2OH group of O6 is presumably performed by an Asp, acting as a general base (Lunin et al., 2004).

Not only kinases, but almost all phosphate-transferring enzymes, have been shown to have Mg^{2+} in their active sites. They interact with both the β - and γ -phosphate to assist the reaction by orienting and stabilizing the terminal phosphate during its transfer to an acceptor (Ito et al., 2001).

As with other kinases, a metal ion, such as Mg^{2+} or Mn^{2+} , is expected to be an essential component of the catalytic machinery. No cocrystal structure of HK or Glk with a bound Mg^{2+} has yet been reported.

The kinetic data support the postulation of a sequential mechanism for the Glk reaction. These data are consistent with an ordered type of mechanism in which the glucose binding is initially observed, ending with G6P dissociation.

Studies of the kinetic mechanisms of both PolyP and ATP of Glk of *Mycobacterium tuberculosis* indicate that the activity follows a steady-state ordered Bi Bi mechanism in both PolyP and ATP dependent reactions. Product and dead-end inhibition studies suggest that PolyP binds to the free enzyme as the first substrate and is released as the first product, after the terminal phosphate is transferred to glucose, in the ordered Bi Bi mechanism. A comparison of efficiencies suggests that PolyP is favored over ATP as the phosphoryl donor for Glk in *M. tuberculosis*. The mechanism of PolyP utilization is nonprocessive, since it requires its dissociation from the enzyme prior to complete utilization (Hsieh et al., 1996).

It is known that *S. cerevisiae* has three distinct enzymes for glucose phosphorylation, the HKs (P1 and P2) and Glk. All three enzymes display a broad specificity towards the sugar substrate, except fructose, which is not phosphorylated by the yeast Glk. Both HKs are dimers of subunits with approximately 52 kDa in molecular weight and share between themselves considerable structural homology. The molecular weight of the Glk subunit was found to be 51 kDa (Maitra and Lobo, 1977) (Table 12.2).

HK binds glucose, mannose, or xylose. Xylose induces the conformation change in the active site, necessary for the interaction of ATP-Mg and the formation of catalytic ternary complexes. The mechanism of the reaction catalyzed by yeast HK seems essentially ordered. The sugar substrate must first bind at its specific site, and then the conformational change induced allows ATP-Mg to interact at the nucleotide site (Roustan et al., 1974).

Trypasosomatids are the only organisms known to possess both HKs and group A Glk enzymes. The *Trypanosoma cruzi* Glk (42 kDa) and HK (50 kDa) differ considerably in their substrate affinity; their K_m values for glucose are 0.7 mM and 0.06 mM, respectively. The crystallographic structure of Glk shows a homodimer in the asymmetric unit. Each monomer forms a complex to β -D-glucose and ADP (Cordeiro et al., 2007) (Table 12.2).

TABLE 12.2 The Molecular Dimensions of Glks

			Molecular mass (kDa)		
Taxon	Organism	Isoenzyme	Subunit	Native	References
Archaea	<i>Thermococcus litoralis</i>	ADP-Glk (Ribokinase family)	54	Monomeric	Ito et al. (2001)
	<i>Pyrococcus horikoshii</i>	ADP-Glk (Ribokinase family)	52	Monomeric	Tsuge et al. (2002)
	<i>Pyrococcus furiosus</i>	ADP-Glk (Ribokinase family)	51	Dimeric	Ito et al. (2003)
	<i>Aeropyrum pernix</i>	ATP-Glk (Ribokinase family)	36	Monomeric	Hansen et al. (2002)
Bacteria	<i>Escherichia coli</i>	ATP-Glk (Hexokinase family, A subgroup)	35	Dimeric	Lunin et al. (2004)
	<i>Streptomyces griseus</i>	ATP-Glk (Hexokinase family, B subgroup)	33	Tetrameric	Miyazono et al. (2012)
	<i>Thermus thermophilus</i> HB8	ATP-Glk (Hexokinase family, B subgroup)	31	Tetrameric	Nakamura et al. (2012)
	<i>Arthrobacter</i> sp. KM	ATP/PP-GMK (Hexokinase family, B subgroup)	30	Monomeric	Mukai et al. (2004)
	<i>Corynebacterium glutamicum</i>	ATP/PP-Glk (Hexokinase family, B subgroup)	27	Dimeric	Lindner et al. (2010)
	<i>Mycobacterium tuberculosis</i>	ATP/PP-Glk (Hexokinase family, B subgroup)	33	Dimeric	Hsieh et al. (1993)
	<i>Microlunatus phosphovorus</i>	PP-Glk (Hexokinase family, B subgroup)	32	Dimeric	Tanaka et al. (2003)
	<i>Streptomyces coelicolor</i>	ATP/PP-Glk (Hexokinase family, B subgroup)	27	Aggregates	Koide et al. (2013)
Fungi	<i>Saccharomyces cerevisiae</i>	PI, PII (Hexokinase family, Hk subgroup)	51	Monomeric or dimeric	Schmidt and Colowick (1973)
	<i>Saccharomyces cerevisiae</i>	ATP-Glk (Hexokinase family, Hk subgroup)	51	Aggregates	Maitra and Lobo (1977)
Protozoa	<i>Trypanosoma brucei</i>	Hexokinase (Hexokinase family, A subgroup)	51	Aggregates	Misset et al. (1986)
	<i>Trypanosoma cruzi</i>	Hexokinase (Hexokinase family, A subgroup)	50	Aggregates	Cáceres et al. (2003)

12.5 PRODUCTION

Despite their industrial relevance, as well as their importance for clinical analyses and research, to our knowledge, very few methodologies for the large-scale production of Glks have been described and patented. One of the best known is the production of a thermostable Glk by *Bacillus stearothermophilus* UK 788, which is about 10 microns longer than the type culture *B. stearothermophilus* IAM 11001. The new strain, having cells that settle easily and an easily breakable cell wall, was isolated as a result of screening naturally occurring microorganisms by Koch's plate culture. The new strain was isolated from manure in Ogura, Uji, Kyoto, Japan. Besides the discovery of a new strain, the invention provided a process for the industrial large-scale production of a useful enzyme selected from the group consisting of a heat-resistant polynucleotide phosphor-ylase, heat-resistant maleate dehydrogenase, heat-resistant Glk, heat-resistant glucose-6-phosphate dehydrogenase, and heat-resistant pyruvate kinase. In brief, cells in the last stage of the logarithmic growth phase growing in a batch culture in a 30 jar fermentor were collected. Chemostatic fermentation was performed supplying fresh medium (Nakajima et al., 1982) to the fermentor and withdrawing its content with a metering pump at a rate of 24 L/h. The physical parameters were: temperature, 60°C; pH, 6.8–7.0; air supply rate, 20 L/min; and stirring speed, 600 rpm. Throughout the continuous fermentation that lasted for about 4 h, the cell concentration was maintained at the level present at the start of the fermentation (5.8 g of wet cells/L or 0.75 g of dry cells/L), and 550 g of wet cells were centrifuged from 96-L of the fermentation liquor. To collect intracellular protein, cells were subjected to ultrasonic treatment and protein was determined by the biuret method. In this way, the yield was 12.8 U of heat-resistant Glk per gram of wet cells (0.16 U/mg of protein) (Nakajima et al., 1982). Later, considering the high energy needed to industrially produce the thermostable Glk due to its high growth temperature (50–60°C) and also to produce larger amounts, another invention was patented. The goal was to provide genetic material for genetic engineering to produce the thermostable Glk from *B. stearothermophilus* at a larger scale in a mesophylic bacterium such as *E. coli*, lowering the production cost. The authors isolated and cloned on a commercial vector, like pUC29, a gene encoding a thermostable Glk from *B. stearothermophilus* and the construct was introduced in *E. coli*. In regards to cloning, commercial vectors such as pUC19, pKK223-3, pPL-Qt may be used, although the authors suggest using a vector constructed by combining the ori and tac promoters originating in a multicopy vector such as pUC19. The host strains are preferably *E. coli* TG1 or BL21. In detail, the gene isolation was conducted using a chromosomal DNA library from *B. stearothermophilus*. The full length gene was successfully amplified by PCR using a combination of two primers capable of amplifying the N-terminal and C-terminal parts of the Glk gene. The Glk gene-containing DNA fragments were mixed with vector plasmid fragments and subjected to a ligation by using a T4 phage-derived DNA ligase at 16°C for 30 min.

The plasmid with the Glk gene was transformed in *E. coli* TG1 competent cells prepared by the calcium method. The transformant *E. coli* was inoculated into 300 mL of medium containing 50 µg/mL of ampicillin and cultured at 37°C overnight. The overnight culture was inoculated into 20 L of medium supplemented with 50 µg/mL of ampicillin and cultured at 37°C for 10 h. Then, 1 mM isopropyl β-thiogalactopyranoside was added and the culturing was continued for an additional 15 h. The cells were collected and showed 1,000,000 U of Glk activity. The intracellular proteins were obtained by ultrasonic disruption in 25 mM phosphate buffer (pH 8.0). The Glk was purified by affinity chromatography with Blue Sepharose and ion exchange chromatography using DEAE-Sepharose. In this way, 360,000 U of Glk were recovered, which was 50 times as much as the amount of Glk obtained by culturing 20 L *B. stearothermophilus* UK-563 (Kawase and Kurosaka, 2003). This production method reflects a clear cost reduction.

Most probably, heterologous expression may be an important alternative to increase Glk yield, avoiding regulation and energetic cost associated with thermophilic bacteria. For instance, in [Table 12.3](#), examples of cloned genes for enzyme expression of microbial Glk by PCR can be seen.

12.6 POTENTIAL APPLICATIONS IN INDUSTRIAL PROCESSES

Glk functions as the “glucose sensor” in pancreatic β-cells regulating the glucose-stimulated insulin secretion (GSIS) (Matschinsky et al., 2006). Therefore, its structural integrity is determinant for the maintenance of normal glucose homeostasis. In addition, there are several variants of congenital pancreatic diseases affecting Glk, causing either hypo (Njølstad et al., 2001) or hyperinsulinism (Wabitsch et al., 2007). In the opinion of the WHO, the global prevalence of diabetes in 2014 was estimated to be 9% among adults and it is projected that in 2030, it will be the seventh leading cause of death worldwide. Accordingly, Glk has attracted attention as a diagnostic and therapeutic target of diabetes, and is of great significance not only for industrial applications but also for medical purposes (Nakamura et al., 2012).

Glucose monitoring in diabetes. One of the challenges for diabetic patients is the regular monitoring of glucose levels without constant finger needle pricks. Thus, there is an urgent need to develop technology for the in vivo measurement of glucose (D'Auria et al., 2002; Hussain et al., 2005; Pickup et al., 2005a). One of the first assays was the glucose determination by a spectrophotometric method based on the production of NADPH by the coupled action of the enzymes Glk and G6PDH. The formation of NADPH is therefore proportional to the amount of glucose present in the assay, which is measured as a change in absorbance at 340 nm ([Fig. 12.8](#)). For this purpose, the Glk of *B. stearothermophilus* has attracted attention because of its thermal stability, which allows for its use for about 1 month at room temperature (Tomita et al., 1995). A novel, thermostable adaptation of the coupled-enzyme assay (Glk-G6PDH) for monitoring glucose

TABLE 12.3 Examples of Cloned Bacterial Glks

Gram-positive bacteria								
Name	Enzyme	Size (bp)	Accession number	Cloning		Subcloning		References
				Vector	<i>E. coli</i> host strain	Vector	<i>E. coli</i> host strain	
<i>B. subtilis</i>	ATP-Glk	966	AL009126 region 2570606-2571571	pMD492 ^c	UE26	pQE9 ^d	RB791	Skarlatos and Dahl (1998)
<i>C. glutamicum</i>	Pp/ATP-Glk	753	NC_006958 region 1980681-1981433	pEKEx3	LJ142 ^b	pET16b ^d	BL21 (DE3)	Lindner et al. (2010)
<i>L. sphaericus</i> C3-41	ATP Glk	876	EF065663	pUC18	ZSC13 ^b	pET28a ^d	BL21 ^a	Han et al. (2007)
<i>S. inulinus</i> Y2-8	ATP-Glk	975	JN860435	pMD18	DH5 α	pET28a ^d	BL21 (DE3)	Zheng et al. (2012)
<i>S. coelicolor</i> A3(2)	Pp-Glk	741	NC_003888 region 5499055-5499795	pMD19	DH5 α	pColdI ^d	BL21 (DE3)	Koide et al. (2013)
<i>S. coelicolor</i> A3(2)	ATP-Glk	954	NC_003888 region 2285983-2286936	pET15b	DH5 α	pFT61 ^{c,d}	FT1 (pLysS)	Mahr et al. (2000)
<i>T. fusca</i> YX	Pp-Glk	789	CP000088 region 2111660-2112448	pCG	DH5 α	pC- ppgk ^{c,d}	BL21 Star (DE3)	Liao et al. (2012)
<i>T. maritime</i> MSB8	ATP Glk	954	NC_000853 region 1481349- 1482302	pET19b	JM109 BL21 -CodonPlus (DE3)-RIL ^d	-	-	Hansen and Schönheit (2003)
Gram-negative								
<i>E. coli</i> O157:H7 str. EDL933	ATP-Glk	966	NC_002655.2 region 3306555-3307520	pET15 ^d	<i>E. coli</i> BL21 (DE3)	-	-	Lunin et al. (2004)
<i>L. interrogans</i>	ATP-Glk	897	NC_004342 region 1438799-1439695	pET28b ^d	<i>E. coli</i> BL21 (DE3)	-	-	Zhang et al. (2011)

^aFor Genomic DNA library construction.^bStrain without pts and glk genes for complementation analysis.^cPlasmid with cloned glk gene.^dFor protein expression.

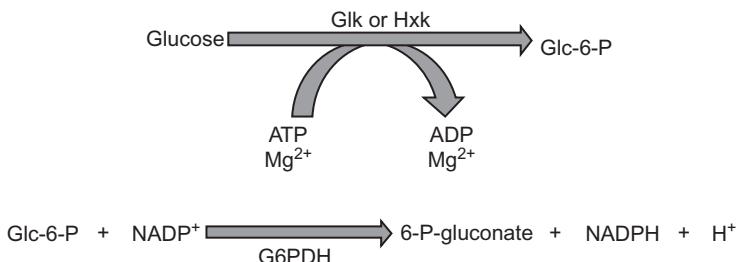


FIGURE 12.8 Diagram of the coupled reaction of Glk/Hxk and G6PDH for the quantification of glucose. Modified from Tomita et al. (1995).

concentrations was recently developed. This thermostable enzyme complex was isolated from the marine hyperthermophile *Thermotoga maritima* and works at 85°C (McCarthy et al., 2012).

Otherwise, the quantification of glucose has also been performed through the use of biosensors. Biosensors have been defined as “analytical devices or units, which incorporate a biological or biologically-derived sensitive recognition element integrated to or associated with a physico-chemical transducer” (Yoo and Lee, 2010). Biosensors are composed basically of (1) a recognition element of biological origin (receptors, enzymes, antibodies, nucleic acids, microorganisms, or lectins) able to differentiate the target molecules in the presence of other chemical agents, (2) a transducer (electrochemical, optical, thermometric, piezoelectric, or magnetic) that converts the recognition of the target molecule into a measurable signal, and (3) a signal processing system that converts the signal into a reading (Yoo and Lee, 2010). The majority of the current glucose biosensors are of the electrochemical type, due to their better sensitivity, reproducibility, and easy maintenance, as well as their low cost (Yoo and Lee, 2010). Electrochemical sensors may be subdivided into potentiometric, amperometric, or conductometric types. Amperometric sensors monitor currents generated when electrons are exchanged either directly or indirectly between a biological system and an electrode. Enzymatic amperometric glucose biosensors are the most common devices commercially available which are mainly based on glucose oxidase (GOD) enzyme activity (Hussain et al., 2005; Yoo and Lee, 2010).

One disadvantage of continuous glucose sensors based on needle-type amperometric enzyme electrodes with immobilized GOD is the need for frequent calibration to compensate for impaired responses and signal drift in vivo; for that reason, new glucose-sensing approaches are being explored (Hussain et al., 2005).

An example of this is the sol–gel coimmobilization of GOD and the Hxk from yeast for the development of an amperometric biosensor for the simultaneous detection of glucose and ATP (Liu and Sun, 2007).

There is one patent for Glk immobilization from *B. stearothermophilus* based on the fact that this enzyme is quite specific for glucose with very low interference derived from other monosaccharides. In addition, the use of this Glk avoids the problems associated with the use of the GOD enzyme, such as low sensitivity due to low oxygen solubility (Iida and Kawabe 1990).

Preliminary studies on the use of Glk for glucose determination through changes in fluorescence were performed using the Glk from the thermophile *B. stearothermophilus* (BSGlk) (D'Auria et al., 2002). In this report, they compared the stability in liquid between the BSGlk and the HXK from yeast. The enzyme from yeast proved to have poor stability over time at room temperature, unlike that of the BSGlk, which showed 100% of activity after 20 days of incubation. However, a disadvantage of the system was the poor fluorescence obtained by the authors with the fluorophore 2-(4 (iodoacetoamido) aniline) naphthalene-6-sulfonic acid (IA-ANS). Despite the low specificity for glucose of the HXK from yeast, it is possible to monitor its conformational changes when bound to the substrate through the use of fluorescence (Hussain et al., 2005; Pickup et al., 2005b). Each subunit of yeast HXK has four tryptophan residues, two surface residues, one quenchable residue in the cleft and one buried (Fig. 12.6). At an excitable wavelength of approximately 300 nm, both monomers and dimers have a steady state fluorescence emission maximum, which is attributable to tryptophan fluorescence (Pickup et al., 2005b). To avoid problems associated with low fluorescence due to the binding of glucose to the enzyme (Pickup et al., 2005b) and those associated with interferences due to the nature of the serum sample, Hussain et al. (2005) conducted a HXK immobilization in a sol-gel matrix. This development is very promising for the in vivo measurement of glucose in diabetic patients.

The Proassay Glucokinase kit from ProteinOne is a convenient, high-throughput method for the enzyme-luminescence detection of Glk activity, which can be used for the screening of Glk modulators (<http://www.proteinone.com/glucokinase-kit-proassay-glucokinase-kit-2-10-137.php>).

Preclinical findings have supported the Glk role as a glucose sensor and suggested that pharmacological activation of GK activity could have important clinical benefits in type 2 diabetes. Therefore, by selectively activating the liver Glk, an increase in glucose utilization and lower blood glucose levels without inducing excessive insulin secretion has been observed. Several groups have identified and developed a new class of drugs—called Glk activators (GKAs)—that increase the efficiency, or activate, this crucial enzyme (Matschinsky et al., 2011). Testing to date shows that oral administration of these compounds reduces blood sugar in preclinical models of type 2 diabetes. As an example, TransTech Pharma has developed several compounds covered by composition of matter patents and applications, which is the case of its lead compound TTP399. Currently, this compound is in Phase-II clinical trials against type 2 diabetes mellitus (Adjunctive treatment) in the United States (PO) (NCT02405260).

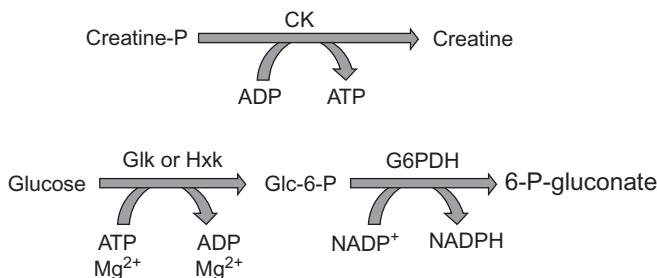


FIGURE 12.9 Diagram of the coupled reaction of creatine kinase (CK) and Glk/Hxk for measuring serum CK activity. Modified from Tomita et al. (1995).

Other clinical analyses. The determination of magnesium in serum and urine could be utilized for the diagnosis of renal diseases and gastrointestinal disorders. For this assay, it is possible to perform a reaction with Glk as stated in Fig. 12.8, as the magnesium ion is required in complex with ATP in order to carry out the phosphorylation of glucose. Thus, the concentration of magnesium can be determined spectrophotometrically by the increase of NADPH at 340 nm. This method has proven to be linear up to 100 mg/mL, which is better than that obtained in the colorimetric Xylidyl Blue method (Tabata et al., 1986; Tomita et al., 1990).

Serum creatine kinase (SCK) activity has been used for a long time to diagnose myocardial infarction or progressive muscular dystrophy (Shiraishi et al., 1991; Tomita et al., 1995). Glk could be used to determine SCK activity in a coupled reaction (Fig. 12.9), measuring at 340 nm the appearance of NADPH.

The determination of α -amylase activity in serum and urine is used to diagnose pancreatic and parotitic diseases (Kondo et al., 1988; Tomita et al., 1990). Alternative methods have presented several problems, such as time-consuming assays or some interference from the production of maltose during the action of α -amylase. The α -amylase assay occurs in two groups of reactions (Fig. 12.10). In the main group of reactions, the action of the α -amylase on its substrate, the maltohexaose (G_6), produces maltose (G_2), which is converted to glucose by maltose phosphorylase (MP). Then, glucose is converted to 6-phosphogluconate (6-P gluconate) by the action of G6PDH producing NADPH, which can be measured by the change in absorbance at 340 nm (Kondo et al., 1988; Tomita et al., 1990). The reaction of elimination converts glucose and maltose to fructose-1,6-biphosphate by the action of the enzymes MP, glucose phosphate isomerase (Glc-P isomerase), and phosphofructokinase (PFK). In a first step, glucose and maltose present in the serum samples are removed. Then, an inhibitor or MP is added and subsequently the α -amylase assay is initiated (Kondo et al., 1988).

In the aforementioned clinical applications, Hxks of yeast origin, or Glks from the thermophile *B. stearothermophilus*, were used. Currently, there is an

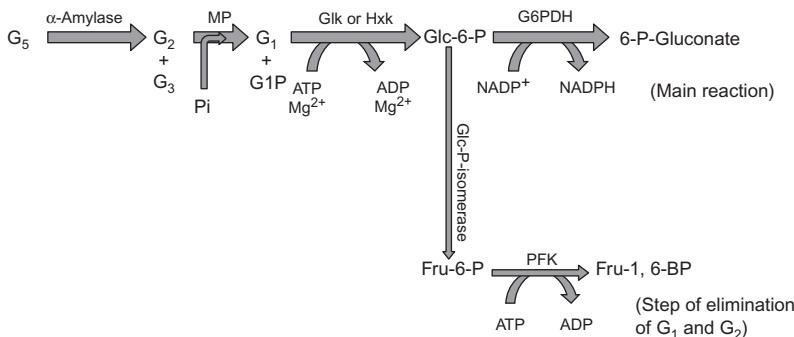


FIGURE 12.10 Diagram of the basis of the assay for α -amylase activity determination using Glk. Darker arrows show the main reaction while lighter arrows show the step of elimination of glucose (G_1) and maltose (G_2). G_5 is for maltopentaose, G_3 for maltotriose, $G1P$ for glucose-1-phosphate, $Glc-6-P$ for glucose 6-phosphate, PFK for phosphofructokinase and MP for maltose phosphorylase. Modified from Kondo et al. (1988).

increased interest in Glks capable of using PolyP instead of ATP which is more labile and unstable. One option is the Pp-Glk from *T. fusca* which has been cloned, expressed, and purified (Liao et al., 2012). It has been mentioned that the low cost generation of G6P by using Pp would be advantageous for the production of metabolites of interest such as hydrogen (Liao et al., 2012).

Other applications requiring immobilized Glks have used immobilization matrices resistant to continuous utilization. In this sense, Liu and Sun (2007) created an electrode based on a silicate hybrid sol-gel membrane for the measurement of glucose and ATP. As the new matrix was biocompatible, the HXK and G6PDH from Baker's yeast and the glucose oxidase from *Aspergillus niger* were immobilized. The resultant electrode showed high sensitivity, fast response, and good stability (Liu and Sun, 2007).

Nonclinical applications. The utilization of less costly PolyP rather than ATP as the phosphate donor for Glk activity is potentially attractive to produce high-yield hydrogen at low cost without ATP (Liao et al., 2012). Another potential application may be cell-free protein synthesis, which requires a large amount of ATP input. By integration of PolyP-Glk that can produce G-6-P from low-cost PolyP with enzymes in the glycolysis pathway in the *E. coli* cell lysate (Calhoun and Swartz, 2005; Wang and Zhang, 2009), it could be possible to synthesize proteins from low-cost substrates rather than from costly substrates such as creatine phosphate, PEP, and acetate phosphate.

A process was recently described for G6P production coupled with an adenosine triphosphate (ATP) regeneration system. In this process, glucose is phosphorylated by using Glk and acetyl phosphate to produce G6P with a conversion yield greater than 97% at 37°C for 1 h (Yan et al., 2014).

In biofuel production, it is possible to lower costs by using cellulose as a substrate. However, the cellulose must be degraded prior to fermentation by an enzymatic process. In order to monitor glucose release during the latter process, McCarthy et al. (2003) developed a continuous assay using the thermostable Glk and G6PDH from *T. maritima*, which can withstand the conditions used to hydrolyze cellulose. The coupled reaction included 1,4- β -D-glucan glucohydrolase (for cellobiose hydrolysis), Glk, and G6PDH and was performed at 85°C and a pH range of 7–8.5. This assay proved to be simple and fast and could also be coupled to glucose oxidase.

The emerging markets for polylactic acid (PLA) are likely to stimulate a significantly increased demand for high optical purity D-lactic acid. *Sporolactobacillus inulinus*, a homofermentative lactic acid bacterium, is widely used for the industrial production of D-lactic acid of high optical purity; it efficiently ferments glucose exclusively to D-lactic acid. Phosphorylation of glucose is the first step of glycolysis for D-lactic acid production. It was found that the Glk pathway was the major route for glucose uptake and phosphorylation in *S. inulinus* D-lactate production. Glk was prominently upregulated followed by the formation of a large transmembrane proton gradient, while the phosphotransferase system (PTS) pathway was completely repressed (Zheng et al., 2012).

12.7 CONCLUDING REMARKS

Glks are responsible for glucose phosphorylation and in addition to their catalytic activity, some Glks also exhibit a regulatory role. They are produced by a great array of microbial systems including bacteria, fungi, and other eukaryotes. Taking advantage of their phosphorylating activity, some Glks, especially those that are thermostable enzymes, have been envisaged for industrial purposes. In clinical analyses, they have been used for the accurate quantitation of glucose in diabetic patients. In a coupled reaction with G6PDH, Glk has been used to determine SCK activity to diagnose myocardial infarction. In another coupled reaction with G6PDH, Glk has been utilized to monitor glucose release in biofuel production from cellulose as substrate. Glk has been also used for D-lactic acid production of high optical purity. Finally, polyphosphate Glks have great potential for the generation of G6P and high-yield hydrogen based on low-cost polyphosphate.

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Chapter 13

Lipase-Catalyzed Organic Transformations: A Recent Update*

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13.1 INTRODUCTION

Development of new synthetic and catalytic methods to access new classes of chemical entities and their diverse analogs that are emerging as the key target of molecular research in drug discovery process is of prime interest and importance to the organic and bioorganic chemists. With the advent of the concept of green and sustainable chemistry, chemists are now also to take care of the increasing constraints imposed by environmental concerns during the incorporation of new catalysts in designing new synthetic protocols (Brahmachari, 2015a,b). This is due to the fact that many of the new reagents and catalysts that have benefited organic synthesis in the last several years have contained transitional metals or heavy elements; in cases where they can be environmentally acceptable, still their uses sometimes impose several disadvantages in regard to handling and disposal issues. Replacement of such metals and their salts with catalysts with better environmental acceptability would almost always be an advantage. Design of synthetic processes may also suffer from several constraints arising out the use of volatile and toxic organic solvents, and thus use of water as a solvent for reactions has already become an attractive and encouraging option. In addition, chemical syntheses with classical catalysts frequently result in undesirable mixtures of racemic components. However, optically pure isomers are particularly important in the synthesis of biologically active compounds and also for other useful purposes.

* This chapter is dedicated to Professor (Dr) Arnold Demain in honor of his profound contribution to the field of microbial enzymes.

To overcome all these constraints, chemists are dedicated to the search for novel alternatives, and it has already been established that enzymes possess enormous possibility and potentiality in emerging as the most suited catalysts (known as biocatalysts) to solve many of the aspects concerned (Halgas, 1992; Tawaki and Klibanov, 1992; Koskinen and Klibanov, 1996; Roberts, 1998, 1999, 2000; Gotor, 1999; Davis and Boyer, 2001; Klibanov, 2001; Liese et al., 2006; Vasic-Racki, 2006; Gröger, 2010; Kamerlin and Warshel, 2010; Paravidino et al., 2010; Faber, 2011; Drauz et al., 2012; Ranoux and Hanefeld, 2013). Enzymes are, in general, natural proteins that catalyze chemical reactions. These are intrinsically environmentally benign materials that operate best in water as well as in organic solvents or under solvent-free conditions (Martinek et al., 1986; Gupta, 1992; Vulfson et al., 2001; Gupta and Roy, 2004). The fact that many enzymes are reported to possess activity against nonnatural substrates in organic media has invoked interest in their potential use to carry out synthetic transformations. The use of enzymes in fine organic synthesis is most feasible in those cases when the molecules subjected to chemical rearrangement are rather complex and contain chemically similar bonds, only one (or several) of which is to be affected without the need of protection of any functional group. That is why enzymes are indispensable for the synthesis of the derivatives of natural products with complex structures (Kobayashi et al., 2003; Träff et al., 2008; Choi et al., 2009; Leijondahl et al., 2009; Krumlinde et al., 2009, 2010; Han et al., 2010a,b; Hoye et al., 2010; Andrushko and Andrushko, 2013; Wever et al., 2015). Enzymes, in a true sense, nowadays offer an important part of the spectrum of catalysts available to synthetic chemistry.

Enzyme catalysis is characterized by two main factors: specificity and rate acceleration. Chemoenzymatic syntheses provide enantioselective products in most of the cases, which are demanding. The active sites of enzymes are chiral, and contain moieties, namely, amino acid residues and, in the case of some enzymes, cofactors, that are responsible for these properties of an enzyme (Rahman and Shah, 1993; O'Brien and Herschlag, 1999; Tsai and Huang, 1999; Arora et al., 2014a). Enzyme catalysis offers several benefits at the same time, such as wide applicability, mild reaction conditions required for complex and chemically unstable molecules, low catalyst loading, good and effective reusability of biocatalyst, desired biodegradability of enzyme (catalyst) to promote green chemistry, its safe and ecofriendly nature, the possibility to reduce or to eliminate reaction by-products, and carrying out a conventional multistage reaction via single stage process without the need for protection/deprotection steps.

During the recent past, lipase (triacylglycerol acyl hydrolase EC 3.1.1.3) has emerged as one of the most promising enzymes for broad practical application in organic synthesis (Theil, 1995; Andersch et al., 1997; Itoh et al., 1997; Schmid and Verger, 1998; Davis and Boyer, 2001; Fan et al., 2012; Adlercreutz, 2013; Stergiou et al., 2013). Unlike many other enzymes, it has extremely broad substrate specificity and the ability to use a wide range of structurally diverse nonnatural compounds as substrates, both in research laboratories and

in industry. So, lipases have been extensively utilized in the synthesis of many biologically active compounds and natural products. In general, lipases are the most frequently used biocatalysts in organic synthesis. There are a variety of natural sources of lipases, such as plants, animals, and microbes (particularly, bacteria and fungi), and the lipases available from various sources have considerable variation in their reaction specificities (referred to as enzyme specificity). There are a number of good reviews and book chapters on their sources, enzyme specificity, biotechnological developments, immobilization of lipases, and reusability (Copping et al., 1990; Tramper et al., 1992; Cedrone et al., 2000; Patel, 2000, 2007; Raillard et al., 2001; Schmid et al., 2001; Sharma et al., 2001; Bolon et al., 2002; Krishna, 2002; Saxena et al., 2003; Ran et al., 2008; Zhao, 2010; Faber, 2011; Sanchez and Demain, 2011; Bornscheuer et al., 2012; Sharma and Kanwar, 2012; Adrio and Demain, 2014); hence, these are not the subject matters of this present article. As mentioned, during the last decade lipases have been finding enormous applications in organic synthesis, and the experimental outcomes have also been summarized on a regular basis in the literature (Saxena et al., 1999; Davis and Boyer, 2001; Hassan et al., 2006; Adlercreutz, 2013). The aim of this present article is to highlight the lipase-catalyzed organic transformations of interest reported in recent times (2013-mid 2015).

13.2 CHEMOENZYMIC APPLICATIONS OF LIPASES IN ORGANIC TRANSFORMATIONS: A RECENT UPDATE

As discussed in the earlier section, lipases find many biocatalytic applications in organic synthesis. At the current scenario of chemoenzymatic organic reactions, lipases stand amongst the most important biocatalysts carrying out such novel organic transformations with efficiency under ecofriendly conditions. This section offers an update on the useful catalytic applications of lipases in synthetic organic chemistry reported during the very recent period (2013-mid 2015).

Among various types of lipases, lipase B from *Candida antarctica* (CAL-B) is regarded as a promising biocatalyst by organic chemists. Branneby et al. (2003) demonstrated the first example of carbon–carbon bond formation by a CAL-B-catalyzed aldol addition of hexanal, and also observed that the mutant Ser 105Ala catalyzed this reaction faster than the wild-type enzyme. Since then various groups of researchers have reported on the direct application of this hydrolytic enzyme in the formation of Michael-type adducts (Torre et al., 2004; Carlqvist et al., 2005; Svedendahl et al., 2005; Lou et al., 2008; Strohmeier et al., 2009). CAL-B belongs to the folding family of α/β hydrolases, and the three amino acid residues, the so-called catalytic triad 187Asp–224 His–105 Ser, are supposed to play a key role in the catalytic process, including normal and promiscuous reactions (Born scheuer and Kazlauskas, 2004; Busto et al., 2010; Wu et al., 2010; Humble and Berglund, 2011). From these reports, it can be suggested that the carbonyl function of a substrate is activated through an oxyanion hole and proton transfer gets facilitated through the basic nitrogen

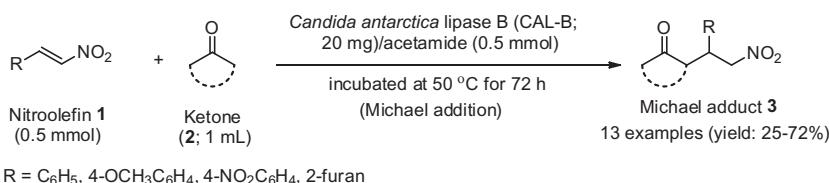


FIGURE 13.1 Michael addition between nitroolefins and less-activated ketones under co-catalytic system.

of the Asp-His pair during the catalytic process. However, such successful examples of CAL-B-catalyzed C-C bond formations via Michael or Michael-type additions were mainly restricted to the reactions between α,β -unsaturated carbonyl compounds and activated carbon nucleophiles such as acetylacetone, acetoacetates, and methyl nitroacetate. Less-activated carbon nucleophiles like cyclohexanone were found to be difficult to add to the Michael acceptor. [Chen et al. \(2013\)](#) have been successful in carrying out such a transformation between a series of aromatic and heteroaromatic nitroolefins (**1**) as the Michael acceptors and less-activated cyclic and acyclic ketones (**2**) as the Michael donors in the presence of (CAL-B)/acetamide as a co-catalytic system to obtain the corresponding Michael adducts (**3**) (Fig. 13.1). Interestingly, it has also been revealed that neither CAL-B nor acetamide can independently catalyze the reaction to any appreciable extent. The investigators have confirmed the involvement of hydrogen bonds between acetamide and oxo functionalities for the observed activation from the experimental outcomes, and they have input new mechanistic insights into CAL-B/acetamide cocatalysis ([Chen et al., 2013](#)).

In addition to the esterification and transesterification reactions, lipases are reported to catalyze amidation reactions as well via ammonolysis and aminolysis ([Zaks and Klibanov, 1985; Adamczyk and Grote, 1996, 1997, 1999](#)). [Gotor-Fernández et al. \(2006\)](#) published an informative review on CAL-B catalyzed ammonolysis and aminolysis. In recent times, aminolyses of carboxylic esters with propargyl amine containing an alkynyl moiety that can be functionalized judiciously was explored for the first time by [Hassan et al. \(2013\)](#). Propargyl amides are particularly interesting as biologically active functionalities and as synthetic building blocks ([Garg et al., 2005; Merkul and Müller, 2006; Bonger et al., 2008; Sanda et al., 2008; Merkul et al., 2009; Grotkopp et al., 2011; Gruit et al., 2011; Arkona and Rademann, 2013](#)). The investigators demonstrated that *Candida antarctica* lipase B (CAL-B) immobilized on an acrylic resin (Novozyme 435) smoothly catalyzes the aminolysis of methyl esters (**4**) with propargyl amine (**5**) affording a series of diverse propargyl amides (**6**) with moderate to good yields (Fig. 13.2). Additionally, the investigators utilized these propargyl derivatives **6** in the synthesis of amide ligated 1,2,3-triazoles in a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click reaction in good to excellent yields in the same reaction vessel ([Hassan et al., 2013](#)).

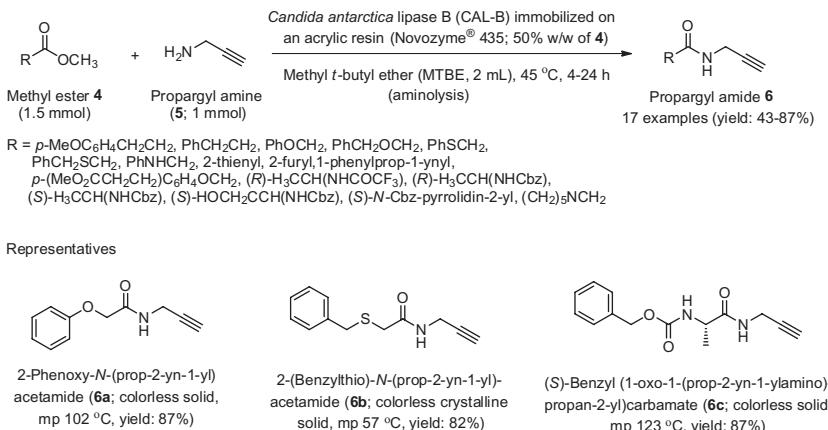


FIGURE 13.2 Aminolysis of methyl esters with propargyl amine.

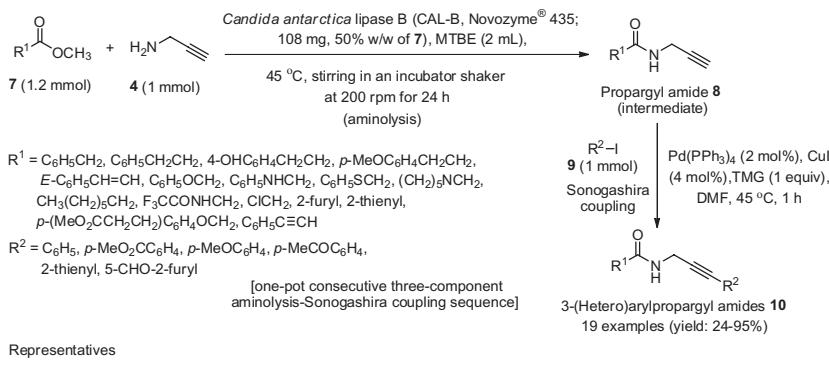


FIGURE 13.3 Consecutive three-component synthesis of (hetero)arylated propargyl amides by chemoenzymatic aminolysis-Sonogashira coupling sequence.

The same group of investigators (Hassan et al., 2015) has further extended their works in this direction, very recently. They have disclosed for the first time a consecutive three-component syntheses of (hetero)arylated propargyl amides (**10**) by CAL-B (Novozyme 435)-assisted aminolysis-Sonogashira coupling sequence from the reaction of substituted esters (**7**), propargyl amine (**4**) and alkyl/aryl/heteroaryl iodides (**9**) (Fig. 13.3). The propargyl amides (**8**) formed in the first step upon CAL-B catalyzed aminolysis of ester (**7**), subsequently

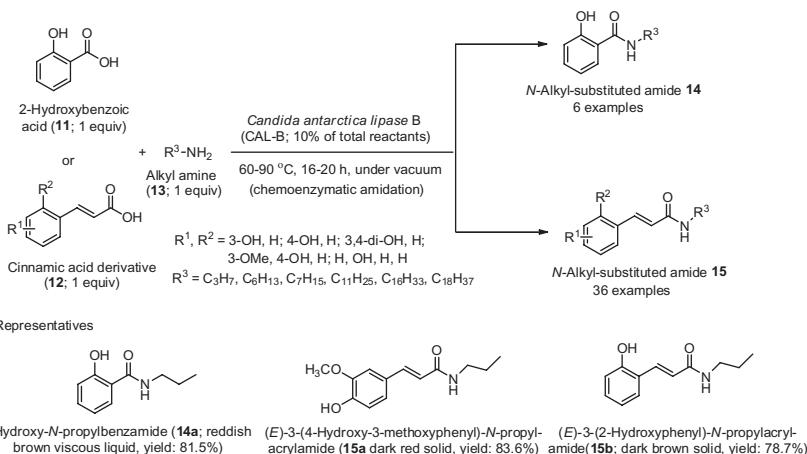


FIGURE 13.4 Solvent-free amidation of phenolic acids.

underwent Sonogashira coupling to finally afford the desired product of (hetero)arylated propargyl amides (**10**) in a one-pot. This combination of enzyme–metal catalyzed methodology may find useful application to more sophisticated peptides and aryl halides as a bioorganic tool for the efficient generation of peptidomimetics in a one-pot fashion.

Amidation of organic acids is an important organic transformation and substituted amides of phenolic and cinnamic acids are known to exhibit a wide range of biological activities, including antioxidant (Moon and Terao, 1998; Pérez-Alvarez et al., 2001; Spasova et al., 2007), anti-inflammatory (Sudina et al., 1993), anti-mutagenic (Namiki, 1990), and antihyperlipidimic (Lee et al., 2007; Stankova et al., 2009) activities. These types of scaffolds are also reported to possess anti-bacterial, anti-fungal, anti-viral, insecticidal, nematicidal, and herbicidal properties (Torres et al., 2004; Christodouloupolou et al., 2005; Lamberth et al., 2006; Cohen et al., 2008; Hu et al., 2008; Debonsi et al., 2009; Vishnoi et al., 2009; Bose et al., 2010). That is why there are so many methods available in literature for their synthesis. With the advent of enzyme-catalyzed organic reactions that have provided a great impetus to organic synthesis during the past two decades, Kaushik et al. (2015) have recently envisioned the use of *Candida antarctica* lipase B (CAL-B) to carry out biocatalytic one-pot amidation of a variety of phenolic/cinnamic acids (**11/12**) with alkyl amines (**13**) in bulk at 60–90°C under solvent-free conditions in vacuum (Fig. 13.4). A series of *N*-alkyl-substituted amides (**14/15**) were synthesized with good yields (75.6–83.5%). The present enzymatic procedure offers some important advantages over the uses of conventional catalysts, which demand for its large-scale application in industrial processes.

An elegant example of the chemoenzymatic application of lipase for regioselective acetylation of diol sugars in multiple-gram scale has recently been demonstrated by Sharma et al. (2014a,b) (Fig. 13.5). Novozyme435 (*Candida*

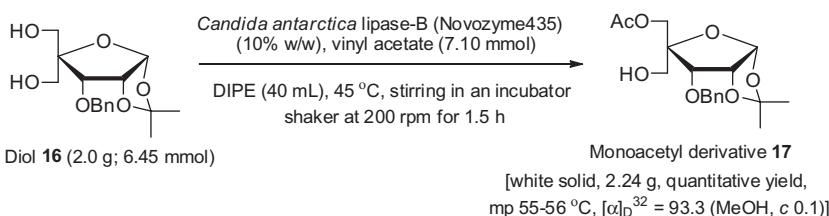


FIGURE 13.5 Chemo-enzymatic regioselective acetylation of one of the two diastereotopic hydroxymethyl functions in 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene- α -D-ribofuranose.

antarctica lipase-B) was found to catalyze regioselective acetylation of the two diastereotopic hydroxymethyl functions in 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene- α -D-ribofuranose (**16**) with vinyl acetate in diisopropyl ether (DIPE) at 45°C (incubator temperature) in an efficient manner to produce the monoacetate derivative, 5-*O*-acetyl-3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene- α -D-ribofuranose (**17**) in quantitative yield. The investigators have also exploited this enzymatic methodology successfully for the first time in the convergent synthesis of bicyclic nucleosides (LNA monomers) T, U, A, and C with relatively shorter routes and with significant improvement in overall yields. They have screened lipases from different sources, such as *Candida antarctica* lipase-B (Novozyme435; CAL-B), *Thermomyces lanuginosus* lipase immobilized on silica (Lipozyme TL IM), *Candida rugosa* lipase (CRL), and porcine pancreatic lipase (PPL) in five sets of organic solvents (tetrahydrofuran, acetonitrile, toluene, diisopropyl ether, acetone) using both acetic anhydride and vinyl acetate as acetyl donor at 45°C and at 200 rpm in an incubator shaker for the study of selective acetylation of one over the other primary hydroxyl function in diol (**16**). Among them, Novozyme435 (CAL-B) came out as the best one to effect the transformation in diisopropyl ether (DIPE) with vinyl acetate as the acetyl donor (Fig. 13.5). They have also demonstrated that the biocatalyst can be used for 10 cycles of the acylation reaction without losing selectivity and efficiency. The newly developed methodology demands useful applications for the commercial synthesis of LNA monomers of current pharmaceutical promise (Koshkin et al., 1998; Wengel, 1999; Hildebrandt-Eriksen et al., 2012; Watts, 2013; Gebert et al., 2014; Sharma et al., 2014a,b).

Recently, Hoang and Matsuda (2015) have reported on the successful utilization of immobilized *Candida antarctica* lipase B (Novozyme435) as a useful biocatalyst for transesterification of *rac*-alcohols in a liquid carbon dioxide medium in a batch reactor (Fig. 13.6). The investigators have also performed a large-scale kinetic-resolution of secondary alcohols by the immobilized lipase successfully with a continuous packed-column reactor that afforded corresponding enantiopure products, thereby achieving waste minimization.

In the previous year, Janssen et al. (2014) demonstrated the preparation of octyl formate (**23**) via immobilized lipase-catalyzed transesterification of ethyl

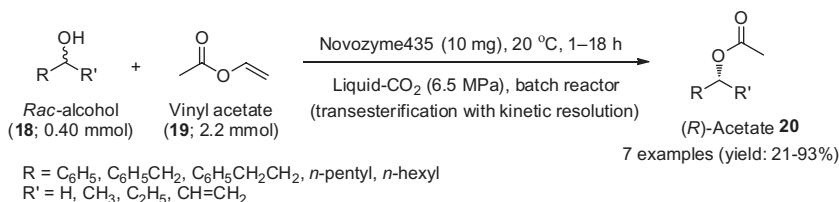


FIGURE 13.6 Transesterification of *rac*-alcohols with vinyl acetate with kinetic resolution.

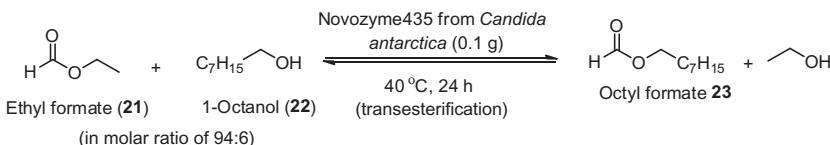


FIGURE 13.7 Transesterification of ethyl formate to octyl formate.

formate (21) with 1-octanol (22) (Fig. 13.7). Although some formyl esters are valuable compounds, eg, ingredients in flavors and fragrances (Herrmann, 2007), the formation of formic acid esters, despite several promising early contributions (Bevinakatti and Newadkar, 1989), has so far failed to be the focus of research. The present method offers simple access to a hydrophobic formic acid ester, which can be used as a reactive organic phase in biocatalytic redox reactions (Churakova et al., 2014). In addition, ethyl formate that was added in surplus to shift the equilibrium of the reaction could be partially recovered for subsequent reactions, and the biocatalyst could also be reused at least 27 times (Janssen et al., 2014).

Lipase-catalyzed synthesis of kojic acid ricinoleate has also been reported in recent times. Kojic acid (24; 5-hydroxy-2-(hydroxymethyl)-1,4-pyrone) is widely used as a food additive to prevent the browning reaction or in cosmetics as a skin whitening agent (Bentley, 2006; Chang, 2009; Kang et al., 2009). The main shortcomings of using kojic acid in industrial purposes are its water-solubility and instability at high temperature, due to which its long term storage as well as direct use in incorporating in oil base cosmetic products are major problems. To improve the kojic acid properties, such as storage stability, compatibility and oil-solubility, many kojic acid derivatives have been synthesized by different groups, usually by modifying the C-5 hydroxyl function to form hydroxyphenyl ethers or esters or by using this function to form glycosides or peptide derivatives (Nishimura et al., 1995; Kadokawa et al., 2003; Kim et al., 2004; Hsieh et al., 2007). Kojic acid possesses two different hydroxyl groups: the secondary hydroxyl group at the C-5 position and the primary hydroxyl group at C-7. The hydroxyl group at the C-5 position of kojic acid is essential to the radical scavenging activity and tyrosinase interference activity (Raku and Tokiwa, 2003). But in practice, the esterification protocol of kojic acid with long

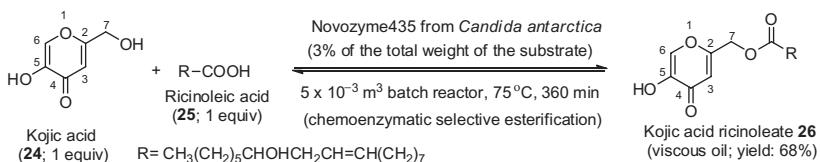


FIGURE 13.8 Solvent-free synthesis of kojic acid ricinoleate, a novel hydroxyl-fatty acid derivative of kojic acid.

chain fatty acids in the presence of acid or alkaline catalysts usually results in a complex mixture and makes easy the formation of esters at C-5, the secondary hydroxyl group of kojic acid. However, the use of immobilized lipases offers a solution to these inherent problems associated with the use of chemical catalysts. Liu and Shaw (1998) improved the lipophilic property of kojic acid by lipase-catalyzed acylation with lauric and oleic acids in the presence of acetonitrile as solvent. In this case, the acylation was also carried out at the C-5 hydroxyl group to some extent. Subsequently, Khamaruddin et al. (2009) tried to improve Liu yields by esterification of kojic acid and oleic acid using lipase from *Candida rugosa* and *Aspergillus niger*, in organic media. The maximum yield did not exceed 45%. Optimized enzymatic synthesis of kojic acid monooleate was reported in the same year by Ashari et al. (2009) but with an unsatisfactory yield (40% after 48 h reaction time). In both cases, kojic acid was also esterified at the C-5 hydroxyl group. Under this background, El-Bouafi et al. (2014) have demonstrated a modified protocol for the enzymatic esterification of kojic acid (24) for the first-time with hydroxyl-fatty acid, ricinoleic acid (*cis*-12-hydroxy-9-octadecenoic acid; 25) using Novozyme435 in solvent-free system. This lipase-catalyzed esterification took place regioselectively with the C-7 hydroxyl group affording kojic acid ricinoleate (26) with good yield of 68% (Fig. 13.8).

The unsaturated chiral γ -lactone, (*S*)- γ -hydroxymethyl- α,β -butenolide (HBO) is an important intermediate for the synthesis of many drugs (such as Burseran or Isostegane) (Tomioka et al., 1979; Enders et al., 2002), flavors (Takashi et al., 1990), and antiviral agents against HIV or hepatitis B virus (Hawakami et al., 1990; Diaz-Rodriguez et al., 2009; Flores et al., 2011). Recently, Flourat et al. (2015) have synthesized this key intermediate from (–)-levoglucosanone (LGO) using a two-step sequence involving a lipase-mediated Baeyer–Villiger oxidation and an acid hydrolysis (Fig. 13.9). This chemoenzymatic synthetic protocol offers a cost-effective, less toxic, and greener alternative.

Arora et al. (2014b) reported that lipase can also catalyze a Cannizzaro-type reaction of substituted benzaldehydes in aqueous medium at 30°C without the addition of any external redox reagent (Fig. 13.10).

In 2014, Zhou et al. (2014) reported for the first-time enzyme-catalyzed direct vinylogous Michael addition reaction of electron-deficient vinyl malononitriles to nitroalkenes. A series of nitroalkenes (34) underwent smooth Michael addition with varying vinyl malononitriles (33) to generate the

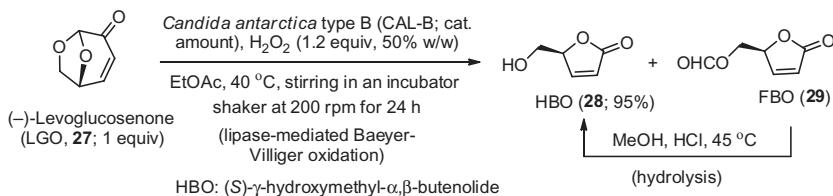


FIGURE 13.9 Chemo-enzymatic synthesis of (S)-γ-hydroxymethyl-α,β-butenolide via lipase-mediated Baeyer-Villiger oxidation of (-)-levoglucosenone.

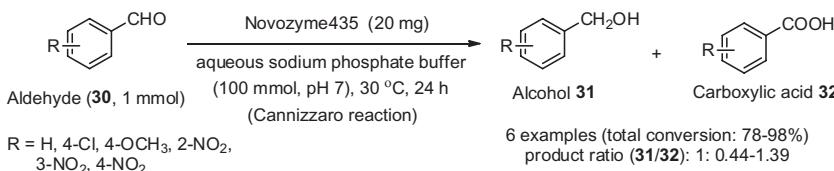


FIGURE 13.10 Cannizzaro-type reaction of substituted benzaldehydes in aqueous medium.

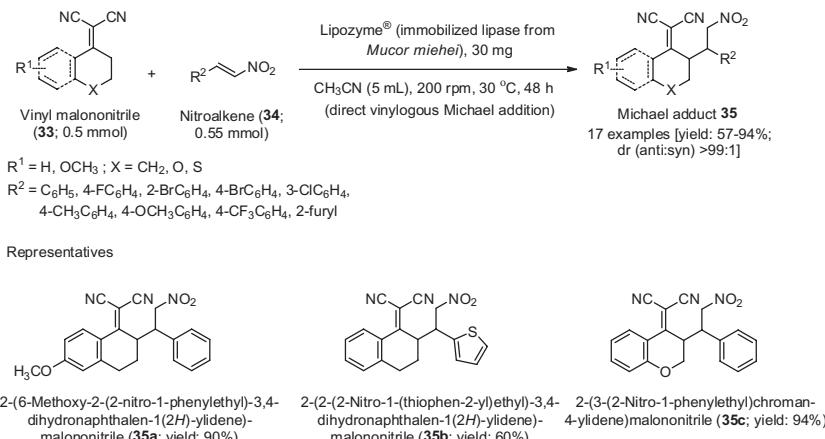


FIGURE 13.11 Lipase-catalyzed direct vinylogous Michael addition reaction.

corresponding products (**35**) with moderate to high yields (57–94%) in the presence of Lipozyme (immobilized lipase from *Mucor miehei*) with excellent diastereoselectivities (Fig. 13.11).

Wang et al. (2014) have also synthesized a series of bis-lawsone derivatives, 3,3'-(arylmethylene) bis(2-hydroxynaphthalene-1,4-diones) (**38**), from the reaction of 2-hydroxy-1,4-naphthoquinone (**36**) with aromatic aldehydes (**37**) using lipase as a green and inexpensive biocatalyst in excellent yields (Fig. 13.12). This new protocol has several advantages over the earlier reported methods, particularly in green chemistry aspects.

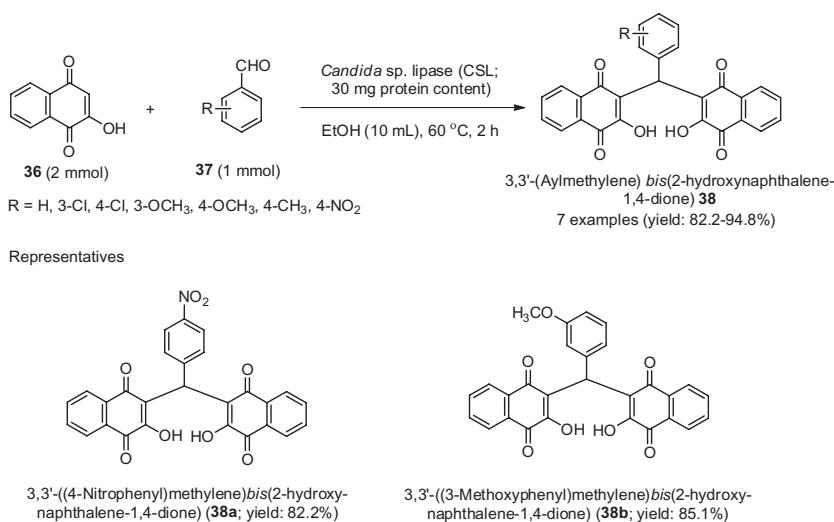


FIGURE 13.12 Synthesis of functionalized 3,3'-(arylmethylene)bis(2-hydroxynaphthalene-1,4-diones).

Among the heterocyclic compounds, thiazole derivatives are also regarded as useful chemical entities in organic and medicinal chemistry for their varied biological activities, such as antitumor, antifungal, antibiotic, and antiviral properties (Alcaide et al., 2007; Diness et al., 2011; Mouri et al., 2012; Bevk et al., 2013). In particular, 2,4-disubstituted thiazoles demand special mention for exhibiting multifarious bioactivities, and hence synthetic methods have been reported continually (Bharti et al., 2010; Chimenti et al., 2011; Naveena et al., 2013). Zhang (2014) has recently developed a novel enzyme-catalyzed multicomponent synthetic method for a series of new 2,4-disubstituted thiazoles (**43**) from the reaction of aldehydes (**39**), amines (**40**), thioacetic acid (**41**) and methyl 3-(dimethyl amino)-2-isocyanoacrylate (**42**) under the catalysis of lipase from porcine pancreas (PPL) in methanol under mild conditions with good yields (Fig. 13.13). This one-pot enzymatic multicomponent conversion method provides a novel strategy and useful tool for the synthesis of 2,4-disubstituted thiazoles offering certain green chemistry perspectives.

1,3-Oxathiolan-5-ones and their related derivatives are also attractive heterocyclic targets not only for their existence in numerous natural products and broad biological activities, but also due to their importance as intermediates for a range of highly successful and useful pharmaceuticals. These chemical scaffolds are reported to possess inhibitory activities towards human type-II (non-pancreatic) secretory phospholipase A2 (PLA2) (Higashiyama et al., 1998). The oxathionyl-nucleosides emtricitabine (Coviracil) and lamivudine (3TC) are two of the most potent antiviral drugs as nucleoside reverse transcriptase inhibitors (NRTIs) for the treatment of diseases, such as human immunodeficiency virus

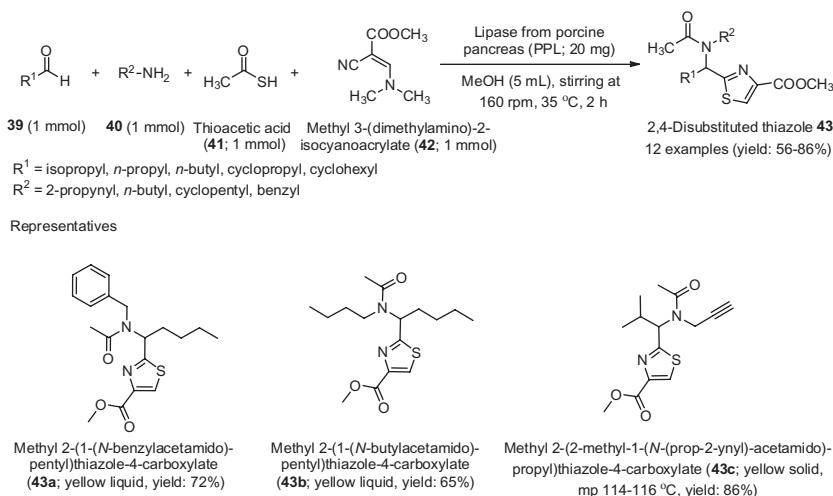
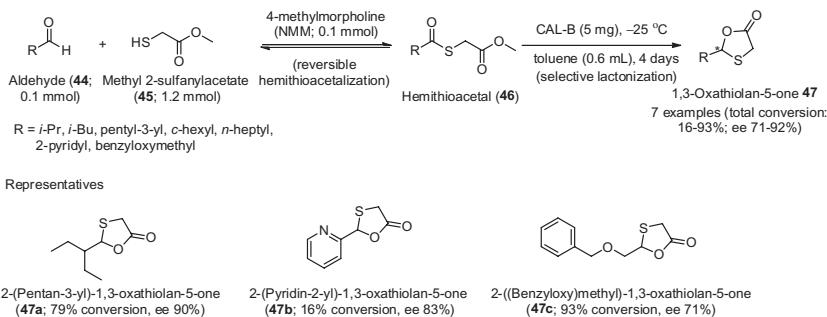
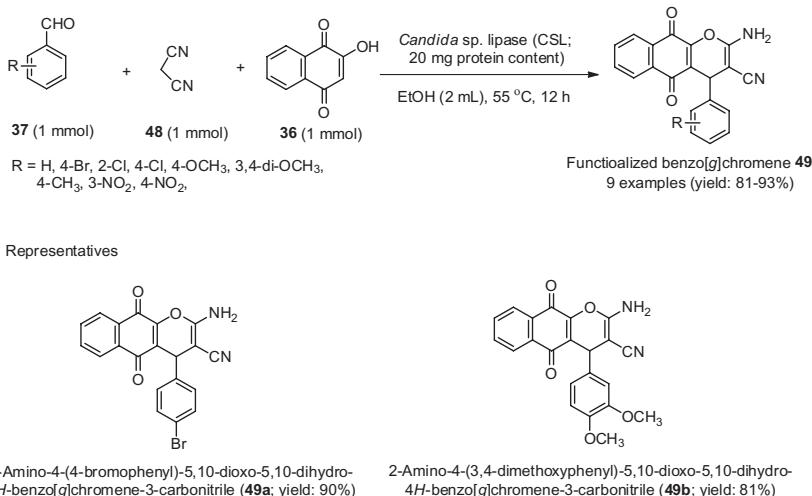


FIGURE 13.13 One-pot multicomponent synthesis of 2,4-disubstituted thiazoles.

(HIV) or hepatitis B (Beach et al., 1992; Jeong et al., 1992). Since the initial discovery of the antiviral activity of this motif, the synthesis of enantiomerically pure 1,3-oxathiolan-5-one derivatives has received significant attention (Chu et al., 1991; Humber et al., 1992; Roy et al., 2009). Zhang et al. (2014) have successfully demonstrated a lipase-catalyzed dynamic covalent kinetic resolution protocol based on reversible hemithioacetal (**3**) formation for the asymmetric synthesis of 1,3-oxathiolan-5-one derivatives. Among a variety of lipases, lipase B from *Candida antarctica* (CAL-B) was found to show the most potent efficiency in carrying out the transformation in good yields with moderate to good enantiomeric excess(ee) for the final products. The investigators exploited such lipase-catalyzed resolution to obtain selective γ -lactonization of the hemithioacetal (**46**) arising out of the reaction between the aldehydic substrates (**44**) and methyl 2-sulfanylacetate (**45**) in a one-pot process (Fig. 13.14). Furthermore, some of the synthesized 1,3-oxathiolan-5-one derivatives showed potential for a simple access to the core structure of active pharmaceutical nucleoside analogs.

Very recently, Yang et al. (2015) have reported on the development of a facile and simple method for the synthesis of biologically relevant benzo[*g*] chromenes (**49**) using lipase as an efficient biocatalyst from the one-pot multicomponent reaction of aromatic aldehydes (**37**), malononitrile (**48**) and 2-hydroxy-1,4-naphthoquinone (**36**) in ethanol medium at 55°C (Fig. 13.15). Benzo[*g*]chromenes are known to exhibit a wide range of bioactivities including antimicrobial, antiproliferative, and antitumor properties (Mohr et al., 1975; Coudert et al., 1988; Tandon et al., 1991; Zamocka et al., 1992; Brunavs et al., 1994; El-Agrody et al., 2000). In their experiment, the present investigators also examined several kinds of lipases such as *Candida antarctica* lipase B

**FIGURE 13.14** Asymmetric synthesis of 1,3-oxathiolan-5-one derivatives.**FIGURE 13.15** Synthesis of functionalized benzo[g]chromenes.

(CAL-B), Porcine pancreas lipase (PPL), *Candida* sp. lipase (CSL), *Candida rugosa* lipase (CRL), *Pseudomonas fluorescens* lipase (PFL), *Pseudomonas* sp. lipase (PSL), and *Bacillus subtilis* lipase (BSL2) to catalyze this multicomponent reaction, and *Candida* sp. lipase (CSL) came out with the highest catalytic activity, thereby suggesting that the catalytic activities depend mainly on the lipase origin. When the denatured lipase or bovine serum albumin (BSA) was used as the catalyst, almost no product could be detected, which suggested a special active conformation of the enzyme plays a crucial role in effecting this multicomponent reaction.

Synthesis of such compounds was reported earlier by using nonenzymatic catalysts (Wang et al., 2009; Yao et al., 2009; Khurana et al., 2010, 2012; Dekamin et al., 2013; Azizi and Heydari, 2014), but this chemoenzymatic route

is more advantageous over the existing protocols in terms of atom economy, environmental friendliness, and operational simplicity.

13.3 CONCLUDING REMARKS

Lipases are amongst the most important biocatalysts that are used to carry out a broad spectrum of organic transformations in both aqueous and nonaqueous media to generate biologically relevant organic molecules of potential practical interest, both in research laboratories and in industry. Lipases have the remarkable ability to carry out a wide variety of chemo-, regio-, and enantioselective transformations, and also have very broad substrate specificity. The present chapter offers a recent update on the lipase-catalyzed organic transformations reported during 2013–mid-2015. This overview reflects the biocatalytic efficacy of the enzyme in carrying out various types of organic reactions, including esterifications, transesterifications, additions, ring-closing, oxidation, reduction, amidation, and many others. Ease of handling, broad substrate tolerance, high stability towards temperatures and solvents, high enantioselectivity, convenient commercial availability, and reusability are the key advantages of choosing lipase as a biocatalyst in a huge number of organic transformations. The author hopes that this overview should boost ongoing research in chemoenzymatic organic transformations, particularly the biocatalytic applications of lipases. It is noteworthy that each lipase has its own unique properties, and that fine-tuning of any methodology employing lipases to suit the individual enzyme should be screened carefully. To widen the usage level of lipases, there is an urgent need to understand the mechanisms behind the lipase-catalyzed reactions in more depth. Protein engineering of lipases and the further improvement of lipase preparations and reaction methodology have great potential to generate even better bioconversions in the future.

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Chapter 14

Enzymatic Biocatalysis in Chemical Transformations: A Promising and Emerging Field in Green Chemistry Practice

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14.1 INTRODUCTION

Biotechnology has become an important economic factor and, in some countries, a significant contributor to the gross domestic product. The use of biotechnology generates over \$250 billion a year with a great variety of products for different markets, and this figure does not include biofuels (Meyer and Schmidhalter, 2014). Although biopharmaceuticals (therapeutic proteins, monoclonal antibodies, vaccines, pDNA) account for more than 50% of the total biotechnology business volume, it is mainly genetically modified plants and animals as well as biofuels which brought biotechnology onto the front pages of newspapers, although not always in a positive context. The switch from a petro-based economy to a bio-based economy is an option which is firmly established in discussions and the first bio-based chemicals are being marketed today. A specific advantage of biotechnology put forward routinely is its sustainability. Keeping in mind that sustainability has three axes (economy, ecology, society), biotechnology is not intrinsically more sustainable than organic chemistry. However, the chemical industry has realized that sustainability is a necessity to be included in long-term strategic considerations. And they have to be periodically reevaluated as new technologies emerge.

Enzymes have also become an irreplaceable economic ingredient, belonging to vertical and horizontally differentiated products. But the benefits of enzymes remain in the dark, although the general public became probably aware of the term “enzymes” through detergent commercials in the 1960s and 1970s and they are still best known for their applications in the commodity business. Estimations vary, but hydrolytic enzymes account for over 90% of the five billion enzyme market (detergents, feed, sugar production, bioethanol, sweetener, beverage, food), and detergent manufacturers are still the most important customers of enzymes buying about one-fifth of their production. Lately, commercial hydrolytic enzyme blends for large-scale production of the first and second generation of biofuels have added considerably to that demand. The global enzyme market was \$3.3 billion in 2010 and expected to reach \$7 billion in 2017, up from \$5.5 billion today ([Freedomia, 2015](#)). Hydrolytic enzymes will remain the backbone of enzyme sales due to a more affluent global population, increasingly able to afford products requiring enzymes such as advanced laundry detergents.

However, new and different enzyme classes than hydrolytic enzymes do emerge for synthetic applications ([Chen and Tao, 2011](#)). It is noteworthy that the first targeted application of enzymes outside of cheese production was in the 1950s for making steroids for the pharmaceutical market. Over 50 years later, the same small molecule market starts to finally reappreciate the advantages of enzymes in chiral building blocks for small molecule pharmaceutical ingredients. The captive use of enzymes for biotransformation by integrated large companies, having in-house manufacturing capabilities of both organic chemistry and biotechnology for the production of complex molecules (eg, Dr Reddys, DSM, Lonza, Merck, and other companies) are not included in the estimates for the global enzyme sales mentioned above. However, specialty enzyme sales will continue to be of minor magnitude compared to bulk enzymes, and this will persist in the future, as biocatalysis will remain a niche technology in organic chemical synthesis for two reasons. First, abiotic methods will continue to deliver the bulk of molecules leaving the introduction of specificity into molecules to enzymes. Second, the ideal manufacturing methods (if possible) is the total biosynthesis by fermentation using advanced genetic engineering and synthetic biology, which will radically change the way we produce chemicals in the longer term future. By the way, these considerations are true not only for the pharmaceutical market, but equally for the production of agrochemicals, flavors, fragrances, and other products, wherever biological activity and molecular complexity or chirality are required. But then again, the truly added value is not in the sales of the enzyme itself, but in the access to sustainable manufacturing methods, using the substrate-, regio-, and enantioselectivity of enzymes. Several routes can lead to the desired enantiomers. In the early 1990s, most chiral drugs were still derived from chiral pool materials and only 20% of all drugs were made via purely synthetic approaches ([Meyer et al., 2009](#)). Today, this ratio is the other way around with most drugs introducing chirality not from a chiral pool but by using chiral technologies.

The yet incomplete toolbox of commercial enzymes from all six enzyme classes, available in industrial quantities, is particularly hampering biocatalysis in the API and intermediate production. Timeline compression in the development cycle of pharmaceuticals, in combination with an insufficient choice of industrially available enzymes (other than hydrolytic enzymes) still results in the fact, that biotransformation often represents the second generation process. But performing route evaluations on existing products shows that there is potential for synthetic alternatives involving biotransformation steps in manufacturing processes. Besides the above mentioned time constraints, another important difference between commodity enzymes and specialty enzymes, for example, for fine chemicals and pharmaceuticals, are the quality and regulatory requirements of the final product (cGMP, optical purity, chemical purity etc.) to be met.

Adding to these challenges is the circumstance that biotransformation processes are much more diverse than recombinant therapeutic protein production. Many different enzymes from all six classes and different host systems are needed to exploit biotransformation for the conversion of a myriad of different substrates into the (typically optically active) desired fine chemicals, intermediates, or APIs for different markets and requirements. Although almost any organic chemical reaction can theoretically be catalyzed by enzymes, directing in the right thermodynamic energy path, only a fraction of all possible organic chemicals' reactions, where enzyme could be applied, are actually implemented. There are examples of well-established chemical reactions routinely used in the organic chemical industry and suggestions as to where biotechnology could replace environmentally burdened reactions impacting the whole spectrum of chemical manufacture (Bornscheuer et al., 2012; Meyer et al., 2009, 2013; Patel, 2011; Sutton et al., 2012; Wells and Meyer, 2014). Enzymes, identified and listed according to their importance, which should be available in commercial quantities for the production of fine chemicals and intermediates include NAD(P)H-dependent dehydrogenases, monohydroxylases, peroxidases, Baeyer Villiger monooxygenases, aldolases, hydroxynitrile lyases, ThDP-dependent lyases, aminolyases (for C-N bond formations), or hydratases (for C-O bond formation). But once enzymes of all six different enzyme classes are available to meet the ever-increasing degree of sophistication of the molecules to be sustainably produced, modified, or functionalized, there will be another advantage when using biocatalysis in organic chemistry: in most cases, enzyme applications can be performed in existing fine chemical installations, if a commercial biocatalyst is available.

Table 14.1 lists examples of enzymes and their application. Hydrolytic enzymes, such as lipases, proteases, glycosidases, and other hydrolases, remain the most important commercial enzymes with well over 90% of market share. These hydrolytic enzymes are mainly used in detergents, animal feed formulations, bioethanol production, sweetener production, beverage and food industry, pulp and paper, and textile industry. Hydrolytic enzymes are also used in

TABLE 14.1 Selection of Enzymes, Their Applications, and Markets. This Nonexhaustive List Contains Close to 70 Enzymes Used in Numerous Applications and Industries. Almost 50 of These Enzymes Belong to the Class of Hydrolytic Enzymes. But Enzymes From the Other Five Enzyme Classes Are Continuously Added. Alcohol Dehydrogenase (ADH), Ketoreductases (KRED) and Transaminases Are Recent Examples of Enzymes Being Established Alongside the Widely-Used Hydrolases, Expanding the Enzyme Toolbox for Organic Chemical Synthesis

Acetolactate decarboxylase lyase used in beer and wine making to reduce maturation time
Adenosine deaminase Adagen (pegademase), a bovine adenosine deaminase as enzyme replacement for patients with an inadequate elimination of 2'-deoxyadenosine
Alcoholdehydrogenase (ADH) both, (S) and (R) specific enzymes for the synthesis of chiral building blocks
Amidases for the enantioselective hydrolysis of racemic amino acids
Amino acid dehydrogenase for the enantioselective reductive amination of ketoacids to (also unnatural) amino acids. A key intermediate for the synthesis of Saxagliptin (type 2 diabetes mellitus) by reductive amination
Amylases and glucoamylases. The most important enzymes (endo- and exoglucanase) by volume and market share with a wide range of applications of the α -, β -, and γ - amylases (including thermophilic enzymes) in biofuels, high fructose corn syrup, detergent formulations, and pancreatic enzyme replacement among others
Asparaginases Oncaspar® (Pergasparargase) is a PEGylated asparagine of Enzon against leukemia. Asparaginase (which breaks down asparagine, a precursor of acrylamide) is used to reduce acrylamide forming during cooking, baking, or frying in the food industry
Baeyer Villiger monooxygenases (BVMO) is a nicotinamide dependent flavoenzyme that converts linear or cyclic ketones into lactones and ketones to esters
Catalase with many applications as antiage enzyme, in cheese production, to prepare cotton fibers for dying, industrial applications, and degradation of hydrogen peroxide after whitening or disinfecting of materials, food, or containers
Cellulase hydrolytic enzyme required for second generation biofuels, animal feed products (in combination with other hydrolytic enzymes) or textile industry (stone washing)
Chitinase processing enzymes in human health care or for the production of <i>N</i> -acetylglucosamine from spent mycelium
Chondroitinase investigated as potential treatment of spinal cord injuries
Chymosin , (Rennin) protease originally isolated from rennet. Today 70% of the chymosin used in cheese making (curdling of milk) is recombinant
Collagenase , metalloprotease used against skin ulcers in ointments, where these enzymes hydrolyze the connective tissue protein collagen. One application discussed was meat tenderization to replace papain which, however, never made it to the market

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Cutinase, hydrolytic properties similar to lipases and esterases. Potential application in areas such as food, cosmetics, fine chemicals, pesticide, and polymer chemistry for the hydrolytic degradation of polyethylene terephthalate (PET)

Deoxyribonuclease (DNase) nonspecifically cleaves DNA, used as a therapeutic enzyme or for biofilm prevention

Enterokinase used for the cleavage of fusion proteins at defined sites; the serine protease recognizes the amino acid sequence Asp-Aps-Aps-Lys-↓-X-

Esterases hydrolytic enzymes used in organic synthesis of optically pure compounds or for pitch (resin) reduction in the pulp and paper industry

Flavourzyme exopeptidase applied in the food industry for the degradation bitter peptides

β-Galactosidase (lactase) in the food industry for lactose-free products (lactose intolerance). Also used in the transfer reaction for the production of galactose oligosaccharides

β-Glucanase degradation of glucans in malt in the food and brewing industry

α-Glucosidase (acid alpha glucosidase) as Pompe disease enzyme replacement therapy

Glucarpidase (Voraxase[®]) recombinant bacterial carboxypeptidase that hydrolyzes the carboxyl-terminal glutamate residue from methotrexate, an anticancer drug

Glucocerebrosidase (Taligurase) an enzyme replacement therapy against Gauchers disease

Glucoseoxydase, an oxidoreductase used in analytics as well as in the food and beverage industry for the elimination of oxygen in juices. Used in sensors, for example, on-line glucose sensor

Glutamate dehydrogenase for the quantitative, enzymatic determination of ammonia in food and biological samples

Glutaminase (and L-asparaginase) amidohydrolase enzyme used against leukaemia. Selectively suppresses the growth of myeloid leukemia cells

Glycosyltransferases to generate glycosidic linkages in one step reactions between an unprotected acceptor and a sugar donor nucleotide for, for example, antiviral nucleosides

Glycosynthases, catalytic mutant glycosidase in which the glycosidase activity is abolished

Halogenases, marine derived halogenases, especially recently discovered fluorinases, are a new alternative to hazardous and low yield chemical halogenation

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Heparinases (Extravase), for the neutralization of the anticoagulant heparin, with a number of other potential medical applications such as wound healing
Hyaluronidase for the depolymerization of the anticoagulant heparin to the pentasaccharide, used in ophthalmology or for subcutaneous delivery of injectable biologics
Hydantoinases in combination with carbamoylases for the production of optically pure amino acids
Invertase hydrolyses sucrose to invert sugar (fructose and glucose) which can be used for glucose-fructose syrup (GFS)
Inulinase for the hydrolysis of inulin into fructose and fructoseoligosaccharides
Isomerase for another route to glucose-fructose syrup and high fructose syrup, by converting some of the glucose resulting from separate starch hydrolysis into fructose, which is twice as sweet as sucrose
Ketoreductases (KREDs) increasingly used for the production of chiral fine chemicals and small molecule pharmaceuticals such as montelukast
Laccase an oxidoreductase used in pulp and paper, textile, flavor & fragrance, fine chemicals, food production or wastewater treatment
Lactoperoxidase in combination with glucose and glucose oxidase in a cascade of linked reactions to generate antimicrobial products <i>in situ</i> used for cosmetics and other applications (Biovert™)
Laronidase or Aldurazyme, this human recombinant hydrolytic enzyme is used for patients suffering from mucopolysaccharidoses
Levensucrase belongs to the glycosyltransferases used in the food industry for the production of fructan oligosaccharides
Lipase Hydrolytic enzyme widely used in food, textile, detergents or organic synthesis for, for example, enantioselective acylation, esterification, or together with other hydrolytic enzymes used for the control of pitch in the pulp and paper industry
Lysozyme this ubiquitous hydrolytic enzyme is used in pharmaceutical products and control in food processing
Nattokinase , protease originally from the fermentation of soy beans with <i>Bacillus subtilis</i> natto. The fibrinolytic enzyme is investigated as antithrombotic (see also urokinase and tPA)

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Malto-oligosyl trehalose synthase is a isomerase used for the production of trehalose, a 30,000 tons per year disaccharide. Mannanase hydrolytic enzymes used, for example, for degumming of guar
Monoaminoxidase used in the Boceprevir production
Naglazyme (galsulfase) recombinant human <i>N</i> -acetylgalactosamine 4-sulfatase (rhASB) for patients suffering from mucopolysaccharidosis
Nitrile hydratase for the biohydrolysis of 3-cyanopyridine to niacinamide, a reaction operated at several thousand tons per year
Nitrilase for the manufacturing of, for example, (<i>R</i>)-mandelic acid
Old Yellow Enzyme OYE flavin dependent redox biocatalyst for the industrial reduction of activated alkenes
Papain protease used to tenderize meat
Paraoxonase is a hydrolytic enzyme able to degrade toxic organophosphates such as the insecticide "Paraoxon," and is used for the degradation of nerve toxin agents
Pectinase , hydrolytic enzyme used in juice manufacturing and detergent formulations
Penicillin G acylase to replace the chemical process for the conversion of penicillin G to 6-APA
Pegademase (Adagen) PEGylated adenosine deaminase of Enzon against combined immunodeficiency
Phytase launched in 1991 growing rapidly and volumes exceeding US\$250 millions. The hydrolytic enzyme is used as animal feed additive for the release of phytate phosphate in the animal digestive tract
Prolyl endopeptidases (Endoproteases) as supplements for the degradation of gluten against Celiac disease
Pullulanase amylopectin hydrolysis in brewing
Sortases , bacterial hydrolytic enzymes that possess transpeptidase activity with potential application for enzymatic ligation
Streptokinase enzyme used against blood clots
Subtilisin also known as savinase, alcalase, protex, etc. is a <i>Bacillus subtilis</i> , serine protease used in many different applications such as cleaning formulations, stain removal, food manufacturing or biocatalysis

(Continued)

TABLE 14.1 Selection of Enzymes, Their Applications, and Markets. This Nonexhaustive List Contains Close to 70 Enzymes Used in Numerous Applications and Industries. Almost 50 of These Enzymes Belong to the Class of Hydrolytic Enzymes. But Enzymes From the Other Five Enzyme Classes Are Continuously Added. Alcohol Dehydrogenase (ADH), Ketoreductases (KRED) and Transaminases Are Recent Examples of Enzymes Being Established Alongside the Widely-Used Hydrolases, Expanding the Enzyme Toolbox for Organic Chemical Synthesis (Continued)

Thermolysine is a metalloprotease used for the production of aspartame
Transaminase an engineered (<i>R</i>)-amine transaminase converts a ketone (Prositagliptin) into Sitagliptin
Transglutaminase has potential for protein drug conjugations (eg, PEG, dextran)
Trypsin another serine protease used in food processing, wound healing and inflammation or in the “trypsination” for the harvest of adherent animal cells. Together with pepsin and α -chymotrypsin, trypsin is a most commonly used protease for the production of protein hydrolysates
Tyrosine phenol lyase for the synthesis of DOPA
Uricase (rasburicase) uric acid oxidase; diagnostics and for the treatment of acute hyperuricemia, the latter being antigenic
Urokinase , serine protease and thrombolytic agent for the lysis of acute massive obstructions of blood
Xylanase hydrolyses beta-1,4-xylan used in fuel ethanol and animal feed additives

organic chemical synthesis, biofilm removal, digestive and enzyme replacement therapies, wound treatment, or analytics, but in much smaller quantities.

Nowadays about 77% of all chemicals are made from crude oil, 10% from natural gas, 2% from coal, and 11% from renewable raw materials. This ratio is expected to change in the future with the expansion of bio-based economies for the production not only of fuels, but of platform chemicals. However, these developments will need mainly whole cell fermentation for the de novo synthesis of, for example, dicarboxylic acids such as succinic acid, which has already been produced in large scale. Consequently, there is not much progress to be expected for the biocatalysis in organic chemical synthesis of complex and chiral molecules, with the abovementioned interest in bulk and platform chemicals. Additionally the amount of enzymes required for complex and chiral molecules is very small, compared to the amount of industrially used hydrolytic enzymes. As a result the economic fallout is limited for an enzyme producer, as the value is more in the access of a sustainable and economically interesting route than in the enzyme itself.

“Biocatalysis for Green Chemistry and Chemical Process Development” ([Tao and Kazlauskas, 2011](#)) gives an overview of how biotechnology can improve the manufacturing footprint for pharmaceuticals, fine chemicals, flavor and fragrance, or agrochemical products. “Green chemistry” is a concept and guiding principle encouraging the development of low impact production processes. The 12 principles formulated by [Anastas and Warner \(1998\)](#) for sustainable production have been followed consciously or subconsciously for a long time for one simple reason: these principles also make a lot of sense from an economic point of view. It is evident that biotechnology should be able to make a great contribution in the future. There are a number of reasons why biotechnology for small molecule pharmaceuticals will remain important in the future.

- Over 70% of all pharmaceuticals remain being small molecules
- Increasing complexity of molecules
- Multiple functional groups
- Requirement of chemo-, regio-, and stereoselectivity reactions
- Keep or make APIs (Active Pharmaceutical Ingredients) orally available
- Need of affordable and effective drugs requires effective synthetic methods

Hand in hand to the growing enzyme toolbox and the increasing use of enzymes in different areas, grows the number of companies. [Table 14.2](#) lists companies involving enzymes and their application in their business activities.

[Table 14.2](#) shows that hydrolytic enzymes still are the core of most enzyme companies, but more and more enzymes, other than lipases, glucosidases, or proteases, become available every year in industrial quantities. This is due to several different reasons related to scientific progress as well as structural improvements. One reason is that today SME companies successfully offer enzyme development including recombinant technology ([Table 14.2](#)). The enzyme development process has now been adapted so well to the needs of the industry and has become so reliable, that companies planning to use enzymes in their technology portfolio do not have to incorporate these activities into their own in-house R&D teams but can source that out to specialized companies. Furthermore, there are also academic and industrial consortia, transnational funding initiatives, and interest groups with the goal to facilitate and accelerate the application of biotechnology in chemical manufacturing processes; examples are listed in [Table 14.3](#).

These joint efforts are vital for the success of the industry. While chemistry had 150 years to develop its toolbox, the biotechnology toolbox for chemical manufacturing has much less time with the pressing need of sustainable processes. Biocatalysis will play a key role in the chemical synthesis and modification of complex molecules. In the following pages, we discuss a few selected and relevant technological aspects.

TABLE 14.2 A Selection of Small and Large Companies Actively Developing, Producing, Selling or Using Enzymes and Enzyme Solutions. Worldwide, There Are Now Close to 30 Larger Companies and Enzyme Producers Acting Globally, However, Mostly for Hydrolytic Enzymes. There Are Probably Also a Number of Companies Producing Small Molecule Pharmaceuticals, Which Have In-House Fermentation Capacity and Produce their Enzyme for Captive-Use. There Are Not Too Many Companies Offering Enzymes From all Enzymes Classes for Fine Chemical Production. Bioethanol and Biosolvents Are a Special Case. Although One of the Oldest Applications, They Have Undergone a Particular Strong Revival Thanks to Government Subsidies of Products and Development Costs

AB Enzymes www.abenzymes.com and **AB Vista** www.abvista.com are integral parts of ABF (Associated British Foods) and number four or five of the commodity enzyme producers. Food enzymes for baking and beverages, textile the detergents industry; big stakeholder in the phytase and xylanase feed market

Advanced Enzymes www.advancedenzymes.com Indian enzyme producer started with extracted papain for medical and pharmaceutical purposes in 1957. Today manufacturing hydrolytic enzymes for various applications using large scale submersed and surface fermentation

Ajinomoto www.ajinomoto.com mainly known as an important producer of amino acids with own brands in the food sector. Founded in 1909, manufactures and also sells enzyme systems for texture improvement in food protein

Almac www.almacgroup.com offers reductases, transaminases, hydrolases, and nitrilases

Amano Enzyme Inc. www.amano-enzyme.co.jp established enzyme producer since the late 1940s starting with digestive enzymes. Still offering mainly hydrolytic enzymes (amylase, cellulases, lipases, lactase, proteases)

Arzeda www.arzeda.com offering computational enzyme design, protein optimization, and metabolic engineering

ASA Spezialenzyme GmbH www.asaenzyme.de offering industrial enzymes such as AOD, laccases, esterases, chitinase, phytase, penicillin acylase, tannase

BASF www.bASF.com the world's largest chemical company produces mainly hydrolytic enzymes for detergents, animal nutrition, with the goal to become a major player in enzyme technology by 2020. This also through acquisitions (Henkel AG, Verenium) or collaborations (Direvo Biotechnology GmbH, Dyadic International Inc.). Number four or five among global enzyme producers

Biocatalysts www.biocatalysts.com in existence for about 25 years, offering speciality food enzymes, and enzyme development services

Biochemtex www.biochemtex.com operates a large biofuels & biochemicals plant in Crescentino in northern Italy. They produce the hydrolytic enzymes in-house for the biomass hydrolysis followed by fermentation of C₅ and C₆ sugars

Biochemize SL www.biochemize.com Spanish SME founded in 2011 offering biocatalysis process development

(Continued)

TABLE 14.2 A Selection of Small and Large Companies Actively Developing, Producing, Selling or Using Enzymes and Enzyme Solutions. Worldwide, There Are Now Close to 30 Larger Companies and Enzyme Producers Acting Globally, However, Mostly for Hydrolytic Enzymes. There Are Probably Also a Number of Companies Producing Small Molecule Pharmaceuticals, Which Have In-House Fermentation Capacity and Produce their Enzyme for Captive-Use. There Are Not Too Many Companies Offering Enzymes From all Enzymes Classes for Fine Chemical Production. Bioethanol and Biosolvents Are a Special Case. Although One of the Oldest Applications, They Have Undergone a Particular Strong Revival Thanks to Government Subsidies of Products and Development Costs (Continued)

Brain www.brain-biotec.de German SME focusing on cooperation to develop bioactive natural substances and enzyme discovery for biocatalytic processes
Buckman www.buckman.com hydrolytic enzymes for drainage or unhairing of hides
CLEA Technologies BV www.cleatechnologies.com Dutch SME commercializing Cross Linked Enzyme Aggregates as well as soluble enzymes (mainly lipases, proteases amidases and nitrilases)
c-Lecta www.c-lecta.com German SME offering recombinant biocatalysis applications for reaction types using lipases, RNAses, asparaginase, nucleases for pharma, food, or destaining applications
Chr Hansen www.chr-hansen.com in Denmark produced the first standardized animal rennet in 1874. Still actively selling among others enzymes (chymosin, lipases, lactases, lactoperoxidases, lysozyme, nisin) now produced by fermentation for various applications in the dairy industry
Codexis www.codexis.com founded in 2002 in California to provide enzyme solutions for industrial biocatalysis. Also enzymes in the manufacturing of chiral small molecule pharmaceuticals including enzyme kits (transaminases, ketoreductases, reductases, Nitrilases) for the chemistry laboratory bench top. Winner of the "Green Chemical" award 2010 with Merck for an improved Sitagliptin synthesis
Daiichi-Sankyo www.daiichisankyo.com , large global Japanese pharmaceutical company founded in 1899 based on a digestive enzyme Taka-Diastase. Today a mainly small molecule pharmaceutical company
Daicel www.daicel.com with an integrated fine chemicals business units using enzymes and having fermentation capacity
Direvo www.direvo.com enzymes formulas for animal feed and anaerobic conversion of nonfood biomass
Dr Reddy's www.drreddys-cps.com offer access to biocatalysis with a variety of recombinant enzymes (hydrolases, oxidoreductases, transferases) though the acquisition of Chirotech Technology Ltd, UK in 2008, producing with a <i>Pseudomonas fluorescens</i> host
DSM www.dsm.com large Dutch global company with a broad product portfolio from building and construction to medical, and global #3 in commodity enzymes. Producing and using enzymes since a long time for many different applications, mostly for the food, feed, beverage and dietary markets

(Continued)

TABLE 14.2 A Selection of Small and Large Companies Actively Developing, Producing, Selling or Using Enzymes and Enzyme Solutions. Worldwide, There Are Now Close to 30 Larger Companies and Enzyme Producers Acting Globally, However, Mostly for Hydrolytic Enzymes. There Are Probably Also a Number of Companies Producing Small Molecule Pharmaceuticals, Which Have In-House Fermentation Capacity and Produce their Enzyme for Captive-Use. There Are Not Too Many Companies Offering Enzymes From all Enzymes Classes for Fine Chemical Production. Bioethanol and Biosolvents Are a Special Case. Although One of the Oldest Applications, They Have Undergone a Particular Strong Revival Thanks to Government Subsidies of Products and Development Costs (*Continued*)

DuPont www.dupont.com US multinational active in many areas from food & personal care to people and process safety (DuPont's origins are as a gun powder producer!). DuPont offers enzymes for food and carbohydrate processing, detergent formulations or animal nutrition. A considerable part of that portfolio came to DuPont by the acquisition of Danisco in 2011, which acquired Cultor and in 2005 Genencor, which made DuPont the second largest enzyme producer after Novozymes

Dyadic www.dyadic.com US SME founded in 1979 offering industrial hydrolytic enzymes for biofuels, food and beverage, feed, pulp and paper, bio-based chemicals

EnginZyme www.enginzyme.com Swedish SME offering enzyme immobilization using porous glass and polymers coating for hydrophilic or hydrophobic surfaces

Enzyme Solutions www.enzymesolutions.com, small enzyme producer in Indiana, USA, of hydrolytic enzymes (proteases, amylases, lipases) and enzyme products

Enzyme Works Inc. www.enzymeworking.com SME close to Shanghai offering the development of biocatalysis for organic chemical synthesis, with in-house fermentation facility to produce enzymes and chemicals at larger scale

Enzymicals www.enzymicals.de esterases, transaminases, BVMO, dehalogenases and aminoacylases for the production of fine chemicals and APIs

Enzymology Research Center Inc. www.ercenzymes.com US SME supplier in microbial, plant and animal derived hydrolytic enzymes

Evocatal www.evocatal.com develops and produces various enzymes (ADHs and KREDS, transaminases, hydroxynitrile lyases, lipases) for the chemical and pharmaceutical industry

Evonik Industries AG www.evonik.com Large German specialty chemical multinational, present in many areas from automotive to food and beverage. A leading L-lysine manufacturer by fermentation. Best known in enzymes for their development of enzyme-membrane-reactor for the production of L-amino acids with Prof. Christian Wandrey

Eucodis Bioscience www.eucodis.com offers a number of mostly hydrolytic enzymes and fermentation up to 1000L volume

Fermenta www.fermentabiotech.com from India, offering mainly hydrolytic enzymes (lipases, Penicillin G acylase, Penicillin amidases)

(*Continued*)

TABLE 14.2 A Selection of Small and Large Companies Actively Developing, Producing, Selling or Using Enzymes and Enzyme Solutions. Worldwide, There Are Now Close to 30 Larger Companies and Enzyme Producers Acting Globally, However, Mostly for Hydrolytic Enzymes. There Are Probably Also a Number of Companies Producing Small Molecule Pharmaceuticals, Which Have In-House Fermentation Capacity and Produce their Enzyme for Captive-Use. There Are Not Too Many Companies Offering Enzymes From all Enzymes Classes for Fine Chemical Production. Bioethanol and Biosolvents Are a Special Case. Although One of the Oldest Applications, They Have Undergone a Particular Strong Revival Thanks to Government Subsidies of Products and Development Costs (Continued)

Fermic www.fermic.com Brazilian enterprise with large fermentation capacity of up to 190 m ³ used to produce enzymes for third parties (eg, Codexis, Verenium)
Fordras SA www.fordras.com extracted animal derived trypsin, chymotrypsin and phospholipase
Greenlight Biosciences www.greenlightbiosciences.com offer a cell-free bioprocessing (CFB) platform using enzymes in dead cells
Inofea www.inofea.com SME offering silica particle based protected immobilization of enzymes
Iogen www.ingen.ca Producer of cellulosic ethanol, sold its industrial hydrolytic enzymes business to Novozymes
Johnson-Matthey www.matthey.com an old company with roots in platinum and related metals became a leading catalyst manufacturer, including enzymes (reductases, transaminases, lipases) in their product offering
Kaneka www.kaneka.co.jp , company with a broad product portfolio (plastics, Life Science products, electronic products, synthetic fibers) using biocatalysis (ADH, hydantoinases, N-carbamoyl-D-amino acid amidohydrolase), with manufacturing capabilities including single reactor fermentation volumes of up to 100 m ³
Kerry www.kerry.com , US specialty enzymes for the food industry
Kyowa Hakko Kirin Co. Ltd www.kyowa-kirin.com , founded in 1885 as brewing company, became a pharmaceutical company since 1951, through various cooperations and acquisitions. First producer of L-glutamic acid and L-tyrosine by fermentation. Enzyme products include L-asparaginase against leukemia
Libragen www.libragen.com French SME and CRO offering metagenomic research and biocatalytic development
Lonza www.lonza.com Swiss company and largest contract manufacturer with large mammalian and microbial fermentation installations and one of the early companies offering biocatalysis for the manufacturing. In-house integrated groups of microbiologists and synthetic chemists, able to identify and develop biocatalytic steps for fine chemicals and scale them up in house to manufacturing scale

(Continued)

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Lyven www.lyven.com produces enzymes on an industrial scale by solid state fermentation for the food and brewing industry

Meiji Saika Kaisha Ltd www.meiji-seika-pharma.co.jp, Japanese company with roots in food products and fermentation capacities, diversified into pharmaceuticals via penicillin fermentation after WWII. Today various products (pharma, feed additives, biocides) include also enzymes such as cellulases with proprietary fungal expression system (MSPEX) offering also contract manufacturing

Metgen www.metgen.com, enzymes for lignocellulosec feedstocks. Specialized in laccases for different applications

Sigma-Aldrich Fine Chemicals SAFC www.sigmaaldrich.com with a whole array of different enzymes for laboratory purposes. In 2014 SAFC was acquired by Merck KGaA

Nagase www.nagase.co.jp Japanese company with activities in chemicals, cosmetics, health food, plastics and electronic materials. Hydrolases and oxidoreductases are used for the production of APIs and intermediates. Acquired Hayashibara in 2011, founded in 1883 and a producer of trehalose, maltose pullulan, vitamin C, enzymes, and other products using microorganism-derived enzyme technology

National Enzyme Company (NEC) www.nationalenzyme.com founded in 1932 for the application of enzymes for human nutrition. Specializing in product formulation of (eg, antioxidant) enzyme blends

Noor Enzymes www.noor-enzymes.com supplier of hydrolytic enzyme concentrates (eg, cellulases, xylanases, amylases), but limited at standard 500L fermentation volume

Novozymes www.novozymes.com, enzyme from all classes except ligases are offered, but the Danish pioneer and probably the world's largest enzyme producer is best known for its industrial hydrolytic enzymes for applications ranging from textiles to biofuels

Provivi www.provivi.com developing novel enzymatic routes for synthesizing biopesticides. The SME was founded in 2013 based on biocatalysis technology, namely biocatalysts catalyzing the transfer of carbenes, nitrenes, and oxenes to olefins and carbon-hydrogen bonds

(*Continued*)

TABLE 14.2 A Selection of Small and Large Companies Actively Developing, Producing, Selling or Using Enzymes and Enzyme Solutions. Worldwide, There Are Now Close to 30 Larger Companies and Enzyme Producers Acting Globally, However, Mostly for Hydrolytic Enzymes. There Are Probably Also a Number of Companies Producing Small Molecule Pharmaceuticals, Which Have In-House Fermentation Capacity and Produce their Enzyme for Captive-Use. There Are Not Too Many Companies Offering Enzymes From all Enzymes Classes for Fine Chemical Production. Bioethanol and Biosolvents Are a Special Case. Although One of the Oldest Applications, They Have Undergone a Particular Strong Revival Thanks to Government Subsidies of Products and Development Costs (Continued)

Prozomix www.prozomix.com United Kingdom, biocatalysts discovery and recombinant small scale contract enzyme manufacturer
Puratos www.puratos.com enzymes and ingredients for bakery, patisserie, and chocolate companies
Purolite www.purolite.com company focusing on resin technology, but offers and realized enzyme immobilization for pharma (transaminase immobilization for Sitagliptin with Codexis), the food (glucoamylase) or the cosmetic industry (lipases)
Promega www.promega.com producer of restriction enzymes
Rakuto Kasei www.rakuto-kasei.com producer of enzymes for different industrial applications (vinification, pharmaceuticals, textiles, paper technology)
Roal Oy www.roal.fi , a joint venture between Finnish Alko Ltd and Röhm GmbH from Germany. Now owned by Altia and ABF (Associated British Food). Roal produces of enzymes for baking, food, feed, and other industrial applications and cooperates with AB Enzymes in marketing and sales of products
Roche Custom Biotech www.custombiotech.roche.com offering in vitro glycosylation of proteins using glycosyltransferases, recombinant porcine trypsin, carboxypeptidase, endoproteinas, catalases
Solvay www.solvay.com , global Belgian company founded in 1863, serving diverse markets. Solvay Pharmaceuticals is marketing an exocrine pancreatic insufficiency drug based on lipase
Specialty Enzymes www.specialtyenzymes.com US manufacturer belonging to the Advanced Enzyme group. Nutraceutical and industrial enzymes mainly for the food, brewing and feed industry. The mainly hydrolytic enzymes are produced using submerged and solid state fermentation
Sunhy www.sunhy.cn founded in 2000 with own production facilities for amylases cellulases, xylanases, phytase, peroxidase for grain processing, animal feed, and the textile industry

(Continued)

TABLE 14.2 A Selection of Small and Large Companies Actively Developing, Producing, Selling or Using Enzymes and Enzyme Solutions. Worldwide, There Are Now Close to 30 Larger Companies and Enzyme Producers Acting Globally, However, Mostly for Hydrolytic Enzymes. There Are Probably Also a Number of Companies Producing Small Molecule Pharmaceuticals, Which Have In-House Fermentation Capacity and Produce their Enzyme for Captive-Use. There Are Not Too Many Companies Offering Enzymes From all Enzymes Classes for Fine Chemical Production. Bioethanol and Biosolvents Are a Special Case. Although One of the Oldest Applications, They Have Undergone a Particular Strong Revival Thanks to Government Subsidies of Products and Development Costs (*Continued*)

Swissastral www.swissastral.ch SME specializing in “extreme biotechnology” and bioprospecting. With offices in Switzerland, the United States, and Chile, they exploit the potential extreme microorganisms and their unexplored metabolic pathways as a source of novel enzymes and other biomolecules

Syncozymes www.syncozymes.com producer of enzymes and coenzymes (including KREDS, ω -transaminases, nitrilase, aldolases, and other enzymes) able to produce them in larger quantities

Toyobo www.toyobo-global.com (Biochemical Department) offering whole range of different diagnostic reagent grade enzymes with large scale fermentation capacity ($2 \times 30 \text{ m}^3$)

Verenium www.verenium.com, develops microbial and fungal enzymes, also from extreme environments for industrial processes. Commercialized products include mainly hydrolytic enzymes (α -amylases, xylanases, cellulases). Now part of BASF

Worthington Biochemical Corp. www.worthington-biochem.com started by a Rockefeller Institute research assistant in 1947 for high purity enzymes. The company does not carry out fermentations but extracts enzymes from various sources including nonrecombinant microbial sources

Wuxi Syder Bio-Products www.syder.com, enzyme factory in China producing hydrolytic enzymes

14.2 BIOPROSPECTATION OF NOVEL MICROBIAL BIOCATALYSTS

Microorganisms possess an outstanding diversity, built over millions of years of adaptive evolution. They constitute an almost unlimited reservoir of enzymes and bioactives with potential biotechnological applications. Indeed, the demand for enzymes for industrial applications is increasing with the advancements of microbiology. The intrinsic properties of microbial enzymes, for example, consistency, reproducibility, specificity, and high yields among many others, have

TABLE 14.3 Examples of Present and Past Consortia, International Research Programs and Centers Offering Services Related to the Application of Enzymes

ACIB Austrian Centre of Industrial Biotechnology www.acib.at center with a broad field of application including biocatalysis and enzyme technology
AMBIOCAS www.ambiocas.eu AMine synthesis through BIOcatalytic CAscades. Enzymatic cascades for the production of amine chemical molecular building blocks, specifically the use of transaminases for the delivery of amine functions was funded by the FP7-KBBE program of the European Union 01-01-2010 to 30-06-2013
Biocore www.biocore-europe.org conceive and analyze industrial feasibility of biorefinery concepts
Bio Base Europe www.bbeu.org Open innovation and education center for bio-based economy with large pilot plant facilities (15 m ³ fermentor)
BioCatalysis 2021 www.biocatalysis2021.net a large consortium with over 60 members from academia, industry, and innovation promotion agencies for novel approaches to industrial biotechnology
BioConSePT EU project for the production of consumer goods out of biomass not competing with the food chain
BIONEXGEN www.bionexgen.eu biocatalyst development for the chemical industry funded by the European Union FP7 program. Running from 2010 to 2014 the project focused on membrane bioreactor processes combined with nanofiltration. 17 companies and universities worked on key enzymes for eco-efficient chemical manufacturing
Biotrains www.biotrains.eu a Marie Curie training program for the application of biocatalysis to sustainable chemistry
Center for Biocatalysis and Bioprocessing (CBB) www.uiowa.edu biocatalysis research group founded in 1983 at the University of Iowa. Iowa is #1 in biofuel and #2 in biodiesel production in the US
Centre of Excellence Biocatalysis (CoEBio3) www.coebio3.org centre for biocatalysis, biotransformation, and biocatalytic manufacture at the University of Manchester
Centre of Process Innovation (CPI) www.uk-cpi.com for proof of concepts and piloting manufacturing processes with fermentation capacity up to 10 m ³
Chem21 www.chem21.eu Europe's largest public-private partnership led by the University of Manchester and GlaxoSmithKline
CLIB2012 www.clib2021.de German cluster in industrial biotechnology
COMENZYMEEVOLUTION http://cordis.europa.eu/project/rcn/104436_en.html harnessing proto-enzymes for novel catalytic functions. 2012–2017
ESAB Biotechnology Section of Applied Biocatalysis www.esabweb.org providing practical guidelines for reporting experiments

(Continued)

TABLE 14.3 Examples of Present and Past Consortia, International Research Programs and Centers Offering Services Related to the Application of Enzymes (*Continued*)

IBLF <https://connect.innovateuk.org/web/industrial-biotechnology> UK group supporting the uptake of industrial biotechnology

Italian Biocatalysis Center (IBC) www.italianbiocatalysis.eu public and private partnership for the advancement of manufacturing processes

OPTIBIOPCAT www.optibiocat.eu enzymes (mainly esterases) for the cosmetic industry funded by the FP7 of the European Industry 01.12.2013–01.12.2017. The industrial partners include Dyadic, Taros, or Supren GmbH

SIBC Swiss Industrial Biocatalysis Consortium, consortium of companies active in the field of biocatalysis http://www.swissbiotech.org/swiss_industrial_biocatalysis_consoritium_sibc

SUPRABIO www.suprabio.eu biorefinery scenarios based on sustainable biomass feedstocks

pushed their introduction into diverse products and industrial processes. Among microorganisms, extremophiles are gaining increased interest as a source of innovation in biocatalysis. They have adapted to “extreme” environments developing different mechanisms and tools to thrive under such conditions. In many cases, their enzymes or extremozymes are also adapted to withstand extreme conditions by their own, such as Taq polymerase, a thermostable DNA polymerase, widely used in molecular biology, purified from the thermophilic bacterium *Thermus aquaticus*. Enzymes derived from extremophiles or “Extremozymes” expand the ranges of enzyme optimum performance enabling enzymatic biocatalysis for current industrial processes where robustness is a principal issue.

Through actively bioprospecting diverse environments, we are able to discover enzymes that can perform novel activities and improve them through the use of protein engineering. These enzymes can accommodate optimally to existing industrial processes or products, which represent a highly attractive, sustainable, cost-effective, and environmentally friendly option to replace chemical catalysis. In order to exploit the unexplored potential of microbial diversity, different approaches for bioprospection or screening of biomolecules and enzymatic activities are currently being used.

14.2.1 Prokaryotic Diversity

Prokaryotic diversity has been underestimated for a long time, due to technical limitations and a dependency on cultivable species (Torsvik et al., 2002). Biochemical calculations and novel molecular techniques, which do not depend on culturing microorganisms, have demonstrated that the prokaryotic diversity

on earth is enormous. For instance, Whitman and collaborators estimated that the number of prokaryotes on earth is $4\text{--}6 \times 10^{30}$ cells, representing the largest source of nutrients (carbon, nitrogen, and phosphorus) in living organisms (Whitman et al., 1998). In addition, by using whole-genome shotgun sequencing, Venter and collaborators identified over 1.2 million previously unknown genes from seawater samples collected at the Sargasso Sea (Venter et al., 2004). Indeed, one gram of soil may contain billions of microorganisms belonging to thousands of different species (Rossello-Mora and Amann, 2001; Torsvik and Ovreas, 2002). Although the diversity of prokaryotes is outstanding, less than 1% of these microorganisms can be cultivated by using current microbiological techniques. To reach the 99% remaining, and explore the untapped diversity of microbial enzymes and bioactives for potential applications, novel tools and techniques are needed.

14.2.2 Metagenomics

Metagenomics is the direct study of genetic material recovered from microbial communities present in environmental samples, which take advantage of the rich diversity of genes and biochemical reactions of millions of noncultivated and uncharacterized microorganisms. Thanks to the advances of molecular techniques and next-generation sequencing technologies, metagenomics has emerged as an alternative approach to the application of direct microbial/enzymatic screening. Its success lies in the ability to survey millions of genetic sequences avoiding microorganism's cultivation. The first ideas about using metagenomes as a resource of great amount of genetic information were proposed in 1998 by Handelsman and collaborators (Handelsman et al., 1998), but it was back in 1995 when the first applications were reported, searching for cellulases encoding genes in a microbial consortia from anaerobic digesters (Healy et al., 1995).

Metagenomic projects are initialized isolating DNA of a high quality, appropriate for cloning and/or sequencing techniques, from the target sample in order to generate DNA libraries. The screening of metagenomic libraries differ in two approaches: function-based or sequence-based screenings (Simon and Daniel, 2011; Lee and Lee, 2013). Function-based screening depends on the cloning and expression of metagenomic genes in hosts such as *Escherichia coli*, *Streptomyces* spp., *Sulfolobus sulfataricus*, and other well-described model microorganisms. The activity of the cloned and expressed metagenomic genes in the hosts may be detected by different methods such as phenotypical detection (Beloqui et al., 2010; Liaw et al., 2010), complementation of host mutant strains (Simon et al., 2009; Chen et al., 2010), and induced gene expression (Uchiyama et al., 2005; Uchiyama and Miyazaki, 2010). On the other hand, sequence-based approaches use conserved regions of known genes as templates to design DNA probes or primers that are applied to find target sequences in metagenomic samples. Additionally, the advances in next generation sequencing have accelerated

the development of this technology towards understanding microbial ecology (Dinsdale et al., 2008; Thomas et al., 2012; Bragg and Tyson, 2014).

Functional and sequence metagenomic screening approaches are powerful tools, but sequence-based screening does not allow to obtain direct conclusions about enzyme functionality. Otherwise, functional-based screening holds the key to discover new biocatalysts and novel versions of known enzymes with potential biotechnological application (Lorenz and Eck, 2005; Ferrer et al., 2009; Iqbal et al., 2012). For example, recent reports describe the identification of β -galactosidases (Wang et al., 2010), proteases (Neveu et al., 2011), xylanases (Verma et al., 2013), pectinases (Singh et al., 2012), and amylases (Sharma et al., 2010) with potential applicability in diverse industrial processes and products. Interestingly, through functional metagenomics screenings, several esterases and lipases with novel sequences and extremophilic characteristics have been found (Hu et al., 2012; Selvin et al., 2012; Fu et al., 2013; Lopez-Lopez et al., 2014; Peng et al., 2014). In addition to identifying novel biocatalysts, metagenomic approaches also found application for identifying new antibiotics, bioactives, and other small molecules with potential application in Pharma and other related areas, such as diagnostics and research (Lorenz and Eck, 2005; Banik and Brady, 2010). A classic example is the isolation of the antibiotics turbomycin A and B by using heterologous expression of metagenomic DNA from soil samples (Gillespie et al., 2002).

In spite of the benefits of this powerful technique, there are significant technical challenges that need to be addressed. For instance, the isolation of high-quality DNA from environmental samples is not always an easy task. Cell recovery biases, complex samples, and contamination with humic acids, which interfere with DNA manipulation, are part of the problem of isolating good DNA samples. This issue becomes even more complicated when isolating DNA from extreme environments, because of the difficulties to lyse extremophilic microorganisms. In addition, a main bottleneck of functional metagenomics is to express recovered genes into cultivable heterologous hosts for activity tests (Uchiyama and Miyazaki, 2009). Because of the significant differences in gene expression among different taxonomic groups, oftentimes the genetic machinery of the hosts does not recognize the sequence information and fails on expressing the enzyme. The expression of metagenomes in *E. coli* is extremely biased. Indeed it has been suggested that only about 40% of the enzymatic activities found in metagenomic samples may be obtained by random cloning in *E. coli* (Gabor et al., 2004). Expanding the range of potential hosts to increase the chances to find active enzymes is not a comprehensive solution, it should be complemented by developing novel molecular tools such as optimal and broad cloning and expression vectors. Other factors that influence the probability of finding a desired activity are the size of the target gene, its abundance in the sample, and the assay method (Uchiyama and Miyazaki, 2009). Because of these technical issues, and keeping in mind that the raw material or source for metagenomic analyses corresponds to millions of environmental sequences, the overall success rate of functional metagenomics in discovering novel biocatalysts is still low.

14.2.3 Classical Enzymatic Screening

The classic alternative to metagenomics is enzymatic functional screenings over cultured microorganisms. Even though this approach is not as powerful as metagenomics, it is still an effective way to find novel biocatalysts with decades of proven success. In general terms, microorganisms are isolated from environmental samples by cultivation under specific growth conditions, such as temperature, pH, and substrate and nutrient composition among others. Once microorganisms have been isolated, they are functionally screened for activities of interest, for example, enzymatic or antimicrobial activities. It is important to note that through a functional approach it is possible to confirm the presence of the target biocatalyst as well as its activity and behavior under previously determined conditions. This is a huge advantage for the development of novel biocatalysts tailored for specific industrial uses. The main drawback of this approach is that its application is limited to only cultivable microorganisms, which, as has been stated above, represent just 1% of the total prokaryotic diversity. Many factors affect the cultivation of microorganisms such as nutrient availability, osmotic conditions, oxygen levels, temperature, and pH (Vartoukian et al., 2010). In addition, several microorganisms require close interactions with other microorganisms in a community, such as the case of *Nanoarchaeum equitans*, an obligate symbiont of the archaeon *Ignicoccus* (Huber et al., 2003). As not every possible variable can be controlled applying enrichment/plate isolation in laboratory conditions, the great majority of the microorganisms in a sample are difficult to grow. Only the microorganisms that are best suited for a particular set of laboratory conditions are the ones that will be culturable, even though they may not be the most representative or dominant species of a specific environmental sample. Several novel techniques have been developed in recent years to improve the classic enrichment/plate isolation of microorganisms (Table 14.4) (Vester et al., 2015). In general, these new approaches are based on the idea of integrating the natural environment into the laboratory, and cultivate the microorganisms using the environmental nutrients and other chemicals, but keeping the microorganisms isolated from the environment. A recent successful example is the use of the isolation Chip (iChip, Table 14.4) for the discovery of teixobactin, a novel antibiotic that avoids the development of antibiotic resistance, from uncultured microorganisms (Ling et al., 2015). Even though techniques such as the iChip are promising for the cultivation of microorganisms, they could prove difficult to apply for microbes from extreme environments, which represent the most prolific natural source for industrial biocatalysts.

In addition to the challenges for cultivating novel extremophiles, there are several other technical challenges that need to be addressed at the moment of screening for activities and developing new biocatalysts toward industrial applications. For example, to test the presence of an enzyme in the crude extract of a microorganism, extremely robust enzymatic assays need to be developed and implemented, which have to be adapted to the required conditions for enzyme activity, a particular complex task when working with extremophiles. Also, it

TABLE 14.4 Novel Methodologies for Cultivation of Microorganisms

Method	Brief description	Citation
Diffusion chamber	A chamber closed with permeable membranes containing separated microorganisms embedded in agar is exposed to the natural environment allowing the diffusion of nutrients, chemicals, and other growth factors.	Kaeberlein et al. (2002)
Isolation chip (iChip)	High-throughput system with hundreds of miniaturized versions of diffusion chambers, each one inoculated with single cells (monospecific cultures).	Nichols et al. (2010)
Hollow-fiber membrane chambers	Inoculated fibers with microorganisms are exposed to the natural environment. The fibers allow the exchange of nutrients and chemicals between the inside cells and the external natural environment.	Aoi et al. (2009)
Microdroplets	Microorganisms encapsulated in gel microdroplets, which are porous allowing nutrient exchange with microorganisms encapsulated in other microdroplets.	Zengler et al. (2002)
I-tip	In situ cultivation device where microorganisms grow on solid support while receiving naturally occurring nutrients and chemicals from the environment.	Jung et al. (2014)
Dilution to extinction	High-throughput extinction culturing of microorganisms in small volumes of low-nutrient media (in situ substrate concentrations)	Connon and Giovannoni (2002)
Resuscitation-promoting factors (Rpf)	The use of resuscitation-promoting factors (Rpf) is used to bring back to life dormant organisms, increasing the chances to cultivate them.	Nichols et al. (2008)
Alternative gelling agents	Use of alternative gelling agents instead of agar, such as gellan gum.	Tamaki et al. (2009)
Extended incubation times	Increasing the cultivation times to even more than 8 months increases the chances to grow slow-growing microorganisms	Vester et al. (2013)
Cocultures	Diverse coculture efforts have improved the culturability of microorganisms that needs interaction with other microorganisms in the environment.	Stewart (2012)

Modified from Vester et al. (2015).

is necessary to develop enzymatic activity assays under miniaturized and automated conditions to allow massive and rapid discovery of efficient biocatalysts. Currently, High Throughput Screening (HTS) technologies are being implemented for searching new drugs or chemicals by pharmaceutical companies (Zhu et al., 2010), which, if applied for enzymes discovery, will help to move forward this field. Finally, as there are several limitations to grow extremophiles directly in bioreactors for mass production because of the extreme conditions for their growth and the low cell mass yields, the current strategy is to clone and express the genes encoding for the desired product in mesophilic hosts as a prior step to the operation in a bioreactor (Karlsson et al., 1999). However, *E. coli* and other well-known bacterial hosts pose some problems in expressing genes from several types of extremophiles, because of misreading or intervened genes, which are translated into low level or nonexpression of the target enzyme. There is a clear need for new hosts able to properly express extremophilic enzymes, such as the recent efforts reported for the expression of hyperthermophilic cel-lulases of the archaea *Pyrococcus* sp. into the fungus *Talaromyces cellulolyticus* (Kishishita et al., 2015). Beyond these technical limitations, classical enzymatic screening over culturable microorganisms is still an effective way to find novel biocatalysts for diverse industrial applications. Recent reports successfully demonstrates the discovery and testing of novel extremophilic enzymes from isolated microorganisms for possible application in diverse industries, such as cold-active protease (Alias et al., 2014), cold-active salt-tolerant amylase (Qin et al., 2014), and thermostable laccase (Zheng et al., 2012), among many other examples.

Even though extremophiles are an interesting natural source to discover novel biocatalysts, very few companies around the world dedicate exclusively their efforts to discover new biocatalysts from them. Swissaustral, a company with headquarters in Santiago, Chile operating in the United States and Switzerland uses classical microbial isolation techniques and functional enzymatic screening for the discovery of novel biocatalysts. These new enzymes are then examined for application in industry and research. Extreme microorganisms provide unexplored metabolic pathways as a unique feature to enable industrial innovation. Their proprietary collection of extremophiles has over 300 isolates, which have been collected from a broad range of extreme environments in Chile (Figs. 14.1–14.3).

In addition, thanks to years of experience working with extremophiles and extremonzymes, they have developed an Extremophilic Bioprospection and Development (EBD) platform to discover and develop novel biocatalysts from their collection of extremophiles. This platform, which is based in classical microbiology, biochemistry, and molecular biology, is part of their internal pipeline and R&D service. Through this platform, a novel recombinant catalase from a psychrophilic microorganism was developed. This enzyme is active in a wide range of temperatures, between 20°C and 70°C, and keeps 50% of its activity after 7 h of exposure at 50°C. They have also developed a natural



FIGURE 14.1 Extreme environments screened by Swissaustral in diverse expeditions. Fumarole at the Copahue volcano, south of Chile. The Copahue volcano is an active stratovolcano which has very active acidic fumaroles and solfataras. From its environmental samples thermoacidophiles have been isolated which contain interesting acidic and thermophilic enzymes. *From Copyright@Swissaustral.*



FIGURE 14.2 Another extreme environment of interest for sampling and screening, the Ascotan salt flat in the north of Chile. This salty and acidic natural environment has interesting halophiles which possess several polymer degrading enzymes as well as compatible solutes or osmolytes such as ectoine, betaine and threulose. *From Copyright@Swissaustral.*

purple pigment violacein, with proven interesting antimicrobial, antitumoral, and antiparasitic properties ([Duran et al., 2007](#)), from a novel nonpathogenic psychrotolerant microorganism. Both products are now commercially available in Europe and the United States. In addition, they are about to launch a novel microbial recombinant thermostable glutamate dehydrogenase, isolated from a novel thermophilic bacteria, and they are developing several other extremophilic enzymes which are envisioned to be launched between 2016 and 2018.



FIGURE 14.3 A cold extreme environment for sampling and isolation of diverse extremophiles. Paradise Harbor, located in the Gerlache Strait, Antarctica. From this area, several psychrophiles, which possess diverse enzymes with potential industrial applications, have been isolated from environmental samples. *From Copyright@Swissaustral.*

The application of novel culturing techniques, optimal molecular tools, and applied comprehensive efforts like Swissaustral's model will maintain classic microbial isolation and functional enzymatic screening as an extremely valuable approach for discovering novel biocatalysts for industrial use.

14.2.4 Protein Engineering

Even though enzymes are extremely versatile and efficient catalysts that have been optimized through millions of years of evolution, they cannot always be applied to solve industrial problems because of the differences between native function and human-designed industrial needs. Many enzymes discovered in nature could be applied to diverse industrial processes, but they do not efficiently perform under a particular condition or do not recognize the target substrate. In those cases, the enzyme properties or its activity have to be improved. Protein engineering allows the design and construction of enzymes or proteins with novel functions or improved characteristics. Thanks to the advances in diverse biological sciences, many different protein engineering methods are available today, which are exhaustively reviewed by Turanli-Yildiz and collaborators ([Turanli-Yildiz et al., 2012](#)).

Among protein engineering methodologies, directed evolution represents the most successful strategy when little or no *a priori* knowledge of the target structure or function is available. Directed evolution was started with the pioneering work of Stemmer and Arnold in the early 1990s ([Born scheuer et al., 2012](#)) and it works by mimicking natural selection to evolve enzymes/proteins or genes *in vitro* towards a tailored goal. This technology involves iterative

rounds of random amino acid/nucleotides changes in a protein/gene, followed by selection or screening of the resulting libraries for variants with improved properties, such as enzyme stability, substrate specificity, and enantioselectivity (Born scheuer et al., 2012). Over the years, this technology has been further developed and improved towards higher efficiency for the creation of improved enzymes.

Codexis, Inc. (Redwood City, California, USA) is one of the largest companies dedicated to protein design and improvement for pharmaceutical and chemical production. Their model starts with the design and development of novel biocatalysts, but their technology enables scale-up and implementation of biocatalytic solutions to optimize full processes. Codexis has applied its protein engineering platform, CodeEvolver, and their ProSAR and MOSAIC directed evolution technologies to improve biocatalysts, such as halohydrin dehalogenase (Fox et al., 2007) and carbonic anhydrase (Alvizo et al., 2014), and also in full biocatalytic processes (Savile et al., 2010; Li et al., 2012).

Another interesting company working in protein engineering is Novici Biotech, located in the California Bay Area. They developed a proprietary gene shuffling technology, called Genetic ReAssortment by MisMatch Resolution (GRAMMR), that overcomes the limitations of conventional variant gene library construction approaches to produce large (10^3 – 10^6) and high-quality shuffled gene libraries with exceptional crossover frequencies (10–20 per kb) and extremely granular crossover resolution (Padgett et al., 2010). This technology streamlines directed evolution workflows with rapid construction of initial libraries and production of subsequent libraries of reshuffled hits at much lower per-gene cost and higher diversity content than can be achieved by other shuffling methods or by gene synthesis.

GRAMMR is an in vitro enzymatic process that resolves sequence mismatches in heteroduplex DNAs through the coordinated action of several enzyme activities. Mismatch endonuclease nicking at a given mismatched site is followed by excision and localized resynthesis to transfer sequence information from one strand to the other (Padgett et al., 2010). Independently resolved sites are often as close as one or two nucleotides apart (such as between adjacent codons), and the high crossover frequency of the process ensures a thorough blending of diversity that is incorporated into the initial library. These libraries are constructed to manage varying degrees of diversity input. For protein engineering applications, library strategies can be employed to interpolate sequence space between naturally occurring genes, either pairwise, or with larger numbers of starting genes. Protein alignments often contain contiguous strings of dissimilar sequences that the GRAMMR technology splits into blocks of varying size for maximal incorporation of diversity into the library. Since this shuffling technology operates full-length sequences that remain intact throughout the process, large genes or multigene targets are efficiently chimerized with minimal damage from the process itself. Beyond protein engineering, this technology enables to construct large libraries of codon substitution variants to drive

the process of empirical codon optimization. This directed evolution approach for codon optimization is unique to GRAMMR and allows optimal codon usage to be evolved for any gene in any host in which expression and screening can be performed.

Despite all the technological advances that science is currently bringing for the development of novel biocatalysts, nature itself is still the best source to find truly novel enzymes, and techniques like classical microbial/functional enzyme screening and functional metagenomics, which explore nature's diversity, are still the best tools to find novel enzymatic activities. However, the advances in protein engineering are tremendous and its application to improve existing sequences leads to the development of enzymes with novel features and even novel activities. All these technologies are powerful and increase the velocity of discovery, development and improvement of novel biocatalysts. The future is to improve each approach and to adapt them to work together towards a comprehensive solution for creating novel biocatalysts that can replace current chemical processes for more sustainable and environmentally friendly solutions.

14.3 INDUSTRIAL BIOTECHNOLOGICAL PROCESSES AND THEIR GREEN CHEMISTRY RELEVANCE

Green biotechnological processes and products find more and more applications in various sectors like food production and treatment, synthesis of active pharmaceutical ingredients, chemicals, detergents (eg, low temperature washing detergents), treatment of textile fibers and their bleaching, pulp and paper treatment, and many other applications (Straathof et al., 2002; Eş et al., 2015). In comparison to the traditional techniques used in chemistry, the bioprocesses are often less demanding with respect to process energy, and raw materials.

Two main categories of bioreactions are differentiated: *biocatalysis* with enzymes and *biotransformation* using cells. In contrast to *biosynthesis*, where a product (eg, an enzyme) is completely synthesized from unrelated substrates, *biotransformations* are always related to the supply of a starter molecule that is chemically modified and an additional carbon and energy source provided in the growth medium (Ladkau et al., 2014). Consequently, *biocatalysts* may consist of free enzymes, immobilized enzymes or (living or dead) cells, and planktonic cells. Optimally, the biocatalyst is very selective with respect to the substrate for its conversion with high chemo-, regio-, and stereoselectivity, and it should remain highly active over long periods of time, broadly applicable, safe to use and, last but not least, cost-efficient (Liese and Villela Filho, 1999; Mateo et al., 2007; Choi et al., 2015). Consequently, much emphasis is put on the selection of the best performing biocatalyst under the preferred process conditions. This task is not easy since a scale-up has to be carried out in order to achieve a cost-efficient industrial production. Obstacles are loss of performance because of scale-up problems, and complex process control (Tufvesson et al., 2010). A first approach is the improvement of the biocatalyst (eg, broader range

of pH, better reaction kinetics). Novel tools have been developed to evolve better performing enzymes and cells (eg, directed evolution using genetic engineering) that are finally evaluated by automated screening platforms (Kumar & Clark, 2006). A second approach is the selection of the optimal bioprocess. Besides the technical aspects of the scale-up, also the choice of the biocatalyst (enzyme or cell) and its implementation (eg, planktonic state, immobilized on a surface or part of a lattice) plays a significant role in selecting the appropriate bioprocess (Hanefeld et al., 2009). As a consequence, the production process needs to be carefully designed (batch, fed-batch or continuous mode) concomitantly considering the most efficient isolation of the final product (Tufvesson et al., 2010). The following section focuses on the different options that are available to maintain a high performance of biocatalysts and what kinds of restrictions have been identified.

14.3.1 Bioprocesses and Bioreactors Used in Biocatalysis and Biotransformation

Nonimmobilized biocatalysts are usually difficult to recycle in bioprocesses and their use clearly depends on the chosen bioprocess, for example, batch, fed-batch, or continuous.

14.3.1.1 Batch Bioprocesses

The batch method is the most applied bioprocess when planktonic cells or soluble or immobilized enzymes are applied as biocatalysts. Generally stirred tank reactors STR (Fig. 14.4) are used when the optimal process conditions have to be accurately controlled and maintained throughout the entire batch process, for example, mixing, pH, and temperature control. Most bioreactors used for enzymes are slightly modified reactors designed for chemical reactions and often are not run under sterile conditions. In many batch processes the enzymes are not recycled because of their low production cost (DiCosimo et al., 2013).

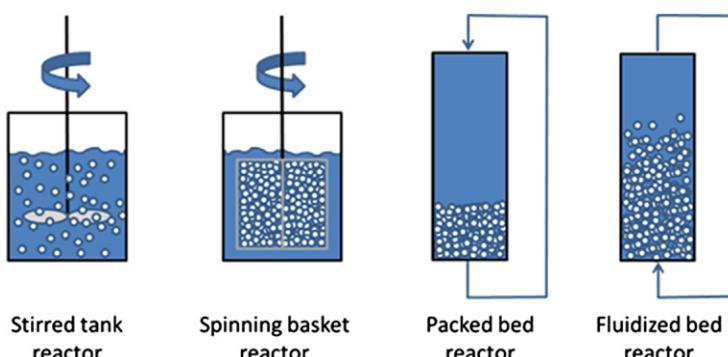


FIGURE 14.4 Different types of batch reactors.

Nevertheless, the recycling of costly enzymes or valuable cells for several production cycles is worth being considered; however, to maintain their full active state is difficult (see section Immobilization of Biocatalysts). Here the biocatalyst's immobilization on beads or its encapsulation in a polymer lattice represents an interesting option facilitating the recycling and handling.

The simplest approach to recycle cells in a bioreactor is the spinning basket reactor ([Sheelu et al., 2008](#)) (Fig. 14.4), where the catalyst is retained within a closed sieve which is only permeable for solubilized substrates and products. Interestingly, such containment may also be part of the mixing system (stirrer or baffles) and therefore have a higher reactivity because of the enhanced fluid dynamics that can be achieved in the microenvironment of the biocatalyst. The superior performance of a spinning basket reactor was successfully demonstrated for the degumming of rice bran oil with immobilized lecitase (phospholipase A1) in cross-linked gelatin particles with 1–2 mm in diameter ([Sheelu et al., 2008](#)). The loss of enzyme activity was only in the range of 80% after six batch cycles.

A different set-up than the stirred reactors is the plug-flow batch reactor. The best known types are the packed bed or the fluidized bed reactors (Fig. 14.4), where the liquid is circulated through a side-loop in order to avoid the formation of concentration gradients of substrate and product ([Brady and Jordaan, 2009](#); [Veny et al., 2014](#)). These plug-flow reactors are typically used when a single pass through the reactor is not sufficient to achieve the targeted product yield or to determine kinetic data, for example, decontamination of toxic compounds ([Cassidy et al., 1996](#)).

A recent study showed that the transesterification of jatropha oil could be best performed in circulated batch packed bed reactor (CBPBR). An immobilized lipase from *Rhizomucor miehei* showed stability in CBPBR by maintaining 70% of its relative activity up to 10 cycles ([Veny et al., 2014](#)). Fluidized bed reactors are clearly more favored because they are easier to operate and form fewer flow blockades caused by biocatalyst plugs in the cycle loop. Jakovetić and coworkers compared the formation of ethyl cinnamate, an ester commonly used as an aroma and fragrance component, in two bioreactors with immobilized Novozyme435 lipase: one utilized in batch mode, and the other one in fluidized bed mode ([Jakovetić et al., 2013](#)). The initial kinetics showed that in bioreactors the reaction followed the ping-pong bi-bi mechanism, but substrate inhibition occurred only in the batch bioreactor. In addition, the overall reaction was 6.5 times faster in the fluidized bed bioreactor, despite the fact that in both set-ups internal and external mass transfer limitations were negligible. Thus, this approach was found to be scalable for industrial scale production of phenolic acid ester ([Jakovetić et al., 2013](#)).

14.3.1.2 Fed-Batch Bioprocesses

Interestingly, there are not many processes with enzymes run in semicontinuous or fed-batch mode. One reason is the higher tolerance of purified enzymes towards elevated substrate and product concentrations ([DiCosimo et al., 2013](#)).

This is in contrast to (multistep) biotransformations that are performed with living cells. In general, the biotransformation using fed-batch technology is carried out in two time-separated bioprocesses: in a first step the biomass concentration is significantly increased with a pulsed, linear, or exponential feed of highly concentrated solution of the growth-limiting carbon source. At high cell densities, the substrate that needs to be produced is added and is then converted preferentially. Frequently, the biomass is also harvested after the first step, concentrated and subsequently exposed to the substrate in a second stirred tank bioreactor. This approach of separation of biomass growth from biotransformation does not need to be followed always, as has recently been shown by a study carried out by Le Meur and coworkers ([Le Meur et al., 2013](#)). In order to produce poly(4-hydroxybutyrate) (P4HB), a suture material approved by the FDA, *E. coli* JM109 was engineered to carry the PHA synthase gene (*phaC*) from *Ralstonia eutropha* and a 4-hydroxybutyric acid-coenzyme A transferase gene (*orfZ*) from *Clostridium kluyveri* ([Hein et al., 1997](#)). When the strain was provided with 4-hydroxybutyrate, it accumulated the polyester P4HB inside the cell independently of the growth phase of the recombinant strain. Interestingly, the strain could also be grown on glycerol as growth substrate to a high cell density of 43 g/L. The addition of acetic acid and a concomitant limitation by amino acids was beneficial for the biosynthesis, but were not modifying the P4HB composition. A P4HB concentration of 15 g/L was obtained leading to a productivity of 0.207 g/L/h, which has been the highest productivity for P4HB reported so far ([Le Meur et al., 2014](#)).

14.3.1.3 Continuous Bioprocesses

Over the years different techniques and bioreactor types have been developed for continuous processes ([Wiles and Watts, 2014](#)). This is not surprising, since most of the studies revealed that higher volumetric productivities can be achieved in comparison to batch processes. In addition, the ease of automatic control, ease of operation, and good production performance with a high reproducibility are very appealing attributes.

A well-established system based on the continuous stirred tank bioreactors (CSTR) is the chemostat. In research, also multistage chemostat fermentation has been proposed as a valid production method suitable for industrial production of polymers ([Koller and Muhr, 2014](#)). Under constant feed and growth conditions, the chemical environment and biomass concentration comes near to a steady-state situation that can theoretically last forever. A clear advantage of the chemostat is the full control of the cell activity: the ratio of the feed rate to the volume of the culture broth determines the dilution rate, D, or the specific growth rate of the cell in steady-state. With appropriate selection of the medium composition, simultaneous biotransformation of a second substrate can be achieved. This combination of growth and simultaneous product synthesis was used for the production of poly(3-hydroxy-5-p-methylphenylvalerate) (PMePV) ([Hany et al., 2009](#)). Lactic acid was continuously supplied to *Pseudomonas putida* GPo1 as the only carbon source, thus enabling a constant specific growth (and

dilution) rate of $D = 0.2 \text{ h}^{-1}$. Under nitrogen-limited growth conditions, the substrate for PMePV, 5-*p*-methylphenylvaleric acid, was continuously added. The biotransformation resulted in the polymerization and accumulation of PMePV inside the cell. For the first time a highly crystalline aromatic polyhydroxyalcanoate could be biosynthesized (Hany et al., 2009).

The chemostat cultivation can be modified in such a way that cells are looped back to the bioreactor while the product can pass a ceramic filter. Lonza evaluated such a cell recycling system for the continuous production of L-carnitine using a knock-out strain of *Agrobacterium/Rhizobium* HK4 lacking the L-carnitine dehydrogenase (Hoeks et al., 1992; Meyer and Robins, 2005; Robins and Gordon, 2011). The volumetric productivity of a 450L chemostat was found to be significantly better than a corresponding fed-batch process. However, in this continuous process the substrate 4-butyrobetaine was not fully converted into optically active L-carnitine and therefore the downstream processing costs were increased in comparison to the fed-batch process which had a purity of more than 99% L-carnitine. Even though a technical solution was found, the implementation of an additional chemostat stage, the continuous production was not followed any further.

When a high reactivity of the immobilized biocatalyst is given, also packed bed (Hama et al., 2011) and fluidized bed reactors (Poppe et al., 2015) may be used in a continuous way (also frequently termed continuous plug-flow bioreactors). In theory these techniques are superior to the ones based on CSTR because the time of interaction can be easily adjusted by the length of the reactor and the flow rate of the reaction liquid. Since there is no agitation system installed, the conditions in the bioreactor are never uniform. Adverse factors are usually observed such as temperature and velocity gradients normal to the flow direction and substrate gradients along the reactor.

During the past years the membrane technology has made significant progress and different materials (eg, PTFE) are now used in bioprocesses showing a low membrane fouling rate (Carstensen et al., 2012). The principle is that the substrates and products are freely crossing the membrane but at the same time the biocatalyst is retained due to size exclusion. The membrane technology has been applied in different bioreactor designs (Carstensen et al., 2012). A rather simple, but very effective application is the combination of a tangential flow membrane, which can be combined in case of microbial biotransformation with a CSTR (Giorno and Drioli, 2000). The advantage of this approach is that a product inhibition may be significantly reduced and the immobilized biocatalyst recycled easily. A disadvantage of such a design is that typically the shear force on the membrane surface needs to be maintained at a high level in order to avoid plugging of the membrane pores (Moueddeb et al., 1996). Consequently, the efficacy of the biocatalyst may be reduced over time due to this issue. In order to enhance the surface available for the exchange and to reduce the hydraulic pressure on the membrane, also cartridges containing membrane tubes were implemented (Pollice et al., 2005).

The biotransformation using biofilm cells in membrane bioreactors is not yet that far developed and is still largely at a research state. However, the results are very promising and reveal a large potential of reducing total bioprocess costs ([Straathof et al., 2002](#); [Ladkau et al., 2014](#)). As an example silicone tubes were used as tubular membrane bioreactor to cultivate the engineered *Pseudomonas* sp. strain VLB120ΔC in order to continuously produce (S)-styrene oxide from styrene that diffused through the membrane. The oxygen transfer and the flow rate were found to determine the productivity. In addition, a linear relationship was identified between the specific membrane area and the tube wall thickness. Nevertheless, the bioprocess could be maintained for c. 50 days. A particular advantage of such an application of biofilm cells is the fact that they are much more resistant to toxic compounds than planktonic cells. Consequently, the substrate inhibition is significantly reduced ([Halan et al., 2012](#)).

14.3.2 Immobilization of Biocatalysts

As previously mentioned, the immobilization of enzymes and whole cells have a cost advantage because of the better recycling options and better stability of the biocatalyst ([Es et al., 2015](#)). Therefore, immobilization techniques have not only acquired an important role in research and development dedicated to analytical and medical applications (eg, as biosensors ([Vashist et al., 2014](#))), but also to improve well-established industrial bioprocesses ([Table 14.5](#)). In short, the immobilization can also be defined as a confinement of a biocatalyst inside a bioreaction system with retention of its biocatalytical activity over several production cycles.

Since the first reports in the 1960s ([Doscher and Richards, 1963](#); [Quiocho and Richards, 1964](#)), much progress has been achieved in immobilizing different biocatalysts ranging from crude and highly purified enzymes, microbial cells, to animal and plant tissues ([Cassidy et al., 1996](#); [Sheldon, 2007](#); [Rathore et al., 2013](#)). In many cases the applied techniques are quite costly because often the biocatalysts are unstable and need regeneration systems for NADH/NAD⁺. In the case of the biotransformation with whole cells, sufficient nutrition to maintain viability and full biocatalytic activity is needed ([Liu and Wang, 2007](#)).

Different immobilization techniques are explained in the following sections.

14.3.2.1 Biocatalyst Modification by Cross-Linking

Biocatalysts can be covalently cross-linked with active reagents containing aldehydes or amines to form larger, three-dimensional structures. However, these chemicals can also inhibit catalytic activity. Consequently this method can only be applied to enzymes and nonviable cells. To date, glutaraldehyde has been used as the main chemical because it reacts easily with lysine residues of the enzyme forming Schiff's base. This also means that enzymes can be engineered in such a way that a poly(L-lysine) tail is part of the enzyme, interacting preferentially

TABLE 14.5 Advantages and Disadvantages of Immobilization Techniques

Advantages	
Retention of bioactivity in the bioreactor	Continuous processes are enabled
High concentration of biocatalyst	Increases volumetric productivity Efficient conversion of unstable substrates and intermediates
Control of biocatalytic microenvironment	Protection from shear forces and air bubbles Limitation of side reactions Multienzyme immobilization Charge of matrix can enhance substrate affinity
Higher long-term stability	pH range is better defined Higher temperature stability
Easier product isolation	Minimization of product contamination by biocatalyst Reduction of downstream processing costs
Disadvantages	
Immobilization costs	Carriers may be not cheap Immobilization techniques need fine tuning for each enzyme/cell type The immobilization of biocatalysts results in a noncatalytic dry mass of more than 90% and therefore reduces the volumetric and space-time yields
Loss of biocatalyst activity during immobilization	Catalytic sites may be affected or become inactivated
Restriction to smaller molecules	Immobilization in particles may result in reduced access of macromolecules
Instability of support material	Loss of biocatalyst

with the cross-linker ([Shih et al., 2006](#)). Although only one chemical reagent, glutaraldehyde, is required, the reaction conditions for the crosslinking are quite delicate: the concentration of the reaction components, the temperature and the reaction time ([Sheldon, 2007](#)). The availability of the biocatalyst needs to be

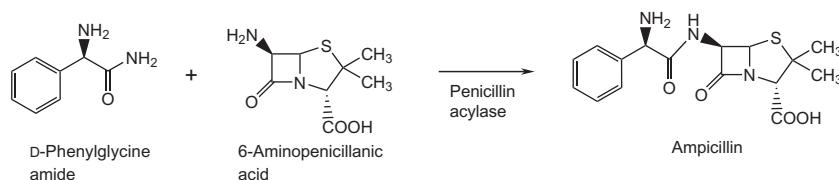
large because a large fraction will be inactivated; generally a loss of the catalytic activity in the range of 90–99.9% has to be considered as normal (Sheldon et al., 2005). Several other drawbacks could be found as well, such as poor reproducibility, low mechanical stability, and difficulties in handling of the gelatinous product (Hanefeld et al., 2009). A potential solution to this problem is to start off with a significantly more active enzyme.

Depending on the biocatalyst's starting condition, one speaks of carrier-free immobilized enzymes and differentiates between cross-linked enzymes (CLEs) produced from soluble state, cross-linked enzyme from aggregates (CLEAs), cross-linked spray-dried enzymes (CSDEs), and cross-linked enzyme crystals (CLECs). All these biocatalysts are more stable against heat and organic solvents than in their native form. Typically trypsin, and papain as CLEs, lipase and penicillin G amidase are used as CLECs, penicillin acylase as CLEA (see Fig. 14.5). For more details on CLEAs the reader is referred to the review by Sheldon and coauthors (Sheldon et al., 2005).

14.3.2.2 Immobilization of Enzymes on Surfaces

The principal interactions of biocatalysts with a support material ranges from simple van der Waals interaction to irreversible covalent bonding (Brady and Jordaan, 2009). The support itself may have various morphologies and one differentiates between surface attachment and lattice entrapment (Brown et al., 1968).

In the case of surface attachment, a polymer particle or an inorganic particle serves as support and anchor material. As polymeric materials polysaccharides, proteins and carbon materials are frequently used, but also synthetic polymers, such as polystyrenes, polyacrylates, polyurethanes, polyamides, polypeptides,



Biocatalyst	Conversion (%)	S/H ^a	V ^b _{syn} (μmol U/h)	Relative productivity ^c
Free enzyme	88	2.00	25.5	100
CLEC	72	0.71	39.6	39
T-CLEA ^d	85	1.58	38.2	151
PGA450	86	1.56	15.6	0.8

^aSynthesis hydrolysis molar ratio at the conversion listed.

^bInitial reaction rate.

^cRelative productivity at the point of maximum conversion, free enzyme set at 100.

^dCLEA produced using tert-butanol as precipitant.

FIGURE 14.5 Effect of different preparation methods of penicillin G acylase on the ampicillin synthesis (Cao et al., 2000).

maleic acid anhydride polymers and polyamides, are employed. Beside organic polymers, also inorganic materials are used. Some are of natural origin, such as kieselgur, bentonite, attapulgite clays, and pumic stone. Also synthetic materials, such as porous and nonporous glasses, metal oxides, and silicates, have been studied and are applied today. Permeabilized or nonviable cells, multienzyme complexes, enzyme-cofactor pairs or simply purified enzymes are either covalently or noncovalently bound to the support material. For covalent binding the Schiff's base formation, alkylation and arylation, amide and peptide formation, diazotization as well as amidination have been described as suitable methods. The noncovalent binding can be established by a specific interaction among binding partners (hydrophobic interaction, antibody-antigen, lectin-carbohydrate, polyphenol-protein, biotin-streptavidin) or rather unspecific by free-energy reduction, induced dipole interaction or ionic interaction.

With the onset of the era of nanotechnology, also new technologies in biotechnology have evolved, coining the term nanobiotechnology. A new industry has emerged over the past fifteen years and many large companies have entered into this field but also new, innovative companies have been founded. Inofea, a Swiss company founded in 2011, has successfully entered into the field of biocatalysis focused on the production of novel biocatalysts for food and beverage, pharma and biotech, as well as for the cleantech market. Their choice of using magnetic carrier materials enables the customer to completely recover the valuable catalysts from the reaction broth. With the application of an additional matrix coating on the surface (see also next section), the nano-sized catalysts became significantly more robust with respect to temperature, pH, and harsh treatments (ultrasound, urea, SDS, freeze-thawing, and protease). Thus, novel fluidized bed reactors can be established where the biocatalyst is fully retained. With respect to immobilization, it was found that the coating on the nano-magnets plays a significant role for the production efficiency of covalently bound enzymes ([Zlateski et al., 2014](#)).

14.3.2.3 Immobilization of Whole Cells in a Polymer Network

As previously mentioned, an alternative type of immobilization is the lattice or network entrapment. Hereby the biocatalyst is immobilized in the lattice while substrate and product can freely diffuse through the network ([Tischer and Wedekind, 1999](#); [Cao et al., 2000](#)). The method can also be applied for cross-linked enzymes (CLEs, CLEAs, CSDEs, and CLECs).

The polymers used need to be versatile and usually they belong to three major groups: proteins (gelatin, albumin, and collagen); polysaccharides (cellulose, gellan, chitosan, alginates, dextran, starch, agar, carrageenans); and synthetic polymers (nylon, polyacrylamides, polyacrylates, silicones, polyurethanes, polyvinylalcohols, polyvinylpyrrolidone, and epoxy resins). The entrapment takes place in different ways, whereas the biocatalyst is generally neither subject to strong binding forces nor resulting in structure distortion (blocking of active site). The polymer matrix/lattice is formed in presence of the biocatalyst and

may differ significantly: *in situ* polymerization starting from monomers, precipitation through ionic interaction (alginates), or covalent cross-linking (chitosan, polyvinyl alcohol, polyurethane), cooling (agarose, gelatin), drying, or induced precipitation.

14.3.3 Effect of Immobilization on Enzyme Kinetics and Properties

The immobilization has an influence on kinetics and other properties of the enzyme ([Tischer and Wedekind, 1999](#)). The reason for these observations is explained by the following factors:

- conformational changes and steric effects
- partitioning effects
- diffusional effects affecting the mass transfer

The key elements of these aspects are detailed below.

14.3.3.1 Conformational and Steric Effects

Along with the treatment of the enzyme for immobilization, conformational changes in its tertiary structure also take place. This is particularly the case when the enzyme is covalently linked to a surface. This new interaction results often in stretching the enzyme and thus modifies the active center. In some cases the immobilization reagents (eg, radical polymerization ([Tischer and Wedekind, 1999](#))) may partially denature the enzyme and even cause a complete loss of the enzyme's activity. This shape modification by partial denaturation usually results in a steric hindrance that limits the accessibility of the substrate to the active site of the enzyme. One method to avoid such modifications of the active site of the enzyme is the addition of specific enzyme inhibitors prior to the immobilization processes. Alternatively, so called spacers can be integrated to reduce enzyme kinetic loss. A frequently used spacer is 1,6-diaminohexane and was successfully used to couple glucoamylase on a plastic support ([Roig et al., 1995](#)).

14.3.3.2 Partition Effects

An effect that is frequently neglected in the immobilization of biocatalysts is the partitioning effect ([Tischer and Wedekind, 1999](#)). This is caused due to the fact that the support material influences the chemical environment of the enzyme by addition of different charges or chemical moieties having a more hydrophilic or hydrophobic character. As a consequence, the optimal conditions for the kinetic reaction can be shifted towards a different pH. Interestingly, this effect can also be used on purpose, when the chemical environment can be tuned in such a way that it results in an attraction of the reaction substrate and therefore leads to higher concentrations of the substrate and improved kinetics ([Brady and Jordaan, 2009](#)).

14.3.3.3 Mass Transfer Effects

A quite obvious modification of the enzyme's activity is the influence of the matrix on the diffusional process of substrates and products. This limitation of mass transfer is significant when the enzyme is not only bound on a surface but rather integrated in a lattice. In the latter case, stagnant fluids in the pores or network can significantly reduce the kinetics by gradient formation. Thus the distance from the surface influences the local activity significantly (Choi et al., 2015).

14.3.4 Industrial Scale Production

Finally, in order to depict the industrial reality, Figs. 14.6–14.9 show selected installations for the industrial scale biocatalysis. The large scale equipment is operated by Lonza, one of the pioneers in combining organic chemistry with biotechnology. In over 40 years, the Swiss company has developed several large-scale biocatalytic and biosynthetic processes for the production of fine and specialty chemicals. As mentioned above, standard chemical installations can be used for the biocatalytic step itself. However, for the production of enzymes, fermentation equipment is needed.



FIGURE 14.6 Fermenters, such as this 75 m³ bioreactor, which are ex-proof and with high gas, mass and momentum transfer characteristics are ideally suited multipurpose containments, which can be used for enzyme production including methanotrophic yeast as host organisms, and subsequent biocatalytic steps requiring solvents. However, Lonza has also exceptionally adapted 12 m³ chemical tanks for fermentation purposes, not only enzymatic conversions. *From Copyright@ Lonza, Kouřim s.r.o.*



FIGURE 14.7 When it comes to cell separation, centrifugation (disk stack centrifuge, *left*) and filtration (ultrafiltration, *right*), also often in combination, remain the preferred methods. *From Copyright@Lonza, Kouřim s.r.o.*



FIGURE 14.8 Downstream processing steps must conserve value created upstream. Efficient purification methods reducing the number of required steps and maximum yield losses are essential for commercial biotransformation or fermentation processes. Column chromatography is increasingly used using various resins and partitioning principles. Columns with 2 meters in diameter and well controlled liquid are among the largest used in the industry. This method is a one preferred choice when dealing with fragile and complex molecular structures or natural compounds. Besides novel stationary phases, also novel column operation procedures, such as SMB (Simulated Moving Bed) chromatography have been developed which are not discussed in this chapter. *From Copyright@Lonza, Kouřim s.r.o.*

Modeling and simulations are used to reduce development time and costs for scale-up in addition to on-line control with process specific algorithms which allow manufacturing processes hitherto not possible to be operated, for example, the possibility to carry out fed-batch fermentation with multiple substrate feeds and tight control of all fermentation parameters and eventually avoiding



FIGURE 14.9 Quality and regulatory requirements often ask for specifically designed closed dosing containments for material transport of solids in manufacturing, including specific washing areas allowing the automated and validated cleaning of these containments. *From Copyright@ Lonza, Kouřim s.r.o.*

ISPI (in situ product inhibition). Consequently, recycling immobilized biocatalysts may help in controlling the overall reaction and reducing the production cost, which is of great importance when large volumes of product have to be synthesized (see Table 14.6). However, the aftermath has to be considered, too: the disposal of deactivated, immobilized biocatalysts has to be taken into account. Yearly, many hundreds of tons of carrier bound enzymes end up as waste material.

14.4 GREEN CHEMISTRY AND RECENT NEW REACTIONS AND DEVELOPMENTS IN ENZYMATIC CATALYSIS

14.4.1 Green Chemistry Metrics in Industrial Enzyme Catalysis

Enzyme catalysis can significantly increase greenness in industrial chemical production (Schepens et al., 2003; Meyer et al., 2013). Today, a set of tools is available to assess the sustainability in biotransformations. These methods are based on the 12 principles of green chemistry (Anastas and Warner, 1998). The tool that is easiest to use is atom economy, as different reaction pathways can be compared on a sheet of paper. A more sophisticated approach is to estimate waste/product ratios known as E-factor calculations (Eq. 14.1). It shows how much waste per kg product is generated. Similar but more detailed analyses are Process Material Impact (PMI) assessments (Eq. 14.2). For each component a number is calculated which allows detailed greenness analysis as all numbers can be summed up providing an overall PMI. The lower the PMI, the greener is the process. It is noteworthy that greenness calculations also need to include the energy to produce the required materials and their processing. Other assessment tools are very common in diverse industrial settings such as Life Cycle Assessment (Cowan et al., 2008) and the less reported Environmental Impact Assessment (Jegmannathan and Nielsen, 2013). For such analyses, software

TABLE 14.6 A Selection of Large Scale Production Processes Using Immobilized Biocatalysts

Product	Production scale (t/a)	Biocatalyst	Substrate	Company
6-Aminopenicillanic acid	1000	Penicillin acylase	Penicillin G/V	DSM
Nicotinamide	4000	<i>Rhodococcus rhodochrous</i> J1 cells entrapped in polyacryl-amide	3-cyanopyridine	Lonza
Aspartame	1000	Thermolysine	N-protected L-aspartic acid, D/L-phenylalanine methyl ester	Holland Sweetener Company
S-Ibuprofen	<1	Lipase (in fiber membrane)	Racemic Ibuprofen	Sepracor
Acrylamide	20,000	<i>Rhodococcus rhodochrous</i> J1 entrapped in polyacrylamide	Acrylonitrile	Mitsubishi Rayon Co., Ltd
L-Alanine	1000	<i>Escherichia coli</i> and <i>Pseudomonas dacunhae</i> cells entrapped in κ-carrageenan	Fumaric acid and ammonia	Tanabe

tools are available and designed for chemical and biochemical engineering. One of them is EATOS ([Eissen and Metzger, 2002](#)) and another one is the Umberto/Sabento program ([Brinkmann et al., 2010](#)).

$$\text{Environmental Factor E} = \sum \frac{\text{Waste}}{\text{Product}} \quad (14.1)$$

$$\text{Process Material Impact} = \sum \frac{\text{Input}}{\text{product}} \quad (14.2)$$

14.4.2 Green Chemistry Developments

14.4.2.1 The 12 Principles of Green Chemistry Applied to Atorvastatin Production

Atorvastatin is a cholesterol-reducing drug commercialized as Lipitor (Pfizer). The production is realized at a multiton scale but greenness was not optimized. For the key intermediate (*S*)-ethyl-4-cyano-3-hydroxybutyrate ([Fig. 14.10](#)) an improved three enzyme two-step process was developed ([Sheldon, 2012](#)).

Although substrate conversions were high, a large amount of wild-type *ketoreductase* (KRED) was needed causing difficulties during downstream processing. With directed evolution using DNA shuffling technology the *ketoreductase* became significantly more reactive. (*S*)-ethyl-4-chloro-3-hydroxybutyrate was obtained in excellent yields >95% and at high enantiomeric purity ee >99.5%. The second enzyme, the *halohydrin dehalogenase* (HHDH), was equally a wild-type enzyme and needed in large quantities. Again DNA shuffling improved the enzymes activity by 2500 times. The lower enzyme load also significantly contributed to reduced emulsion problems during work-up and reduced DSP cost. These improvements were compared to the 12 principles of green chemistry and according to them, the process became greener and

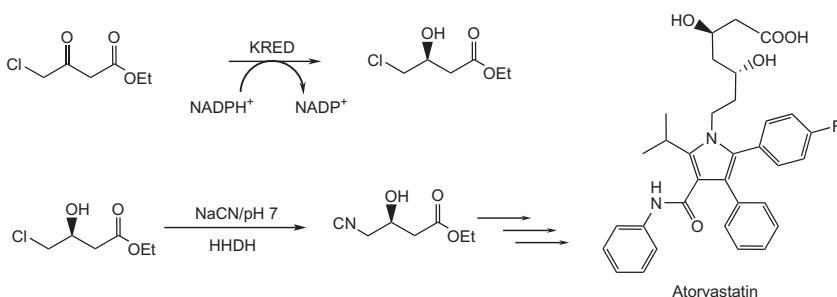


FIGURE 14.10 Atorvastatin production became more sustainable after directed evolution of the wild type enzymes (KRED and HHDH) needed in the syntheses of the intermediates (*S*)-ethyl-4-chloro-3-hydroxybutyrate and (*S*)-ethyl-4-cyano-3-hydroxybutyrate. KRED, *ketoreductase*; HHDH, *halohydrin dehalogenase*.

more economic ([Sheldon, 2012](#)). This is a metrics example that is not based on detailed calculations but nevertheless it represents a compelling case, showing that the application of green chemistry concepts results in economically well improved biotransformations. A more advanced metric tool is E-factor calculation described in the following.

14.4.2.2 Reducing the E-Factor in Enzymatic β -Amino Acid Synthesis

Optically pure β -amino acids are of interest for various pharmaceuticals ([Jurasti and Soloshonok, 1997](#)). The synthesis of (*S*)-3-aminobutanoic acid was a process with a very high E-factor of 359. A key step for a greener process consisted of a new one-pot reaction where a Michael addition was followed by an enzymatic aminolysis using immobilized *Candida antarctica* lipase B (Novozyme435) ([Fig. 14.11](#)). With this one-pot reaction and other improvements the E-factor was reduced from 359 to 41 ([Weiß et al., 2010](#)). This E-factor is still high and typically found in pharmaceutical manufacturing. This shows that notable E-factor improvements are possible, but from a green perspective there is still room for further action. This concerns also the following peptide synthesis which is not a green process and progress is needed.

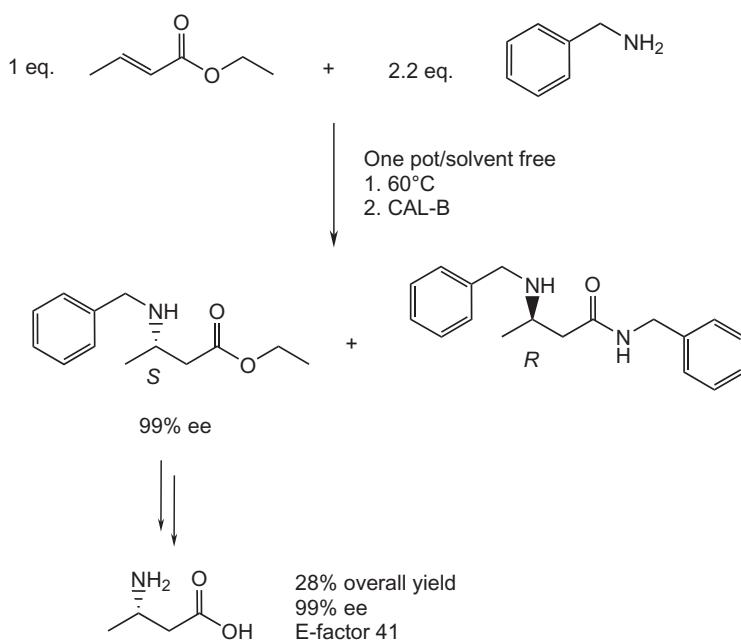


FIGURE 14.11 Improved greenness in chemoenzymatic β -amino acid synthesis. It consisted of a Michael addition and a subsequent enzymatic aminolysis. The E-factor was reduced seven times from 359 to 41.

14.4.2.3 L-Dopa Biotransformation Lowers Process-Material-Impact

L-dopa is used in Parkinson treatment and manufactured at an industrial scale. There are four different production processes established and used today (Fig. 14.12). These processes comprise pure chemical synthesis and an entire biotransformation.

But there are also combinations of both as enzymatic resolution in a multistep chemical synthesis of L-dopa (*S*)-2-Amino-3-(3,4-dihydroxyphenyl) propanoic acid. Over time, the number of reaction steps was reduced for the synthesis of L-dopa from seven to four chemical steps and finally to a single biotransformation (Li and Li, 2014). The largest producer of L-dopa is Shandong Xinhua Pharmaceuticals in China, which surprisingly uses the seven chemical steps synthesis. To compare the environmental impact of the four manufacturing processes, a process material impact (PMI) assessment was realized. The least green process, as expected, was the seven step chemical production, the four step Hoffman La-Roche process was also found to be hardly green with a PMI of 438. This synthesis includes classical resolution chemistry to recover enantiomerically pure L-dopa. This was changed with the Monsanto process, where an asymmetric hydrogenation reduced waste generation resulting in a clearly lower PMI of 104. The single step biotransformation of Ajinomoto was identified as a low environmental impact process (PMI = 39.8). Here the waste originated mostly from enzyme production and media preparation.

Route	Steps	PMI	Stereocenter generation
Ajinomoto	1	39.8	Enantioselective biocatalysis
Monsanto	4	104	Asymmetric hydrogenation
Hoffmann La-Roche	4	438	Classical resolution
Sankyo	7	--	Enzymatic resolution

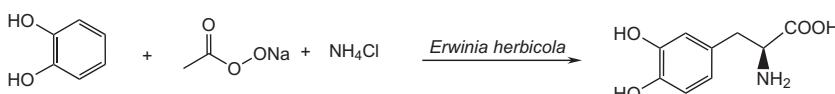


FIGURE 14.12 Industrial L-dopa production and process material index evaluation. The one pot biotransformation of Ajinomoto was the greenest according to PMI calculations.

14.4.3 New Enzymatic Reactions

New enzymatic selectivities are a source for innovation in industrial biotransformation. Nevertheless the asymmetrication of prochiral compounds or deracemization of reacemates still dominate the industrial sector. The stereo selectivity remains the most remarkable capacity of enzyme catalysts. Other selectivities of industrial interest are chemoselective oxidation and the regioselectivity of hydrolases. The industrial enzymatic catalysis has not advanced very much in recent years because chemical production is very competitive, as long as stereochemistry and protection group chemistry are not complicating syntheses. Enzyme use in commodity manufacturing opens new fields for novel biotransformations. However, only a limited number of successful production processes are described. A long-standing example is the saccharification of corn starch into glucose, fructose, and bioethanol. With the emergence of biorefineries, biotransformation of renewable commodities into products is an option, since many of the produced renewables are polyfunctional and enzymes are the logical catalysts for their conversion into fine chemicals. The simplest commodities with a large future market are biodiesel, surfactants, and lubricants. On the other side, because chemical manufacturing is much cheaper and the catalytic step is just one element in the production process, the current state of these enzymatic catalysis' demonstrate how much still needs to be done to develop cost-effective processes for bulk chemical production.

14.4.3.1 Enzymatic C-C Bond Formation

There are enzymes that catalyze nonnatural reactions, also called promiscuous enzyme reactions. Most interestingly C-C bond forming organic Name-Reactions were catalyzed by enzymes. These comprise aldol couplings with the following condensation, Michael additions, Mannich reactions, Henry reactions, and Knoevenagel condensations. Such reactions are catalyzed by lipases, proteases, nucleases, acylases, and transglutaminases (Miao et al., 2015). In addition, they revealed surprisingly high enantioselectivities. Michael additions, Mannich reactions, Henry reactions, and Knoevenagel condensations are extremely rare or nonexistent in natural systems while aldolases are found as biocatalysts in nature (Ferrara et al., 2011).

14.4.3.2 Size Selectivity in Lipase-Mediated Inverse Hydrolysis

Size selectivity is, in comparison to stereoselectivity or regioselectivity, less researched. The size selectivity of enzymes can be engineered (Brundiek et al., 2012). Size selectivity is a means to tailor or select fat lubricant molecules. For example, a chiral pentaeryritol was partially or fully esterified with oleic acid using *Candida antarctica* lipase B. The tetrol was esterified three or four times depending on the applied conditions. The on/off size selectivity was stimulated in the presence and absence of tert-butanol. With solvent conditions the triester was accumulated in excess, while under solvent-free conditions the selectivity was lost and no mono- and diesters were obtained (Happe et al., 2014).

14.4.3.3 Redox Enzyme Cascade for Profenol Deracemization

Chemically produced racemic profenol needs to be deracemized before further use (Díaz-Rodríguez et al., 2015). In a first step profenol was oxidized to the aldehyde using laccase/air and TEMPO as mediator (Fig. 14.13). This oxidation generated also some carboxylic acid with subsequent decarboxylation. The obtained profenol racemized spontaneously and an aldehydedehydrogenase reduced it to enantiomerically pure profenol (with 94% ee). The *R* and *S* enantiomers where both accessible due to the use of a *R* or *S* selective aldehydedehydrogenase. The process is potentially very useful in similar situations, such as ibuprofen and naproxenol reacemization, but solubility problems prevented for the moment the application in larger processes. All in all, the combination of a laccase with an aldehydedehydrogenase is an interesting option in the deracemization of 2-methyl-alkan-1-ol motifs.

14.4.3.4 Direct Oxidation of Alkanes and Cycloalkanes

The C–H activation of alkanes is an old stimulating challenge in metalorganic chemistry and today is also investigated for enzymatic catalysis to achieve a so-called “dream reaction” (Staudt et al., 2013). The enzyme for such an outstanding biocatalytic oxidation is P450-monoxygenase, which ideally uses pure air as an oxygen source. The hydroxylation of cyclohexane is among the most interesting objectives in view of subsequent possible biotransformations (Fig. 14.14). The next step of broad interest is cyclohexanone formation, which is well possible. Cyclohexanone itself is further transformed by the biocatalytic Bayer–Villiger oxidation into a lactone, an important precursor for industrial polymers such as Nylon-6 and others. This process is described below.

14.4.3.5 Enzyme Cascade Synthesis of Oligo ϵ -Caprolactone

Polymeric ϵ -caprolactone is a bulk chemical produced today by using peroxy acids, which results in toxic wastes. A greener process is sought and the

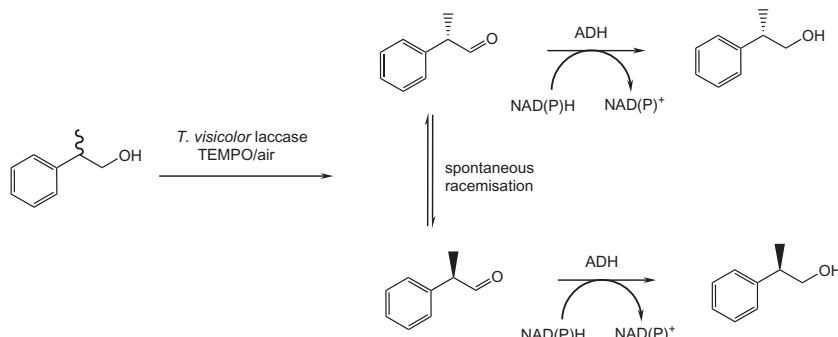


FIGURE 14.13 Deracemization of chemically produced racemic profenol: Laccase oxidation yielded profenol that spontaneously racemized and stereo selective reduction with an aldehydedehydrogenase provides chiral profenol with either *R* or *S* chirality.

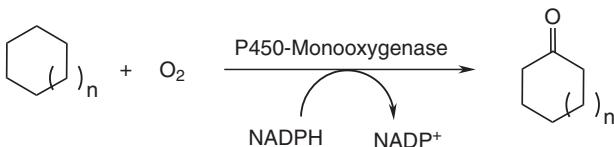


FIGURE 14.14 Direct oxidation of cyclohexanes by P450-monoxygenase and oxygen from air is an attractive alternative to chemical oxidation.

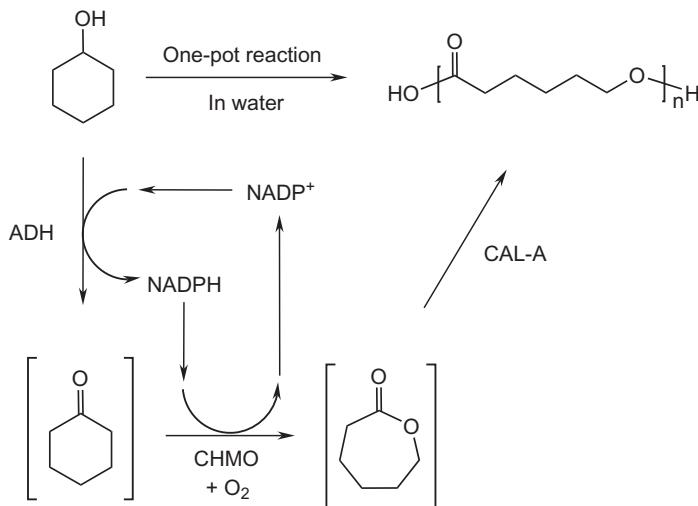


FIGURE 14.15 The one pot oligocaprolactone synthesis using an enzyme cascade consisting of cyclohexane monooxygenase (CHMO), alcoholdehydrogenase (ADH), *Candida antarctica* Lipase A (CAL-A). No product inhibition was observed from the lactone intermediate.

enzymatic Bayer-Villiger oxidation is an envisaged possibility but suffers from inhibition and low efficiency. Combining an alcohol-dehydrogenase, a cyclohexanone monooxygenase, and a *Candida antarctica* lipase the process could overcome these limitations. The inhibiting ϵ -caprolactone was directly transformed to its oligomeric polyester and the inhibition was overcome (Fig. 14.15). The resulting oligo ϵ -caprolactone is then easily polymerized into bulk polymers by chemical catalysis (Schmidt et al., 2015). The cofactor NADPH/NADP⁺ is regenerated within the reaction cascade, however, an additional regeneration step was integrated to enhance efficiency. This shows that cofactor regeneration is another key problem that needs to be tackled when working with redox enzymes.

14.4.4 Cofactor Regeneration

Cofactors in enzymatic redox catalysis need to be regenerated continuously. This renders enzymatic redox catalysis more challenging than, for example,

using hydrolases. Progress in cofactor regeneration has been sought for a long time to reduce the related complexity in reaction design and improve atom economy by eliminating auxiliary substrate use and the need of a regeneration enzyme. In some cases cofactors are recycled within a reaction cascade, as was the case in the aforementioned oligo ϵ -caprolactone synthesis and other examples. But even in such synergistic recycling systems, cosubstrate and recycling enzymes are needed as electron transfer efficiencies lag well behind theory. The state-of-the-art in large-scale regeneration systems is using whole cell and crude cell extracts, which are currently the most effective approaches in working with redox enzymes (Dascier et al., 2014). Electrocatalytic cofactor regeneration is an interesting alternative to whole cell and cell extract use, as atom economy proved to be unbeatable as no cosubstrate is needed. Moreover, side reactions are reduced and work-up is easier and therefore greener. However, there is no breakthrough in electrocatalytic cofactor regeneration so far. The major hindrance is that electrode cofactor interaction is a diffusion-limited process. The emergence of bioelectrical systems in wastewater treatment and other applications provide more and more insight into how low-power electrochemical devices work efficiently. This will potentially influence the research in electrochemical cofactor regeneration (Ruinatscha et al., 2014). A direct use of bioelectrical systems, such as microbial fuel cells, is therefore proposed as it transforms organic compounds into protons and electrons, which are then transferred into the cathode and used there for cofactor or mediator reduction (Fischer et al., 2012). Another idea is to regenerate cofactors by sun light as proposed by Choudhury et al. (2014) who examined regeneration of NADPH photo-catalytically on graphene electrodes to reduce ketones into chiral alcohols. This sun light powered reduction was also considered for CO₂ transformation into formic acid and subsequent product generation (Yadav et al., 2012).

14.5 GREEN SOLVENTS FOR BIOTRANSFORMATION

14.5.1 Green Neoteric Solvents

Modern green solvents potentially enhance sustainability in biotransformation. Besides water, green solvents are needed in specific consumer product syntheses, such as cosmetics. Green solvents are more expensive than petrol derived organic solvents but higher prices are accepted for cosmetics. The definition of what is a green solvent, however, is far less clear than one thinks. This is based on the fact that the synthesis pathways for their generation become more and more elaborate but greenness may be lost with some multistep procedures. A good example for the complexity of what is green are chemically produced ionic liquids which are considered to be green solvents as they do not evaporate and in some cases improve enzyme stability and accelerated enzymatic reactions (Moniruzzaman et al., 2010; Fischer et al., 2011).

Another emerging class of green solvents are supercritical fluids like scCO₂. Once the catalysis is terminated CO₂ is evaporated through the opened reactor

vessel. The CO₂ recovery is technically not resolved for small-scale chemical production. But one may expect that CO₂ will become easily recoverable as CO₂ concentrators are a widely investigated subject for gas to fuel production. Another class of neoteric solvents are perfluorinated hydrocarbons, which has gained not too much attention in the past decade. This is certainly due to the difficult handling of the Teflon-like liquids and the stability of perfluorinated compounds, which are even found in food products. The origin of this pollution problem is not well understood but as perfluorinated compounds do not degrade fast enough in nature, they remain in the environment and accumulate in the food chain (Martin et al., 2013).

Simpler solvents are nonvolatile liquid polymers, such as polyethyleneglycols (PEG), produced in large quantities but not widely described for biotransformation use. Last but not least, solvent-free processing is an option applicable to a small number of products and applications, such as biolubricants, surfactants, and biodiesel manufacturing. All in all, a solvent should be easily removable from products to be useful in manufacturing.

14.5.2 Biomass Derived Organic Solvents

Organic solvents derived from biomass are considered also as green solvents, but pose the typical hazards of organic solvents (Fig. 14.7). They are of interest as they are well compatible to industrial manufacturing in contrast to many aforementioned neoteric solvents. They are extracted from food waste or produced by microbial cultivations (Paggiola et al., 2014). Such solvents are for example p-cymene and limonene which were extracted from citrus fruit waste (Fig. 14.16). 2-Methylfuran is another organic solvent that was obtained from lignocellulosic biorefining (De et al., 2015). A very often used solvent is acetone, which is considered green when produced by the ABE fermentation

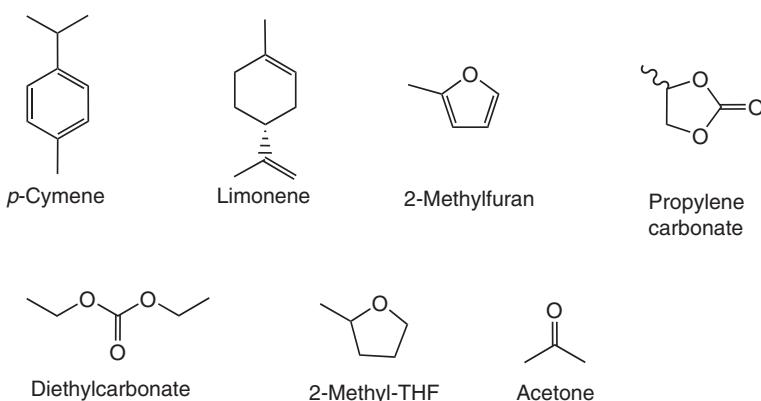


FIGURE 14.16 Bioderived organic solvents from fruit waste, wood, and microbial cultivation.

(Gao and Rehmann, 2014). Recent research shows that even *Clostridium* sp. MAceT113 produces acetone in high excess (Berzin et al., 2012). As indicated, acetone is a versatile solvent and broadly applied in lipase catalysis but also useful for product isolation, as it is easily removed by evaporation from reaction mixtures (Fischer et al., 2013).

14.6 CONCLUDING REMARKS

Biotechnology is becoming increasingly relevant, and it generates over \$250 billion worth of products through biosynthesis or fermentation, biocatalysis, and biotransformation. However, the latter two have more the character of an important technological niche in organic chemical synthesis due to two reasons. First, abiotic methods will continue to deliver the bulk of molecules, leaving the introduction of specificity into molecules to enzymes. Second, the ideal manufacturing method (if possible) is the total biosynthesis by fermentation by advanced genetic engineering and synthetic biology, which will radically change the way we produce chemicals in the longer term future. Hydrolytic enzymes continue to lead the list of the most frequently used enzymes. Enzymes from the other five classes are scarce for biotechnological applications, but novel advances in biochemistry, microbiology, protein engineering, and biotechnology are helping to increase the diversity of the commercially available enzyme toolbox. Prokaryotes are a rich source of industrially interesting enzymes, yet less than 1% of these organisms have been cultivated and explored for novel enzymes. Enzyme bioprospecting by using microbiological techniques for cultivation and functional enzymatic screenings, or functional metagenomics remain important tools to identify and isolate truly novel enzymes from nature. Extreme environments are a particularly promising natural habitat to find new enzymes for industrial organic chemical synthesis. Immobilization allows not only cost advantages but also facilitates separate cascade reactions with different enzymes. Finally, enzyme catalysis can increase greenness in industrial chemical synthesis, when measured with assessment tools such as E-factor, process material impact, life cycle analysis, or environmental impact assessments.

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Chapter 15

Microbial Enzymes for Glycoside Synthesis: Development of Sucrose Phosphorylase as a Test Case

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15.1 INTRODUCTION

The attachment of a carbohydrate moiety drastically changes the physicochemical and biological properties of small organic molecules. Consequently, glycosylation can be used to enhance the solubility of hydrophobic compounds, to improve the pharmacodynamics of drugs, to optimize the activity spectrum of antibiotics, and to modulate flavors and fragrances (Fig. 15.1). This is nicely illustrated by flavonoids (eg, quercetin) and stilbenoids (eg, resveratrol), as their pharmaceutical properties can only be efficiently exploited in the form of hydrophilic derivatives (Křen, 2008). Glycosylation can also be used to improve the stability of labile molecules. A famous example is ascorbic acid, a very sensitive vitamin whose long-term storage can be increased by glycosylation, resulting in high-value applications in cosmetics and tissue culture (Yamamoto et al., 1990a,b). Another important application of glycosylation is the reduction in skin irritation caused by hydroquinone and it is also useful in cosmetics for its skin whitening effect (Kurosu et al., 2002). Glycosides with flavors and fragrances, in turn, can function as controlled release compounds. The α -glucoside of L-menthol, for example, is only slowly hydrolyzed in the mouth, resulting in a prolonged sensation of freshness (Nakagawa et al., 1998). Most importantly, it has been possible to modulate the activity spectrum of glycopeptide antibiotics by varying their carbohydrate moiety, in a technique known as “glycorandomization” (Křen and Rezanka, 2008).

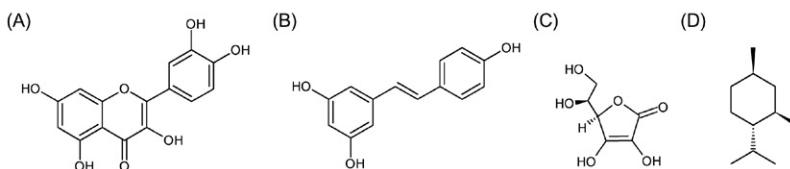


FIGURE 15.1 Some interesting targets for glycosylation: (A) the flavonoid quercetin; (B) the stilbenoid resveratrol; (C) the antioxidant ascorbic acid; and (D) the flavoring agent menthol.

Glycosylation reactions can be performed by means of conventional chemical catalysis, but this suffers from a number of serious drawbacks ([Nicolaou and Mitchell, 2001](#)). First of all, the need for tedious activation and protection procedures results in long multistep synthetic routes with a low overall yield. Furthermore, the enantioselectivity is difficult to control and a mixture of α - and β -glycosides is, therefore, often produced. Finally, the chemical processes require the use of toxic catalysts and generate a considerable amount of waste, thereby causing adverse effects to the environment. To overcome these limitations, the use of enzymes would be a major step forward. [De Roode et al. \(2003\)](#) calculated that enzymatic glycosylation reactions generate 5-fold less waste and have a 15-fold higher space-time yield, a tremendous improvement in eco-efficiency. However, enzymes are not yet routinely applied for glycoside synthesis at an industrial scale, in contrast to the opposite hydrolytic reaction (cleavage of glycosidic bonds with water as acceptor substrate).

There are basically four different types of carbohydrate-active enzymes (CAZymes) that can be used for glycoside synthesis, each with specific characteristics ([Seibel et al., 2006](#)) (Fig. 15.2). Nature's catalysts for such reactions are known as "Leloir" glycosyl transferases (GTs). Although very efficient, these enzymes require expensive nucleotide-activated sugars (eg, UDP-glucose) as glycosyl donors, which hamper their industrial application. However, two special types of GTs are the proverbial "exception to the rule" and are active with low-cost donors. Glycoside phosphorylases (GPs), on the one hand, only require glycosyl phosphates (eg, glucose 1-phosphate) as donors, compounds that can be easily obtained in large quantities. Transglycosidases (TGs), on the other hand, even employ nonactivated carbohydrates (eg, sucrose) for the transfer of a glycosyl group. Additionally, glycoside hydrolases (GHs) can also be used for synthetic purposes, when applied under either kinetic (transglycosylation) or thermodynamic (reverse hydrolysis) control.

In recent years, GP enzymes have attracted a lot of attention because they can produce various di- and oligosaccharides in large amounts ([Puchart, 2015](#)). However, sucrose phosphorylase (SP) is the only GP specificity with known activity toward noncarbohydrate acceptors ([Goedl et al., 2010](#)). Indeed, this enzyme is able to glycosylate aliphatic, aromatic and sugar alcohols, ascorbic and kojic acid, furanones and catechins ([Kitao and Sekine, 1994b](#); [Kitao et al.,](#)

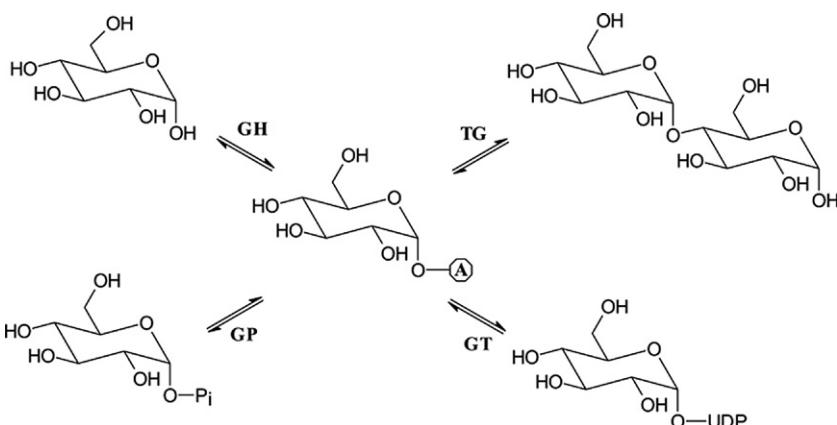


FIGURE 15.2 Four types of enzymes capable of synthesizing glycosidic bonds (GH: glycoside hydrolase, TG: transglycosidases, GP: glycoside phosphorylase, GT: glycosyl transferase, A: acceptor).

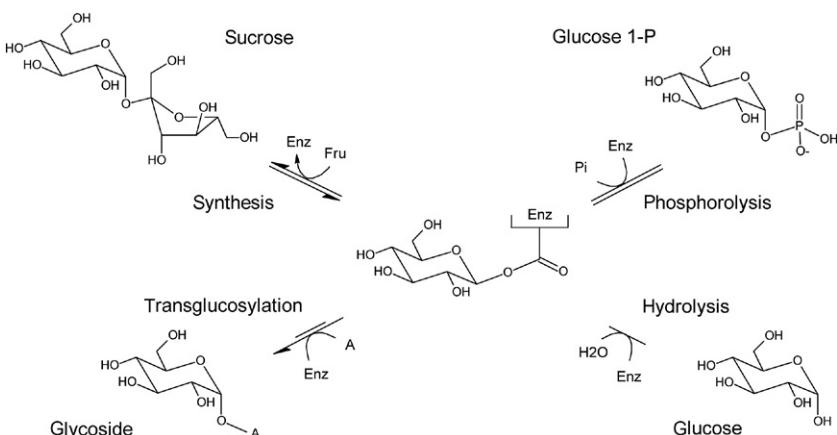


FIGURE 15.3 Different reactions catalyzed by SP. Through a double displacement mechanism, the glucosyl moiety of sucrose can be transferred to a variety of acceptors.

1993, 1995; Kwon et al., 2007; Mieyal et al., 1972). Even a carboxyl group (eg, acetic and caffeic acid) can be used as a point of attachment, resulting in an ester instead of an ether bond (Shin et al., 2009; Sugimoto et al., 2007). Because SP follows a double displacement mechanism, it can also be applied as a transglycosidase without the participation of (glycosyl) phosphate. In that case, sucrose is used as donor substrate, from which a glucosyl group is transferred to an acceptor substrate (Fig. 15.3). It should be noted that sucrose not only is cheap and abundant, it is also very reactive and has an energy content that is comparable with that of nucleotide sugars (Monsan et al., 2010; Skov et al., 2006). High

yields can thus be obtained, as has already been demonstrated for the production of 2-*O*-(α -D-glucopyranosyl)-*syn*-glycerol (Goedl et al., 2008). This product is commercially available under the trade name Glycoin and can be used as a moisturizing agent in cosmetic formulations.

Although the glycosylation potential of SP is very interesting, obtaining high yields with noncarbohydrate acceptors is always a challenge because of the parasitic hydrolytic reaction that consumes the donor substrate (Fig. 15.3). That is particularly true for hydrophobic acceptor molecules, which are not able to compete with water because of solubility limitations. To develop SP into a practical biocatalyst for glycoside synthesis, significant efforts thus needed to be devoted to protein and/or process engineering. In this chapter, a work plan will be discussed that was used successfully to optimize the glycosylation potential of SP, by addressing the different aspects of development in a systematic and chronological fashion.

15.2 A PIPELINE FOR ENZYME DEVELOPMENT

A general overview of the pipeline for enzyme development in this direction is given in Fig. 15.4, and the different steps are mentioned below.

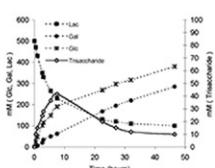
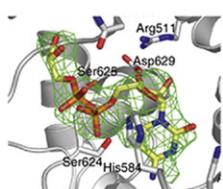
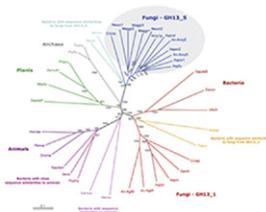
Exploration of natural diversity—A thorough evaluation of the SP enzymes available in nature was first performed to identify the best starting point for further development. To that end, homologous sequences were selected from different microbial phyla and recombinantly expressed in *E. coli*. The resulting enzymes were then compared with respect to expression, protein stability, and acceptor promiscuity.

Improvement of protein stability—The most promising enzyme was then used as starting point for engineering the stability at high temperatures (needed to avoid microbial contamination) and in the presence of organic cosolvents (needed to solubilize hydrophobic acceptors). A stable enzyme also provides a more robust template for the subsequent engineering of specificity, and should thus be obtained before the latter is addressed.

Improvement of substrate specificity—Next, the most stable sequence was used as a template for engineering the substrate specificity. To that end, a hotspot analysis was first performed by substituting specific amino acids in the active site (alanine scanning) and by varying the structure of the substrate (building blocks). This information was then exploited for the rational redesign of the active site to enable binding of polyphenolic acceptors with useful affinities.

Optimization of reaction conditions—Finally, a cosolvent system was developed to enable the use of higher acceptor concentrations, thereby favoring the transglycosylation reaction over the competing hydrolytic reaction. Furthermore, these solvents also formed the basis of a simple purification procedure, which always is a critical step for cost-efficient production.

All these steps are now discussed in detail in separate subsections.



Exploring natural diversity

- selection of diverse representatives
- evaluating stability and promiscuity
- identifying most promising sequence



Improving protein stability

- avoid denaturation under process conditions
- enable long-term use of the biocatalyst
- robust template for specificity engineering



Improving substrate specificity

- performing a hotspot analysis
- rational design of mutant (libraries)
- evaluating product yield and purity



Optimizing reaction conditions

- evaluating medium compositions
- developing a downstream process
- delivering proof-of-concept

FIGURE 15.4 A general pipeline for enzyme development. These steps have been successfully applied to SP, but should also work with other enzyme types.

15.2.1 Exploring Natural Diversity for SP

Already more than 70 years ago, SP activity was demonstrated for the first time in cultures of *Leuconostoc mesenteroides* (Kagan and Latker, 1942). Since then, the SP from *L. mesenteroides* has been intensively studied (Goedl et al., 2010) and it is currently employed for the industrial production of glucosyl glycerol, a natural osmolyte which is commercially available under the trade name Glycoin (Goedl et al., 2008). In the meantime, SP enzymes from other

organisms like *Streptococcus mutans* (Ferretti et al., 1988; Robeson et al., 1983; Russell et al., 1988), *Lactobacillus acidophilus* (Aerts et al., 2011a,b), *Pelomonas saccharophila* (*Pseudomonas saccharophila*) (Doudoroff, 1943; Doudoroff et al., 1943), *Bifidobacterium adolescentis* (Van Den Broek et al., 2004), *Bifidobacterium longum* (Choi et al., 2011), and an uncultured sample of a metagenomes analysis of a sucrose-rich environment (Du et al., 2012) have been characterized, but the SP from *L. mesenteroides* is still the most widely used. The preference for this enzyme is, however, historical rather than rational, and therefore expression, stability, and substrate scope of SPs from different sources were compared to find the most suitable for industrial purposes.

High-level expression of soluble protein is not only a prerequisite for the development of a cost-effective process, but also facilitates screening for mutants with improved properties. Expression is influenced by several parameters like host organism, codon usage, temperature, time, and type and strength of the promoter. Microbial enzymes are typically recombinantly expressed in *E. coli* or *Bacillus* and codon optimized synthetic genes are nowadays readily available at affordable prices. Optimizing the other parameters or comparing different enzymes can easily be performed in 24- or 96-deep well plates, since similar growth conditions in these minicultures as in Erlenmeyer flasks can be achieved (Duetz, 2007; Duetz et al., 2000; Waegeman et al., 2010). That way, timescales can be significantly reduced. A strong promoter is often the first, but not always the best choice for soluble expression, and especially for minicultures (eg, for screening) it can be advisable to lower the promoter strength (Makrides, 1996; Sorensen and Mortensen, 2005). Several inducible promoters are available (eg, with IPTG, rhamnose or arabinose) (Tawfik, 2003), but a convenient alternative is a constitutive expression system. Indeed, the amount of consumables and the number of manipulation steps is lower while the ease of use is higher, which is particularly useful for high-throughput screening (Aerts et al., 2011a). To evaluate the expressing capability and to find out the optimal conditions for different SP enzymes (from *L. mesenteroides* ATCC 12291, NRRL B1355 and B1149 (LMG 18967) (LmSP), *L. acidophilus* LMG 9433 (LaSP) and *B. adolescentis* LMG 10502T (BaSP)), 4 constitutive promoters were selected from a set of 54 with gradually varying strength: a strong (P14), 2 intermediate (P34 and P22), and a weak promoter (P78) (Aerts et al., 2011a; De Mey et al., 2007). Additionally, three different temperatures (25, 30, and 37°C) were evaluated for each promoter-gene combination. The optimal conditions were derived from 96-deep well experiments and these were found to be different for each of the enzymes (Fig. 15.5). *B. adolescentis* and *L. acidophilus* SP's were expressed most efficiently with a strong promoter, at respective temperatures of 37°C and 30°C, while intermediate promoters and a temperature of 25°C was preferred for those from the *L. mesenteroides* strains. The profit of varying promoter strength and temperature is nicely illustrated by these latter. For the same temperature, changing the strength from strong to intermediate resulted in up to five times more protein, while the same could be achieved by lowering

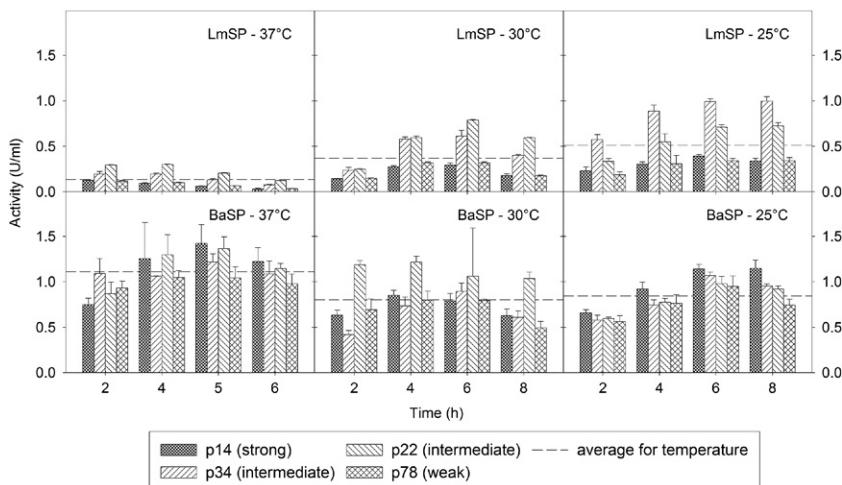


FIGURE 15.5 Total activity of SPs from *B. adolescentis* and *L. mesenteroides* ATTC 12291, expressed with different promoters and at different temperatures.

the temperature from 37°C to 25°C. Nevertheless, the highest level of soluble protein was observed for SP from *B. adolescentis*.

The next step in the evaluation process was to explore the substrate scope of the different SP enzymes. The acceptor promiscuity of SP was already recognized in 1944 by Doudoroff and colleagues, who found that L-sorbose could act as an alternative acceptor (Doudoroff et al., 1944). Thereafter, SP's ability to glycosylate other compounds was further investigated, and observed that transfer activity of SP on a wide variety of mono-, di-, and trisaccharides (Kitao and Sekine, 1992; Lee et al., 2006) and noncarbohydrates molecules like (poly) phenolics, furanones, and even carboxylic acids (Goedl et al., 2010; Kitao and Sekine, 1994a; Kitao et al., 2000; Shin et al., 2009) was demonstrated by different groups. Such developments were excellently reviewed by Goedl et al. (2010) and the information are valuable; still the data are heterogeneous and difficult to compare, because experiments were performed with varying concentrations of enzyme, donor, and acceptor, or at a different pH and temperatures. Moreover, transglycosylation activity was usually expressed as transfer ratio or yield. This parameter, however, reflects the thermodynamic equilibrium of the substrate and product and does not provide any information about the efficiency of the enzyme itself. We therefore performed an extended study comparing the initial transglycosylation velocities of six different SP enzymes on 80 putative acceptor molecules, all under the same conditions, and showed that despite the sometimes high transfer ratios reported, almost all compounds are poor substrates in terms of catalytic activity (Aerts et al., 2011b). Indeed, for all six SP enzymes, only on L-arabinose and L-sorbose was considerable activity measured, while the vast majority of the acceptors were glycosylated very slowly, at a rate that

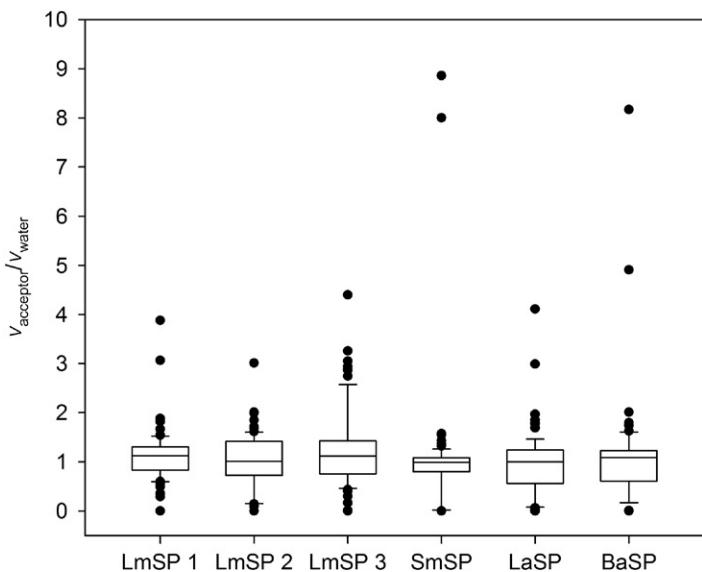


FIGURE 15.6 Relative transglucosylation activity on 80 different acceptors, with hydrolysis as reference ($v_{\text{acceptor}}/v_{\text{water}}$) (at 37°C and pH 7; LmSP1: SP from *L. mesenteroides* NRRL B1149, LmSP2: SP from *L. mesenteroides* ATTC 12291, LmSP3: SP from *L. mesenteroides* B1355, SmSP: SP from *S. mutans* SP, LaSP: SP from *L. acidophilus*, BaSP: SP from *B. adolescentis*).

was comparable to the contaminating hydrolytic side reaction (Fig. 15.6), (Table 15.1). None of the SP's is thus more promiscuous than the other nor displays a higher activity toward alternative acceptors.

During the screening, relatively low concentrations (1.5–65 mM) of acceptor were used, because phenolic acceptors are typically very insoluble. At these conditions, the unwanted hydrolytic side reaction was unfortunately very prominent. This was for instance also the case for glycerate where the relative transglycosylation activity was only 1.4. Optimization of the reaction conditions (0.8 M sucrose and 0.3 M glycerate), however, could increase the yield to 91% (Sawangwan et al., 2009). A similar finding was observed for the glycosylation reaction of glycerol, where 2M of acceptor was necessary to yield quantitative conversion (Goedl et al., 2008). This shows that improving the substrate concentration could have a profound influence on the transglycosylation over hydrolysis ratio, a topic that we wish to discuss further in this chapter.

Stability is another important property of industrial biocatalysts and especially for industrial carbohydrate conversions since these are preferably performed at 60°C, mainly to avoid microbial contamination (Bruins et al., 2001; Eijsink et al., 2005; Vieille and Zeikus, 1996). All currently known SPs, however, originate from mesophilic organisms and accordingly have optimal temperatures of 30–37°C and half-lives of less than a minute at 60°C (Goedl et al.,

TABLE 15.1 Relative Transglucosylation Activity on Phenolic Acceptors, with Hydrolysis as Reference ($v_{\text{acceptor}}/v_{\text{water}}$)

Acceptor	LmSP1	LmSP2	LmSP3	SmSP	LaSP	BaSP
Water	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.13	1.00 ± 0.14	1.00 ± 0.12
Phenol ^a	0.74 ± 0.06	0.77 ± 0.07	0.69 ± 0.06	0.88 ± 0.04	0.61 ± 0.11	0.89 ± 0.09
Catechol ^b	0.25 ± 0.09	0.35 ± 0.04	n.d.	n.d.	n.d.	n.d.
Resorcinol ^a	0.85 ± 0.08	0.60 ± 0.10	0.97 ± 0.07	n.d.	1.41 ± 0.05	n.d.
2-Phenylethanol ^c	0.75 ± 0.21	0.81 ± 0.18	0.83 ± 0.13	0.87 ± 0.26	0.75 ± 0.07	0.52 ± 0.06
2-Nitrophenol ^d	0.58 ± 0.11	1.14 ± 0.09	0.73 ± 0.04	0.78 ± 0.24	0.82 ± 0.02	0.61 ± 0.07
p-Nitrophenol ^a	n.d.	0.55 ± 0.12	1.28 ± 0.10	n.d.	0.76 ± 0.02	n.d.
2-Phenylphenol ^d	0.70 ± 0.09	1.31 ± 0.25	1.69 ± 0.19	0.62 ± 0.11	0.48 ± .02	0.58 ± 0.07
4-Phenoxyphenol ^d	0.52 ± 0.12	0.69 ± 0.06	2.53 ± 0.07	1.57 ± 0.14	0.62 ± 0.03	0.63 ± 0.11
p-Hydroxybenzoic acid methylester ^a	0.54 ± 0.12	0.49 ± 0.08	0.58 ± 0.04	0.98 ± 0.08	0.56 ± 0.05	0.40 ± 0.03
3-Hydroxybiphenyl ^d	0.70 ± 0.04	0.72 ± 0.12	0.76 ± 0.03	0.83 ± 0.14	0.62 ± 0.02	0.39 ± 0.04
p-Hydroxybenzoic acid ^a	n.d.	1.13 ± 0.17	0.47 ± 0.10	n.d.	0.57 ± 0.05	n.d.
3,4-di-Hydroxybenzoic acid ^a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Salicylic acid ^a	0.12 ± 0.03	0.35 ± 0.05	0.85 ± 0.07	0.82 ± 0.07	0.67 ± 0.03	1.80 ± 0.14
Shikimic acid ^e	1.33 ± 0.08	1.32 ± 0.14	1.33 ± 0.12	0.99 ± 0.05	0.86 ± 0.08	0.78 ± 0.04
Gallic acid ^a	n.d.	0.65 ± 0.13	0.30 ± 0.06	n.d.	0.18 ± 0.15	n.d.

(Continued)

TABLE 15.1 Relative Transglucosylation Activity on Phenolic Acceptors, with Hydrolysis as Reference ($v_{\text{acceptor}}/v_{\text{water}}$) (Continued)

Acceptor	LmSP1	LmSP2	LmSP3	SmSP	LaSP	BaSP
Ethyl Gallate ^b	0.85 ± 0.15	0.69 ± 0.05	n.d.	n.d.	n.d.	n.d.
Vanillin ^a	0.61 ± 0.01	0.74 ± 0.05	0.66 ± 0.01	0.79 ± 0.06	0.26 ± 0.04	0.82 ± 0.01
Pyridoxine ^a	n.a.	n.a.	0.99 ± 0.08	0.83 ± 0.08	0.21 ± 0.08	0.77 ± 0.12
3,4-Dimethoxybenzyl-alcohol ^a	0.90 ± 0.11	1.51 ± 0.08	1.37 ± 0.20	1.43 ± 0.04	1.05 ± 0.12	0.56 ± 0.06
p-Hydroxy-benzoic acid n-butyl ester ^d	0.70 ± 0.11	0.87 ± 0.12	0.60 ± 0.13	0.92 ± 0.25	n.d.	0.46 ± 0.08
Daidzein ^e	0.70 ± 0.08	1.09 ± 0.12	0.75 ± 0.33	0.79 ± 0.19	n.d.	0.43 ± 0.05

37°C, pH 7.0, 50 mM sucrose as donor; LmSP1: SP from *L. mesenteroides* NRRL B1149, LmSP2: SP from *L. mesenteroides* ATTC 12291, LmSP3: SP from *L. mesenteroides* NRRL B1355, SmSP: SP from *S. mutans* SP, LaSP: SP from *L. acidophilus*, BaSP: SP from *B. adolescentis*.

Acceptor concentration:

^a65 mM,

^b32.5 mM,

^c15 mM,

^d25 mM,

^e1.5 mM.

n.d.: no detectable activity;

n.a.: data not available.

2010). The only exception is the SP from *B. adolescentis*, which has a remarkable optimal temperature of 58°C and a half-life at 60°C of 12–24 h (depending on the protein concentration) (Cerdobbel et al., 2010b; Cerdobbel et al., 2011). The enzyme is, in addition, also more stable in organic cosolvents like ethanol, isopropanol, and DMSO, making it an attractive biocatalyst for glycoside synthesis where cosolvents are often required to dissolve the hydrophobic acceptor compounds (Aerts et al., 2011b). In conclusion, the SP from *B. adolescentis* is the most solvent and thermostable, is easily expressed, and displays (low) activity on a variety of compounds and was therefore selected for further process development.

15.2.2 Improvement of Protein Stability

To ensure the robustness of an industrial process, a biocatalyst should be as stable as possible. In case of SP, stability could be drastically improved by means of immobilization (Cerdobbel et al., 2010a,b) and mutagenesis (Cerdobbel et al., 2011). Multipoint covalent immobilization on amino-epoxy (EC-HFA) Sepabead carriers is capable of increasing the optimal temperature from 58°C to 65°C and of broadening the pH range (Fig. 15.7) (Cerdobbel et al., 2010b). The immobilized enzyme retained 65% of its activity after 16 h of incubation at 60°C, and even 75% in the presence of 500 mM sucrose. Many factors influence the efficiency of immobilization (Mateo et al., 2007) and therefore pH and ionic strength of the immobilization buffer as well as temperature were first carefully optimized. Under the best suited conditions, a yield of 70% was obtained based on activity measurements (10% activity was lost during washing steps and 20% due to suboptimal conformation of the protein on the carrier and/or to diffusional problems). The loss of activity caused by immobilization could, however, be compensated by the higher substrate turnover that is achieved at the higher

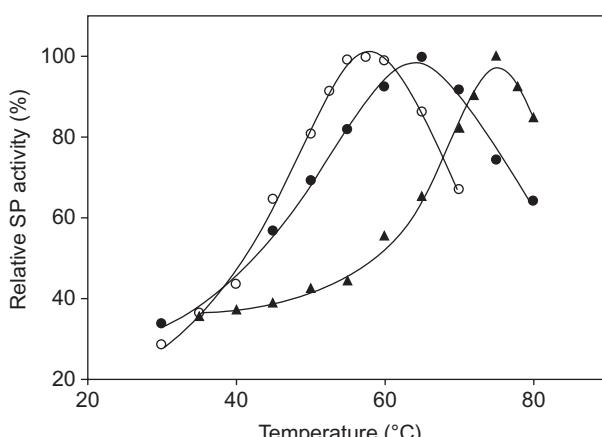


FIGURE 15.7 Temperature profile of SP: the soluble enzyme (open circle), the immobilize (black circle) and the CLEA (triangle).

optimal temperature (65°C compared to 58°C). Immobilization on another type of epoxy Sepabeads (EC-EP) was found to be much less efficient, indicating the importance of selection of the right type of carrier.

A different class of immobilized biocatalysts are cross-linked enzyme aggregates (CLEAs) (Cao et al., 2000). Such preparations are obtained by the physical aggregation of the enzymes followed by chemical cross-linking. This procedure allows enzymes to be immobilized without the use of a carrier, which not only decreases the cost, but also avoids “dilution” of the enzymes’ activity (Cao et al., 2003). CLEAs of *B. adolescentis* SP displayed a temperature optimum of 75°C , which is respectively 17°C and 10°C higher than that of the soluble and the Sepabeads immobilized enzyme (Fig. 2.7) (Cerdobbel et al., 2010a). They furthermore had an exceptional thermostability, retaining all of their activity after 1 week of incubation at 60°C , and could be used in at least ten consecutive batches. Preparation of the CLEAs was done by first precipitating (aggregating) the enzyme with 60% *tert*-butanol without loss of activity (ammonium sulfate, which is a widely used precipitant, did not give satisfactory results) and subsequently cross-links it with glutaraldehyde that contains two aldehyde groups forming imine bonds with lysine residues from two enzyme molecules. The optimal incubation time (1 h) and glutaraldehyde/protein ratio (0.17 mg/mg) were determined and a maximal immobilization yield of 31% was achieved.

Although immobilization is often employed in industrial processes to enable recovery of enzymes, the procedure can be time-consuming and expensive, especially when carriers are involved. Creating inherently stable variants, by means of protein engineering, is thus still an attractive alternative. Furthermore, the resulting proteins can serve as robust templates for altering the specificity, because stable enzymes are more tolerant toward introduction of amino acid substitutions (Bloom et al., 2006; Tokuriki and Tawfik, 2009). Several strategies are available to stabilize proteins, but random mutagenesis followed by screening vast numbers of mutants is still often applied (Bommarius and Paye, 2013; Bornscheuer and Kazlauskas, 2011). The stability of *B. adolescentis* SP is however strongly dependent on the protein concentration (Cerdobbel et al., 2010b) and the varying levels of expression during high-throughput screening complicate the detection of improved variants. It would thus be convenient to limit the number of mutants and therefore more focused approaches were applied. Fortunately, high-resolution crystal structures are available (Mirza et al., 2006; Sprogøe et al., 2004), allowing the rational prediction of (potentially) stabilizing point mutations and identification of positions with high flexibility. Indeed, the latter should be mutated first because they lower the overall stability of a protein (Reetz et al., 2006). Residues that occur more frequently at the corresponding positions in a set of related proteins could be introduced instead, as these have been propagated through natural selection and should thus be favorable to stability and/or activity (Lehmann et al., 2000; Steipe et al., 1994). Next to these so-called “consensus” mutations, amino acid substitutions that

stabilize the network of electrostatic interactions on the protein surface can also be introduced (Fig. 15.8).

Based on these considerations, 14 enzyme variants were created and out of them 5 variants displayed a considerable improvement in thermostability (136–143% residual activity compared to wild type, after 24 h incubation at 60°C) (Cerdobbel et al., 2011). The introduction of consensus residues at the most flexible positions (445–446) had indeed a positive effect, although a pairwise mutation was required. The rational design, in contrast, proved to be a more challenging task. Nevertheless, creating additional salt bridges at the protein surface was found to be a successful strategy in about half of the cases. Particularly, combining all beneficial mutations in a single sequence generated a biocatalyst with a half-life at 60°C of 62 h, which is more than twice as long as the wild-type enzyme (Fig. 15.9). The final variant was moreover not only more thermostable,

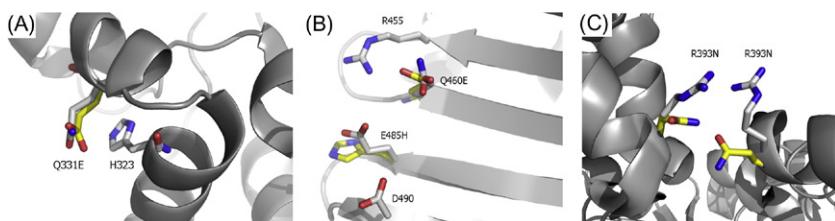


FIGURE 15.8 Rational mutations that increased the stability of SP: (A) Variant I; (B) variant L; and (C) variant N.

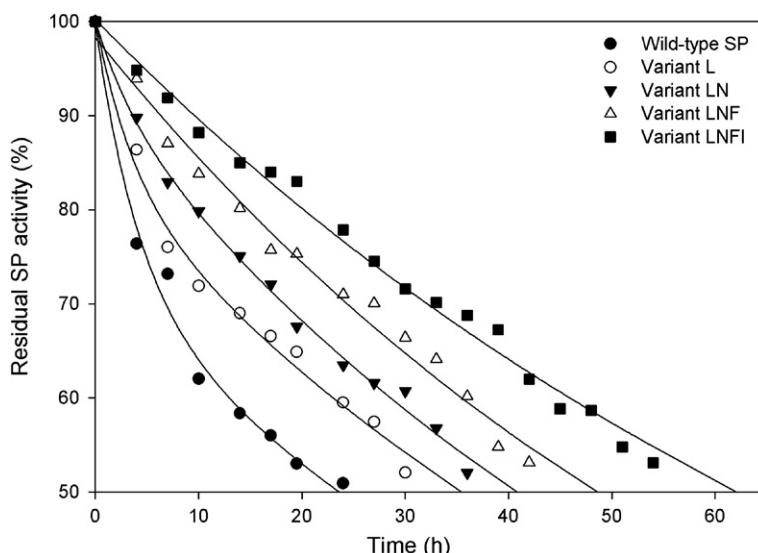


FIGURE 15.9 Kinetic stability at 60°C of improved SP variants.

but also more tolerant to cosolvent and the increased stability did not come at the expense of activity (catalytic activity on sucrose had remained constant).

Consensus mutations at the most flexible positions were found to be effective, albeit only if two positions were mutated together. This suggests a large degree of synergism and hence it could be interesting to extend the principle to the entire protein. Indeed, the semirational consensus approach takes advantage of purifying or negative selection that nature already has applied to naturally occurring sequences (Lehmann et al., 2000; Steipe et al., 1994). Destabilizing random mutations are highly probable and are purged by nature if the overall stability falls below a certain threshold. As a result, residues that stabilize a protein tend to be more prevalent than other amino acids at any given position in a protein family (Steipe et al., 1994). For the full-length consensus design of an SP, known sequences were aligned with the 3DM-software (Kuipers et al., 2009, 2010) and those sharing more than 95% sequence identity were discarded (Aerts et al., 2013). For each position in the multiple sequence alignment, the most occurring amino acid at each position was taken, unless a gap was more prevalent than an amino acid (except for a small highly variable C-domain where the sequence of the closest homolog was used). Consensus sequences were derived from all sequences (74 unique sequences from 37 different species) as well as a subset of lactic acid bacteria-like (20 sequences) or *Bifidobacteria*-like (12 sequences) SPs (Fig. 15.10). These synthetic new-to-nature SP enzymes

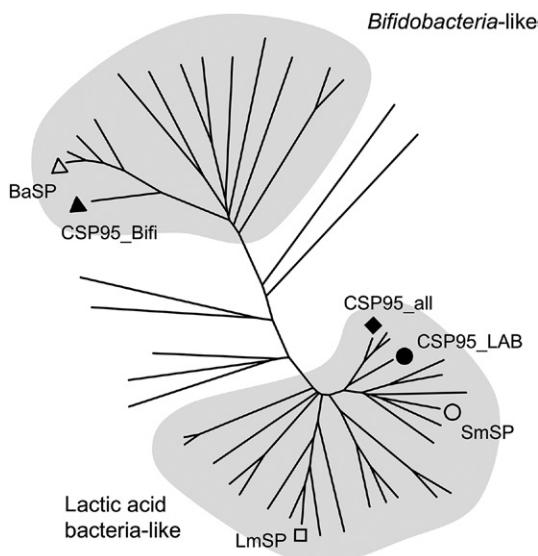


FIGURE 15.10 Phylogenetic tree of SPs used to derive the consensus sequences (LmSP: SP from *L. mesenteroides* ATCC 12291; SmSP: SP from *S. mutans* SP; BaSP: SP from *B. adolescentis*; CSP95_all: consensus SP from all sequences; CSP95_LAB: consensus SP from lactic acid bacteria-like sequences; CSP95_Bifi: consensus SP from *Bifidobacteria*-like sequences).

folded correctly and displayed native-like activity, although they differed from their closest natural homolog at about 70 positions (around 14% of the total sequence). They were more stable than the widely used *L. mesenteroides* SP, but not compared to that of *B. adolescentis* and their stabilities also differed from one another (Table 15.2). The consensus enzymes thus displayed an “average” thermostability, that is, one that is higher than some, but not all known representatives and the difference in thermostability can most likely be attributed to the intrinsic difference in thermostability of the parent sequences in the respective alignments. Nevertheless, this could be an interesting strategy, for example, when no stable variants are known or when a lot of sequences are available but not characterized.

15.2.3 Improvement of Substrate Specificity

Naturally occurring SPs are able to make a glucose moiety attached to a variety of (small) hydroxyl-bearing aromatic compounds, as discussed above. These poorly soluble molecules, however, have to compete with an excess of water to attack the covalent glycosyl-enzyme intermediate (Fig. 2.3) and this has been shown to be difficult. Indeed, transglycosylation often did not exceed hydrolysis and in order to obtain higher product yields the balance should be tilted in favor of the former. One solution could be to increase the affinity for alternative acceptors by changing the biocatalyst’s protein sequence. In the case of SP, the number of mutants that can be screened is however limited, because most acceptors are not compatible with the available assays or transglycosylation is

TABLE 15.2 Thermal Properties of Wild-Type and Consensus SPs

Enzyme	$T_m(^{\circ}\text{C})$	$t_{50}(\text{min})$	$T_{\text{opt}}(^{\circ}\text{C})$
Wild-type			
LmSP	n.a.	<1	37
SmSP	57.6 ± 0.1	3.0	50
BaSP	78.1 ± 0.1	1260	60
Consensus			
CSP95_all	64.1 ± 0.4	7.0	55
CSP95_LAB	60.9 ± 1.0	3.4	50
CSP95_Bifi	68.9 ± 0.2	21.9	60

LmSP: SP from *L. mesenteroides* ATTC 12291; *SmSP*: SP from *S. mutans* SP; *BaSP*: SP from *B. adolescentis*; *CSP95_all*: Consensus SP from all sequences; *CSP95_LAB*: Consensus SP from lactic acid bacteria-like sequences; *CSP95_Bifi*: Consensus SP from Bifidobacteria-like sequences; n.a.: not available.

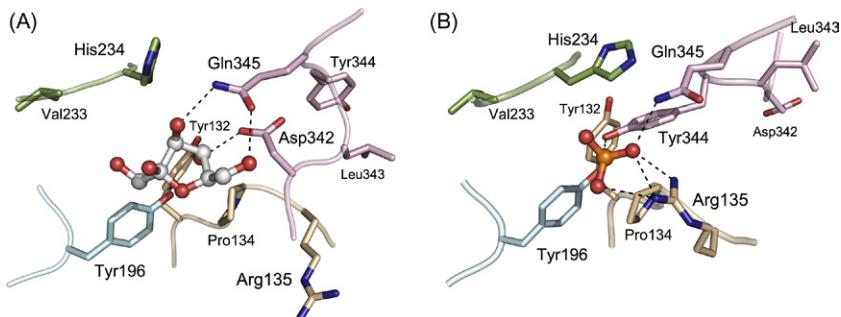


FIGURE 15.11 Acceptor site residues of SP from *B. adolescentis*. Acceptor site loops undergo structural rearrangements to form a (A) fructose (pdb entry 2GDU) or (B) phosphate (pdb entry 2GDV) binding pocket.

too poor to be detected in high-throughput screens. Knowledge of the determinants of substrate specificity would accordingly be very valuable, as this would allow applying (semi)rational mutagenesis strategies. Previously, some structure–activity relationships for acceptors had already been derived (Goedl and Nidetzky, 2010). In addition, a complete map of the acceptor site of the SP from *B. adolescentis* was now created by so-called “alanine scanning” (Fig. 15.11) (Verhaeghe et al., 2013). By replacing each amino acid individually with an alanine, its influence on acceptor specificity could be determined, as this accounts to the removal of the side chain without affecting the main chain conformation (Cunningham and Wells, 1989).

All residues that shape the acceptor site were found to strongly contribute to the affinity for either phosphate (Arg135, Leu343, Tyr344) or fructose (Tyr132, Asp342) or both (Pro134, Tyr196, His234, Gln345), and mutation to alanine did not have a profound influence on the rate of hydrolysis (Table 15.3). Alternative acceptors that are glycosylated rather efficiently (eg, d-arabitol) were found to interact with the same residues as fructose, whereas poor acceptors like pyridoxine do not seem to make any specific interactions with the enzyme. As a consequence, the best engineering strategy to increase the transglycosylation over hydrolysis ratio would probably be to improve the affinity/activity for alternative acceptors (increase transglycosylation) rather than trying to lower the hydrolysis, although that would probably require a drastic remodeling of the acceptor site in most cases. This implies, together with the difficult high-throughput measurement of improved activity, a significant screening effort and therefore it could be wise to explore the possibilities of process engineering first.

Very recently, a new phosphorylase that is involved in a new breakdown route of sucrose has been discovered in *Thermoanaerobacterium thermosaccharolyticum* (Verhaeghe et al., 2014). It actually prefers sucrose 6'-phosphate

TABLE 15.3 Importance of the Acceptor Site Residues for Affinity and/or Activity on Wild-Type and Alternative Acceptors and Water

Residue	Wild-type acceptors		Alternative acceptors			Water
	Fructose	Phosphate	Arabitol	Pyridoxine		
Tyr132	+++	-	-	-	-	-
Pro134	++	+	-	-	-	-
Arg135	-	+++	-	-	-	-
Tyr196	++	++	-	-	-	-
Val233	-	-	-	-	-	-
His234	+++	+++	+++	-	-	-
Asp342	+++	-	+++	-	-	-
Leu343	-	++	-	-	-	-
Tyr344	-	+++	-	-	-	-
Gln345	++	++	++	-	-	-

+++: crucial; ++: important; +: slightly important; -: not important; numbering according to SP from *B. adolescentis*.

as substrate (EC 2.4.1.329), but it can still efficiently use sucrose as glycosyl donor ($K_M=77\text{ mM}$). Its stability is moreover excellent, with an optimal temperature of 55°C and a half-life at 60°C of 50 h, and it can glycosylate a wider range of phenolic substrates compared to SP from *B. adolescentis* (Fig. 15.13C) (Dirks-Hofmeister et al., 2015). As discussed in the introduction, resveratrol (3,5,4'-trihydroxystilbene) is regarded as a very interesting compound, as several studies state it to be highly beneficial for human health and nutrition (Baur and Sinclair, 2006; Fremont, 2000; Jang et al., 1997; Quideau et al., 2012). Neither BaSP nor TtSPP display significant activity on resveratrol, but the latter is considerably more active on the smaller building blocks resorcinol (benzene-1,3-diol) and orcinol (5-methylbenzene-1,3-diol) (Fig. 15.13A). Additionally, its gradual decrease in activity with increasing acceptor size points to a limitation in the size of the acceptor subsite. Molecular modeling revealed that the pocket is not extended enough for binding of resveratrol and in particular residue Arg134 seems to act as a “gatekeeper” that closes the entrance to the active site and thus limits glycosylation of large acceptors (Fig. 15.12A). In silico mutagenesis and docking studies indeed indicated that substitution of Arg134 with smaller residues (eg, alanine) would leave an opening, enabling the second ring of resveratrol to be accommodated (Fig. 15.12B).

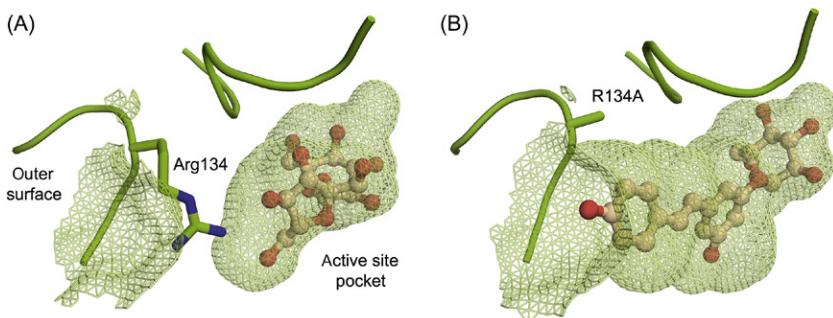


FIGURE 15.12 Structure-based rationale for site-specific loop mutation at position 134 in the sucrose 6'-P phosphorylase of *T. thermosaccharolyticum*: (A) positioning of active site loops in TtSPP with sucrose bound in the active site, showing arginine 134 to cause a closed active site and (B) variant R134A with resveratrol 3- α -glucoside docked in the unbolted active site.

A good number of in vitro experiments confirming these predictions and transglycosylation activity on resveratrol are available in literature. The feasibility to use variant R134A as a biocatalyst for the glycosylation of resveratrol in an aqueous system was demonstrated by the full conversion of 10 g/L resveratrol into 17 g/L resveratrol 3- α -glucoside (55°C, 24 h) (Fig. 15.13B). This new biocatalyst is thus able to (1) reach full and specific conversion of resveratrol in, (2) an aqueous system without the need for solvent additions, and (3) use sucrose as a cheap and renewable glycosyl donor. Comparison of its activity on 45 different acceptors with that of the wild-type enzymes of *B. adolescentis* and *T. thermosaccharolyticum* furthermore showed that it is much more promiscuous (Fig. 15.13C). Notable is the activity on quercitin, a bulky representative of the flavonol class. No transfer activity could be detected for the wild-type enzymes, whereas the R134A variant produced significant amounts of quercitin glucoside. The same moreover holds true for other (industrially relevant) polyphenols and phenolic glycosides. Hence, the R134A variant is a promiscuous biocatalyst that has opened up the potential to discover and exploit novel identities, properties, and applications of glycosylated products. It furthermore proves, together with other recent studies, the importance of active-site loops (Nestl and Hauer, 2014) and enzyme gates (Gora et al., 2013; Kingsley and Lill, 2015) for substrate specificity and accordingly these have become interesting targets for enzyme engineering.

15.2.4 Optimization of Reaction Conditions

In parallel with enzyme engineering for improved binding of noncarbohydrate acceptors, process engineering was also performed to maximize the product yields. In particular, solvent systems have been developed to enable the addition of higher acceptor concentrations, thereby favoring the transglycosylation reaction over the competing hydrolytic reaction. For these experiments, the SP

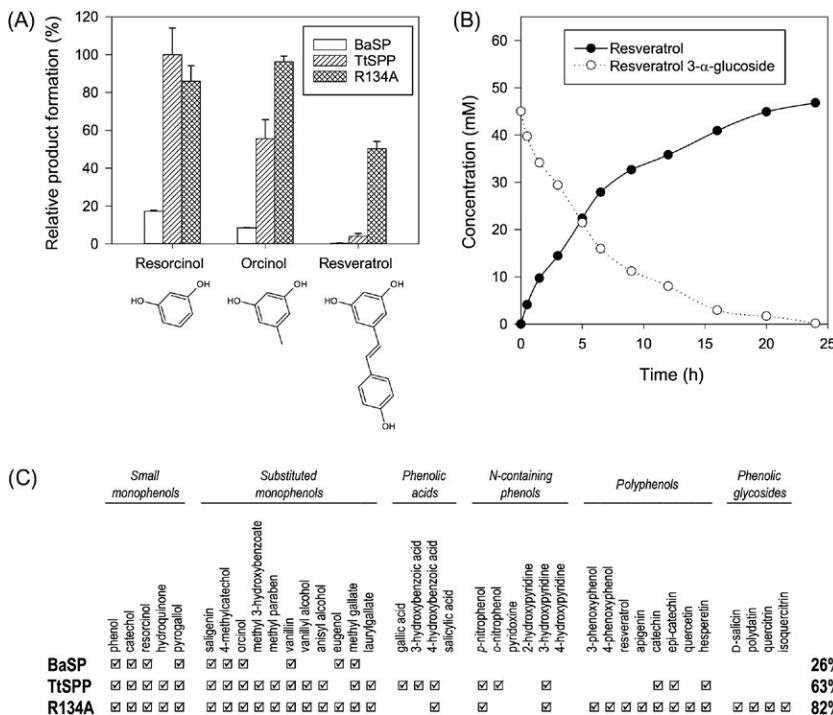


FIGURE 15.13 Activity of the SP from *B. adolescentis* (BaSP), the wild-type sucrose 6'-P phosphorylase from *T. thermosaccharolyticum* (TtSPP) and the engineered variant (R134A): (A) activity on resveratrol and its smaller building blocks; (B) full conversion of ~45 mM resveratrol (10 g/L) into resveratrol 3- α -glucoside (17 g/L) using the engineered variant; and (C) promiscuity toward different phenolic acceptors.

from *B. adolescentis* was used as biocatalyst, as it was the most stable enzyme available at that time.

For most enzymatic conversions, DMSO is the preferred cosolvent as it typically does not influence the activity up to a concentration of 20%. However, this set up does not allow to solubilize very hydrophobic acceptors like resveratrol or quercetin. Therefore, ionic liquids (ILs) have now been tried as alternative solvents that are much more environmentally friendly. In that case, acceptor concentrations up to 2 M could be achieved, which is more than enough to develop a practical glycosylation process (Table 15.4). The best results were obtained with the quaternary ammonium salt TEGO K5, which is produced by Evonik as a cleaning agent that is available in bulk quantities for a cheap price. Consequently, the stability of SP in the presence of this IL was assessed, with very good results (Fig. 15.14) (De Winter et al., 2013). Indeed, the enzyme displayed a half-life of 2 days when mixed with 20% TEGO and 1 M sucrose at 50°C. The benefits of TEGO K5 were then unambiguously proven with

TABLE 15.4 Solubility of Various Compounds at 60°C and pH 6.5, in the Presence of Cosolvent

Compound	Solubility (mM)					
	Buffer	DMSO		TEGO K5		
		20%	40%	20%	40%	
Resveratrol	0.2 ± 0.1	37.5 ± 2.9	97.5 ± 6.9	246 ± 15	1166 ± 95	
Quercetin	<0.1	9.4 ± 0.9	22.1 ± 1.3	21.2 ± 1.1	76.5 ± 5.2	
Curcumin	<0.1	8.2 ± 0.5	19.1 ± 1.1	13.5 ± 0.8	45.9 ± 1.8	
Dodecanol	1.3 ± 0.2	3.6 ± 0.2	18.1 ± 1.7	268 ± 18	508 ± 39	
Hexanol	6.3 ± 0.8	12.3 ± 0.9	31.1 ± 2.2	1664 ± 106	3108 ± 159	
Geraniol	4.3 ± 0.3	9.3 ± 0.3	19.1 ± 1.4	403 ± 26	1861 ± 94	
Linalool	5.2 ± 0.6	7.3 ± 0.4	16.1 ± 1.2	294 ± 18	1130 ± 82	
3-Hydroxypyridine	255 ± 20	457 ± 26	923 ± 68	654 ± 51	1395 ± 109	
3-Hydroxy-2-nitropyridine	261 ± 18	602 ± 54	1356 ± 96	968 ± 63	2652 ± 153	
Saligenin	352 ± 16	409 ± 11	920 ± 48	721 ± 52	2560 ± 192	
Salicylic acid	7.3 ± 0.7	61.8 ± 3.6	162 ± 12	271 ± 21	854 ± 66	
Vanillyl alcohol	70.4 ± 5.9	452 ± 11	796 ± 66	817.3 ± 75.6	1018 ± 72	
Cinnamyl alcohol	17.4 ± 1.3	40.6 ± 2.9	137 ± 9	937 ± 46	2751 ± 196	
4-Nitrophenol	167 ± 12	187 ± 16	442 ± 31	642 ± 52	1219 ± 86	

quercetin as acceptor, since it was the only cosolvent that enabled the formation of a glycosylation product. Other cosolvents either inactivated the enzyme too rapidly or were not able to solubilize enough of the acceptor substrate.

Although the use of IL was very promising, the downstream processing is somewhat complicated because these solvents have no vapor pressure and thus cannot be removed by evaporation. Therefore, the use of true organic solvents in a two-phase system has also been evaluated (De Winter et al., 2014). As proof-of-concept, the glycosylation of phenylethanol was tested using a system containing the enzyme and the donor in the lower (aqueous) phase, and the acceptor in the upper (hydrophobic) phase. Although the reaction was rather slow, a clear product spot on TLC could be observed after 72 h incubation. Subsequent optimization of the reaction identified the following conditions as optimal in terms of productivity and yield: a solvent ratio of 5/3, 2 M sucrose, pH 7.5, 50 U/mL SP, and 50°C. In addition, EtOAc was selected as the best solvent since it does

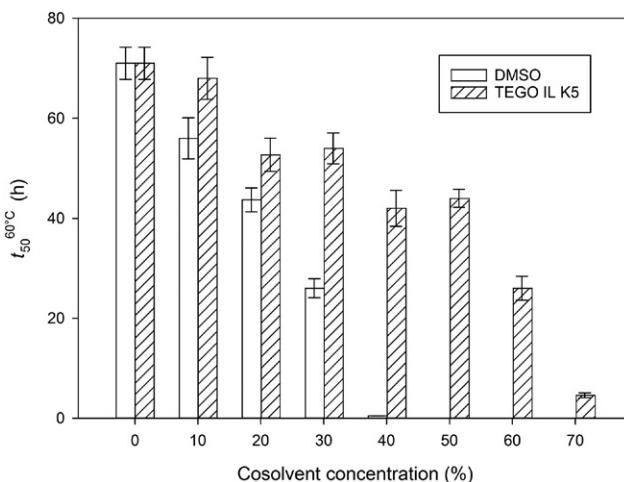


FIGURE 15.14 Half-life of SP at varying cosolvent concentrations (pH 6.5 and 60°C, in the presence of 1 M sucrose).

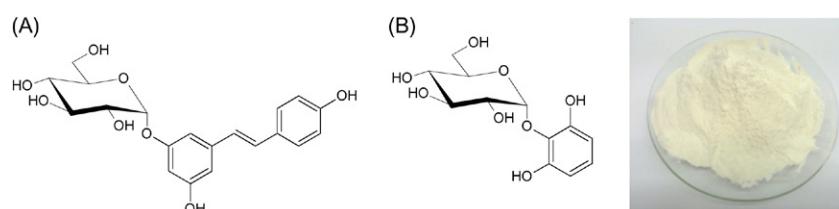
not affect the enzyme's stability and has a very low boiling point. Such a system was found to easily outperform the IL system described above, both in terms of yield and number of acceptors that can be converted by the enzyme (Table 15.5). As proof-of-concept, the glycosylation of pyrogallol (PG) was performed at a scale of 1 L. Starting from an acceptor concentration of 100 g/L, almost 51 g/L of pyrogallol glucoside could be produced, which corresponds to a product yield of nearly 60%.

For the downstream processing, the two phases are first to be separated by decanting and then dried, after which the product can be isolated by selective extraction using a solvent with the appropriate polarity. If needed, additional cleanup can be accomplished by flash chromatography although that would increase the price of the final product. Several glycosides have meanwhile been produced at the gram scale, with a purity of more than 95% after extraction. Excellent selectivity was observed in all these cases, as evidenced by NMR analysis. With pyrogallol, for example, the α -glucosyl moiety is only attached to the middle of the three hydroxyl groups, whereas resveratrol is only glycosylated at the 3'-position (Fig. 15.15).

Finally, the environmental impact of this new technology was evaluated by performing a Life Cycle Assessment, using the production of pyrogallol glucoside as a case study. All production steps were included in the analysis (“from cradle to gate”) as well as the processing of waste streams. The enzymatic process was found to perform much better than the corresponding chemical process, as the overall environmental impact was nine-times lower. Specific improvements were apparent in the endpoint-categories human health (factor 12), ecosystems (factor 9), and resources (factor 6). However, a hot spot analysis revealed that

TABLE 15.5 Glycosylation Potential of SP in a Biphasic System (Based on TLC Analysis, after 48 h Incubation at 50°C)

Acceptor	Product spot intensity	Acceptor	Product spot intensity
Pentanol ^a	+++	Cinnamyl alcohol ^a	+++
Hexanol ^a	++	Menthol ^b	-
Heptanol ^a	+	Saligenin ^b	++++
Octanol ^a	+	p-Nitrophenol ^b	+
Nonanol ^a	-	Phenol ^b	-
Decanol ^a	-	Hydroquinone ^b	+
Dodecanol ^a	-	Catechol ^b	+++
Cyclohexanol ^a	++++	Resorcinol ^b	+++
2-Hexanol ^a	+++	Pyrogallol ^b	+++++
Linalool ^a	-	Gallic acid methyl ester ^b	++++
Eugenol ^a	++	Gallic acid ethyl ester ^b	++++
Nerolidol ^a	-	Gallic acid propyl ester ^b	+++
β-Citronellol ^a	+	Gallic acid lauryl ester ^b	-
Geraniol ^a	++	Salicylic acid methyl ester ^a	+
2-Phenylethanol ^a	+	Curcumin ^b	-
R-1-Phenylethanol ^a	+++++	Resveratrol ^b	+
S-1-Phenylethanol ^a	+++++	Quercetin ^b	+
Benzyl alcohol ^a	++	Vanillin ^b	++
Anisyl alcohol ^a	+	Vanillyl alcohol ^b	+++

^aGlycosylation with the acceptor as organic phase.^bGlycosylation with EtOAc and 100 mg/mL acceptor as organic phase.**FIGURE 15.15** Glycosylated products generated by SP: glucoside of (A) resveratrol and (B) pyrogallol.

further improvements might be realized by lowering the solvent consumption in the reaction and extraction steps, and by lowering the aqueous waste production in the decanting steps. Nevertheless, our results clearly demonstrate that the glycosylation reactions catalyzed by SP can be economically and ecologically attractive if they are carefully optimized.

15.3 CONCLUDING REMARKS

In this chapter, various steps needed to develop SP into a practical biocatalyst for glycoside synthesis are described. The integration of enzyme and process engineering proved to be essential for success, as these complementary approaches were able to solve different aspects of the problem. Crucially, the industrial relevance of the developed technology has meanwhile been demonstrated by scaling-up selected reactions at pilot plant facilities, with several products now being commercially available in kilogram scales. The engineering pipeline presented here is based on a systematic and logical sequence of steps that should be useful for the development of other biocatalytic systems as well.

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Chapter 16

Industrial Applications of Multistep Enzyme Reactions

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16.1 INTRODUCTION

In 1897, Eduard Buchner found that sugars can be converted into ethanol by a cell-free extract of yeast, demonstrating that living cells are not necessarily required for alcohol fermentation. This finding opened the door to a new research field, namely enzymology, and Buchner was awarded the Nobel Prize in Chemistry in 1907. In his Nobel lecture, Buchner said “We are seeing the cells of plants and animals more and more clearly as chemical factories.”

In fact, living cells are often compared to chemical factories. Both facilitate a number of chemical reactions in a coordinated fashion to produce a wide variety of compounds. However, aside from the differences in their spatial scales, they vary in terms of their number of reaction compartments. In chemical manufacturing processes, each reaction is normally separately operated in a distinct reactor and step-by-step separation and purification of intermediates are needed. In contrast, thousands of reactions composing the natural metabolic network are carried out in a single cell, which is compartmentalized into a limited number of organelles or is even uncompartimentalized in prokaryotic cells. Such a remarkable orchestration of enzymatic reactions is attributed to the excellent substrate specificity of enzymes, by which they can recognize their physiological substrates among the large number of intracellular compounds. Thus, in addition to classically well-interpreted features, such as their high regioselectivity, enantioselectivity, and catalytic ability under benign conditions, the industrial application of enzymes offers a significant operational advantage that enables us to implement multistep cascade reactions in a single reactor. In this chapter, the current status of commercially exploited multistep enzymatic reactions and ongoing challenges for expanding the industrial applicability of multienzyme systems are reviewed.

16.2 MULTISTEP REACTIONS WITH A SMALL NUMBER OF ENZYMES

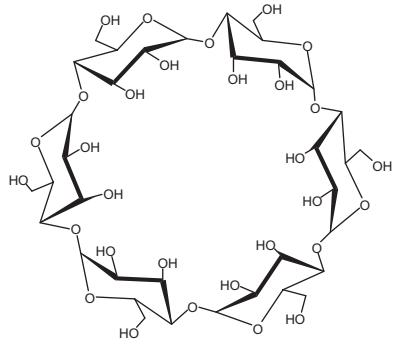
The world demand for industrial enzymes will rise 6.3% per year, approaching nearly \$7.0 billion in 2017 (Freedonia Group, 2014). Biocatalytic conversion with single or a small number of enzymes has matured as a well-developed technology and is practically employed in a wide range of industrial fields, including food processing, diagnosis, and chemical manufacturing. Several representative examples where two to three enzymes are coordinately used for conversion are introduced in this section.

16.2.1 Starch Processing

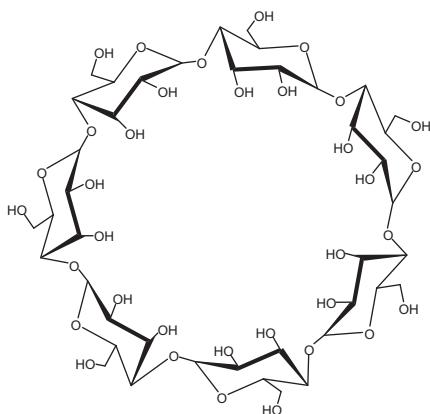
Food processing is the largest segment of industrial application of enzymes. Starch processing enzymes are widely exploited in various sectors of this field, and are often coupled with other types of enzymes. Starch is digested by an amylase to oligosaccharides and subsequently hydrolyzed by a glucoamylase. The resulting monomeric sugar (ie, glucose) can be further processed by additional enzymes to give a series of commercially valuable products. A combination of fungal α -amylase, glucoamylase, and a bacterial glucose isomerase is used to produce high fructose corn syrup. Enzymatic isomerization of glucose to fructose gives a mixture of these monosaccharides. The relative sweetness of the resulting mixture is 1.25–1.5 times higher than that of pure glucose and comparable to that of table sugar (sucrose), which is a disaccharide of glucose and fructose. Owing to its low price compared to sucrose, high fructose corn syrup is widely used in processed foods and has an annual market of \$1 billion (Adrio and Demain, 2014).

Cyclodextrins, which are cyclic oligosaccharides typically consisting of six to eight molecules of (α -1,4)-linked D-glucose, are also commercially produced from starch using an enzyme pair of α -amylase, and bacterial cyclodextrin glycosyltransferase (Biwer et al., 2002) (Fig. 16.1). Cyclodextrins contain a hydrophilic outer surface and a relatively hydrophobic central cavity, in which other hydrophobic molecules can be included as guest molecules. Owing to this feature, cyclodextrins can solubilize hydrophobic compounds in aqueous media. Particularly, α -, β -, and γ -cyclodextrins (6-, 7-, and 8-membered glucose ring, respectively) are generally recognized as safe (GRAS) and are widely applied in food industries for the solubilization and stabilization of hydrophobic colorant and fragrance compounds. They are also used in pharmaceuticals to increase the bioavailability of hydrophobic drugs (Hamada et al., 1975; Nambu et al., 1978; Uekama et al., 1983).

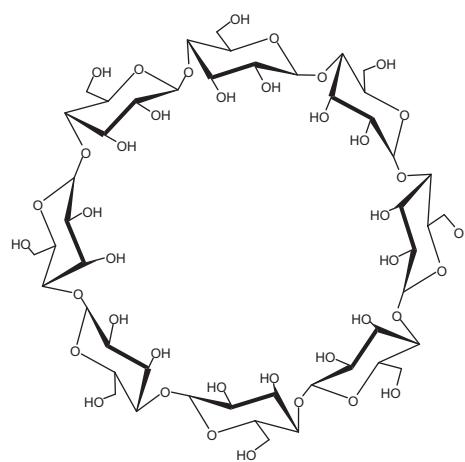
Another unique example of starch processing is the enzymatic production of trehalose, which is a disaccharide consisting of (α -1,1)-linked two glucose units. While the relative sweetness of trehalose is 45% of that of sucrose, it is more thermostable and has a wide pH-stability range than other saccharides.



α-Cyclodextrin



β-Cyclodextrin



γ-Cyclodextrin

FIGURE 16.1 Chemical structures of α-, β-, and γ-cyclodextrins. In industrial processes, starch is first liquefied at a high temperature using thermostable α-amylase, acids, or mechanical disintegration. The liquefied starch is converted into cyclodextrins by a cyclodextrin glycosyltransferase. Alternatively, starch is directly processed with a thermostable cyclodextrin glycosyltransferase.

Additionally, since it is a nonreducing sugar, trehalose does not undergo the Maillard reaction with amino compounds, which often cause an undesired color change in processed foods. These features of trehalose make it an attractive material in the food industry (Higashiyama, 2002). Although trehalose is widely distributed in various organisms and can be extracted from baker's yeast by suspension in a hot ethanol–water solution, this method is not suitable for industrial mass production. In 1995, Murata and coworkers isolated a trehalose-producing bacterium, *Arthrobacter* sp. strain Q36, and identified the key enzymes involved in trehalose production in the bacterium, namely maltooligosyl trehalose synthase and maltooligosyl trehalose trehalohydrolase (Fig. 16.2) (Murata et al., 1995). On the basis of this finding, the Hayashibara Company (Okayama, Japan) started the commercial production of trehalose from starch in 2000, and the current production scale is assumed to be more than 30,000 tons/year.

16.2.2 Clinical and Biochemical Analysis

The functional diversity and strict substrate specificity of enzymes are also beneficial for the determination of specific compounds, ranging from small metabolites (eg, sugars, amino acids, and coenzymes) to macromolecules (eg, viral antigens, antibodies, and serum proteins), contained in blood, serum, and urine for diagnostic purposes (Bergmeyer., 1983). NAD(P)⁺- and NAD(P)H-dependent dehydrogenases are often employed as diagnostic enzymes since the reduced forms of nicotinamide cofactors can be easily determined using a spectrophotometer at a wavelength of 340 nm. While these enzymes are directly applicable in colorimetric assays, they are also coupled with other enzymes to enhance the sensitivities of the assays. Diaphorases, which are also referred to as NAD(P)H dehydrogenases, are a ubiquitous class of flavin-containing enzymes that catalyze the reduction of various compounds, including dyes and their precursors, by using NAD(P)H as reducing cofactors. For example, Yamasaki-Yashiki et al. (2012) developed an enzymatic assay system for the determination of L-methionine levels in blood samples (Fig. 16.3). The methionine dehydrogenase, which was obtained by the directed mutagenesis of a phenylalanine dehydrogenase from *Bacillus sphaericus*, was coupled with *Clostridium kluyveri* diaphorase. In their system, NADH generated by the methionine dehydrogenase is used as a reductant for the diaphorase-mediated reduction of resazurin to resorufin, a pink colored and highly red fluorescent compound.

Peroxidases are another class of enzymes widely used for clinical and other analytical purposes. They catalyze the oxidation of various organic compounds using hydrogen peroxide as an oxidant. In particular, horseradish peroxidase has long been investigated and exploited in many commercial diagnostic reagents. The enzyme catalyzes the oxidation of chromogenic substrates, such as 3,3',5,5'-tetramethylbenzidine (TMB), 3,3'-diaminobenzidine (DAB), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) into colored

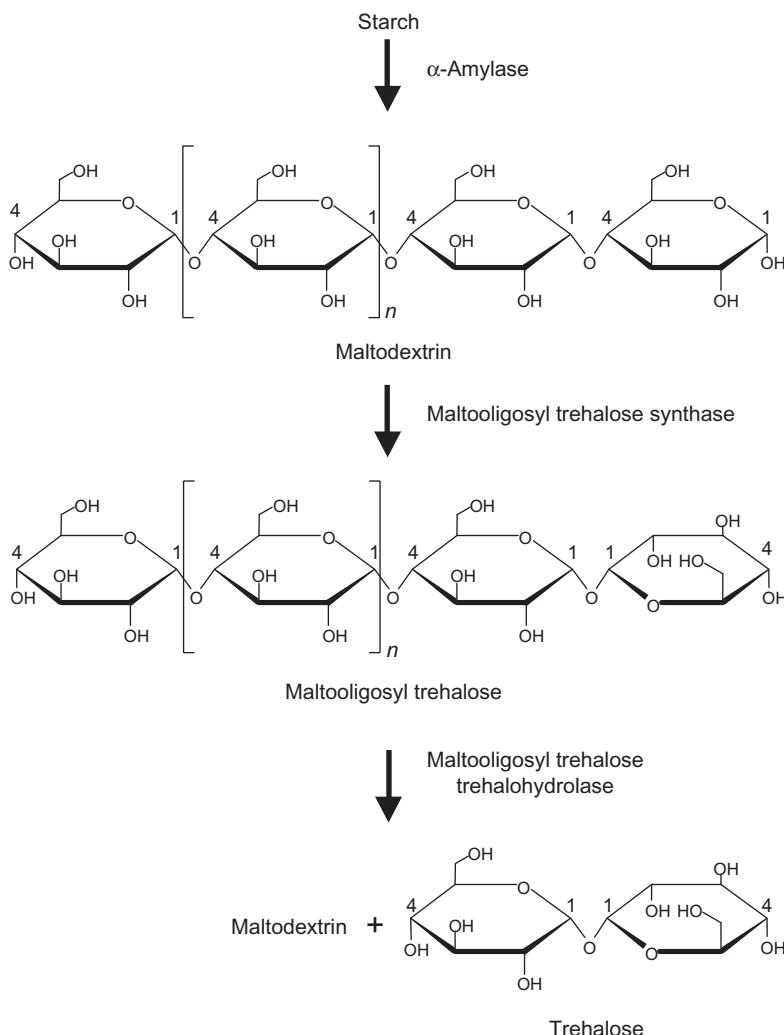


FIGURE 16.2 **Enzymatic production of trehalose from starch.** Starch is partially digested into maltodextrin by α -amylase. Subsequently, maltooligosyltrehalose synthase catalyzes the intramolecular transglycosylation to produce maltooligoxylyl trehalose, which is then hydrolyzed by maltooligosyltrehalose trehalohydrolase to release trehalose.

products. These color reactions are coupled with specific oxidases (eg, glucose oxidase, alcohol oxidase, and cholesterol oxidase) and used to quantify the concentration of hydrogen peroxide generated by the oxidase reactions. A recent successful example in this area is the identification of a novel fructosyl peptide oxidase and its clinical application to diabetes diagnosis (Hirokawa et al., 2003,

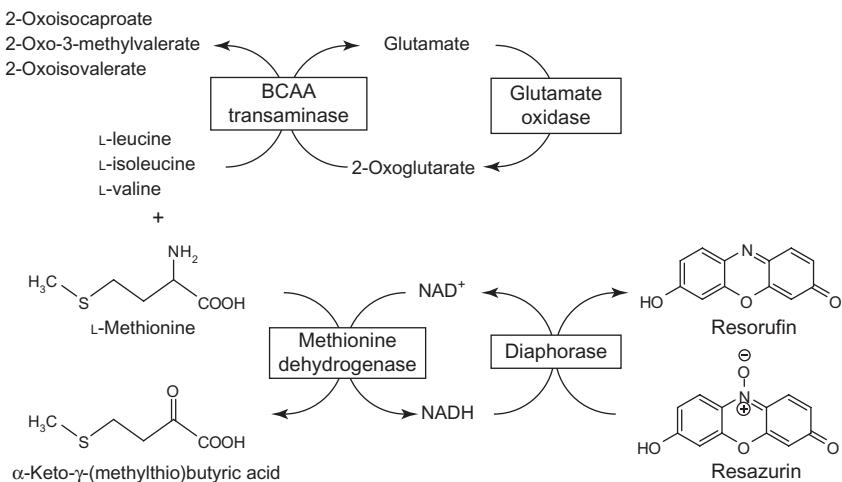


FIGURE 16.3 Enzymatic determination of L-Methionine using a coupled enzyme system. Methionine dehydrogenase catalyzes the NAD⁺-dependent oxidation of L-methionine. NADH released by the enzyme was used as a reductant for the diaphorase-mediated reduction of resazurin to resorufin. Since the methionine dehydrogenase exhibits significant activity toward branched-chain amino acids (BCAAs), these amino acids are eliminated by a BCAA transaminase. L-Glutamate oxidase is used to regenerate 2-ketoglutarate which is consumed by the transaminase reaction.

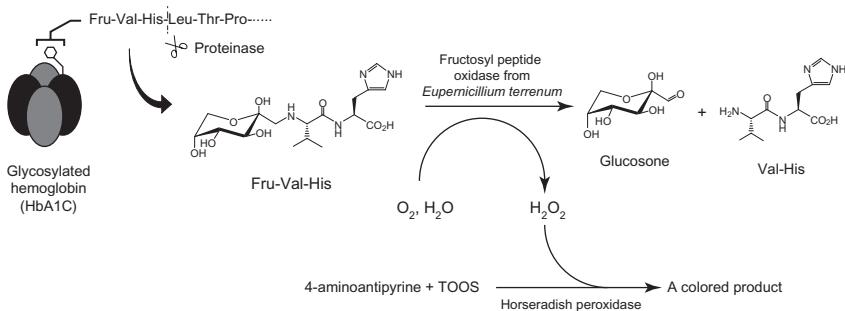


FIGURE 16.4 Diabetes diagnosis using the fructosyl peptide oxidase. The fructosyl peptide released from HbA1c is specifically oxidized by the *Eupenicillium* oxidase. The reaction is colorimetrically determined through the oxidative conjugation of 4-aminoantipyrine and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) catalyzed by the horseradish peroxidase.

2004). Three enzymes, namely an *Aspergillus* protease, *Eupenicillium* oxidase, and horseradish peroxidase, are used in this system. The glycopeptide, which is cleaved from a glycohemoglobin (HbA_{1c}) by the protease, is specifically oxidized by the *Eupenicillium* oxidase. Subsequently, hydrogen peroxide generated by the oxidase reaction is quantified by the colorimetric reaction mediated by the horseradish peroxidase (Fig. 16.4).

16.2.3 Chemical Manufacturing

Enzymatic synthesis of value-added chemicals is recognized as a practical alternative to traditional metallo- and organocatalysis-based manufacturing processes (Born scheuer et al., 2012). Particularly, owing to their excellent enantioselectivities, enzymes are preferably used for the synthesis of enantiomerically pure compounds. For instance, the ketoreductase-catalyzed asymmetric reduction of prochiral ketones to optically active alcohols is intensively studied for the production of a range of chiral compounds, such as cholesterol-lowering statin drugs and their precursors (Kataoka et al., 2003; Born scheuer et al., 2012) (Fig. 16.5). Ketoreductases require the reducing cofactors, NADH and NADPH, and release NAD⁺ and NADP⁺ upon catalyzing the reduction of prochiral ketones. Since these cofactors are generally expensive, integration of another enzyme reaction, by which NAD(P)⁺ can be regenerated to NAD(P)H upon the oxidation of a cheap sacrificial cosubstrate, is indispensable to maintain the economic feasibility of the ketoreductase processes. In addition to ketoreductases, NAD(P)H play a pivotal role in a wide range of enzymatic redox reactions, including other industrially important ones, such as asymmetric saturations of C = C bonds by enoate reductases (Stuermer et al., 2007), and regioselective hydroxylation of nonactivated carbon atoms by cytochrome P450 monooxygenases (Urlacher et al., 2004). As a result, NAD(P)H regenerating enzymes are one of the most important coupling enzymes in enzymatic chemical manufacturing.

Several NAD(P)H-regeneration enzymes have been well characterized and commercially used. Among them are glucose dehydrogenases, which catalyze the oxidation of glucose to gluconolactone using NAD(P)⁺ as a cofactor. Since gluconolactone is spontaneously hydrolyzed to gluconate, the overall reaction (glucose + NAD(P)⁺ + H₂O = gluconate + NAD(P)H + H⁺) proceeds in the direction of NAD(P)H formation in aqueous solution. Since glucose is

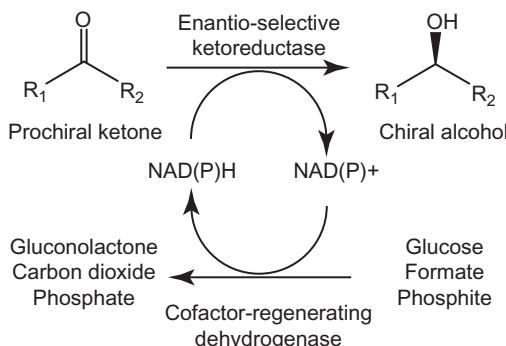


FIGURE 16.5 Schematic representation of ketoreductase-catalyzed asymmetric reduction of prochiral ketones. NAD(P)H consumed by the enantio-selective ketoreductase is regenerated by the NAD(P)⁺-dependent dehydrogenation of a sacrificial cosubstrate.

readily available, stable, and inexpensive, glucose dehydrogenases are broadly used for NAD(P)H regeneration in industrial processes. Furthermore, many glucose dehydrogenases accept both NAD⁺ and NADP⁺ as cofactors. Therefore, they can be used for the regeneration of both types of nicotinamide cofactors (Weckbecker and Hummel, 2005). However, the decrease in the reaction pH caused by the accumulation of gluconate is not negligible when a high concentration of substrate is used. Hence, pH-maintaining operations are required when using glucose dehydrogenase. Formate dehydrogenases are another class of enzymes which have also been widely used for the regeneration of nicotinamide cofactors (Wichmann et al., 1981). The enzymes catalyze NAD⁺-dependent oxidation of formate ($\text{formate} + \text{NAD}^+ = \text{CO}_2 + \text{NADH} + \text{H}^+$). The reaction product, carbon dioxide, is released from the reaction mixture, and therefore the NADH-regenerating reaction proceeds in an irreversible manner. However, naturally occurring formate dehydrogenases are specific to NAD⁺, prohibiting their application in NADPH regeneration. Accordingly, many protein-engineering efforts have been made to alter the cofactor specificity of this group of enzymes as well as to improve their stability and catalytic ability (Tishkov and Popov, 2006). A relatively newly recognized NADH-regenerating enzyme is the phosphite dehydrogenase (Vrtis et al., 2002). This enzyme catalyzes the oxidation of phosphite, an inexpensive and stable inorganic compound, to phosphate with the concomitant reduction of NAD⁺ to NADH. The equilibrium constant for the oxidation of phosphite by NAD⁺ is estimated as 10^{11} , indicating that the cofactor-regenerating reaction proceeds in a thermodynamically preferable manner. Since the reaction can be performed in phosphite buffer which is simply converted to phosphate buffer, both the substrate and product are innocuous to most enzymes. Although phosphite dehydrogenase can use both NAD⁺ and NADP⁺, its activity with NADP⁺ is considerably lower than that with NAD⁺. Therefore, similar to the case of formate dehydrogenase, engineering of phosphite dehydrogenase would be needed to relax its cofactor specificity (Johannes et al., 2007).

ATP is also involved in many synthetically important enzyme reactions and a number of studies have been reported for the enzymatic regeneration of ATP from its dephosphorylated derivatives, ADP and AMP. Several ATP-forming kinases, including pyruvate kinase, acetate kinase, and creatine kinase, have been conventionally used for ATP regeneration with their substrates, namely phosphoenol pyruvate, acetyl phosphate, and creatine phosphate, respectively (Chenault et al., 1998). However, in many cases, these phosphorylated compounds are too expensive and/or unstable to be used as sacrificial substrates. Hence, a combination of polyphosphate and polyphosphate kinase has been preferably employed as a less expensive enzymatic ATP-regeneration system. Polyphosphate is a linear polymer of orthophosphate containing a high-energy phosphoanhydride linkage and is a suitable phosphate donor in ATP regeneration systems owing to its high stability and low price (Zhao and van der Donk, 2003). Polyphosphate kinases catalyze the transphosphorylation from polyphosphate to ADP in a reversible fashion (Ishige et al., 2002). They are also

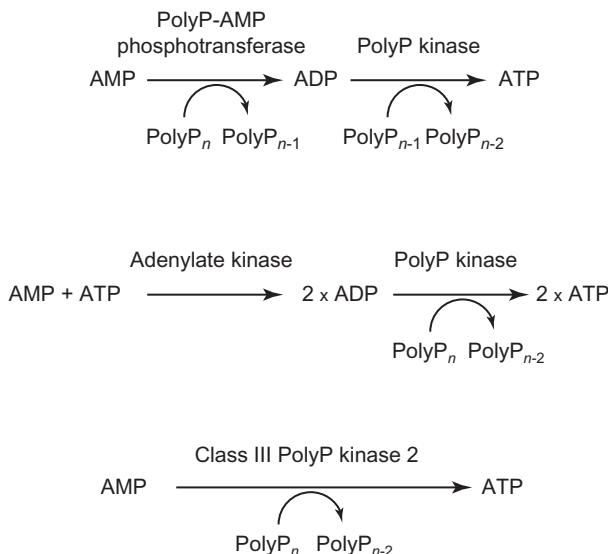


FIGURE 16.6 Enzymatic regeneration of ATP from AMP using polyphosphate (PolyP) as a phosphate donor.

used for the regeneration of ATP from AMP by coupling with other enzymes, such as polyphosphate-AMP phosphotransferase (Kameda et al., 2001), and adenylate kinase (Satoh et al., 2004) (Fig. 16.6). Recently, Motomura et al. (2014) identified a novel subfamily of polyphosphate kinases (class III PPK2) from *Meiothermus ruber* and taxonomically related strains. These enzymes catalyze the phosphorylation of both nucleoside monophosphate and nucleoside diphosphate using polyphosphate as a phosphate donor, and thus are suitable for the direct regeneration of ATP and other nucleoside triphosphates from corresponding nucleotide monophosphates.

16.3 MULTISTEP REACTIONS WITH LARGER NUMBER OF ENZYMES

In 1985, Welch and Scopes reported the *in vitro* reconstitution of the glycolytic pathway using individually purified yeast enzymes (Welch and Scopes, 1985). The reconstituted pathway was capable of converting 1 M (18% [w/v]) glucose to ethanol in 8 h with a nearly 100% molar yield. Similar work using the cell-free extract of *Zymomonas mobilis* resulted in the conversion of 2 M glucose to 3.6 M ethanol (Algar and Scopes, 1985). The final ethanol concentration (nearly 20% [v/v]) was higher than any natural fermentation system can achieve. Despite these highly motivating achievements, industrial application of *in vitro* multienzyme systems consisting of more than three enzyme reactions has been limited because of the complexity of the process including the

enzyme-purification procedures. Alternatively, recent advances in genetic and enzyme engineering technologies provide us more convenient and less expensive approaches for purifying and assembling multiple enzymes, and potentially enable us to develop an economically feasible bioconversion system consisting of a number of enzymes. In this section, the current state-of-the-art of in vitro bioconversion systems using a moderate and large number of enzymes is reviewed and the remaining challenges in this field are highlighted.

16.3.1 In Vivo and In Vitro Bioconversion

Engineering of the metabolic routes of living microorganisms has emerged as a powerful strategy to improve the fermentative ability of microbial cells for the production of various useful chemicals, and is generally called “metabolic engineering” (Stephanopoulos et al., 1998). In this context, in vitro construction of an artificial synthetic pathway using multiple enzymes can be regarded as an analogy of metabolic engineering. However, chemical conversions through in vitro synthetic pathways offer several theoretical advantages over in vivo fermentation systems. Since bioconversions using in vitro pathways can be implemented without being affected by cell growth, synthetic pathways can be more flexibly designed than engineering of living organisms. Ye et al. (2012) constructed a chimeric glycolytic pathway, in which the consumption and regeneration of ATP and ADP were balanced, by integrating an archaeal phosphate-independent glyceraldehyde-3-phosphate dehydrogenase (GAPN) into a bacterial glycolytic pathway (Fig. 16.7A). Such a non-ATP-forming glycolytic pathway can no longer play a physiological role (ie, energy production particularly under anaerobic conditions) and thus would not be available for fermentative reactions in vivo. They combined this artificial pathway with other enzymes and demonstrated the one-pot conversion of glucose to L-lactate (Ye et al., 2012), L-malate (Ye et al., 2013), and 1-butanol (Krutsakorn et al., 2013). Guterl et al. (2012) designed an ATP-independent glycolytic pathway using only four enzymes, namely glucose dehydrogenase, dihydroxy-acid dehydratase (DHAD), 2-keto-3-deoxygluconate aldolase, and glyceraldehyde dehydrogenase. In this pathway, an archaeal DHAD bifunctionally catalyzes the dehydration of gluconate to 2-keto-3-deoxygluconate and glyceraldehyde to pyruvate (Fig. 16.7B). This pathway could be used for the in vitro production of ethanol and isobutanol. Bogorad et al. (2013) constructed a cyclic pathway for the non-oxidative conversion of C6, C5, and C3 sugars to C2 metabolites (Fig. 16.7C). In vitro reconstitution of the designed pathway with eight enzymes led to the stoichiometric conversion of one molecule of fructose-6-phosphate (C6) to three molecules of acetyl phosphate (C2) without any carbon loss. Furthermore, they demonstrated that their pathway was also functional in vivo. Installation of the pathway in engineered *Escherichia coli* cells led to the catabolism of xylose (C5) to acetate (C2) with a molar yield of 2.2 mol acetate/mol xylose, approaching a theoretical yield of 2.5.

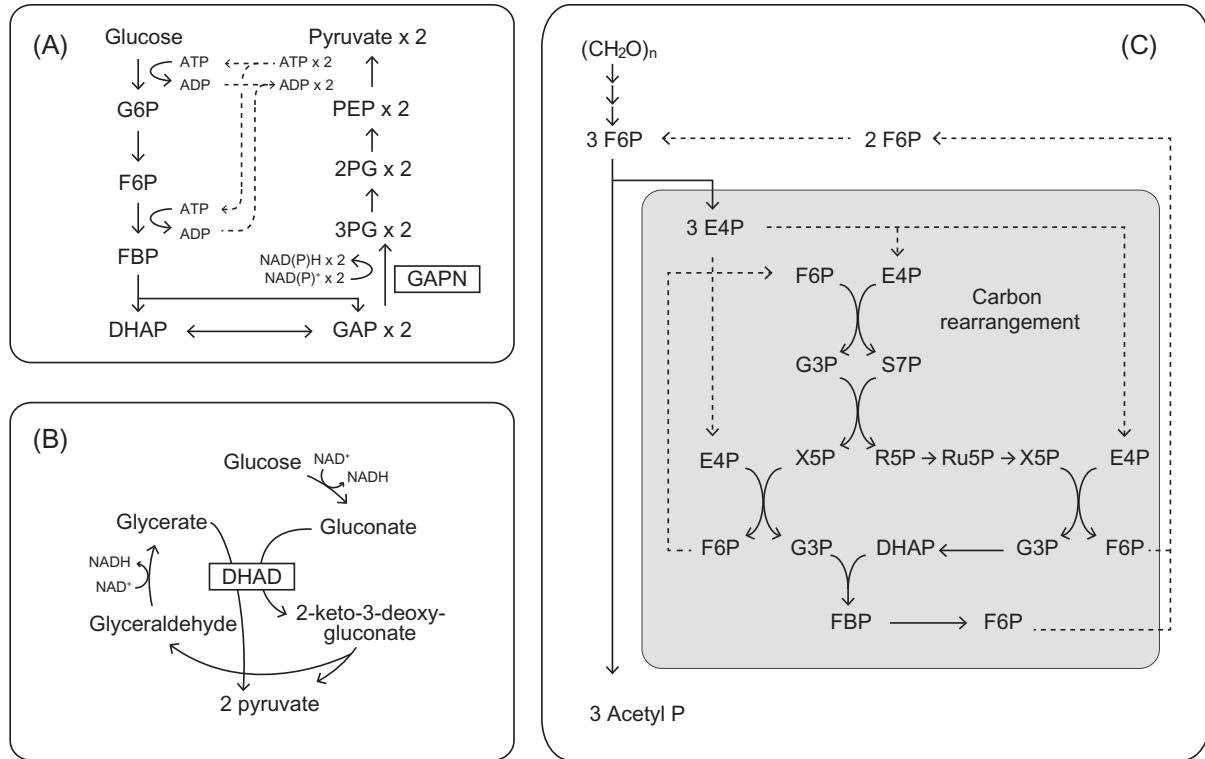


FIGURE 16.7 Representative examples of artificial glycolytic pathways. (A) A non-ATP-forming chimeric glycolytic pathway (Ye et al., 2012). An archaeal GAPN enzyme is integrated in the bacterial Embden-Meyerhof pathway to achieve the intrapathway balance between ATP and ADP. (B) ATP-independent glycolytic pathway (Guterl et al., 2012). Dihydroxy-acid dehydratase (DHAD) operates bifunctionally in the pathway. (C) Nonoxidative glycolytic pathway (Bogorad et al., 2013). Three molecules of erythritol-4-phosphate (E4P) are rearranged into two molecules of fructose-6-phosphate (F6P) through the carbon rearrangement pathway.

Since *in vitro* pathways consisting of only a limited number of enzymes, substrates, and intermediates can be directly converted to the products of interest without being routed into undesired metabolites, and thus, high-yield bioconversion can be accomplished. Myung et al. (2014) assembled 15 enzymes for the high-yield production of hydrogen from sucrose *in vitro*. Nearly 12 mol of hydrogen was produced from each glucose unit of the disaccharide; this value is considerably higher than the theoretical yield (four H₂/glucose) of biological hydrogen fermentation. More recently, the same group reported that a similar *in vitro* system is capable of utilizing real-biomass-derived sugars, which are mainly composed of glucose and xylose, for the high-yield hydrogen production (Rollin et al., 2015).

Another practically important aspect of *in vitro* multistep enzyme systems is their ability to implement metabolism-like cascade reactions in a simple inorganic buffer. Hence, the product recovery steps can be greatly simplified compared with those in conventional fermentation systems. This point would be particularly advantageous in the production of biomolecules which are difficult to separate from other cellular molecules because of their low concentration or the presence of analog compounds in living cells. Schultheisz et al. (2011) demonstrated the *in vitro* de novo synthesis of pyrimidine nucleotides using 16 enzymes. By the combinatorial use of isotopically labeled substrates, they succeeded in the selective production of a series of pyrimidine nucleotides which are specifically labeled at different positions.

16.3.2 Enzyme Production

Low-cost preparation of enzymes is the most critical issue for expanding the applicability of multiple-enzyme systems. In most cases, enzymes are produced as recombinant proteins. The recent price reduction in commercial gene-synthesis services extends the availability of codon-optimized genes and improves the production titer of recombinant proteins in heterologous hosts. Recombinant techniques also enable the introduction of affinity tag sequences, such as His-tag, FLAG, and Streptag II, to the enzyme of interest (Arnau et al., 2006; Young et al., 2012). Resulting tag-fused enzymes can be easily recovered and purified from the crude extract of host cells through adsorption to specific affinity ligands. Additionally, sequence-specific endoproteases, which are often introduced in the linker region between the affinity tag and target enzyme, can be used for the tag removal when their addition produces a negative effect on the function of isolated enzymes. In addition to enzyme-purification purposes, affinity tags can be used for the spatial organization of multiple enzymes. Colocalization of a series of enzymes involved in a biosynthetic pathway is believed to allow substrate channeling between the enzymes and consequently improve the flux through the synthetic pathway. Dueber et al. (2009) demonstrated that the spatial organization of three mevalonate biosynthetic enzymes resulted in a drastic improvement of mevalonate production through the *in vitro*

pathway. The enzymes were aligned on a synthetic protein scaffold via specific attachments between protein–protein interaction domains. Combinatorial optimization of the relative number of interaction domains on the scaffold protein led to a 77-fold increase in the production titer. Similarly, [Chun et al. \(2012\)](#) constructed a self-assembled enzyme complex based on the specific interaction between cohesins and dockerins from natural cellulosomes. Assembly of three glycolytic enzymes (triose isomerase, fructose bisphosphate aldolase, and phosphofructokinase) on a scaffold protein resulted in 33-fold higher catalytic ability (k_{cat}/K_m) than that with the mixture of free enzymes. Furthermore, integration of a cellulose binding domain into the scaffold protein enabled a simple purification of the enzyme complex through its adsorption to amorphous cellulose.

Another promising approach is the employment of thermophilic enzymes as catalytic modules. Heterologous expression of thermophilic enzymes in mesophilic hosts (eg, *E. coli*) followed by heat treatment of the crude extract of the resulting cells leads to the denaturation of thermally unstable host-derived enzymes. Consequently, highly selective thermophilic biocatalysts comparative to purified enzymes can be readily obtained. [Ninh et al. \(2015\)](#) reported the co-expression of nine thermophilic enzymes comprising a non-ATP-forming chimeric glycolytic pathway in a single recombinant *E. coli* strain. Genes encoding the thermophilic enzymes were assembled in an artificial operon and introduced into *E. coli*. Gene expression levels were controlled by their sequential order in the artificial operon. The in vitro chimeric glycolytic pathway was prepared by simply heating the crude extract of the co-expression cells and used for the one-pot conversion of glucose to lactate.

16.3.3 Optimization of Enzyme Concentration

Unlike highly branched metabolic pathways in living organisms, in vitro synthetic pathways, in which a limited number of enzyme reactions are sequentially aligned, appear to be less sensitive to the imbalance in enzyme concentrations. Although the existence of a rate-limiting enzyme leads to the accumulation of the specific intermediate (ie, the substrate of the rate-limiting enzyme), it is eventually converted by the enzyme without being consumed by other coexisting enzymes. However, the accumulation of chemically labile intermediates will result in their spontaneous degradation and decrease in the overall conversion yield. Additionally, when the designed pathway has bifurcating branches, competition for node intermediates will occur between the downstream pathways. In such cases, enzyme concentrations should be properly tuned to achieve a balanced flux through the constructed pathway. For the optimization of enzyme concentrations, a computer-aided modeling analysis is often employed. [Bogorad et al. \(2014\)](#) used an ensemble modeling analysis to determine the robustness of their methanol-condensation cyclic pathway against the loss of steady state. The modeling analysis resulted in the identification of the enzyme which has the most significant effect on the pathway robustness, and was followed

by a simulation analysis to predict the optimum concentration of the identified enzyme. [Rollin et al. \(2015\)](#) used a genetic algorithm approach, by which parameters of a nonlinear kinetic model of hydrogen-production enzymes were fitted with experimental data. On the basis of the updated kinetic parameters, they conducted a global sensitivity analysis to identify the enzymes having the highest impact on the reaction rate and yield. By optimizing the concentration of the key enzyme, volumetric hydrogen productivity by the in vitro enzyme system was increased by threefold.

In contrast, we proposed an experimental approach for the optimization of enzyme concentrations in an in vitro pathway. The in vitro pathway was divided into several partial pathways, in which NAD(H)-dependent enzymes were assigned to be the last steps. By doing this, the flux through the partial pathways could be spectrophotometrically determined in a real-time manner. This real-time monitoring technique enabled experimental optimization of the flux through the divided pathways by directly modulating the concentrations of enzymes in a spectrophotometric cuvette ([Ye et al., 2012](#); [Krutsakorn et al., 2013](#)).

16.3.4 Cofactor Issue

In in vitro bioconversion systems, energy and redox cofactors, such as ATP and NADH, are exogenously added to the reaction mixture. Since these cofactors are expensive compounds, it is vital to keep the overall stoichiometry between the consumption and regeneration of cofactors and to continuously use them in catalytic amounts. In their pioneering work, Welch and Scopes demonstrated that the imbalance of ATP in their in vitro glycolytic pathway can be prevented by adding an excess amount of arsenate to the reaction mixture. Glyceraldehyde-3-phosphate (GAP) dehydrogenase (GAPDH) can accept arsenate instead of phosphate to form 1-arseno-3-phosphoglycerate, which is simultaneously broken down to arsenate and 3-phosphoglycerate. Consequently, the ATP-generating reaction catalyzed by phosphoglycerate kinase, which converts 1,3-bisphosphoglycerate to 3-phosphoglycerate, was skipped, and no net ATP was yielded through the reconstituted pathway ([Welch and Scopes, 1985](#)). Recently, [Opgenorth et al. \(2014\)](#) developed a molecular purge valve module to maintain the NADP⁺/NADPH balance in in vitro synthetic pathways. Their purge valve module consists of a wild-type NAD⁺-dependent pyruvate dehydrogenase (PDH^{NADH}), a mutant PDH that utilizes NADP⁺ (PDH^{NADPH}), and a H₂O-forming NADH oxidase (NoxE). Under low-NADPH, high-NADP⁺ conditions, the PDH^{NADPH} mainly converts pyruvate to acetyl-CoA with a concomitant generation of NADPH. Under high-NADPH, low-NADP⁺ conditions, the PDH^{NADPH} activity is shut down and the wild-type PDH^{NADH} is preferably used to produce acetyl-CoA. NoxE catalyzes the reoxidation of NADH to NAD⁺ but not NADPH to NADP⁺ ([Fig. 16.8](#)). The purge valve system was demonstrated to be highly robust to variations in cofactor levels and applicable to the stoichiometric conversion of pyruvate to poly(hydroxybutyrate) (PHB) and isoprene.

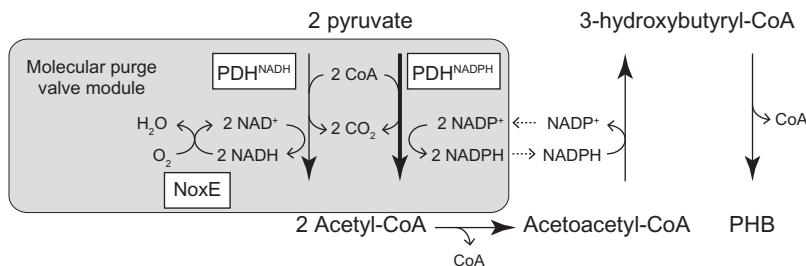


FIGURE 16.8 Molecular purge valve system for maintaining the $\text{NADP}^+/\text{NADPH}$ balance in an in vitro PHB production system. When the NADP^+ concentration is high, $\text{PDH}^{\text{NADPH}}$ primarily catalyzes the oxidative decarboxylation of pyruvate using equimolar amounts of NADP^+ . Resulting acetyl-CoA molecules are conjugated to acetoacetyl-CoA, which is subsequently reduced by a NADPH-dependent reductase to give 3-hydroxybutyryl-CoA. The imbalance between the consumption and regeneration of NADP^+ leads to a high-NADPH, low- NADP^+ condition. Consequently, $\text{PDH}^{\text{NADPH}}$ activity is shut down and the wild-type PDH^{NADH} is preferably used to produce acetyl-CoA. NADH generated by PDH^{NADH} is reoxidized by NoxE .

However, the high prices of the cofactors still tend to be a major obstacle to developing an economically feasible in vitro biocconversion system. Additionally, the relatively low thermal stability of nicotinamide cofactors often hampers the long-term operation of in vitro systems particularly when thermophilic enzymes are used as catalytic modules (Ye et al., 2012; Krutsakorn et al., 2013). One of the promising strategies to overcome this problem is the development of low-cost and stable biomimic analogs. A series of biomimic cofactors has been developed and demonstrated to be utilized by natural and engineered enzymes (Rollin et al., 2013). However, the catalytic performances of most enzymes with these biomimics is still far lower than those with natural cofactors, indicating that further improvements are indispensable for their practical application. Another possible approach would be the in vitro construction of a salvage synthetic pathway of the cofactors. The thermally unstable nature of nicotinamide cofactors creates a demand for the salvage of their degradation products particularly in thermophilic microorganisms. Hence, these organisms seem to be equipped with a series of enzymes involved in the resynthesis of the cofactors from the degradants. Identification and assembly of these enzymes would enable the construction of an in vitro pathway for the salvage synthesis of the cofactors.

16.4 CONCLUDING REMARKS

Rapidly advancing “omics” technologies have accelerated the exploration of novel biocatalytic molecules with unique properties. Structural and functional information of these biomolecules is systematically incorporated into web-accessible public databases, such as KEGG, BRENDA, and MetaCyc. For example, more than 8000 enzyme reactions can be found in the KEGG

database, indicating that nearly infinite numbers of combinations are possible to design multistep enzymatic cascade reactions. A computational method may be a promising approach for mining the comprehensive biochemical information databases and for rationally designing a synthetic enzymatic pathway (Araki et al., 2014). Besides these bioprospecting approaches, the integration of water-compatible metal- and organo-catalysts into biocatalytic conversion systems may also greatly expand the repertoire of multienzyme systems (Gröger and Hummel, 2014). As well as our expanding knowledge about natural biocatalytic molecules, advancements in these relevant areas will further inspire us to develop more diverse, feasible, and sustainable bioconversion systems.

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Chapter 17

Biocatalysis for Industrial Production of Active Pharmaceutical Ingredients (APIs)

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17.1 INTRODUCTION

From an industrial point of view, biocatalysis is particularly important in the production of active pharmaceutical ingredients (APIs), offering increasingly high demands for regio-, stereo-, and enantioselectivity of drugs. Biocatalytic processes are more ecofriendly, sustainable, and profitable, and hence biocatalysis is proving to be key for the development of the so-called bioeconomy. Thus, in the manufacturing of APIs, as either pharmaceutical products or intermediates, biocatalysis is considered as a “green” technology to efficiently discriminate between isomers in a racemic mixture under mild conditions, compared to chemical salt resolutions which may involve contaminants and are expensive. In addition to enantioselectivity, regioselectivity on complex molecules is another inherent feature of chemoenzymatic processes, which avoids the need of protecting groups, and reduces the number of synthetic steps. Finally, biocatalysis can also be used to produce achiral APIs where classical chemical methods are too complicated. Therefore, a huge number and quantity of enzymes are now available in the market (Reetz, 2013). Although isolated enzymes are mainly chosen for their simple implementation in biocatalyzed processes, whole-cell biotransformations are generally preferred for complex reactions involving more than one enzyme or cofactor, or for reactions where enzymes are not

suitable for isolation. We herein provide examples of the application of isolated enzymes (wild-type or mutants) and whole cells, either in soluble or immobilized form, in the synthesis of some valuable APIs.

17.2 SOURCE, QUALITY, AND SPECIFICATIONS OF BIOCATALYSTS EMPLOYED FOR THE SYNTHESIS OF APIs

Biocatalysis is becoming a widespread methodology for the synthesis of chiral compounds, not only at laboratory scale, but also at industrial scale (Solano et al., 2012). Microorganisms are the main source of industrial enzymes, although recombinant enzymes (higher productivity levels) are used in the synthesis of different APIs rather than their wild-type counterparts. Among them, microbial hydrolases (eg, lipases, esterases, acylases, amidases, proteases) and oxidoreductases (eg, reductases, monooxygenases) are some examples of useful enzymes in the pharmaceutical industry.

Lipases are extremely helpful since they can catalyze many enantioselective reactions involving carboxylic groups (esterifications, transesterifications, and aminolysis) under very mild reaction conditions, and they are highly stable and active in organic solvents (Gotor-Fernández et al., 2006; Reetz, 2002).

Optically active alcohols are useful and important starting materials for the synthesis of various pharmaceuticals and fine chemicals (Kataoka et al., 2003). Asymmetric reduction of prochiral carbonyl compounds using isolated enzymes (mainly aldo-keto reductases, including aldehyde reductases and carbonyl reductases) or whole-cell systems is an attractive alternative for the production of chiral alcohols instead of chemical methods, showing advantages such as low cost, high yield, environmentally friendly, and remarkable chemo-, regio-, and stereoselectivity. In these bioreductions, expensive cofactors such as NAD(P)H/NAD(P)+ can be conveniently recycled through enzyme-coupled and substrate-coupled systems (Goldberg et al., 2007a,b; Ni & Xu, 2012). Nevertheless, cofactor dependency still represents a major impediment to true preparative applicability of reductases as well as many interesting enzymes for API synthesis, such as monooxygenases, dehydrogenases, oxidases, and peroxidases (Hall & Bommarius, 2011; Hollmann et al., 2010).

Chiral diols are another group of key building blocks valuable for preparing a variety of important chiral chemicals and pharmaceuticals. While the preparation of optically pure diols is generally not an easy task in synthetic organic chemistry, three classes of enzymes, namely dicarbonyl reductase, dioxygenase, and epoxide hydrolase, display remarkable ability to stereoselectively introduce two hydroxyl groups in a single-step enzymatic conversion (Chen et al., 2012).

An enzyme may sometimes have to tolerate harsh conditions in an industrial process, including high temperatures, extreme pH values, high substrate and product concentrations, oxidants, and presence of organic solvents. Immobilization can be considered the most traditional way to increase the robustness of enzymes in such conditions (Hanefeld et al., 2009). In addition,

immobilization has become mandatory in industry in order to use repeatedly these biocatalysts, making it a cost-effective process.

On the other hand, molecular engineering techniques, such as directed evolution of enzymes (Behrens et al., 2011), have also contributed to create new biocatalysts with improved activity, stability, and selectivity (Born scheuer et al., 2012; Denard et al., 2015).

Toxicological issues due to unwanted contaminants should be taken into account in the production of enzymes to be used in APIs manufacture, trying to avoid pathogenic microbial sources, and the presence of infectious agents if enzymes are obtained from mammalian origin (Wells et al., 2012).

17.3 EXAMPLES OF BIOCATALYSTS APPLIED TO THE SYNTHESIS OF APIs

In this section we will review some of the most important biocatalysts that are used for the production of pharmaceutical compounds in order to exemplify their versatility in different industrial processes.

17.3.1 Enzyme Inhibitors with Therapeutic Effect

17.3.1.1 Cyclooxygenase Inhibitors

Profens (2-aryl-propionic acids, such as ibuprofen, naproxen, ketoprofen, flurbiprofen, etc.) are considered one of the most important nonsteroidal anti-inflammatory drugs (NSAIDs) used in the treatment of inflammation associated with tissue injury. Although the anti-inflammatory activity of profens is mainly due to the capacity of their (*S*)-enantiomer to inhibit cyclooxygenase (COX), these drugs are commercially available as racemates. Enantioselective resolution of racemates of profens can be carried out by microbial lipases, mainly those ones from yeasts, such as *Candida antarctica* or *Candida rugosa*, and fungi, such as *Rhizomucor miehei* or *Mucor javanicus*. In this sense, there are two enzymatic approaches to perform the racemate resolution in order to obtain the optically pure (*S*)-acids (Fig. 17.1): an enantioselective hydrolysis of 2-aryl-propionate ester racemates in aqueous media catalyzed by microbial lipases with preference for the (*S*)-isomer; or an enantioselective esterification of 2-aryl-propionic acid racemates in organic media catalyzed by microbial lipases with preference for the (*R*)-isomer (Qin et al., 2013; Sikora et al., 2014).

Both approaches have been successfully employed in the efficient production of (*S*)-ibuprofen (Habibi et al., 2013; Long et al., 2005; Siodmiak et al., 2012), (*S*)-naproxen (Battistel et al., 1991; Ozylmaz & Sayin, 2013; Takac & Bakkal, 2007; Yilmaz et al., 2011), (*S*)-ketoprofen (Kato et al., 2000; Liu et al., 2004; Ong et al., 2006; Xi & Xu, 2005), and (*S*)-flurbiprofen (Morrone et al., 1995; Tamborini et al., 2012; Zhang et al., 2007) with high enantiomeric excess (ee > 99%) and good enantioselectivity (E > 100). Likewise, directed enzyme evolution has been demonstrated to be a useful tool to improve significantly

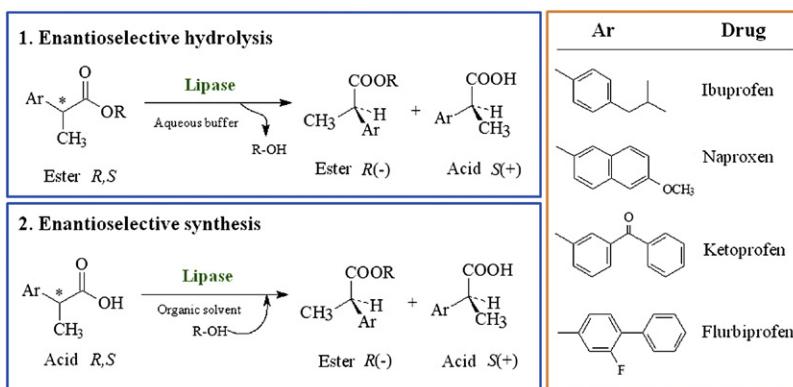


FIGURE 17.1 Lipase-catalyzed resolutions of profens.

both hydrolytic activity and enantioselectivity of *C. antarctica* lipase B toward profen esters of flurbiprofen and ketoprofen (Qin et al., 2013).

Another API for NSAIDs synthesis is the enantiopure *S*-isomer of mandelic acid (hereafter (*S*)-MA), which is considered an important intermediate for the synthesis of selective COX-2 inhibitors such as celecoxib (Celebrex®) and deracoxib (Deramaxx®). The production of (*S*)-MA via deacylation with (*S*)- α -acetoxyphenylacetic acid can be performed either by whole *Pseudomonas* sp. cells (Ju et al., 2010) or enantioselective lipase from *Aspergillus fumigatus* (Shangguan et al., 2012).

17.3.1.2 Angiotensin-Converting Enzyme Inhibitors

Angiotensin-converting enzyme (ACE) inhibitors are widely used in the treatment of hypertension and congestive heart failure. Ethyl (*R*)-2-hydroxy-4-phenylbutyrate (hereafter (*R*)-HPBE) and (*R*)-2-hydroxy-4-phenylbutyric acid (hereafter (*R*)-HPBA) are important chiral precursors in the manufacture of many important ACE inhibitors, such as benazepril, delapril, enalapril, and ramipril. Such intermediates have been successfully prepared by several biocatalytic approaches. In this sense, asymmetric reduction of 2-oxo-4-phenylbutyric acid (OPBA) to yield (*R*)-HPBA has also been successfully performed by whole-cell *Escherichia coli* biocatalyst expressing the highly stereoselective Y52L/F299Y mutant of NAD-dependent D-lactate dehydrogenase (D-nLDH) from *Lactobacillus bulgaricus* as well as the formate dehydrogenase (FDH) as the cofactor regeneration system (Fig. 17.2) (Sheng et al., 2014).

In addition, a recombinant *E. coli* strain coexpressing both NADPH-dependent carbonyl reductase and glucose dehydrogenase from *Bacillus subtilis* has shown excellent catalytic activity in (*R*)-HPBE production by asymmetric reduction of ethyl-2-oxo-4-phenylbutyrate (OPBE) (Ni et al., 2013). Likewise, enzymatic reduction of OPBE to yield (*R*)-HPBE catalyzed by extracellular carbonyl reductase from *Candida glabrata* expressed in *Pichia pastoris* has

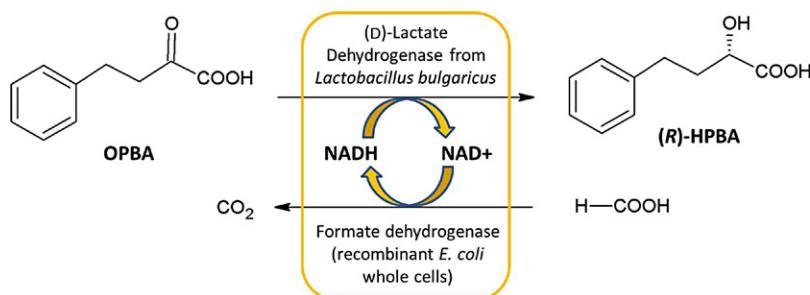


FIGURE 17.2 (*R*)-HPBA production from OPBA by employing a coupled enzymatic system of (D)-lactate dehydrogenase and formate dehydrogenase.

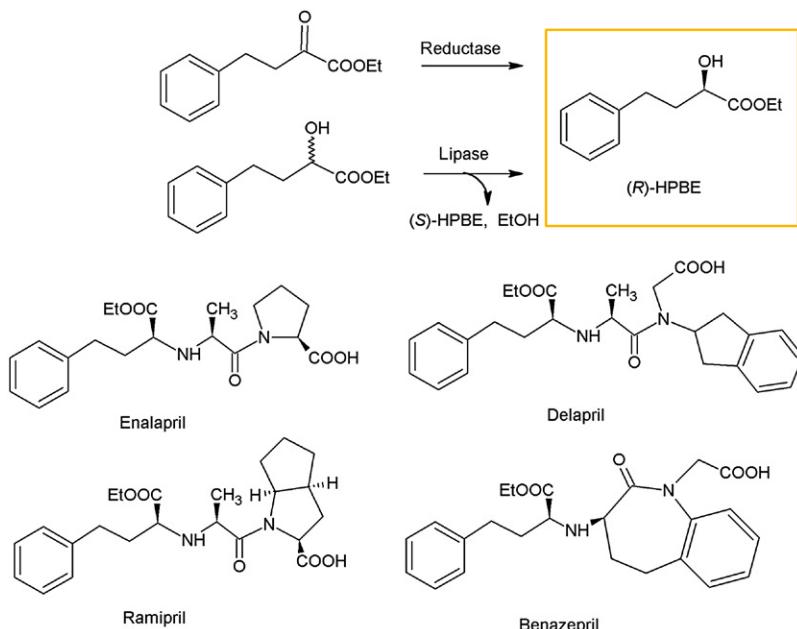


FIGURE 17.3 Biocatalytic approaches for the preparation of ethyl (*R*)-2-hydroxy-4-phenylbutyrate as a chiral intermediate for the synthesis of several ACE inhibitors such as enalapril, delapril, ramipril, and benazepril.

demonstrated significant advantages such as high conversion, eco-friendliness (neutral pH and room temperature), and remarkable stereoselectivity (Qian et al., 2014). Another possible way for producing (*R*)-HPBE is the kinetic resolution of the racemic 2-hydroxy ester catalyzed by *Pseudomonas* lipases (Fig. 17.3) (Liese et al., 2002).

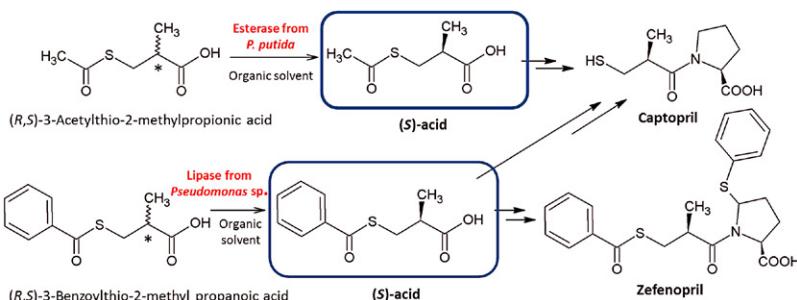


FIGURE 17.4 Enzymatic synthesis of chiral intermediates for the preparation of captopril and zefenopril.

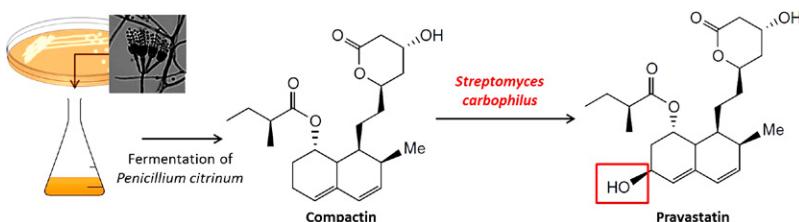


FIGURE 17.5 Biotransformation of compactin to pravastatin.

D- β -Acetylthioisobutyric acid (DAT) also known as *(S)*-(-)-3-acetylthio-2-methylpropionic acid, is a key intermediate for the synthesis of other ACE inhibitors such as captopril and alacepril. DAT synthesis can be performed through the enzymatic resolution of methyl (D,L)- β -acetylthioisobutyrate (DL-MATI) catalyzed by microbial lipases or esterases (Shaw et al., 2006b; Tai et al., 1993). Hence, immobilized esterase from *Pseudomonas putida* IFO12996 on magnetic nanoparticles hydrolyzed DL-MATI to give DAT with high enantioselectivity (ee = 97.2%, E = 245) (Shaw et al., 2006a). Similarly, other microbial lipases have been successfully used for the enantioselective synthesis of the side-chain of other ACE inhibitors such as zefenopril (Patel et al., 1991) and fosinopril (Patel et al., 1990) (Fig. 17.4).

17.3.1.3 HMG-CoA Reductase Inhibitors

Pravastatin is a highly potent and specific inhibitor of HMG-CoA reductase, a key enzyme in cholesterol biosynthesis. Because of its pharmaceutical value as a cholesterol-reducing agent, pravastatin production can be accomplished by compactin hydroxylation in actinomycetes strains. The industrial manufacturing of pravastatin has been performed in *Streptomyces carbophilus* (Fig. 17.5), in which a cytochrome P450 monooxygenase (P450sca-2) was able to catalyze the compactin hydroxylation (Watanabe et al., 1995). Some other

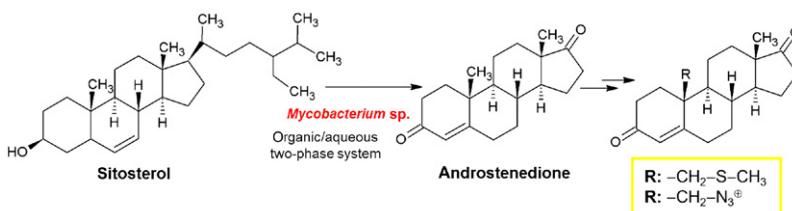


FIGURE 17.6 Bioconversion of sitosterol to androstenedione.

biotransformations for pravastatin production by *Actinomadura* sp. (Peng & Demain, 2000), *Streptomyces* sp. (Park et al., 2003), *Streptomyces griseolus*, and *Pseudonocardia autotrophica* (Park et al., 2003) have also been reported.

17.3.1.4 Aromatase Inhibitors

A relevant industrial biotransformation for the pharmaceutical industry is the selective cleavage of the side-chain of natural phytosterols (eg, sitosterol) by *Mycobacterium* sp. to produce androstenedione (AD) and androsta-1,4-diene-3,17-dione (ADD). These steroids are important intermediates for the synthesis of corticosteroids, mineralocorticoids, oral contraceptives, and several aromatase inhibitors (AIs) used for the treatment of breast cancer and ovarian cancer in post-menopausal women and gynecomastia in men (Fig. 17.6). The AD/ADD market size has been evaluated at over \$1 billion annually. The major shortcomings that exist in sterol biotransformation are the low solubility of steroid compounds in aqueous systems ($1 \mu\text{M}$) due to their strong hydrophobicity, and the toxicity of steroid product AD(D) on microbial cells. One of most efficient approaches used to solve these two problems is a whole-cell bioconversion in an organic/aqueous two-phase system (Cruz et al., 2001; Donova & Egorova, 2012; Xu et al., 2014).

17.3.1.5 14α -Demethylase Inhibitors

Available antifungal drugs show limited utility in the treatment of invasive and systemic fungal infections due to toxicity and resistance. Many azole antifungal agents, such as fluconazole and itraconazole, are believed to inhibit 14α -demethylase, one of the key enzymes involved in the fungal ergosterol biosynthesis. Different enzymes such as lipase B from *C. antarctica* (Morgan et al., 1997) and epoxy-hydrolase from *Aspergillus niger* (Monfort et al., 2002) have been demonstrated to be useful in the preparation of enantiopure building blocks for the synthesis of novel triazoles derivatives with antifungal activity (Fig. 17.7).

17.3.1.6 Inhibitors of the Bacterial Cell Wall Synthesis Machinery

17.3.1.6.1 Semisynthetic β -Lactam Antibiotics

Penicillin and cephalosporins are the most prescribed and effective drugs for bacterial infections. These agents are chemically characterized by the presence of a

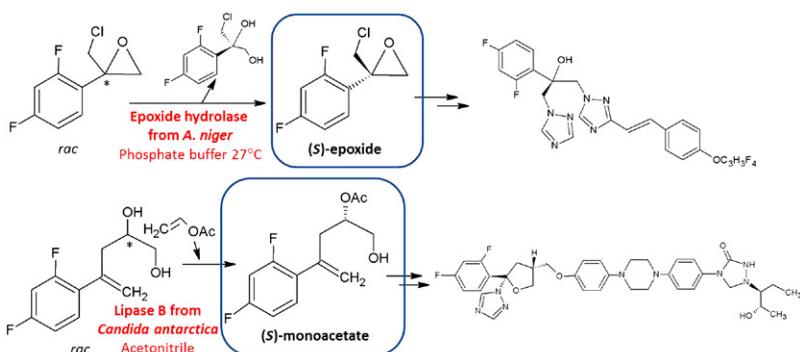


FIGURE 17.7 Enzymatic preparation of enantiopure building blocks for the synthesis of antifungal agents.

common four-membered heterocyclic ring, so-called β -lactam nucleus, which is responsible for the inactivation of the D-Ala-D-Ala-transpeptidases involved in the biosynthesis of the bacterial cell wall. High consumption of β -lactam antibiotics is leading to the accelerated emergence of bacteria which are resistant to the action of these drugs, mainly due to the presence of β -lactamases that hydrolyze the β -lactam ring. As a result, semisynthetic β -lactam antibiotics have been introduced in response to the evolution of β -lactamases and to the demand for antibiotics with broader antibacterial spectrum and/or improved pharmacological properties. The discovery of methods to produce the nuclei of the penicillin molecule (6-amino penicillanic acid, 6-APA), and those of the cephalosporin molecules (7-amino cephalosporanic acid [7-ACA] and 7-amino deacetoxycephalosporanic acid [7-ADCA]) has enabled the production of semisynthetic penicillin and cephalosporins on a large scale. Chemical methods for producing 6-APA, 7-ACA, and 7-ADCA are polluting and expensive, requiring the use of hazardous chemicals and organic solvents. In contrast, enzymatic cleavage of natural penicillin and cephalosporin C to obtain the β -lactam nuclei has been demonstrated to have a positive impact on both the economics of the process and the environment (Arroyo et al., 2003; Maresova et al., 2014; Pollegioni et al., 2013; Srirangan et al., 2013). Natural penicillins (mainly penicillins G and V) and cephalosporin C are produced in bulk by fermentation of *Penicillium chrysogenum* and *Acronymium chrysogenum*, respectively. Then, these fermented products are deacylated enzymatically to generate the respective β -lactam nuclei (Fig. 17.8).

The enzymes which are able to cleave the amide bond between the β -lactam nucleus and the carboxylic acid functionality leaving the cyclic β -lactam amide bond intact are grouped under the general name of β -lactam acylases (also known as β -lactam amidohydrolases). The penicillin G acylase from *E. coli* was quickly recognized as a robust biocatalyst for the industrial production of 6-APA from penicillin G (Maresova et al., 2014). In addition, penicillin V acylases from

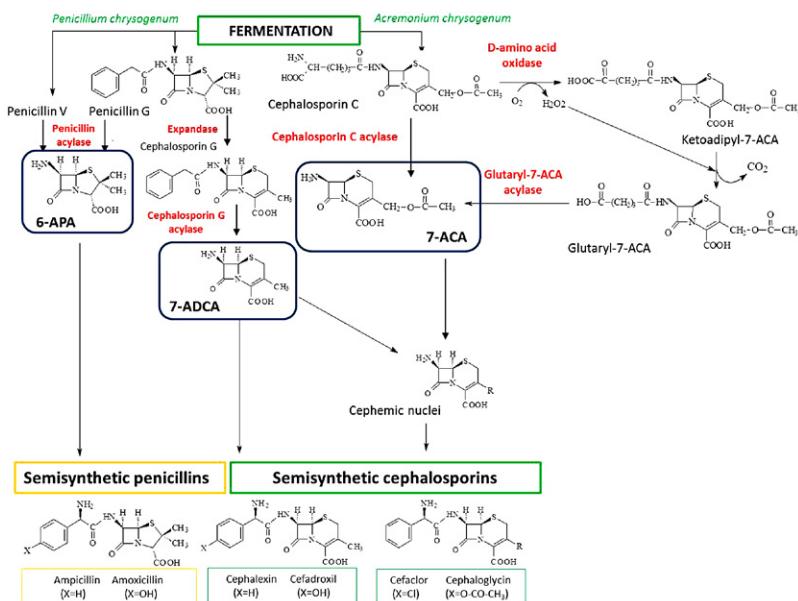


FIGURE 17.8 Enzymatic methods for the production of β -lactam nuclei.

actinomycetes, such as *Streptomyces lavendulae* (Torres-Bacete et al., 2015; Torres-Guzman et al., 2002) and *Actinoplanes utahensis* (Torres-Bacete et al., 2007), have also been established as a promising alternative to 6-APA production from penicillin V. Increasing demand for semisynthetic β -lactam antibiotics has resulted in an exhaustive search for many other microbial enzymes, such as glutaryl-7-ACA acylase, D-amino acid oxidase, and expandase, for the preparation of cepham nuclei (Barber et al., 2004). Optically active D-4-hydroxyphenylglycine (D-HPG), a side-chain of both amoxicillin and cefadroxil, can be produced from racemic DL-5-hydroxyphenylhydantoin (DL-5-HPH) by a two-step enzymatic route which includes the hydrolytic ring cleavage of DL-5-HPH by D-hydantoinase to generate N-carbamoyl-D-p-hydroxyphenylglycine (N-CHPG), and subsequent decarbamoylation of N-CHPG by N-carbamoylase to generate D-HPG (Fig. 17.9) (Nandanwar et al., 2005). Recently, an efficient and cost-effective production of DHPG has been reported by using a whole-cell bioconversion with recombinant *E. coli* which expresses hydantoinase and carbamoylase enzymatic activities from *Agrobacterium* (Zhang & Cai, 2014).

17.3.1.6.2 Fosfomycin

Fosfomycin is a broad-spectrum antimicrobial which irreversibly inhibits phosphoenolpyruvate UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), an enzyme that catalyzes the first step of peptidoglycan biosynthesis. Although

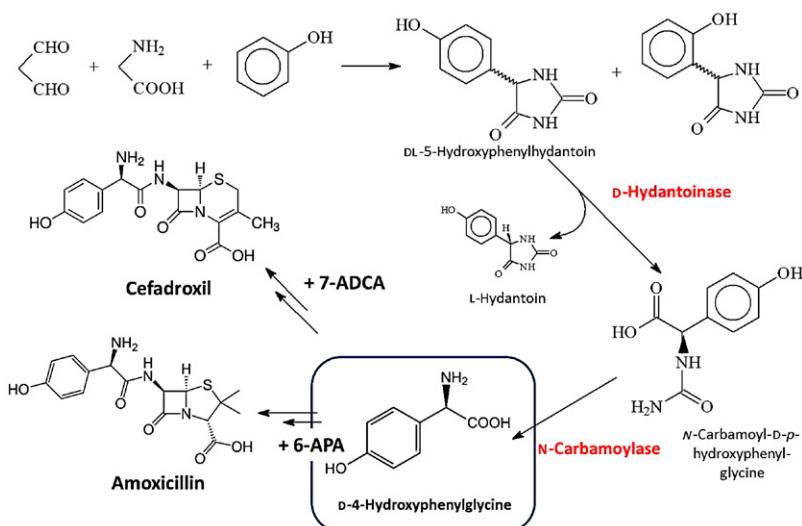


FIGURE 17.9 Biocatalytic process for the production of D-4-hydroxyphenylglycine, intermediate for the synthesis of amoxicillin and cefadroxil.

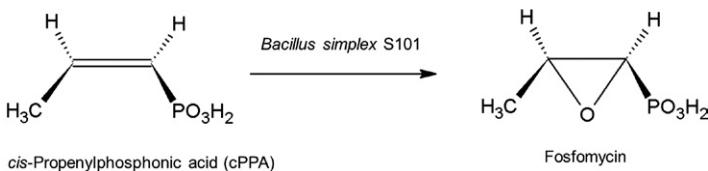


FIGURE 17.10 Biotransformation of *cis*-Propenylphosphonic acid (cPPA) to fosfomycin.

fosfomycin is a secondary metabolite of many strains of *Streptomyces* and *Pseudomonas* and could be obtained by fermentation, it is mainly prepared via chemical epoxidation of *cis*-Propenylphosphonic acid (cPPA). The conversion yield of fosfomycin is less than 50% in the whole chemical process, and a large quantity of waste is produced. Thus, biotransformation has been established as an eco-friendly alternative method of preparation of fosfomycin from cPPA with higher product yield. A number of microorganisms can catalyze this kind of epoxidation (Fig. 17.10), such as *Penicillium spinulosum* (White et al., 1971), *Cellvibrio gilvus* (Aisaka et al., 1992), *Bacillus simplex* (Xie et al., 2009), and some other aerobic bacteria and actinomycete strains (Itoh et al., 1995).

17.3.1.7 Inhibitors of the Fungal Cell Wall Assembly

Despite their relatively narrow spectra and weak solubility, echinocandins have proven to be promising antimycotics due to their strong anti-*Candida* and anti-*Aspergillus* activities, their unique mode of action, and their remarkable

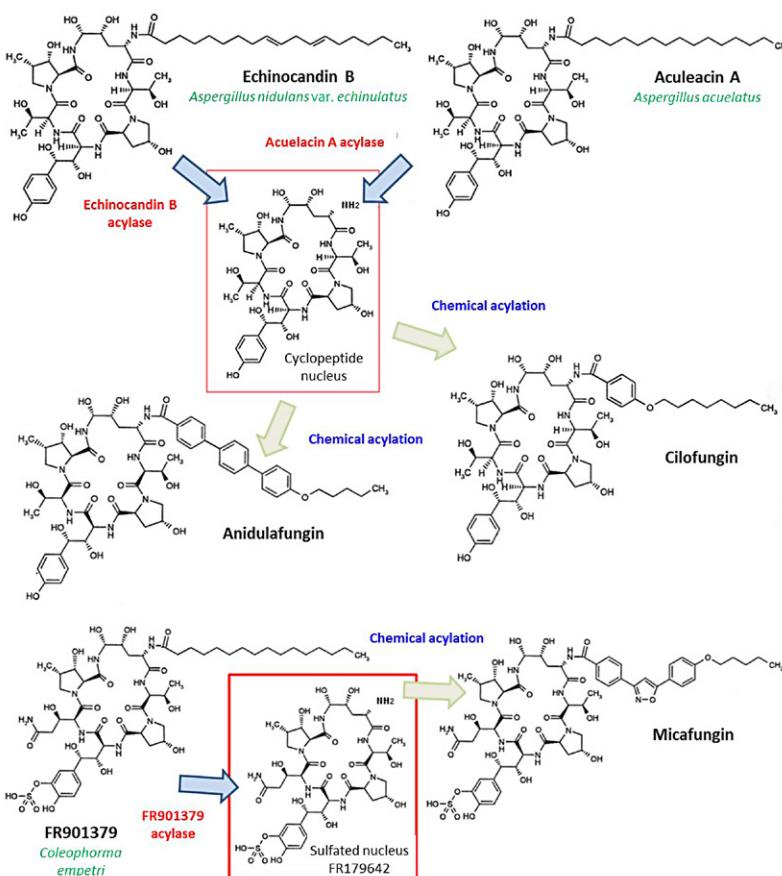


FIGURE 17.11 Enzymatic processes for the production of cyclopeptidic nuclei for the preparation of semisynthetic echinocandins.

efficiency against azole-resistant strains (Emri et al., 2013). Echinocandins inhibit the β -1,3-glucan synthase complex which is involved in the obstruction of the biosynthesis of the fungal cell wall.

Modifications of natural echinocandins are focused on the improvement of their water solubility and/or the reduction of their hemolytic activity, which also increase both the antifungal activity and spectra against sensitive fungi. Some FDA-approved semisynthetic echinocandins, such as anidulafungin (Eraxis[®]) and micafungin (Mycamine), are synthesized via chemical acylation of the corresponding deacylated cyclohexapeptidic nuclei, which are previously produced by enzymatic hydrolysis of the fatty acid-ornithine amide bond of natural echinocandins (Fig. 17.11). Hence, the most relevant biocatalyst in the semisynthesis of echinocandins is represented by acuelacin A acylase from *Actinoplanes utahensis* (Hormigo et al., 2010), which also shows penicillin acylase activity

(Torres-Bacete et al., 2007), and its encoding gene has been suggested to codify for echinocandin B acylase as well (Velasco-Bucheli et al., 2015). Other similar enzymes have been isolated from *Streptomyces* spp. (Ueda et al., 2011), and even from filamentous fungi (Ueda et al., 2010).

17.3.1.8 Inhibitors of DNA Synthesis

Modified nucleosides are important molecules which display antiviral and anti-tumoral activity due to their capacity for inhibiting DNA synthesis. In all cases, acquired resistance and/or undesired side effects of these modified nucleosides are major problems that have encouraged the development of new analogs with therapeutic activity. Nucleoside analogs have been traditionally synthesized by different chemical methods, which often require time-consuming multistep processes, including protection and deprotection reactions on the heterocycle base and/or the pentose moiety to allow the modification of naturally occurring nucleosides (Boryski, 2008). Biocatalysis should be considered as an eco-friendly and cost-effective alternative in nucleoside synthesis (Ferrero & Gotor, 2000; Li et al., 2010). Several types of reactions can be carried out to transform nucleoside derivatives, which include acylation, deacylation, glycosylation, halogenation, and deamination. On one hand, enzyme-catalyzed transfer of glycosyl residues to acceptor bases can be performed by one-step transglycosylations mediated by nucleoside 2'-deoxyribosyltransferases (NDTs), which are actually far more advantageous than the two-step reactions catalyzed by combination of different nucleoside phosphorylases (NPs) (Fresco-Taboada et al., 2013) (Fig. 17.12). Both approaches with NPs or NDTs have been accomplished by

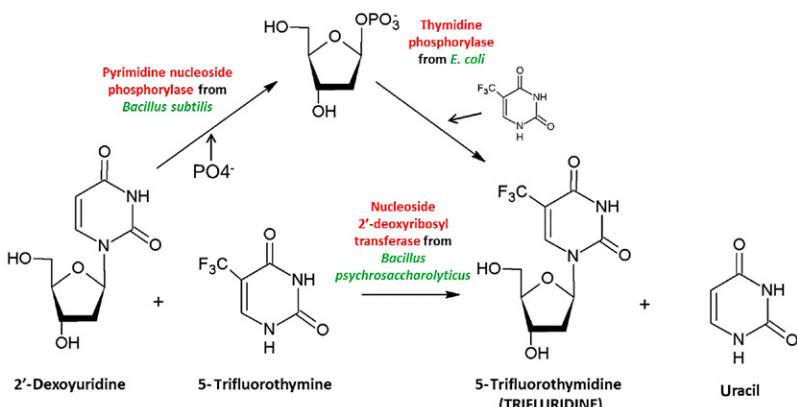


FIGURE 17.12 Two approaches for the enzymatic synthesis of 5-trifluorothymidine (trifluridine) employing (1) nucleoside 2'-deoxyribosyltransferase (Fresco-Taboada et al., 2014) or (2) nucleoside phosphorylases (Serra et al., 2013).

employing soluble and immobilized enzymes, or whole cells of microorganisms containing high amount of the required enzyme. For instance, immobilized NDT from *Lactobacillus reuteri* (Fernández-Lucas et al., 2011; Fernández-Lucas et al., 2013), and NDT form *Bacillus psychrosaccharolyticus* (Fresco-Taboada et al., 2014) have been successfully used in the enzymatic synthesis of different modified nucleosides with antitumoral and antiviral effect.

Likewise, AMP deaminase (AMPAD) from *Aspergillus* species (Margolin et al., 1994) and adenosine deaminase (ADA) from calf intestinal mucosa (Alessandrini et al., 2008; Ciuffreda et al., 2002) may be considered as other valuable biocatalysts in nucleoside chemistry. These enzymes can be used to carry out the transformation of purine nucleosides that are modified in the base or the ribose moiety, and their application can also be extended to carbocyclic nucleosides or acyclonucleosides (Santaniello et al., 2005).

17.3.2 Drugs that Alter Transport Across Membranes

17.3.2.1 Calcium Channel Blockers

Calcium channel blockers, also called calcium antagonists, prevent calcium from entering cells of the heart and blood vessel walls, resulting in lower blood pressure and a slower heart rate. Therefore, these drugs are mainly prescribed to relieve chest pain (angina) and to control an irregular heartbeat. Some chiral intermediates for the synthesis of different calcium channel blockers like diltiazem and 1,4-dihydropyridine derivatives (such as nifedipine and nimodipine) can be prepared through lipase-catalyzed resolution of racemic precursors. In this sense, lipase from *Serratia marcescens* ECU1010 has been considered as a useful biocatalyst for the kinetic resolution of racemic trans-3-(4'-methoxyphenyl)-glycidyl methyl ester [(\pm)-MPGM], a key intermediate for the synthesis of diltiazem hydrochloride (Wang et al., 2010b). Likewise, enzyme-catalyzed resolution and asymmetric synthesis of 1-aryl-1,4-dihydropyridine-3,5-dicarboxylic diesters have been carried out by the lipase from *Pseudomonas fluorescens* (Achiwa & Kato, 1999).

17.3.2.2 Potassium Channel Openers

ATP-sensitive potassium channel openers (PCOs) are a group of compounds with a wide spectrum of potential therapeutic applications, and a few drugs are currently used as antihypertensive agents. Interest in the PCOs was triggered in the early 1980s by the discovery of cromakalim (CRK), which is a powerful smooth muscle relaxant. A highly pure chiral (+)-*trans*-diol intermediate for the synthesis of CRK was obtained by the stereoselective microbial epoxidation of 2,2-dimethyl-2*H*-1-benzopyran-6-carbonitrile using *Mortierella ramanniana* whole cells as biocatalyst (Patel et al., 1994) (Fig. 17.13).

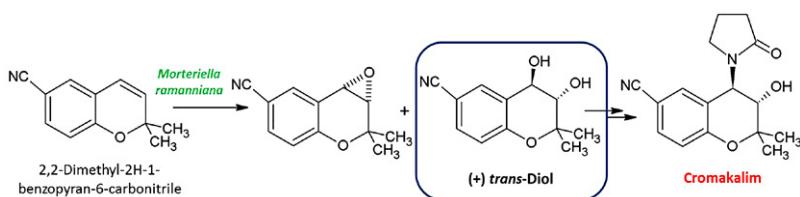


FIGURE 17.13 Whole-cell biotransformation for the production of *trans*-diol precursor of cromakalim.

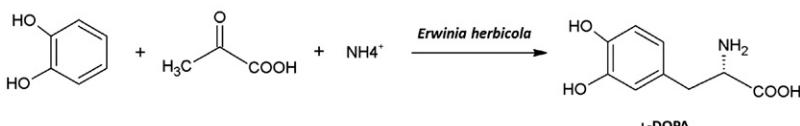


FIGURE 17.14 Biotechnological method for L-DOPA production.

17.3.3 Drugs that Interact with Membrane Receptors

17.3.3.1 Presynaptic Adrenergic Drugs

L-DOPA (3,4-dihydroxyphenyl-L-alanine) has been widely used as a drug for the treatment of Parkinson's disease caused by deficiency of the neurotransmitter dopamine. The world market of L-DOPA is about 250 tons per year, and the total market volume is about \$101 billion per year (Min et al., 2015). Asymmetric hydrogenation for the synthesis of L-DOPA shows critical limitations such as poor conversion rate and low enantioselectivity. Accordingly, improved alternative biotechnological methods for L-DOPA production have been established such as microbial fermentation using whole-cell microorganisms with tyrosinase (Ali et al., 2007; Surwase et al., 2012), tyrosine phenol-lyase (Tpl) (Koyanagi et al., 2005), or *p*-hydroxyphenylacetate 3-hydroxylase (PHAH) activity (Lee & Xun, 1998), and enzymatic conversion by immobilized tyrosinase (Ates et al., 2007; Seetharam & Saville, 2002). *Erwinia herbicola* is the most favorable strain due to its high Tpl activity, and has been used for L-DOPA production by Ajinomoto Co. Ltd since 1993 (Fig. 17.14).

Another example of API for the synthesis of presynaptic adrenergic pharmaceuticals is L-phenylacetylcarbinol also known as L-PAC or (*R*)-1-hydroxy-1-phenyl-propan-2-one. This compound is a chiral intermediate in the production of bronchodilators L-ephedrine and D-pseudoephedrine (Shukla & Kulkarni, 2000), which is currently obtained industrially via a biotransformation of benzaldehyde by yeast cultures expressing pyruvate decarboxylase activity (Khan & Daugulis, 2010; Rosche et al., 2001; Shukla et al., 2002) (Fig. 17.15).

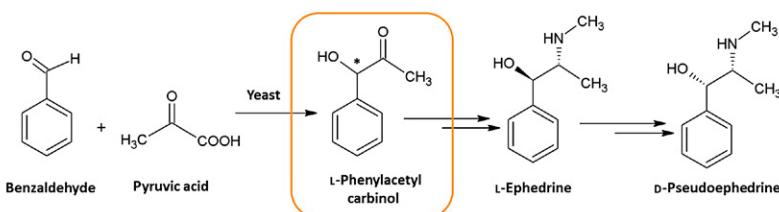


FIGURE 17.15 Biotransformation of benzaldehyde and pyruvic acid into L-PAC for the synthesis of L-ephedrine and D-pseudoephedrine.

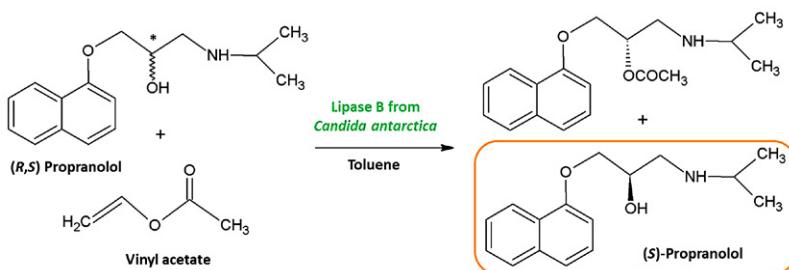


FIGURE 17.16 Lipase-catalyzed transesterification for the resolution of (R,S)-propranolol.

17.3.3.2 Postsynaptic Adrenergic Drugs

Propranolol is a β -adrenergic blocking agent, used for the treatment of arterial hypertension and other cardiovascular disorders, whose therapeutic effect is associated with the S-enantiomer, and administration of the racemic mixture causes serious side effects such as bronchoconstriction or diabetes. Hence, different chemoenzymatic approaches have been tried to obtain single-enantiomer β -blockers like S-propranolol with high enantiomeric purity (Agustian et al., 2010). For instance, enzymatic resolution of (R,S)-propranolol has been accomplished by different hydrolases (lipases, amidases, and esterases) through hydrolysis of the racemic acetate in aqueous buffer (Wang et al., 2010a) or transesterification of the racemic alcohol in organic media (Fig. 17.16) (Barbosa et al., 2010) at moderate temperatures.

17.3.3.3 Serotonergic Drugs

Chiral γ -amino alcohols are valuable building blocks for the synthesis of selective serotonin reuptake inhibitors (SSRI), such as fluoxetine and paroxetine, which are key drugs in the treatment of depression, obsessive compulsive disorder, and panic disorder. Enantioselective reduction of β -amino ketones by carbonyl reductases can provide a straightforward enzymatic method to obtain these chiral compounds (Zhang et al., 2015). Likewise, optically pure

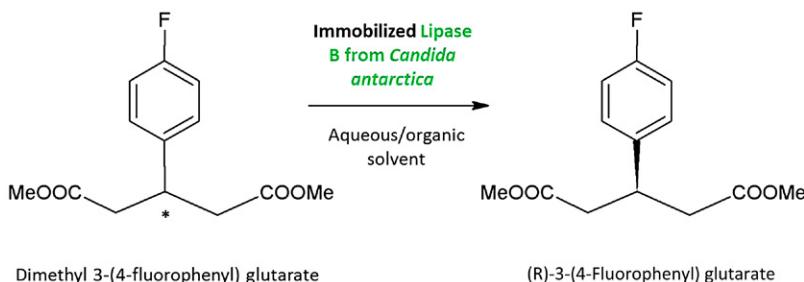


FIGURE 17.17 Enantioselective hydrolysis of 3-DFG to (R)-3-MFG catalyzed by immobilized lipase B from *C. antarctica* (Novozyme 435).

methyl (R)-3-(4-fluorophenyl) glutarate [(R)-3-MFG] is an attractive building block for the synthesis of different SSRI. (R)-3-MFG can be prepared by enzymatic desymmetrization of the prochiral dimethyl 3-(4-fluorophenyl) glutarate (3-DFG) catalyzed by immobilized lipase B from *C. antarctica* in an aqueous–organic solvent biphasic medium (Liu et al., 2012) (Fig. 17.17).

17.3.4 Drugs that Interact with Intracellular Receptors

17.3.4.1 Drugs Targeting Ribosome

Thiamphenicol (a methyl-sulfonyl analog of chloramphenicol) is a potent antibiotic for veterinary applications against infectious diseases, with remarkable activity against both Gram-negative and Gram-positive microorganisms, but negligible solubility in water. Thiamphenicol derivatives with improved solubility can be prepared by regioselective modifications catalyzed by lipase B from *C. antarctica* (da Silva et al., 2014). A similar enzymatic approach has been reported for the regioselective production of chloramphenicol palmitate esters, prodrugs without a bitter taste (Bizerra et al., 2011).

17.3.4.2 Mitosis Inhibitors

Paclitaxel was developed by Bristol-Myers Squibb for ovarian and metastatic breast cancer treatment. Originally, paclitaxel was isolated and purified from the bark of Pacific yew trees, but this source was considered to be economically and ecologically unsuitable since wood cutting of the yew trees was required. Alternative methods for the production of paclitaxel have been reported, including the application of biocatalysis (Patel, 1998). Different enzymes have been described to convert taxanes in extracts of a variety of *Taxus* cultivars to 10-deacetylbaicatin III (Fig. 17.18), a precursor for paclitaxel semisynthesis. Biocatalytic processes have also been described for the preparation of C-13 paclitaxel side chain synthons.

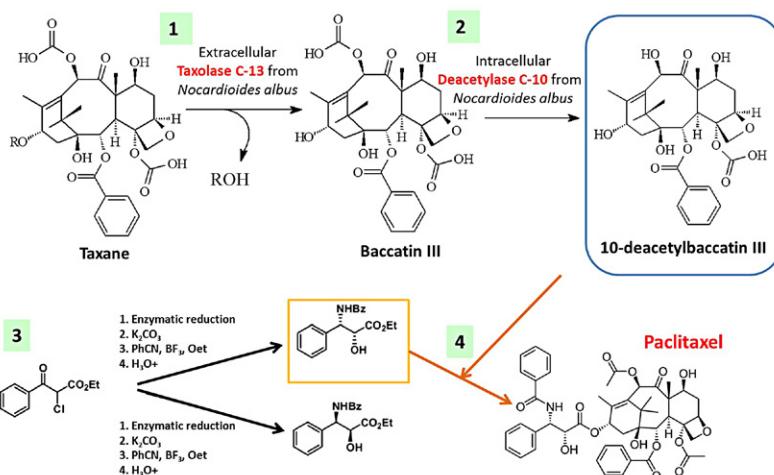


FIGURE 17.18 Semisynthesis of paclitaxel through biocatalytic methods.

17.4 CONCLUDING REMARKS

Biocatalysis has become a relevant tool in the synthesis of APIs, being recognized by many pharmaceutical companies which have integrated this green technology with traditional medicinal chemistry. Biocatalysis has demonstrated that it can provide more sustainable, efficient, and less polluting methods for the production of APIs and advanced pharmaceutical intermediates. In conclusion, the pharmaceutical industry should utilize the advantage of the progress of biochemistry, molecular cloning, random and site-directed mutagenesis, enzyme-directed evolution, immobilization, and fermentation technologies to gain access to a new wave of enzymes and microbial cultures to be used as biocatalysts in the manufacture of APIs and intermediates on an industrial scale, improving the quality of the final products and saving costs (at least 10–15%) as well.

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Chapter 18

Agro-Industrial Residues and Microbial Enzymes: An Overview on the Eco-Friendly Bioconversion into High Value-Added Products

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18.1 INTRODUCTION

Biotechnology has passed through a maturation phase, in which research and development have discovered new applications for biocatalysts in chemical, pharmaceutical, and food industries. The advances in biotechnology, particularly in genetics, protein engineering, and direct evolution, have contributed a new era for enzymes, and the use of enzymes has increased so far in research laboratories as well as at the industrial scale due to their ability to carry out a variety of reactions under varied environmental conditions. Moreover, the capacity to replace harmful chemical reaction conditions and the trend for developing cleaner technologies have enhanced the importance of enzymes for the green chemistry and the bio-based economy society towards the creation of new processes and product innovation in the market. The use of enzymes results in many benefits, such as higher product quality, lower manufacturing costs, less waste, and lower energy consumption (Binod, 2013).

Enzymes have been applied in biotechnology since the earliest of times for the manufacturing of foods, chemicals, and biofuels. For their empirical usages,

enzymes were generated by spontaneously growing microorganisms or by animal and vegetable preparations (Kirk et al., 2002).

The industrial production of enzymes have been reported since 1874, when the Danish scientist Christian Hansen manufactured cheese, using rennin (Chymosin) obtained from calves' stomachs (Nielsen et al., 1994). This enzyme is currently produced using the recombinant DNA technique with the bovine chymosin gene expressed in *Escherichia coli* K-12. This was the first recombinant enzyme approved by the U.S. Food and Drug Administration (FDA) for use in food (Flamm, 1991). Pectinases were used for juice clarification in the 1930s; and for a short period during World War II, invertase was used for sucrose hydrolysis to produce inverted sugar syrup, pioneering the application of immobilized enzymes. The application of enzymes on a large scale began in the 1960s, when glucosidase was used for starch hydrolysis to produce glucose syrups (Fernandes, 2010). This process was used to substitute the acid hydrolysis due to many advantages, that is, higher product yields, increased degree of purity, and easier crystallization. In 1973, the development of immobilized glucose isomerase enabled the industrial production of fructose syrup. Since then, the industrial application of immobilized enzymes has become possible.

The necessity of product innovation as a trend for a sustainable market has inspired technological development, stimulating the creation of new applications for enzymes in different industrial sectors in recent years. There are four major sectors of industrial enzymes: detergent (household care), technical (textile enzymes, leather, pulp and paper, fuel ethanol, and others), food, and feed enzymes.

A wide variety of natural solid supports have been used for biotechnology, with crops and agro-industrial residues being the most studied; these supports supply the main nutrients needed for growth (minerals, vitamins, etc.). In the field of enzyme production, several natural solids have been successfully employed including: wheat, corn, rice, beet, banana waste, potato, tea, coccus, apple, citrus fruits, wheat flours, corn bagasse, and sugarcane bagasse. Of these supports, corn and sugarcane bagasse have been the most widely investigated.

This chapter aims to provide an updated and succinct overview of agro-industrial residues, applications of enzymes in lignocellulosic materials, and production chemical compounds with potential applications from such biomass.

18.2 AGRO-INDUSTRIAL RESIDUES AND THE GENERATION OF HIGH VALUE-ADDED PRODUCTS

Agro-industrial residues are defined as many different wastes from the food and agriculture industry. In recent years, environmental problems have boosted their importance, and a growing interest in the efficient use of various agro-industrial residues. This concern has resulted in an important source for the production of new materials, chemicals, and energy (Rosa et al., 2011). As a result, an extensive range of valuable and usable products have been recovered

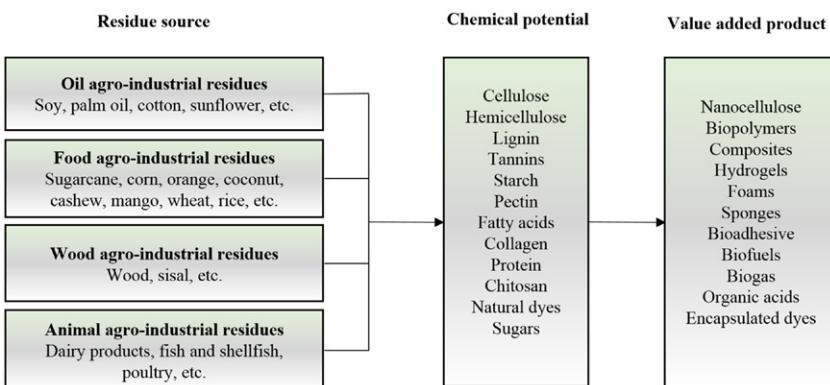


FIGURE 18.1 Appreciation potential of agro-industrial residues of plant and animal origin. Adapted from [Rosa et al. \(2011\)](#).

from what was previously considered waste, which encompasses a huge area of microbial-biotechnology with many possibilities that have been researched, and such findings have shown the massive potential when they are practically and economically applied. ([Singh Nee Nigam and Pandey, 2009](#)).

Agricultural waste is also a broad term in meaning, and it can refer to any lignocellulosic residue produced by agri-food industries in their daily operations such as: leaves, roots, stalks, bark, bagasse, straw residues, seeds, wood residues, and animal residues ([Forster-Carneiro et al., 2013](#)). All of these residues represent a source of billions of tons biomass per year, largely available and renewable ([Forster-Carneiro et al., 2013](#)). Several bioprocesses, such as fermentation processes, have been developed using these residues as substrates for the production of various molecules with high added value, such as microbial proteins, organic acids, ethanol, enzymes, and biologically active secondary metabolites ([Alexandrino et al., 2007](#)). The primary examples of these agro-industrial residues, their chemical potential, and high value-added products are highlighted in Fig. 18.1.

A large amount of agro-industrial residue generated annually, all over the world, contains high lignocellulosics and starch levels. Lignocellulose is the major component of biomass, comprising around half of the plant matter produced by photosynthesis and representing the most abundant renewable organic resource in soil ([Singh et al., 2012](#)). In this context, biomass can be defined as “all organic material of vegetal or animal origin, which is produced in nature or managed ecosystems (agriculture, aquaculture, forestry), all or not industrially transformed” ([Singh Nee Nigam and Pandey, 2009](#)). Sugarcane bagasse, wheat bran, rice bran, corncob, and wheat straw are examples of the cheapest and most abundantly available natural carbon sources from biomass ([Singh et al., 2012](#)). The total amount of lignocellulose compounds have a large variability in each biomass residue from the agro-industry, as shown in [Table 18.1](#).

TABLE 18.1 Composition of Lignocellulosic Compounds of Several Agro-Industrial Residues

Composition wt%	Agro-industrial residue										
	Switchgrass	Rice straw	Wheat straw	Bagasse	Corn stover	Corn cob	Beet pulp	Coconut husk	Hardwood stem	Softwood stem	Silver grass
Cellulose	31–45	35–38	35–40	41–50	27–48	34	23	44	40–55	45–50	37
Hemicellulose	24–31	25–26	21–27	25–30	13–27	34	36	12	24–40	25–35	29
Lignin	12–23	13–25	15–25	18–25	14–31	18	1	33	18–25	25–35	19

Source: [Kobayashi and Fukuoka \(2013\)](#).

Microorganisms degrade lignocellulosic and starchy materials because of their highly efficient enzymatic system. At least two types of extracellular enzymatic systems are present in microorganisms: the hydrolytic system responsible for polysaccharide degradation, and ligninolytic system that degrades lignin and opens phenyl rings (Singh et al., 2012). Both systems are being used in biotechnology and biorefinery approaches, applying multiple biomass resources for generating fuels, chemical products, and energy sources. The most recent studies are showing the production of subproducts from the fermentation of lignocellulosic compounds, obtaining a large range of bioproducts of resins, polymers and film barriers, biodegradable plastic, dispersers, flocculants, as well as other more traditional uses such as cellulose, paper, and textile fibers (Forster-Carneiro et al., 2013). Another applicable example is the recent use of orange residue (generated after the removal of juice), as a substrate to obtain hydrolytic and oxidative enzymes involved in the degradation of lignocellulosic materials, including laccase, manganese peroxidase, xylanase, and endo-1,4-glucanase by the basidiomycetes, *Pleurotus ostreatus* cultivated in a solid state (Alexandrino et al., 2007).

Research of the key and novel microorganisms for degradation of biomass in a biotechnology outlook does not only depend on the microorganisms' hydrolytic systems, but also on the main characteristics of the specific biomass residue. Thus, what should agro-industrial residues possess in order to be a good substrate for microorganisms? Smolinska et al. have listed several conditions of organic waste materials which can be applied as carriers for antagonistic microorganisms. A list of four requirements are outlined in the work: they should be (1) cheap (or redundant) and readily available; (2) a good medium for fungal growth; (3) granulated easily (have proper proportion of dry and wet components); and (4) should favorably affect the soil and plants (Smolinska et al., 2014).

Following this perspective, a demonstrative example from Brazil can be mentioned, where sugarcane has been used as a raw material for ethanol for several years and has had the highest growth of this culture. Each ton of processed sugarcane generates around 140kg of bagasse and between 60% and 90% of this waste is used by the sugar industry as fuel for power generation, heat (Rosa et al., 2011), and as an ideal substrate for microbial processes that produce value-added products such as bioethanol (Pandey et al., 2000). The percentages of cellulose, cellulose conversion to ethanol, and annual production of bagasse and other agro-industrial residues are reported in Table 18.2.

Looking at the other side of the coin, the surplus of the main agro-industrial residues generates serious environmental and storage issues. Most agro-industrial residues, in a certain way, may represent a loss of biomass, nutrients, and problems stemming from their accumulation in nature, increase in potential pollution associated with inadequate provision, in addition to the pollution of soil and water bodies when compounds are leached, which may also cause public health problems (Rosa et al., 2011). On the other hand, the high cost

TABLE 18.2 Chemical Composition of Biomass Used for Bioethanol Production in Brazil

Agro-industrial residue	Cellulose (%)	Cellulose conversion to ethanol (%)	Annual residual production (ton)	Bioethanol production (L)
Sugarcane straw	39	85	208 millions	87.38 billions
Wheat straw	35	89	6 millions	2.37 billions
Rice straw	38	80	3 millions	1.15 billions
Sugarcane bagasse	43	89	208 millions	100.88 billions
Banana pseudostem	46	61	50 millions	17.78 billions

Source: Nunes et al. (2013).

of treatment, transportation, and final disposal of generated waste has a direct effect on the price of the final product (Rosa et al., 2011).

Once again, in the case of Brazil, the price of bagasse varies significantly, depending on the unpredictable harvest period. The price of bagasse was between USD 43 and 52 per metric ton in 2014, significantly higher than the price of USD 11–13 per metric ton in 2009 (IRENA, 2015). Biomass power plants require sustainably sourced, low-cost, adequate, and predictable biomass feedstock supplies. The range of costs for feedstock is highly variable, from zero for wastes produced as a result of industrial processes—and even negative prices for waste that would otherwise have incurred disposal costs (eg, black liquor from pulp and paper mills)—to potentially higher prices for dedicated energy crops, if productivity is low and transportation costs are high (IRENA, 2015).

Feedstock costs typically account for 20–50% of the final cost of electricity based on biomass technologies. Agricultural residues, such as straw and sugarcane bagasse, tend to be the least expensive feedstocks, as they are a harvest or processing by-product, but are correlated with the price of the primary commodity from which they are derived, and they have registered increased costs over the 5 years from 2010 to 2015 (IRENA, 2015).

18.3 ENERGY FROM AGRO-INDUSTRIAL RESIDUES

A sustainable way of processing biomass into a spectrum of marketable products is biorefining (Poggi-Varaldo et al., 2014). Most lignocellulosic biorefineries are using microbial enzymes to deconstruct plant polysaccharides into sugars.

For instance, sugar compounds obtained from nonfood biomass can be the first platform chemical in the biorefinery, being an interesting platform chemical for plastics and fuels (Kobayashi and Fukuoka, 2013).

Biohydrogen for example, is a sustainable form of energy that can be produced from organic waste through fermentation processes involving dark fermentation and photofermentation. This bioenergy is quite often included as a part of a biorefinery approach, which reclaims organic wastes that are abundant sources of renewable and low-cost substrate that can be efficiently fermented by microorganisms (Poggi-Varaldo et al., 2014).

Bioenergy, the renewable energy released from biomass, is certainly expected to contribute significantly in the mid- to long-term. According to the International Energy Agency (IEA), bioenergy offers the potential to meet 50% of our world energy needs in the 21st century (Singh Nee Nigam and Pandey, 2009). About 140 billion tons of biomass from the agriculture sector are generated annually across the world (Forster-Carneiro et al., 2013). This biomass volume can be converted into great amounts of energy and feedstock—equivalent to approximately 50 billion tons of oil—which could substantially decrease the use of fossil fuels and reduce the emission of greenhouse gases (Forster-Carneiro et al., 2013).

Currently, the first generation of biofuels uses food crops such as sugarcane, corn, sugar beet, and sweet sorghum for bioethanol; waste vegetable oils, animal fats, rapeseed (canola), soybean, oil palm, and *Jatropha* are converted into food ingredients, oleo-chemicals, and biodiesel (Canadell and Schulze, 2014; Singh Nee Nigam and Pandey, 2009). On the other hand, the use of certain food crops for the production of biofuels may represent a negative feature wherein fuels may replace food, depending on market fluctuations, which could cause a social problem. To complicate this issue, in addition to the recent imbalance in the oil market, the hike in fuel costs have initiated a global challenge for biofuel production from lignocelluloses, the second generation of biofuels (2G) (Menon and Rao, 2012). The 2G production has been considered feasible, provided that there is a thorough analysis of the pretreatment, hydrolysis, and fermentation processes (Caldeira-Pires et al., 2013). In numbers, biomass as a bioenergy source provides about 10.2% (50.3 EJ/year) of the annual world's total primary energy supply (TPES) from a wide variety of biomass sources (Menon and Rao, 2012). Nowadays, in the race towards sustainable development, the increased use of biomass is associated with significant environmental gains (Caldeira-Pires et al., 2013).

Therefore, the success of microalgae-production systems is key to developing the third generation of biofuels. Algae are able to accumulate large quantities of lipids for biodiesel production with little or no requirement of productive land (Canadell and Schulze, 2014). The third generation of biofuels produced by algae are obtained with three distinct algae production mechanisms, photoautotrophic, heterotrophic, and mixotrophic can be used, all of which follow natural growth processes (Singh et al., 2011). Algae, especially microalgae that can tolerate high

levels of CO₂, utilize much of the carbon and can also utilize CO₂ from industrial emissions, hence minimizing GHG emissions (Singh et al., 2011).

18.4 BIOTECHNOLOGICAL PROCESSES APPLIED IN THE USE OF VEGETABLE BIOMASS

Lignocellulosic biomass is the most abundant and inexpensive nonedible biomass and can be an excellent source for the production of fuels and value-added compounds. The composition of lignocellulosic materials, in general, primarily consists of cellulose, hemicellulose, pectin, and lignin; and the interactions of these components create a highly resistant and recalcitrant biomass structure (Climent et al., 2014; Petruccioli et al., 2011).

18.4.1 Cellulose

Cellulose is the main constituent of virtually all plant cell walls, thus making this compound one of the most abundant (renewable) polymers on the planet. Structurally, the cellulose is a simple homopolysaccharide composed of D-glucoses linked by β -1,4 glycoside bonds. This linear polymer presents hydrogen bonds between the cellulose fibers forming a crystalline structure, which offers structural strength to the plant (Lavoine et al., 2012). The β configuration of the glucose residues creates a structure with physical properties that make the polysaccharide nearly indigestible for most animals (except ruminants) since special enzymes, known as cellulases, are required to hydrolyze the covalent bonds. The cellulose hydrolysis requires several cellulases to perform this function (Table 18.3) (Horn et al., 2012): exoglucanases acts on the reducing (EC 3.2.1.176) or nonreducing (EC 3.2.1.91) end producing cellobiose residues; endoglucanases (EC 3.2.1.4) acting on internal bonds of cellulose polymer producing oligomers and cellobiose; β -glucosidases (EC 3.2.1.21) that hydrolyze cellobiose in two molecules of glucose; and oxidative enzymes that enhance cellulose degradation (Dimarogona, 2012; Levasseur et al., 2013).

18.4.2 Hemicelluloses

Hemicellulose is the second most profuse polysaccharide in the plant cell wall, diversifying the composition according the plant species and tissue (Scheller and Ulvsiov, 2010). The polysaccharides in the hemicellulose are named according to the backbone sugar in each polymers: xylan is composed of xylose, mannans of mannose, arabinoxylan and arabinan of arabinose, and xyloglucans of glucose (Du Toit et al., 1984; Scheller and Ulvsiov, 2010). This backbone could be branched with monomers of D-galactose, D-xylose, L-arabinose, L-fucose, and D-glucuronic acid that preclude the formation of crystalline structures reinforced by hydrogen bonding, unlike cellulose (Brodeur et al., 2011; Sawatdeenarunat et al., 2015). Furthermore, hemicellulases regularly has added

TABLE 18.3 Enzymatic Degradation of Lignocellulosic Biomass

Structure	Substrate	Active enzymes	Products
Cellulose	Glucose β -1,4 Glucose	Endoglucanases	Oligomers of cellulose and cellobiose
	Glucose β -1,4 Glucose	Exoglucanases	Cellobiose
	Glucose β -1,4 Glucose (Cellobiose)	β -Glucosidases	Glucose
Hemicellulose	Xylose β -1,4 Xylose	Endoxylanase	Oligomers of xylan to xylose
<i>Xylan</i>	Xylose β -1,4 Xylose	β -Xylosidases	Xylose
	Xylose α -1,2 Arabinofuranose	α -Arabinofuranosidases	Arabinofuranose
	Xylose α -1,3 Arabinofuranose	α -Arabinofuranosidases	Arabinofuranose
	Xylose α -1,2 Glucuronic acid	α -Glucuronidase	Glucuronic acid
	Arabinofuranose O-5 Ferulic acid	Feruloyl esterases	Ferulic acid
	Acetylated xyloses	Acetyl xylan esterases	Acetyl group
<i>Arabinan</i>	Arabinofuranose α -1,5 Arabinofuranose	Arabinanases	Oligomers of arabinofuranose to arabinofuranose
	Arabinofuranose α -1,2 Arabinofuranose	Arabinofuranosidases	Arabinofuranose
	Arabinofuranose α -1,3 Arabinofuranose	Arabinofuranosidases	Arabinofuranose
<i>Mannan</i>	Mannose β -1,4 Mannose	Endomannananase	Oligomers of mannose
	Mannose β -1,4 Mannose	Exomannananase	Mannose
	Galactose α -1,6 Galactose	α -Galactosidases	Galactose

(Continued)

TABLE 18.3 Enzymatic Degradation of Lignocellulosic Biomass (*Continued*)

Structure	Substrate	Active enzymes	Products
<i>Xyloglucan</i>	Glucose β -1,4 Glucose	Xyloglucan-specific endoglucanase	Xyloglucan oligomers
	Glucose α -1,4 Xylose	α -Xylosidase	Xylose
	Xylose β -1,2 Galactose	β -Galactosidase	Galactose
	Xylose α -1,2 Arabinoxylanose	α -Arabinofuranosidases	Arabinofuranose
	Xylose α -1,3 Arabinoxylanose	α -Arabinofuranosidases	Arabinofuranose
	Galactose α -1,2 Fucose	α -Fucosidase	Fucose
Pectin	Galacturonic acid α -1,4 Galacturonic acid	Polygalacturonases	Oligomers of Galacturonic acid to free galacturonic acid
<i>Homo-galacturonan</i>	Galacturonic acid α -1,4 Galacturonic acid	Pectin lyases	Oligomers of Galacturonic acid
	Galacturonic acid α -1,4 Galacturonic acid	Pectate lyases	Oligomers of Galacturonic acid
	Galacturonic acid α -1,4 Galacturonic acid	Exopectate lyase	Galacturonic acid
	Acetylated galacturonic acid	Pectin acetyl esterases	Acetyl group
	Methylated galacturonic acid	Pectin methyl esterases	Methyl group
<i>Rhamnogalacturonan</i>	Galacturonic acid α -1,2 Rhamnose	Rhamnogalacturonases	Rhamnose
	Rhamnose α -1,4 Galacturonic acid	Rhamnogalacturonan lyases	Galacturonic acid
	Acetylated galacturonic acid	Rhamnogalacturonan acetyl esterases	Acetyl group
	Arabinofuranose α -1,5 Arabinofuranose	Arabinanases	Oligomers of arabinofuranose to arabinofuranose

(Continued)

TABLE 18.3 Enzymatic Degradation of Lignocellulosic Biomass (Continued)

Structure	Substrate	Active enzymes	Products
	Arabinofuranose α-1,2/3 Arabinofuranose	Arabinofuranosidases	Arabinofuranose
	Galactose β-1,4/6 Galactose	Galactanases	Oligomers of galactose
	Galactose β-1,4/6 Galactose	β-Galactosidases	Galactose
	Galactose β-1,4 Glucuronic acid	β-Glucuronidases	Glucuronic acid
	Arabinofuranose O-5 Ferulic acid	Feruloyl esterases	Ferulic acid
	Metilated galacturonic acid	Pectin methylesterases	Metil group
Lignin	Ca-Cβ nonphenolic arylglycerol, β-aryl ether, aromatic amines, H ₂ O ₂	Lignin peroxidase	Benzaldehyde derivatives and phenoxy radicals
	Phenolic compounds, H ₂ O ₂ and Ca- Cβ nonphenolic arylglycerol	Laccase	Semiquinones and phenoxy radicals
	Phenolic compound, aromatic amines, Mn ²⁺ and H ₂ O ₂	Manganese peroxidase	Phenoxy radicals intermediates
	Ca-Cβ nonphenolic arylglycerol, Phenolic compound, Mn ²⁺ and H ₂ O ₂	Versatile peroxidase	Benzaldehyde derivatives, phenoxy radicals intermediates
	β-Aryl ether of phenolic compounds	β-Etherase	Quinones intermediates
	Glyoxal+ O ₂	Glyoxal oxidase	Glyoxylic acid and H ₂ O ₂
	Aromatic primary alcohol+ O ₂	Aryl alcohol oxidase	Aromatic aldehyde and H ₂ O ₂

acetyl, feruloyl, or *p*-coumaroyl residues (Ebringerová and Hromádková, 1999; Ebringerová et al., 1990).

18.4.2.1 Xylan

Xylan is the major polysaccharide in primary walls, about 20%, and its composition depends on the origin (Scheller and Ulvskov, 2010). Structurally, xylan is a polysaccharide with a backbone composed of β -1,4 linkages between D-xylose residues (Ebringerová and Heinze, 2000; Heinze et al., 2004; Mussatto et al., 2008). In addition, the xylan backbone could be branched with D-glucuronic acid and D-glucuronic acid 4-*O*-methyl α -1,2 linked or D-arabinofuranose α -1,2 or α -1,3 linked, and *O*-acetyl groups can also be found in the main chain of the xylan polymer (Vries et al., 2001). Some ferulic acid esters, from lignin, attached to the O-5 of some of the arabinofuranose can also be found linked in the xylan polymer. Xylan hydrolysis requires a variety of enzymes (Table 18.3): endoxylanases (EC 3.2.1.8) acts on the inside of xylan main chain producing xylan oligomers and small moiety of free xylose (Damásio et al., 2011; De Vries et al., 2000; Squina et al., 2009); β -xylosidases (EC 3.2.1.37) hydrolyzes the β -1,4 linkage from the nonreducing terminal releasing D-xylose residues; α -arabinofuranosidases (EC 3.2.1.55) acts on the nonreducing terminal releasing arabinofuranoside residues; α -glucuronidase (EC 3.2.1.139) hydrolyzes α -1,2 bonds between glucuronic acid and xylose; feruloyl esterases (EC 3.1.1.73) detaches the ferulic acid from arabinofuranose sugar, and the deacetylation of xylan are conducted by acetyl xylan esterases (EC 3.1.1.72).

18.4.2.2 Arabinan

Arabinan is formed by α -1,5-linked arabinose backbone branched by α -1,2- or α -1,3-linked arabinofuranose side chains (Amarasekara, 2014; Cordeiro et al., 2012). The hydrolysis of this structure is conducted by arabinanases (EC 3.2.1.99) acting on α -1,5-L-arabinofuranosidic bonds and arabinofuranosidases (EC 3.2.1.55) catalyzing the hydrolysis of α -1,2, α -1,3 bonds (Table 18.3) (Cantarel et al., 2009; Sørensen et al., 2003).

18.4.2.3 Mannan

The mannan consists of a backbone of β -1,4-linked to D-mannose residues, in spite of the fact that this substrate could be branched with D-galactose by a α -1,6 linkage, calling it galactomannan. The proportion of mannose and galactose in the polymer can change according to the source of the polysaccharide (Scheller and Ulvskov, 2010; Waller, 1980). The hydrolysis of this polymer is conducted by endomannanases (EC 3.2.1.78) acting on the inside of the backbone of mannose on β -1,4-linkages, exomannanases (EC 3.2.1.25) acting on the nonreducing terminal releasing a D-mannose, and α -galactosidases (EC 3.2.1.22) acting on a α -1,6 linkage on the nonreducing terminal of the α -D-galactose residues (Table 18.3) (Moreira and Filho, 2008; Van Zyl et al., 2009).

18.4.2.4 Xyloglucan

In addition to xylan, xyloglucan is one of the main hemicelluloses of the primary cell wall of dicots and half of monocots, approximately 25% of the primary cell wall. The structure is similar to cellulose with a backbone of glucose residues bonded by β -1,4 linkages that exhibit a repeating sequence of four glucose residues (Baumann, 2007). On the repeating sequence, three glucoses from the reducing end are branched with a xylose α -1,6 linked. The xylose sugar can be substituted with D-galactose β -1,2 linked or D-arabinofuranose α -1,2/ α -1,3 linked; moreover, the galactose residue can be substituted with L-fucose α -1,2 linked (Glass et al., 2013; Laine, 2005). The enzymatic attack of the xyloglucan needs a diverse repertoire to complete hydrolysis: xyloglucan-specific endoglucanase (EC 3.2.1.151) acting on the backbone of the β -1,4 linkages; α -xylosidase (EC 3.2.1.177) hydrolyzing the D-xylose residues linked on the backbone by the nonreducing end; β -galactosidase (EC 3.2.1.23) releasing the D-galactose residue linked by the nonreducing end; α -arabinofuranosidases (EC 3.2.1.55) acting on nonreducing terminal releasing D-arabinofuranose; and α -fucosidase (EC 3.2.1.51) which removes L-fucose α -1,2 linked on galactose residue (Table 18.3).

18.4.3 Pectin

Pectin presents a very complex structure and despite its low ratios in sugarcane bagasse, sorghum, and wood (Segato et al., 2014), this heteropolysaccharide is very abundant in citrus peels and fruits (Benoit et al., 2012). The major pectin structures are homogalacturonan, rhamnogalacturonan, and xylogalacturonan. The homogalacturonan and xylogalacturonan backbone are composed of α -1,4-linked D-galacturonic acid (can be methylated or acetylated), and the last is branched with β -1,3-linked D-xylose residues. The backbone of rhamnogalacturonan consists of repeating units of D-galacturonic acid α -1,2 linked in a L-rhamnose α -1,4, linked in the next D-galacturonic acid residue (Glass et al., 2013; Guillou et al., 1989; Mohnen, 2008; Ridley et al., 2001). Furthermore, the L-rhamnose can be substituted with arabinan, galactan, or arabinogalactan side chains. Thereby the pectin efficient deconstruction includes the action of polygalacturonases (EC 3.2.1.15), rhamnogalacturonases (EC 3.2.1.171), pectin lyases (EC 4.2.2.10), pectate lyases (EC 4.2.2.2), exopectate lyase (EC 4.2.2.9), rhamnogalacturonan lyases (EC 4.2.2.24), pectin methylesterases (EC 3.1.1.11), pectin acetyl esterases (EC 3.1.1.-), rhamnogalacturonan acetyl esterases (EC 3.1.1.-), arabinanases (EC 3.2.1.99), arabinofuranosidases (EC 3.2.1.55), galactanases (EC 3.2.1.164), β -galactosidases (EC 3.2.1.23), β -glucuronidases (EC 3.2.1.31), and feruloyl esterases (EC 3.1.1.73) (Table 18.3).

18.4.4 Lignin

Lignin, the last main constituent of the lignocellulose structure, is an essential part of the biomass as it provides structural support, resistance against microbial

attack, and water impermeability to the secondary cell walls of plants. However, lignin also serves as both a physical and biochemical barrier that impedes most biomass-to-bioproducts conversion processes (Akhtar et al., 2015; Hendriks and Zeeman, 2009; Rencoret et al., 2011; Vioxeur et al., 2015). Structurally, lignin is a highly complex phenylpropanoid polymer, synthesized from three monomeric precursors: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These monomeric units when incorporated into the lignin polymers are known as *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, and are connected through β -aryl ether (β -O-4) bonds by resistant carbon–carbon and biphenyl-ether linkages forming six or more substructures (Himmel, 2012; Munk et al., 2015; Vioxeur et al., 2015). During plant development, the lignin composition and structure may change drastically (Rencoret et al., 2011). For many years, the lignin deconstruction was unknown, but nowadays some enzymes that play this role are already known. The most well-characterized enzymes involved in the degradation of the lignin structure are laccase (EC 1.10.3.2), manganese peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.1.14), versatile peroxidase (EC 1.11.1.16), and β -etherase (EC 1.14.16.5). Furthermore, some accessory enzymes, such as glyoxal oxidase (EC 1.2.3.5) and aryl alcohol oxidase (EC 1.1.3.7), are involved in the generation of hydrogen peroxide, essential for cleavage of the lignin structure (Table 18.3) (Akhtar et al., 2015; Burns, 2004; Hammel and Cullen, 2008; Himmel, 2012).

18.5 CONVERSION OF BIOMASS IN INDUSTRIALLY SIGNIFICANT PRODUCTS

Nowadays, among the biotechnological processes applied to the use of lignocellulosic biomass, the most promising is the production of second-generation ethanol. The major focus in this area is due to the depletion of fossil fuels, together with the global economic and environmental consequences that encourage the search for renewable energy sources (Singhania et al., 2013). Vegetable biomass represents a potential source of polymers containing fermentable sugars. Conversion of lignocellulosic biomass into second-generation ethanol consists of the following steps: (1) initial pretreatment of lignocellulose (eg, biological, physical, chemical, and physicochemical) to reduce recalcitrance of biomass, (2) enzymatic hydrolysis of cellulose and hemicellulose to fermentable sugars (eg, hexoses and other fermentable pentoses), and (3) microbial fermentation of the sugars to ethanol by specific microorganisms (Subhedar and Gogate, 2013; Sánchez and Cardona, 2008). Among the microorganisms that convert sugars to ethanol, yeasts have been reported to have higher yields and productivity. Currently, there are many reports that highlight on the ethanol production capacity from the fermentation hexoses and pentoses by *Saccharomyces cerevisiae* (Ishola et al., 2015), *Scheffersomyces* (formerly *Pichia*) *stipites* (Slininger et al., 2015), *Candida shehatae* (Ge et al., 2011), *Pachysolen tannophilus* (Cheng et al., 2012), and *Kluyveromyces marxianus* (Lin et al., 2013) with relatively high yields and rates.

Sweeteners are another important product that have been notably highlighted for their wide range of applications in different markets. The use of sweeteners are gaining attention due to their description as a way to avoid the problems of health risks associated with caloric sugars (Chattopadhyay et al., 2014; Pinheiro et al., 2005). In this context, the demand for sweeteners (eg, xylitol, sorbitol, and mannitol) should stimulate the growth of the market for these alternative sugars, which can facilitate the production of sweeteners via sugars obtained from the enzymatic hydrolysis of biomass.

Xylitol is a five-carbon sugar alcohol or polyol with established commercial uses in different healthcare sectors, especially as an alternative sweetener. It is suitable for diabetes and recommended for oral health and parenteral nutrition (Mäkinen, 2000; Rao et al., 2006). Currently, xylitol is one of the most expensive polyol sweeteners, having a global market estimated at 161,500 metric tons, valued at USD 670 million in 2013 and expected to reach 242,000 metric tons valued at just above USD 1 billion by 2020 (Shaw, 2014). On an industrial scale, xylitol is currently produced through chemical reduction of xylose, but the biotechnological processes of xylitol production have been reported as economically viable and attractive (Rafiqul and Mimi Sakinah, 2012). With the increase of interest in exploring more environmentally-friendly and economical xylitol production methods, the biosynthesis of xylitol by microorganisms is becoming increasingly popular. Many studies have investigated the use of the hemicellulose portion of agro-industrial residues like corn stover, sugarcane bagasse, *Eucalyptus*, spent grain, and corncob for xylitol production (Canilha et al., 2004; Carvalho et al., 2005; De Mancilha and Karim, 2003; Mussatto et al., 2005; Latif and Rajoka, 2001). Xylose obtained from the hemicellulose structure by enzymatic hydrolysis can be converted to xylitol by several microorganisms including: *Candida guilliermondii* (Carvalho et al., 2005; Mussatto et al., 2005), *Debaryomyces hansenii* (Parajó et al., 1997), *Pachysolen tannophilus* (Converti et al., 1999), *Candida tropicalis* (Ping et al., 2013), recombinant *E. coli* (Cirino et al., 2006), recombinant *S. cerevisiae* (Lee et al., 2003), and recombinant *Corynebacterium glutamicum* (Sasaki et al., 2010). Among these microorganisms, the *Candida* genus has been the most widely used with good yield and productivity. Ping et al. (2013) analyzed the efficiency of xylitol production from nondetoxified corncob hemicellulose acid hydrolysate by *C. tropicalis*. *C. tropicalis* showed good potential for xylitol production with significant values for xylitol concentration (38.8 g/L), yield (0.7 g/g), and volumetric productivity (0.46 g/L h) (Ping et al., 2013).

Sorbitol, another important artificial sweetener, is a popular six-carbon sugar alcohol (a stereoisomer of mannitol) widely used in food, pharmaceutical, and cosmetic industries and as additives in many end-products (Gallezot, 2012). Sorbitol sweetener provides a good example of a commodity that can be produced from glucose, obtained from biomass hydrolysis with a well-established market and market price (Luterbacher et al., 2014). A global market for sorbitol is estimated at 800,000 tons per year, with a value of USD 600–800 per ton in

2013 (Luterbacher et al., 2014). Sorbitol production can be obtained from the transformation of glucose to sorbitol from different microorganisms (Ladero et al., 2007; Nissen et al., 2005). Ladero et al. (2007) demonstrated sorbitol production by a *Lactobacillus plantarum* strain using glucose as substrate. The authors reported that sorbitol was produced by this strain with remarkably high efficiency (61–65% glucose conversion).

Mannitol sweetener presented a global market growth rate of 5–6% annually between 2005 and 2009 and with market estimate around USD six billion for 2015 (Bhatt et al., 2013). In general, mannitol can be produced by a variety of organisms including bacteria and plants, and *Candida magnoliae* has been used for the industrial production of mannitol. Other organisms currently targeted as microbial hosts for mannitol production include: lactic acid bacteria, *Lactobacillus reuteri*, *E. coli*, *Bacillus megaterium*, *S. cerevisiae*, and *C. glutamicum* (Akinterinwa et al., 2008; Lee et al., 2003; Papagianni and Legiša, 2014). Papagianni and Legiša (2014) demonstrated mannitol production by *L. reuteri* strain, using elevated glucose concentrations in the presence of fructose with efficient conversion of mannitol.

Besides the biofuels and sweeteners mentioned above, biomass sugars can be converted to other industrially significant products such as furfural and organic acids. Furfural is an organic compound produced from lignocellulosic biomass, a precursor of many other molecules. The major production method is acid hydrolysis of the xylose; however, it could be obtained in the fermentation of xylose by *S. cerevisiae* in contact with strong acids produced in a bioreactor (Taherzadeh et al., 1999). This compound has a current annual production of approximately 200,000–300,000 tons, which with modern technologies, is produced at USD 366 per ton; the market price was around USD 1700 per ton as of 2002 (Kabro et al., 2012; Mamman et al., 2012; Vázquez et al., 2007). Furfural could be applied in plastic, agrochemical, pharmaceutical, and nonpetroleum-derived chemical production. About 200,000 tons of furfural is consumed in resins production (Mamman et al., 2012; Vázquez et al., 2007).

Lactic acid, a small organic compound with great industrial potential, can be produced by several microorganisms in an economically viable process (Gruber et al., 2006). Discovered in sour milk at the end of 18th century, it was not produced on an industrial scale until 100 years later (Castillo Martinez et al., 2013; Hofvendahl and Hahn-Hägerdal, 2000). This compound could be used as a precursor of propylene glycol and acrylic polymers, which are biodegradable (packaging and labeling) and biocompatible, being useful for manufacturing prosthetic devices, structures, and internal drug dosing. Furthermore, lactic acid could be applied in food, cosmetic, textile, medical, and pharmaceutical industries. The great range of applications has risen with worldwide demand. In 2007 demand was estimated to be 150,000 metric tons per year with prices around USD 1.54 per kg (88% purity) (Castillo Martinez et al., 2013). The annual world

lactic acid production in 2012 had reached 259,000 metric tons per year ([Kamm et al., 2006](#)). Due to the worldwide requirements of lactic acid, many studies have been undertaken related to the optimization of production and purification in order to increase the quantity and quality of its manufacture ([Abdel-Rahman et al., 2013](#); [Gruber et al., 2006](#)).

18.6 IMPORTANT COMPOUNDS OBTAINED DIRECTLY FROM ENZYMATIC HYDROLYSIS OF BIOMASS

18.6.1 Compounds Derived from Cellulose and Hemicellulose

The growing demands of novel food products for well-being and age-related issues, coupled with increasing healthcare expenditures, have attracted global attention to prebiotics. Prebiotics are nondigestible food components that increase the growth of the probiotic organisms in the gastrointestinal tract. These sugars are neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract, and they affect the host by selectively stimulating the growth of a limited number of bacteria such as *Bifidobacterium* and *Lactobacillus* ([Gibson and Roberfroid, 1995](#); [Sousa et al., 2011](#)). The major advantages of prebiotics consumption, apart from being used as a low-calorie sweetener, include reduction of blood glucose and cholesterol, maintenance of gastrointestinal health, enhanced mineral absorption from large intestine, and immune-stimulation ([Samanta et al., 2015](#)). The global market expects that the prebiotic segment will grow around 5.2% between 2013 and 2019, reaching a market value estimated at USD 15.90 billion per year in 2019 ([Cavalcante Fai and Pastore, 2015](#)).

Production of prebiotics from agricultural residues offers great scope to the nutraceutical industries, as the raw material is cheap and abundantly available. Important prebiotics can be produced from the hydrolysis of lignocellulosic biomass using hydrolytic enzymes. The conversion of xylan, major constituent of hemicelluloses, into value-added useful products by enzymatic processes, holds a strong promise for the use of a variety of unutilized and underutilized agricultural residues ([Samanta et al., 2015](#)). [Yang et al. \(2007\)](#) analyzed xylanase enzyme exposure on xylan originated from corncob, wheat bran, peanut shell, bagasse xylan, oat spelt; the yield of xylo-oligosaccharides, that is, xylobiose (3.42–4.70 mg/mL), xylotriose (0.21–3.66 mg/mL), and xylotetrose (not detected-1.99 mg/mL), varied greatly. Similarly, another kind of functional oligosaccharide (eg, cello-oligosaccharides) can be produced by the enzymatic hydrolysis of cellulose by specific cellulases. [Kuba et al. \(1990\)](#) studied cellobiooligosaccharides produced by enzymatic hydrolysis of cellulose in the presence of activated carbon. In the work, the oligosaccharides produced were mainly cellobiose and a small amount of cellotriose, with the maximum conversion ratio of 30% ([Kuba et al., 1990](#)).

18.6.2 Compounds Derived from Lignin

Through the lignin oxidation, some lignin derived compounds are released, especially phenolic compounds (Tien and Kirk, 1983). These compounds could be applied in cosmetics, foods, and pharmaceutical industry, due to several physiological properties described, such as anti-allergenic, antiatherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective, and vasodilator effects. Phenolic compounds have been shown to be essential in the human diet, because of the beneficial effects derived from phenolic compounds, and some studies have showed their use in cancer prevention and treatment (Balasundram et al., 2006; Cai et al., 2004; Moure et al., 2001; Zheng and Wang, 2001). Another lignin derivate compound is vanillin (4-hydroxy-3-methoxybenzaldehyde) that has the same physiological properties already described, but is famous for its flavor. Vanillin has been used in ice creams, chocolates, cakes, soft drinks, pharmaceuticals, liquors, perfumery, and in nutraceuticals (Sinha et al., 2008). The production of this compound was through coniferin, the glucoside of coniferyl alcohol that is oxidized to glucovanillin, and then cleaved into glucose and vanillin or ferulic acid converted by bioconversion (Hansen et al., 2009; Rana et al., 2013). Based on the potential applications of vanillin, the global market is around 180 million dollars per year. Natural vanillin is commercialized to prices of USD 1200–4000 per kg and the bioconverted vanillin at USD 700 per kg (Hansen et al., 2009). In addition to the products mentioned, some lignin-derived compounds cannot be overlooked, for example, ferulic acid (Rice-Evans et al., 1997), phenolic resins, activated carbon, carbon fibers, and phenol derivatives (Smolarski, 2012).

18.7 LIPASE APPLIED IN BIODIESEL GENERATION

Lipases are glycerol ester hydrolases (E.C.3.1.1.3) with molecular weight between 16 and 670 kDa, produced by plants, animals, and microorganisms, which act on carboxyl ester bonds in triglycerides resulting in fatty acids and glycerol (Barros et al., 2010; Gopinath et al., 2013; Villeneuve et al., 2000). This hydrolytic reaction can be reversed under a microaqueous environment, such as in organic solvents, making lipases able to perform reactions of esterification, interesterification, alcoholysis, acidolysis, and aminolysis at the oil–water interface (Couturier et al., 2009; Desai et al., 2006; Jenab et al., 2014; Lopes et al., 2011; Wang et al., 2012). Usually, the higher lipase catalytic activity occurs at the lipid water interface through the phenomenon called interfacial activation, which happens due to the presence of a “lid” in the enzyme active site (Brocca et al., 2003; Jegannathan et al., 2008). When the lipase is in an aqueous environment, the lid is in the closed form, avoiding contact of the substrate with the enzyme and reducing its activity. In a hydrophobic substrate or in the oil–water interface, lipases undergo conformational changes, and this lid opens, increasing the accessibility to substrate, and therefore, their activity (Brady et al., 1990; González-Navarro et al., 2001; Jaeger et al., 1999).

In the huge market of valuable enzymes, lipases are the fastest growing segment and are the third most commercialized, due to their versatile nature, just after proteases and carboxylases (Gupta et al., 2015; Ray, 2012). Recently, microbial lipases assumed a prominent place in the world market, mainly evidenced by the increase in the amount of information reported in the literature, which includes an average of a thousand publications per year (Gupta et al., 2015; Naqvi et al., 2011; Sharma and Kanwar, 2014). The growing demand for these natural catalysts justified the pursuit of new lipases, with the improvement of existing properties for technical applications, and established the production of new enzymes tailored to entirely new areas of application. The growth in the volume of commercial enzymes is around 4–5% per year, which comes with decreasing prices due to the increase in the manufacturing industry (Guldhe et al., 2015; Sharma et al., 2011). The main applications of lipases are as catalysts in food processing, detergent, pharmaceutical, paper, cosmetics, and chemical synthesis industries (Freire and Castilho, 2008; Gupta et al., 2004, 2015; Shah et al., 2003; Sharma et al., 2001, 2011).

Industrially, microorganisms represent the most important lipases source because of the following reasons: they are easy to produce in bulk amounts because of their extracellular nature, can be obtained either by solid-state or submerged fermentations, and present a high substrate conversion product yield (Azeredo et al., 2007; Freire and Castilho, 2008). Additionally, the production of microbial enzymes is unaffected by seasonal fluctuations, grows rapidly in low-cost media, and demonstrates high stability, selectivity, and specificity (Sharma et al., 2011; Singh and Mukhopadhyay, 2012; Wiseman, 1995). Some of the most important sources of this enzyme are of the genera: *Candida* (Tan et al., 2003), *Saccharomyces* (Ciafardini et al., 2006), *Yarrowia* (Brígida et al., 2014), *Rhodotorula* (Portumarthi et al., 2008), *Geotrichum* (Burkert et al., 2004), *Rhizopus* (Koblitz and Pastore, 2006), *Aspergillus* (Contesini et al., 2010), *Rhizomucor* (Rodrigues and Fernandez-Lafuente, 2010), and *Penicillium* (Wolski et al., 2009).

One of the most important and promising applications of lipases nowadays has been in biodiesel production; a mixture of fatty acid alkyl esters, which is a potential alternative energy source derived from vegetable oils, that could reduce the use of petroleum-based diesel (Muhammad et al., 2015). This occurs because of the benefits offered by enzymatic processes, like mild reaction conditions and less wastewater generation, besides being an environmentally-friendly process and a reduced or nontoxic method (Haas et al., 2009; Olsen, 2008). Furthermore, biodiesel and glycerol achieved by lipase conversion have displayed a higher purity, when compared to that acquired by other conversion techniques (Guldhe et al., 2015). Brazil, the United States, France, Germany, Japan, and other countries already use a blend of biodiesel and petroleum fuels in transportation vehicles (Atabani et al., 2012; Singh et al., 2014).

The chemical process is still the most popular for this purpose, using acids and alkalis catalysts, such as H_2SO_4 and $NaOH$, because of its high yield

production and economic viability at the industrial scale (Abdelmoez and Mustafa, 2014; Sharma et al., 2008). Despite these great advantages, chemical processes have disadvantages, such as high energy consumption, large volume of wastewater, formation of undesirable by-products that are difficult to separate, and the necessity of removal of the chemical catalyst, since it can damage engine parts because they are acids (Abbaszaadeh et al., 2012; Meher et al., 2006; Vyas et al., 2010). These drawbacks stimulate the use of enzymatic processes even more, as well as encourage the pursuit of solutions to reduce the costs with enzymes.

Therefore, lipase immobilization becomes a very important step, since it allows more tolerance to organic solvents, and the repeated use of the enzyme, reducing operation costs; moreover, the separation of products are easier (Bajaj et al., 2010; Zhao et al., 2015). The immobilizing steps also make the lipases become more stable towards temperature, chemical, and shear denaturation (Fjerbaek et al., 2009). Many materials have been used to immobilize lipases, including microspheres (Zhang et al., 2012), carbon nanotubes (Tan et al., 2012), magnetic particles (Ren et al., 2011), glass beads (Pinto et al., 2012), celite, ceramics, silica, and so on (Stoytcheva et al., 2011). Chen et al. (2011) used the commercially immobilized lipase Novozyme 435 and soybean oil in a continuous packed-bed reactor for the biodiesel production in the presence of *tert*-butanol as a solvent, achieving 83.31% of conversion yield in an optimized process. Another recent strategy is to use immobilized lipases with complementary position specificity, instead of just one lipase, which makes the yield increase significantly (Huang et al., 2010). Lee et al. (2011) optimized the biodiesel production, using a mixture of immobilized *Candida rugosa* and *Rhizopus oryzae* lipases (1:1) in a supercritical carbon dioxide process, resulting in a conversion yield of 99% after 2 h of reaction.

Ionic liquids have also been tested to improve enzymatic biodiesel production. They are classified as green solvents because of their unique properties, like nonflammability, low vapor pressure, excellent chemical stability, and so on, and are used to increase the conversion yield since they play important roles in changing the activity, stability, and structure of enzymes and reduce environmental pollution (Gao et al., 2015). They also grant less denaturing properties, high enantioselectivity, and improve the recoverability and recyclability (Abe et al., 2008). However, there are many enzymes showing the same performance with the use of ionic liquids or traditional organic solvents (Gao et al., 2015). It corroborates with the fact that larger studies are needed to prove the real improvement of ionic liquids in biodiesel production.

In conclusion, the enzymatic process for the production of biodiesel has become a very interesting topic in recent years, since it allows the use of mild reaction conditions that are consistent with a green process. However, new techniques to improve the conversion yield with a low cost are still needed, thereby allowing a bright future for the enzymatic processes.

18.8 PROTEASES APPLIED IN PROTEIN RESIDUES AND BY-PRODUCTS

Proteins correspond to another important group of compounds that are found in different residues, and therefore, can be utilized to obtain high value-added products. Several types of proteins are limitedly hydrolyzed, resulting in peptides, also called protein hydrolysates, with remarkable biological activities, such as antihypertensive, immunostimulating, antimicrobial, and antioxidant activities (Danquah and Agyei, 2012). These peptides are sequences 2–30 amino acids in length and can be obtained from different sources including: milk (casein and whey), egg (albumin), soy, and vegetable and animal residues. They are produced from precursor proteins using enzymatic hydrolysis by microbial proteases (Korhonen and Pihlanto, 2006). Peptides must be resistant to potential endogenous degradation, absorbed through the enterocytes to the serum and reach the target site intact in order to exhibit bioactivity in vivo. In addition, the length of the amino acid chain and its composition are of key importance for aiding absorption and resistance to degradation (López-Barrios et al., 2014).

Proteases are a highlighted group of enzymes that correspond to approximately 60% of the total worldwide enzyme sales, representing one of the most important enzymes due to their applications in detergent, food, pharmaceutical, chemical, leather, paper and pulp, and silk industries. Although these enzymes break peptide bonds, they are classified based on different criteria, and therefore, have different numbers in the Enzyme Commission. Among the different sources of proteases, microorganisms are industrially preferable since they can produce enzymes easily and faster in comparison to mammalian and plant cells, and the enzyme production is influenced neither by climatic conditions or seasonal changes, nor by regulatory or ethical issues related to animal slaughter or tree or plant felling. Furthermore, extracellular enzymes produced by microorganisms are preferred since this simplifies downstream processing; hence, further lowering costs (Tufvesson et al., 2011). Literature reports several microorganisms capable of producing extracellular proteases, such as *Aspergillus oryzae* (Belmessikh et al., 2013), *Aureobasidium pullulans* (Chi et al., 2007), *Serratia marcescens* (Bach et al., 2012), and *Bacillus subtilis* (Helal et al., 2012).

Oilseed meal is composed of more than 40% of protein and is a by-product of the processing of oil seeds (Young, 1982). This meal is treated as waste and used as animal feed, and hence, could be used as an economically viable protein food source. In the work of Chatterjee et al. (2015), different proteases were applied in the hydrolysis of sesame proteins. The rate of the degree of hydrolysis (DH) reached a maximum (25–30%) within the first time fragment, but 80% of hydrolysis was obtained in 120 min with Alcalase (*Bacillus licheniformis*). SDS-PAGE of hydrolysates obtained using different proteases resulted in bands of low molecular weight (14.3 kDa) after 120 min. Hydrolysates

presented improved functional properties, evident from their emulsifying and foaming properties, as well as significantly enhanced protein solubility at pH 7. Moreover, these peptides were analyzed by MALDI-TOF and matched to the previously identified ACE inhibitory peptides. The authors affirmed that these highly bioactive protein hydrolysates produced from waste sesame meals can be successfully employed in various functional food formulations.

The olive oil industry also generates a large volume of residues in the processing of this fruit, resulting in solid wastes, also known as olive pomace or olive oil cake, and aqueous liquor, known as olive mill wastewater. These residues result in pollutive products that are phytotoxic, hardly biodegradable, and difficult to be treated (Fiorentino et al., 2003; Khoufi et al., 2009; Vlyssides et al., 2004). In this context, microbial proteases could be applied to olive stone, which has been proposed as a cheap source of protein (up to 22%) (Rodríguez et al., 2008) for obtaining bioactive peptides. Esteve et al. (2015) reported the use of commercial proteases for the revalorization of residual materials from table-olive and olive oil production, based on the extraction of bioactive peptides. Enzymatic hydrolysates of olive seed protein isolate were obtained by treatment of the proteases Alcalase, Thermolysin, Neutrerase, Flavourzyme, and PTN. The authors observed that in spite of the fact that all hydrolysates presented antioxidant properties; the Alcalase yielded the hydrolysate with the highest antioxidant capacity. In addition, all peptides presented antihypertensive capacity and there was further attention on thermolysin, which produced the highest ACE-inhibitory capacity of hydrolysates. After fractionation of the peptides by ultrafiltration, it was possible to observe a high concentration of short chain peptides, which exhibited significantly higher antioxidant and antihypertensive capacities than fractions with higher molecular weights.

The fishing industry is an important area that also generates by-products that can be used for bioactive peptides. Much of this material has been converted to powdered fishmeal by a combined process of cooking, separation of soluble from insoluble, concentration of the soluble and dehydration of the insoluble (Ferreira and Hultin, 1994). Enzymatic hydrolysis is an alternative approach for recovering biomass from fish, resulting in a soluble product known as fish protein hydrolysate. The soluble hydrolysate is subjected to dehydration, resulting in a more stable, powdered form with high protein content (Diniz and Martin, 1997). In the work of Guerard et al. (2002), a waste product from the tuna canning industry was hydrolyzed using a commercial protease "Umamizyme" (protease mixture from *Aspergillus oryzae*), and enzymatic hydrolysis was investigated. A degree of hydrolysis (DH) of up to 22.5% was observed with an enzyme/substrate ratio of 1.5%, after 4 h of hydrolysis. A linear correlation was found to exist between the DH and the nitrogen recovery.

Protein hydrolysates from tuna heads with different degrees of hydrolysis were produced using protease from *Bacillus mojavensis* A21 and Alcalase. All protein hydrolysates produced by the A21 protease presented higher antioxidant

activity than those of the Alcalase protein hydrolysates. The highest DPPH radical-scavenging activity was observed with a DH of 15%. The protein hydrolysates obtained with Alcalase (DH = 12%) and A21 (DH = 15%) contained glutamic acid/glutamine and arginine as the major amino acids, followed by lysine, aspartic acid/asparagine, histidine, valine, phenylalanine, and leucine. Furthermore, the peptides had a high percentage of essential amino acids, which comprised 50.47% and 50.52% of the protein hydrolysates obtained from the Alcalase and A21 proteases, respectively. The results indicated that these protein hydrolysates could be used as a promising source of bioactive peptides ([Bougatef et al., 2012](#)).

As already mentioned, these protein hydrolysates are compounds used in diagnostics and as therapeutic agents in a variety of illnesses, as well as inhibitors of bacterial growth in the food industry. Recent technological advances in delivery and formulation tools have brought attention to this field, resulting in approximately 60 approved peptide drugs, and a predicted annual growth rate of the market of approximately 10% ([Wegmüller and Schmid, 2014](#)). Therefore, it justifies the use of several types of protein wastes and by-products for obtaining bioactive peptides.

18.9 GENETIC ENGINEERING IN IMPROVING ENZYME QUALITY

The catalysis derived from enzyme activity is attractive in industrial production, because it can be selective, efficient, and minimize waste generation. However, there are several challenges associated with the use of enzymes in obtaining products of industrial interest, since they have applications in different conditions from those which the enzymes are naturally found ([Woodley, 2013](#)). Some of the challenges include substrate inhibition or product instability, low specificity to the substrate, and low number of enzymes produced by wild strains ([Adrio and Demain, 2014; Marrs et al., 1999](#)). Thus, enzymes can be genetically selected and modified to fit a specific process in order to develop their full capacity.

To improve the production of industrial enzymes of interest, different strategies, including optimization of fermentation conditions, have been used. However, with the development of molecular techniques that can be applied in the host or in the enzyme, the production of enzymes has exponentially increased ([Demain and Vaishnav, 2009](#)).

In the host, different strategies can be used to improve the production of enzymes, such as the use of strong promoters, increasing the copy number of genes, codon optimization, and changing the signal peptide sequence.

The promoter is classified as strong according to its affinity for RNA polymerase sigma factor based on ideal consensus sequence for the polymerase ([Rossi et al., 1983](#)). This technique maintains the gene encoding the enzyme of interest constitutively expressed during growth of the host

([Nevalainen et al., 2005](#)). Besides strong promoters, inducible promoters may also be used as the promoter gene encoding cellobiohydrolase I from *Trichoderma reesei* ([Madhavan and Sukumaran, 2014](#)). Many enzymes used in industrial waste have been successfully obtained using this technique. As an example, the cDNA of the gene encoding the laccase from the white-rot fungi *Ganoderma lucidum* was cloned under the control of alcohol oxidase I gene (AOX1) strong promoter and inserted into *Pichia pastoris*. The increase in enzyme activity occurred up to 50 times more when cultured in L-alanine ([You et al., 2014](#)). The optimal pH and temperature were specific at pH 3.5 and in a special range of 60–90°C. Fuentes-Garibay and collaborators cloned one *A. niger* tannase under the same AOX1 promoter in *P. pastoris*. The purified enzyme had an optimum pH of 5 and optimum temperature of 20°C. An increase of up to 100-fold in enzyme production was observed when the yeast was grown in a shake flask ([Fuentes-Garibay et al., 2015](#)).

Another strategy is to increase the copy number of the gene of interest into the genome. Researchers have observed that there is a correlation between the secreted proteins of interest and a greater number of copies in the genome. A β-mannase from *T. reesei* was inserted into *P. pastoris* and variants with one and five copies were obtained. The variant with five copies produced two times more enzymes when compared with the variant with a single copy ([Mellitzer et al., 2012](#)). In another study, *Acinetobacter radioresistens* alkaline lipase was inserted up to 19 times in the *P. pastoris* genome. The increase in enzyme production was proportional to the increase until seven copies were integrated in genome ([Zhao et al., 2013](#)).

A phenomenon observed from bacteria to mammals is the use of preferred codons. There are many reasons why preferred codons exist, such as the abundance of tRNA and three-dimensional structure of mRNA ([Gustafsson et al., 2004](#)). The expression of heterologous enzymes cannot result in high production due to preferred codons. This can be solved by replacing rare codons in the host by codons that are preferably used in protein-coding genes. As an example, Zhou et al. have optimized codons of *Yarrowia lipolytica* lipase enzyme to be better expressed in *P. pastoris*. This change increased by twofold when compared to the native gene ([Zhou et al., 2015](#)). Jia et al. optimized codons for xylanase gene from the hyperthermophilic *Thermotoga maritime* being expressed in *P. pastoris*. The optimized gene shared 77.8% of nucleotide sequence identity with that of native gene, and the expression level of the codon optimized gene was increased by 2.8-fold ([Jia et al., 2012](#)).

The last strategy to improve the host that will be cited here is changing the signal peptide sequence. The signal peptide is a sequence that contains 16–30 amino acids added to the N-terminus of the protein and is removed while the protein is translocated across the endoplasmic reticulum membrane ([Lodish et al., 2000](#)). To improve the production, the signal peptide from well-expressed gene is inserted into the enzyme of interest. [Madhavan and Sukumaran \(2014\)](#) fused enhanced green fluorescent protein (EGFP) to the *T. reesei* cellobiohydrolase

I signal peptide (cbh1SS) and *S. cerevisiae* α mating factor prepro-leader sequence (α MFSS). Both constructs were inserted in *Kluyveromyces lactis*, and there was an increase of 1.6-fold in extracellular secretion EGFP for the cells harboring the signal peptide of cbh1SS compared to those with the α MFSS. They believed cbh1SS is relatively small, making this a good candidate for driving heterologous protein production in *K. lactis* (Madhavan and Sukumaran, 2014). *Pycnoporus cinnabarinus* laccase gene was overexpressed in *A. niger* in two ways: fused with the laccase signal peptide or the glucoamylase prepro-sequence of *A. niger*. Both constructions allow secretion of laccase; however, the glucoamylase prepro-sequence of *A. niger* allowed an 80-fold increase in laccase production (Record et al., 2002).

Regarding the improvement of enzymes, two main methodologies have been used: rational design approaches and directed evolution.

The rational design approaches result in changes in amino acid sequence, thus requiring detailed knowledge of the three-dimensional structure and mechanism of the enzymatic reaction, some of which may not be available (Woodley, 2013). However, the increasing growth of databases containing protein sequences and structures are helping to overcome this lack of information (Born scheuer et al., 2012). A comparison of the sequence of a new enzyme in a screening program for the thousands deposited in the databases may identify related proteins whose functions and/or structures are already known (Fisher et al., 2014). Many enzymes have been successfully redesigned as ervatamin-C, which is a papain-like cysteine protease from the plant *Ervatamia coronaria*. This enzyme is thermostable, but has low catalytic efficiency. Using ervatamin-A as a basis, three mutants from enzyme type C were generated by site-directed mutagenesis. The three mutants show catalytic efficiency about eight times higher than the wild-type (Dutta et al., 2013). Other enzymes, such as a *Bacillus circulans* xylanase were also successfully redesigned. Site-directed mutagenesis experiments were performed to change the optimum pH by 0.5–1.5 units of the enzyme by introducing acidic or basic residues glutamate close to the catalytic site (Pokhrel et al., 2012).

Unlike rational design approaches, directed evolution does not require any knowledge of sequence, structure, or function of the proteins. This technique mimics natural evolution in vitro, reducing the development timescale of millions of years to months or weeks (Adrio and Demain, 2014). For this method, it is necessary to perform two steps: one is the generation of a bank of random mutants and the other is a screening and selection of enzyme variants having the desired characteristics (viz an increased catalytic activity, increased specificity or stability). For a bank of mutants, techniques such as error-prone polymerase chain reaction (PCR), random priming recombination (RPR), DNA shuffling, combinatorial active site saturation test (CAST) is used (Grunwald, 2015). After cloning and expression of mutated genes, a large collection of enzyme variants (10^4 – 10^6) is generated and is subjected to screening or selection, which is one of the disadvantages of the method (Adrio and Demain, 2014).

A lipase from *Candida antarctica* (CalA) was optimized with this method using CAST as strategy. A triple-mutated variant of CalA obtained showed an increase in 30% of the activity, and also increased the enantioselectivity and substrate scope (Engström et al., 2010). The β -1,3-1,4-glucanase enzyme from *B. subtilis* was subjected to error-prone PCR technique and 800 mutants were screening. Mutants with increased tolerance to acidic (pH 2) higher thermal stability and increased tolerance to some chemicals were obtained (Pei et al., 2015). Another example is an alkaline protease from *Bacillus alcalophilus* that was also subjected to error-prone PCR technique. Mutants with up to 2.7-fold activity higher than the wild-type were obtained (Liu et al., 2014).

Besides these mentioned techniques, many others exist for protein engineering. However, the success in the improvement of some enzymes is dependent on factors beyond the molecular biology techniques, such as intrinsic properties of enzymes.

18.10 CONCLUDING REMARKS

The present-day energy and environmental demands that the world is experiencing are forcing us, among other things, to reevaluate the efficient utilization of or finding alternative uses for natural, renewable resources, especially organic “waste” by means of clean technologies. Lignocellulose biotechnology offers significant opportunities for developing countries to address some of the issues highlighted, since most of the technology is based on the utilization of readily available residual plant biomass considered as “waste” to produce numerous value-added products. Brazil’s success in biofuel is a showcase of only one example of the economic potential for developing countries in the area of lignocellulose biotechnology. The microbial enzyme for obtaining compounds with high benefits seems to be promising. This bioprocess is a clean technology with great potential for the acquisition of biologically active compounds from natural sources. For better procurement of these compounds, factors such as the type of substrates, microorganisms, and classes of enzymes should be efficiently screened through bioprocesses. The key advantages of using enzymes for value-added compounds are their favorable and unique properties, biodegradability, and high selectivity, which enable their progressive implementation in all types of industries.

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Chapter 19

Microbial Enzymes for the Food Industry

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19.1 INTRODUCTION

19.1.1 A Global Perspective on the Use of Enzymes in the Food Industry

For thousands of years man has relied on microorganisms and enzymes for food production. Typical examples include the making of beer, bread, cheese, or wine, where enzymes were unknowingly used for thousands of years. Currently, enzymes, either obtained by microbial fermentation or extracted from plants or animals, are used for production and/or processing of foods, leading to improved or new processes or goods. Enzymes used in food and beverages have a significant market share that is expected to be of about \$1.3 billion by 2015 (Adrio and Demain, 2014). Different enzyme classes find application in food and beverages production and processing, as a diverse set of reactions is required, although hydrolases clearly stand out (Table 19.1). Besides specificity, enzymes may be required to present diverse, if not totally opposite, operational requirements, as a result of the role intended. Thus, high thermal and operational stabilities are required for glucose isomerase used for the isomerization of glucose to fructose in the production of high fructose corn syrup, in order to allow for high volumetric productivity and adequate shift of the thermodynamic equilibrium toward the product (DiCosimo et al., 2013). On the other hand, cold-active pepsins are sought for the riddling process in caviar production, as operation at low temperatures reduces the risk of microbial contamination and thermal degradation of food; also, cold-active collagenolytic proteases are sought for meat tenderization, as these enzymes display high activity at low temperatures, and are denatured during cooking, therefore ruling out the risk of overtenderization (Zhao et al., 2012).

TABLE 19.1 A Brief Overview of the Roles Played by Enzymes Within the Scope of Food Processing

Class	Enzyme	Role
Hydrolases	Amylases	Starch liquefaction and saccharification
		Production of starch-derived goods, processing of bread and juices
	Glucanase	Breakdown of cell walls of cereals, beer making
	Glucoamylase	Saccharification of starch, production of sugar syrups
	Glycosidases (β -glucosidase)	Hydrolysis of β -glycosidic bond. Release of aroma compounds in wine production
	Invertase	Sucrose hydrolysis, production of invert sugar syrup
	Lactase	Lactose hydrolysis (Lactose-free milk), whey hydrolysis, synthesis of galactooligosaccharides
	Lipase	Cheese flavor, in situ emulsification for dough conditioning, support for lipid digestion in young animals, synthesis of aromatic molecules
	Proteases (eg, chymosin, papain)	Protein hydrolysis, milk clotting, low allergenic infant food formulation, enhanced digestibility and utilization, flavor improvement in milk and cheese, meat tenderizer, prevention of haze formation in brewing
	Pectinase	Viscosity reduction, clarification of fruit juices
	Peptidase	Cheese ripening
	Phospholipase	In situ emulsification for dough conditioning
Isomerases	Phytases	Feed processing through the release of phosphate from phytate, enhanced digestibility
	Pullulanase	Saccharification, complementary to glucoamylases
	Xylanases	Viscosity reduction, enhanced digestibility, dough conditioning
Arabinose isomerase		Isomerization galactose to tagatose

(Continued)

TABLE 19.1 A Brief Overview of the Roles Played by Enzymes Within the Scope of Food Processing (*Continued*)

Class	Enzyme	Role
	Cellobiose 2-epimerase	Isomerization of lactose to lactulose
	Xylose (Glucose) isomerase	Isomerization of glucose to fructose
Lyases	Acetolactate decarboxylase	Fastening beer maturation
Oxidoreductases	Glucose oxidase	Improved dough strength and handling properties
	Laccases	Stabilization of color in winemaking, dough strengthener, increased storage life of beer, improved flavor of vegetable oils, preparation of cork stoppers
	Lipoxygenase	Dough strengthening, bread whiting
	Cyclodextrin glycosyltransferase	Production of cyclodextrins
Transferases	Fructosyl transferase	Synthesis of FOS, production of prebiotics
	Transglutaminase	Cross-linking role, modification of viscoelastic and water binding properties. Dough processing, meat processing

19.1.2 Identification/Improvement of the Right Biocatalyst

Enzymes that display the required features toward the application intended may be identified through suitable screening. Improvement or modification of enzymes, aiming for optimization toward the specific goal aimed at, may be achieved through protein engineering using genetic methods and/or adequate formulation, namely immobilization. Some of the key features involved in protein engineering are summarized in Table 19.2. As a whole, the properties of enzymes that are to be tuned in order to increase the successful application in industrial processes include activity, enantioselectivity, specificity, stability, and tolerance toward substrate, product, and solvent used (Singh et al., 2013). Protein engineering can be implemented through rational design, through directed evolution (random design), or through focused directed evolution (semirational design), which is actually a combination of the two former methodologies. In rational design, site-specific mutations are introduced

TABLE 19.2 Key Issues of the Different Methodologies for Protein Engineering

	Rational design	Directed evolution	Focused directed evolution
Protein structure	Detailed knowledge needed	No information needed	Partial information required
Catalytic mechanisms	Detailed knowledge needed	No information needed	Partial information required
Assay system needed	Sensitive method required, no need for HTP method	Cheap, fast, and reliable HTP method	Sensitive method required, HTP method helpful
Synergistic effects of neighboring mutations	Common	Low probability	Intermediate, possible identification of effects missed in rational design

HTP, high-throughput.
Source: From [Chen \(2001\)](#); [Steiner and Schwab \(2012\)](#); [Singh et al. \(2013\)](#).

to replace given residues in the enzyme molecule by carefully selected residues, so as to achieve the intended goal with a minimal number of variants. Conversely, directed evolution is carried out through random modifications in protein structure, as the result of either haphazard changes in single protein sequences (namely error-prone PCR), or of accidental recombination of a set of related sequences (namely gene shuffling). The process typically requires several rounds, since each step often results in small, not all positive, changes, and results in a large number of variants. Focused directed evolution relies on information on biochemical and/or structural data to generate a relatively small library size, and has proved particularly attractive when the amino acids relevant to the property of interest are known ([Reetz et al., 2006](#); [Steiner and Schwab, 2012](#); [Bornscheuer, 2013](#)). Even in this semirational method not all the consensus mutations contribute positively to the envisaged goal, namely stability, and may even have an impact in other properties of the enzyme, namely activity ([Steiner and Schwab, 2012](#)). The roots of the implementation of protein engineering methods in enzymes for industrial applications can be somehow traced back to food enzymes, namely glucose isomerase and the modification of a specific residue to improve the thermostability of this particular enzyme ([Sicard et al., 1990](#)). Given the relevance of the role of glucose isomerase in the industrial production of sweeteners, it is only natural that many engineered mutants have been produced, aiming to improve its activity, thermal and weak acid stability ([Sriprapundh et al., 2003](#); [Xu et al., 2014](#)), and to decrease the requirements/inhibition regarding metal ions ([Hlima et al., 2012](#)). Such variants were obtained using either site-directed mutagenesis ([Hlima et al., 2012](#); [Xu et al., 2014](#)).

2014) or directed evolution (Sriprapundh et al., 2003). The different approaches for protein engineering have been seminal in gaining insight on the design of more efficient glucansucrases, which can be advantageously used for the tailor-made synthesis of oligosaccharides with a prebiotic role. The protein engineering techniques allowed for the identification of the relevant residues for catalysis and substrate specificity, and provided relevant information for understanding the sequence–structure–function relationships of the enzymes, also paving the way for more rational design of improved glucansucrases (Daudé et al., 2014). Amylases have also been focused with the aim of obtaining engineered variants displaying modification in given properties, namely substrate specificity and cleavage pattern, thermal and pH stability, pH/activity profile, pH/stability profile, and metal ion dependency (Andersen et al., 2013). The use of a semirational method, where functionally correlated variation sites of proteins are used as hotspot sites to construct focused mutant libraries, allowed the production of α -amylase mutants with improved thermal stability by 8°C as compared to the native enzymes. Such a goal had proven unfeasible with rational single- and double-point mutations, while requiring a relatively small library (Wang et al., 2012). Protein engineering has also been used to render more cost-effective the production of food enzymes. Thus, a hybrid β -galactosidase was produced, that combined the intracellular β -galactosidase of *Kluyveromyces lactis*, of particular interest for lactose hydrolysis and galactooligosaccharide production (Panesar et al., 2010b), with its extracellular homologue from *Aspergillus niger*, including a heterologous signal peptide for secretion at the N-terminus of the recombinant protein. The resulting variant displayed improved secretion to the fermentation medium, easing downstream processing and making industrial production more competitive. Moreover, the hybrid β -galactosidase displayed an increase in the optimal temperature and enhanced thermal stability, affinity for natural (lactose) and synthetic (*o*-nitrophenyl- β -D-galactopyranoside) substrates, as well as a shift in the optimum pH from 7.0 to 6.5, when compared with the native enzyme from *K. lactis*, all of which make the variant a more effective biocatalyst for processing lactose and whey (Rodriguez et al., 2006).

19.1.3 Enzyme Sources and Safety Issues

Given the particular nature of the applications of enzymes in food industry, with clear implications in public health, these industrially produced enzymes are assessed for safety by regulatory agencies, although the defined criteria are far from universal (Olempska-Beer et al., 2006; Magnuson et al., 2013; Fraatz et al., 2014). The need for such monitoring has become more noticeable since the late 1980s, with the introduction of protein and genetic engineering techniques and recombinant DNA technology into enzyme production, moreover coupled with the intensive screening of enzymes, particularly from extremophiles. Particular care has thus been given to the selection of enzyme sources. Thus, within the wide array of microorganisms used for the production of enzymes for industrial

applications, those labeled as GRAS (generally regarded as safe) are particularly favored within the scope of food industry (Adrio and Demain, 2003; Liu et al., 2013). GRAS labeling is either related to a long track record of safe use or compliance with the outcome of a set of scientific procedures, based on Food and Drug Administration (FDA) regulations. Still, with the relatively recent onset of genetic manipulation of these microbial strains, long-term effects in health and environment of the engineered strains remain yet to be established. Regulatory agencies such as European Food Safety Agency (EFSA) are currently developing efforts for a thorough evaluation of the safety of enzymes used in food production and processing. Strains that secrete enzymes from cells are again favored, as downstream processing complexity and costs are lower, as compared to intracellular produced enzymes, and within those, the capacity to achieve a reasonable concentration of extracellular protein, namely over 50 g/L, are sought. Microbial enzyme producers on an industrial scale that comply with these demands are typically a few strains, such as *Aspergillus oryzae*, *A. niger*, *Bacillus subtilis*, or *B. licheniformis*, that have furthermore proven adequate hosts for expression of homologous and heterologous enzymes (Sarrouh et al., 2012; Liu et al., 2013; Adrio and Demain, 2014).

This chapter offers an overview of the microbial enzymes currently finding potential use in the food industry.

19.2 MICROBIAL ENZYMES IN FOOD INDUSTRY

19.2.1 Production of Enzymes for Food Processing

Recombinant DNA technology and genetic engineering have enabled: (1) the production by industrial microorganisms of enzymes obtained originally from microorganisms which are pathogenic or producers of toxins, difficult to grow or even consider unculturable. An increasing number of these enzymes are obtained from extremophiles; (2) enhanced enzyme productivity through the use of adequate promoters, signal sequences, and multiple gene copies (Adrio and Demain, 2010; Liu et al., 2013; Dalmaso et al., 2015; Neifar et al., 2015). Within this, systems biology has been gaining relevance as a most valuable tool for consistent integration of multi-ome data. As an outcome, detailed insight in the metabolism of microbial industrial workhorses, namely *A. niger* and *B. subtilis*, enables a more rational and cost-efficient approach for massive enzyme production (Zhu et al., 2012; Vongsangnak and Nielsen, 2013; Brandl and Andersen, 2015). Protein engineering has largely contributed to the design and production of enzymes with improved activity, specificity, or stability (Singh et al., 2013; Damborsky and Brezovsky, 2014). The overall production process for enzyme production is depicted in Fig. 19.1, and involves enzyme synthesis through fermentation, recovery of the enzyme from the fermentation medium, purification of the enzyme to remove unwanted contaminants, and formulation according to the intended use (Panesar et al., 2010a; Ramos et al., 2013).

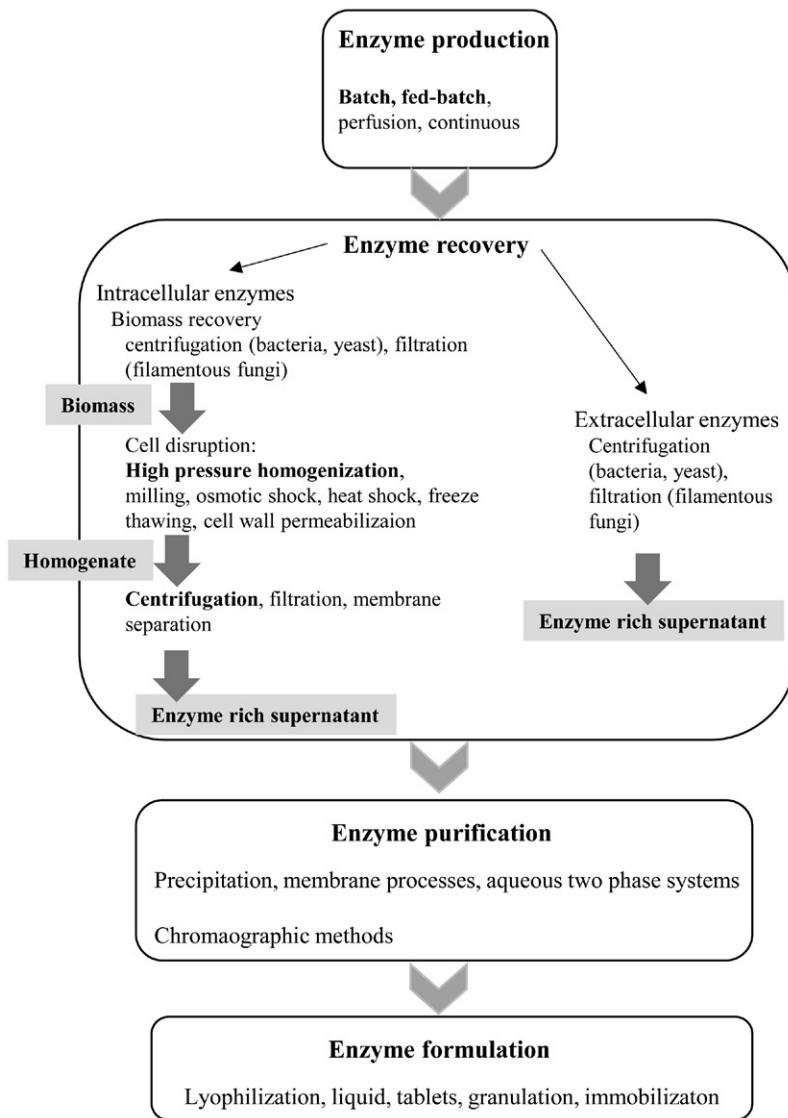


FIGURE 19.1 A simplified flow sheet of enzyme production.

Production of enzymes has been mostly performed through submerged fermentation, although solid state fermentation (SSF) has been gaining relevance in recent years (Singhania et al., 2010; Thomas et al., 2013). Submerged fermentation takes place in vessels of up to 200 m³, with a wide range of substrates, from defined ingredients, such as dextrose, ammonia, and urea, to undefined

ingredients, often by-products from the food industry such as molasses, whey, soybean, fish meal, and yeast extract, and minerals, such as carbonates and phosphates (van den Berg et al., 2010; Ramos et al., 2013). Fermentation is implemented under monitoring and control of variables such as temperature, pH, and dissolved oxygen tension. Batch mode of operation is widely used, since it is a well-established methodology, yet productivity is limited, often as a result of substrate inhibition, a drawback overcome by the fed-batch approach. In the later method, after a given period of batch cultivation, nutrients are fed to the bioreactor according to a given pattern and up to a final volume. This mode of cultivation is considered particularly suitable for the production of enzymes, as operation is relatively straightforward and metabolic responses of the producing cells can be controlled, and has thus been gaining relevance (Illanes, 2008; Chisti, 2010; Ramos et al. 2013). Perfusion cultures, where a cell-free product stream is continuously harvested from the bioreactor, while an equal volume of medium is added to the bioreactor, is also an alternative, since it reduces or prevents the accumulation of inhibitory metabolites within the bioreactor. However, it is somehow cumbersome and expensive for large-scale production of low-value products such as industrial enzymes (Singhania et al., 2010; Salehmin et al., 2013; Spohner et al., 2015). Continuous production is hardly considered a realistic alternative, because in spite of it allowing for steady-state operation, with concomitantly near-balanced growth and little fluctuation in operational parameters, it is most susceptible to microbial contamination (Chisti, 2010).

SSF is carried out in the (near)-absence of free water, yet care has to be taken in order to ensure that the substrate possesses the moisture required to support growth and metabolic activity of the microorganism used for enzyme production. In SSF microorganisms grow in a solid matrix that is either the source of nutrients or an inert support material impregnated with growth solution. Some relevant features of SSF fermentation are summarized in [Table 19.3](#). Given the

TABLE 19.3 Some Significant Aspects of SSF

Concentrated fermentation media, resulting in small reactor volume and low capital investment
Possible use of agro-industrial wastes and low-cost material as substrates, namely defatted soybean cake, rice bran or wheat bran
Low risk of contamination due to low moisture content and substrate complexity
Simple technology and low production of effluents
High product yield and eased downstream processing
Limitations in heat and mass transfer

Source: From Barrios-Gonzalez (2012); Ramos et al. (2013).

particular features of SSF processes, fungal and yeast cultures are deemed the most suitable for growth under such environments. Accordingly, several relevant enzymes in food processing have been produced by SSF (Table 19.4) from bench to large scale (Pandey et al., 2008; Thomas et al., 2013). After fermentation, the enzyme must be recovered. In the case of SSF, enzymes are typically excreted, and the extraction is carried out with either a suitable buffer or with water, followed by solid–liquid separation (ie, centrifugation) for removal of the mycelium. When enzymes are produced by submerged fermentation, recovery depends on the enzyme being of extra- or intracellular origin. In the former case, again a solid–liquid separation is required. In the latter case, cell disruption is required, followed by solid–liquid separation, to remove cell debris. In all cases, the enzyme-rich supernatant proceeds for further steps of purification. A concentration step, usually by ultrafiltration, can be used either prior to further

TABLE 19.4 Some Relevant Enzymes in the Food Industry Produced by SSF

Enzyme	Microbial source	Reference
α -Amylase	<i>Aspergillus oryzae</i> , <i>Bacillus</i> sp., <i>B. subtilis</i>	Dey and Banerjee (2012), Derakhti et al. (2012), Saxena and Singh (2011), Rajagopalan and Krishnan (2010)
Glucoamylase	<i>A. awamori</i> , <i>Aspergillus</i> sp., <i>A. oryzae</i> , <i>A. niger</i>	Kiran et al. (2014), El-Gendy (2012), Parbat and Singhal (2011), Slivinski et al. (2011)
Inulinase	<i>Saccharomyces</i> sp., <i>Kluyveromyces marxianus</i>	Dilipkumar et al. (2013), Onilude et al. (2012), Mazutti et al. (2010)
Invertase	<i>A. niger</i> , <i>S. cerevisiae</i>	Al-Hagar et al. (2015), Al-Saady (2014), Kumar and Kesavapillai (2012)
Lipase	<i>Yarrowia lipolytica</i> , <i>Rhizopus homothallicus</i>	Farias et al. (2014), Velasco-Lozano et al. (2012)
Naringinase	Marine fungi, <i>A. niger</i>	Shehata and El Aty (2014); Shanmugaprakash et al. (2011)
Pectinase	<i>A. niger</i>	Alcântara and da Silva (2014), Heerd et al. (2012), Ruiz et al (2012)
Phytase	<i>Thermomyces lanuginosus</i> , <i>A. niger</i>	Berikten and Kivanc (2014), Rodriguez-Fernandez et al. (2013)
Protease	<i>Penicillium citrinum</i> , <i>Rhizopus stolonifer</i>	Xiao et al. (2015), Kranthi et al. (2012)

Source: From Thomas et al. (2013).

At least, α -amylase, glucoamylase, lipase, pectinase, phytase, protease have been produced in large scale.

processing or if the enzyme proceeds to formulation. Besides the target enzyme, the supernatant contains residual soluble and colloidal components from the fermentation medium, including eventually nonproduct enzymes that are produced by the host organism. Depending on the requirements for the application intended, several purification steps may be needed. Yet it should be taken into account that these add to production costs and decrease efficiency. Precipitation (ie, with ammonium sulfate) followed by ultrafiltration is common. For highly specific application, where a high level of purity is needed, chromatographic processes are used (Dodge, 2010; Ramos et al., 2013). Upon recovery and purification an enzyme concentrate is obtained, which requires a final step of processing, formulation, to be delivered in a suitable form for food processing or production, but also to provide suitable shelf life.

19.2.2 Formulation of Enzymes for Use in Food Processing

Details on formulation of enzyme preparations are rather scarce, yet this is a critical step in the production of industrial enzymes, as it often confers the producer a competitive edge. In the case of food enzymes, they can be delivered in either liquid or solid form. The former allows for simpler dosage while the latter usually extends shelf life (Illanes, 2008; Dodge, 2010). During formulation, several ancillary substances are added to the enzyme concentrate that act as stabilizers and preservatives (Table 19.5). Stabilizers added to liquid formulation help to maintain a soluble product by preventing aggregation. Once stabilizers and preservatives are added, the liquid formulation is filtered to remove undissolved solids (Iyer and Ananthanarayan, 2008; Dodge, 2010; El-Sherbiny and El-Chaghaby, 2011). Until the end of the 1960s, solid formulation consisted almost exclusively of powdered enzyme particles, obtained by spray-drying. The enzyme was mixed with stabilizers and diluents, and the solution or suspension was atomized into small droplets, and exposed to hot air. This resulted into small particles, often with sizes under 10 µm. Complexity in handling and respiratory allergies led to the quest for dust-free alternatives. Still, spray-dried particles can be included in more structured particles. Currently, solid formulation can involve granulates, tablets, or immobilization (Aunstrup et al., 1979; Bayindirli, 2010).

19.2.3 Granulation of Enzymes

Granulation results in the formation of particles with sizes within 425–850 µm. These granules can be engineered as for the enzyme to be encapsulated in a uniform matrix or embedded in a core and shell matrix, where multiple layers of stabilizers and protective agents can be deposited. Several methods can be used to produce enzyme granulate, but only a few are suitable for large-scale processes, namely spray-chilling (prilling), marumerization/spheronization, high shear granulation, and fluid-bed.

TABLE 19.5 Excipients Used in Enzyme Formulations

Excipient	Action	Examples and comments
Stabilizer	Maintain protein structure and prevent denaturation	Carbohydrates: dextrose, sucrose, trehalose, xylan
		Polyols and sugar alcohols: glycerol, mannitol, polyethylene glycols, sorbitol, xylitol
Preservative	Control microbial contamination	Potassium sorbate, sodium benzoate; alternatively, natural inhibitors of microbial growth, namely plant extracts or peptides are looked after. When at high levels, stabilizers help to control activity of water and hence microbial contamination
Diluents, carriers, and fillers	Make the enzyme available at proper rate	Starch, anhydrous, and spray dried lactose, gum arabic, maltodextrins, microcrystalline cellulose, diatomaceous earth, gum arabic, alginates, carrageenans, dairy and soy proteins, emulsifiers, and waxes
Binders	Create liquid bridges which form agglomerates from the powder	Gelatin, starch, polyvinylpyrrolidone, high concentrations of sugar

Source: Based on information from Segura et al. (2007), Rajakari et al. (2013), Lohscheidt et al. (2009).

Prilling involves the incorporation of the enzyme powder in a wax. The mixture is atomized through nozzles or a rotating disk placed on the top of a tower. The falling droplets cool, solidify and harden to yield round particles. Although simple and straightforward, the method is limited by the melting temperature of the wax and by the risk of agglomeration of granules when exposed to moderate to high temperatures.

In marumerization/spheronization either enzyme concentrate or powdered enzyme is mixed with suitable excipients to create a dough-like mass that is extruded through a perforated plate. The resulting cylindrical particles can either be dried in a fluidized bed or converted into small spherical particles using an apparatus known as a marumerizer, consisting of a spheronization plate that spins at 500–2000 rpm. Finally, the spherical particles are dried in a fluidized bed. In high shear granulation the enzyme powder is mixed with stabilizer and diluent with a plough shear mixer and a high-speed mixer. Simultaneously, a binder solution is added. As a result of shear forces, small particles are generated that can be coated with a wax and dried in a fluidized bed (Chotani et al., 2014). The fluidized bed approach, together with high shear,

is considered the most adequate to produce low dust granules. Moreover, fluidized bed is the most flexible technique and the one yielding more uniform granules and smoother coatings. In this method, enzyme powder (or concentrate that undergoes spray-drying) are mixed with suitable excipients in a fluidized bed apparatus. If required, spray-coating can be also carried out, allowing the deposition of sequential layers of the required composition. Examples of food and enzymes formulated as granulates are α -amylases (Duramyl, Termamyl), amyloglucosidase (Amigase), glucose isomerase (Sweetzyme T, Gensweet IGI.), all involved in the production of high fructose syrups from starch; lipases (Lipozyme TL IM), within the scope of the manufacture of healthy oils; and phytases (Lohscheidt et al., 2009; DiCosimo et al., 2013).

19.2.4 Tablets

Tablets can enable the incorporation of enzymes formulated as dry powders or granulates. These are compressed into the intended format and coated with a suitable component. Tablets can include multiple enzyme activities.

19.2.5 Immobilization

Immobilization of enzymes consists in the containment of the biomolecule in a given region of space. This containment can be achieved either through physical or chemical methods, and in the latter case can be carrierless (Fig. 19.2). Entrapment involves containment within a polymeric network, namely polyvinyl alcohol, calcium alginate, chitosan, gelatin, and polyacrylamide. Simple, cheap, and often using mild, biocompatible conditions, it is typically associated with diffusion limitations. In microencapsulation, the enzyme is contained within a semipermeable membrane. Mass transfer limitations are mitigated as compared to entrapment, but the method is more complex. Adsorption involves interactions between the enzyme and the carrier, namely ion-exchange resins, activated charcoal, alumina or celite, through weak forces, namely hydrogen/

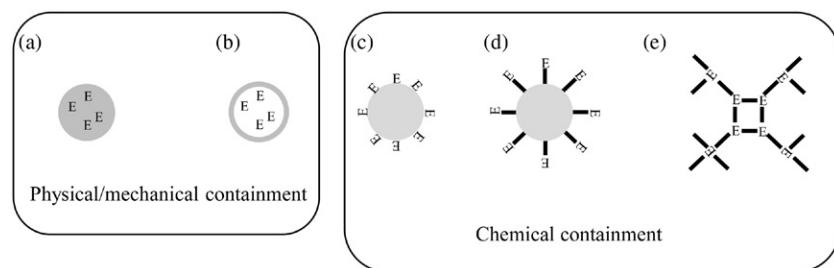


FIGURE 19.2 A pictorial representation of enzyme (E) immobilization methods: (a) entrapment; (b) microencapsulation; (c) adsorption; (d) covalent binding; (e) cross-linking (carrierless).

hydrophobic/ionic interactions and van der Waals forces. Simple and nonaggressive, but it is prone to massive enzyme leakage upon shifts of operational conditions. Covalent binding involves the formation of stable, covalent bonds between the carrier (eg, controlled pore glass, silica, wood chips) and enzyme residues, but for those essential for catalytic activity, a requisite that may prove difficult to comply with. Currently preactivated carriers are available that ease the immobilization process. Cross-linking involves the reaction of enzyme molecules with a bifunctional reagent to yield an insoluble enzyme network, requiring no solid carrier. Typically glutaraldehyde is used to react with NH₂ groups (Torres-Salas et al., 2011; DiCosimo et al., 2013). Immobilization allows for extending shelf life, for the continuous/repeated use of the enzyme and eases the separation of the biocatalyst from the reaction media, hence simplifying downstream processing. These features contribute to making processes more cost-effective. However, there is a downside, as immobilization itself has an added cost; some activity may be lost during the process, namely when chemical methods are involved, and mass transfer limitations may occur as well as enzyme leakage. Moreover, once catalytic activity is lost, the carrier has to be discarded. It is somehow noteworthy that the first report of immobilized enzymes at laboratory scale was on a food-related process, as it involved the adsorption of invertase onto charcoal to hydrolyze sucrose to invert sugar syrup. The same reaction is possibly the first commercial application of immobilized enzymes, using bone char as carrier. Eventually, further developments led to the commercial processes based on invertase adsorbed onto ion-exchange resins or covalently bound to macroporous methacrylate beads, the latter allowing for a productivity of 6000 tons (dry weight) of invert sugar syrup per kg of biocatalyst from sugar beet or cane sugar, with a conversion of 90% (Uhlig and Linsmaier-Bednar, 1998; Swaisgood, 2003). Macroporous methacrylate beads were also used for the immobilization of amyloglucosidase to hydrolyze dextrins to glucose in starch processing. Conversion (94%) was slightly under that achieved with free enzyme (95–96%), due to diffusion-related hindrances. Apparently, this minute difference, together with the cost of the enzyme, prevented the immobilized approach from going beyond pilot scale (Uhlig and Linsmaier-Bednar, 1998). Although currently commercial production of invert sugar syrups relies on chemocatalysis, a wide array of immobilization methods have been assayed using this model system, some up to plant scale with invertase adsorbed onto chitosan beads (Serna-Saldivar and Rito-Palomares, 2008; Kotwal and Shankar, 2009). Currently, and following a trend started in the late 1960s/early 1970s, the most significant commercial scale application of immobilized enzymes within the scope of food industry is by far the isomerization of glucose to fructose for the production of high fructose syrups (DiCosimo et al., 2013). Some other relevant examples on the use of immobilized enzymes in commercial scale processes and prospective developments toward such scale in the short term are given in Table 19.6.

TABLE 19.6 Some Representative Examples on the Use of Immobilized Enzymes in the Food Industry: Current and Emerging Large-Scale Processes

Application	Comments
Glucose isomerization	Granules of glutaraldehyde cross-linked microbial cells homogenates with glucose isomerase activity (current). Adsorption, entrapment, cross-linking, and covalent binding of the enzymes have been tested, some went into production scale but are currently unavailable (DiCosimo et al., 2013)
Lactose hydrolysis/production of whey hydrolysates/GOS (galactooligosaccharides) synthesis	β -Galactosidase entrapped within the microcavities of fibers made from cellulose triacetate or adsorbed and cross-linked to food grade resins for lactose hydrolysis/whey hydrolysates (current); covalent binding to porous silica (discontinued). Several other methods involving adsorption, entrapment, cross-linking, and covalent binding of the enzymes have been tested for both lactose hydrolysis and GOS synthesis and suggest potential development to plant scale (Swaisgood, 2003; Panesar et al., 2010b)
Esterifications within the scope of processing fats and oils	Lipase adsorption or covalent binding to Eupergit like resins. Processes for the interesterification of fats and oils are currently at production scale, namely Betapol, a vegetable fat blend or Crokvitol, a set of fats for margarine and baked goods (current). Perspective developments involve the production of human milk fat analogs and substitutes; processing of anhydrous milk fat and of caprine milk; processing of canola oil; or production of semisolid fats (Swaisgood, 2003; Forde and O’Fagain, 2008; DiCosimo et al., 2013)
Artificial sweeteners (Aspartame)	Thermolysin adsorbed onto polymeric resins flowed by cross-linking (current) (DiCosimo et al., 2013)
Amino acids (L-aspartate)	Microbial (resting) whole cells with aspartase activity either entrapped in polyacrylamide or k-carrageenan cross-linked with glutaraldehyde and hexamethylenediamine or adsorbed to phenolformaldehyde resin; aspartase adsorbed onto weakly basic anion-exchange resin (current) (Wu et al., 2012)
Debittering of fruit juices as alternative to nonspecific neutral or ion-exchange resins; enhancing aroma in wine	Immobilization of naringinase in a wide array of supports, among them alginate, k-carrageenan, celite, chitosan, CLEA, polyvinyl alcohol, perspective implementation to commercial application (DiCosimo et al., 2013; Nunes et al., 2014)

(Continued)

TABLE 19.6 Some Representative Examples on the Use of Immobilized Enzymes in the Food Industry: Current and Emerging Large-Scale Processes (*Continued*)

Application	Comments
Processing of fruit and vegetable juices	Immobilization of pectinases in a wide array of supports, among them alginate, celite, chitosan, Eudragit, ion-exchange resins, gelatin, magnetic particles, silica, ultra-filtration membranes. In particular the advantages of enzyme immobilization during ultrafiltration of food juices have been highlighted at pilot scale. Perspective implementation to commercial application (DiCosimo et al., 2013; Pagán, 2014)
Synthesis of lactulose	β-Galactosidase immobilized in membrane reactors, activated carbon, activated silica gels, CLEA; β-glycosidase immobilized in Amberlite and Eupergit (bench scale) (Wang et al., 2013; Guerrero et al., 2015)

19.2.6 Applications in Food Industries

19.2.6.1 Starch Processing and Sweetener Production

19.2.6.1.1 Starch Industry

Starch is the major form of energy storage in plants. It is essentially a mixture of two polysaccharides, amylose and amylopectin, both composed of glucose units, where the latter polysaccharide is predominant, accounting for around 65–75% of the total, depending on the source. The most common is corn but barley, cassava, rice, sorghum, tapioca, and wheat are also used. From starch several sugar syrups are produced, namely dextrans, dextrose, maltose, high fructose, and fructose; and hydrogenated derivatives, namely hydrogenated starch hydrolysates and sorbitol. The production of these goods involves enzymatic hydrolysis, promoted by amylases, glucoamylases, and pullulanases. When fructose syrups are aimed at, glucose isomerase is also required. Currently, a starch slurry (around 35% dry matter) is prepared and a thermostable α-amylase is added (around 0.5 kg/ton) as the pH is adjusted to 5.0–6.5, in the presence of Ca²⁺. The slurry is pumped through a jet cooker, where steam is injected so that temperature is increased to 105°C. Just 5 min holding time allows for gelatinization of starch to occur (swelling of the granules and breaking up of hydrogen bonds) and the slurry is then cooled to 95°C. The slurry is maintained at this temperature for about 2 h, the time required for liquefaction to proceed until the required dextrose equivalent (DE) value, usually within 8–12 (DE, a measure of the total reducing power of the sugars present, as related to dextrose standard, on a dry mass basis). Further hydrolysis of the liquefied starch, rich in oligosaccharides, proceeds through the

action of either glucoamylase and pullulanase or fungal α -amylase/ β -amylase. In the former case, pH is adjusted to 4.0–5.0 and incubation proceeds at about 60°C for 72 h, until a DE of 97 or higher is achieved, to produce a dextrose syrup. In the latter case, maltose/high maltose syrups are aimed at, requiring pH adjustment to 5.5 and incubation periods of 48 h. Dextrose can then be isomerized to fructose. Dextrose syrup is filtered, processed through activated carbon and ion-exchange resins, and concentrated to about 50% dry solids. Magnesium is then added, pH is adjusted to about pH 7.7, and the liquor is fed to packed bed reactors filled with immobilized glucose isomerase and maintained at 60°C. At this temperature the isomerization of glucose to fructose is governed by the thermodynamic equilibrium between both sugars. Under the conditions used a mixture of 42% (w/w) fructose, 50% (w/w) glucose, and 8% (w/w) of other saccharides is obtained, that can be referred to as HFS42. Further enrichment in fructose to 55% is required for use as sweetener in soft drinks. Thus, HFS42 syrup undergoes column chromatography, so that a syrup with 55% fructose, HFS55, is obtained. This syrup results from the blend of the HFS42 syrup with a fructose-rich syrup (90%), a result of the chromatographic step.

Some improvements on this process are expected to be implemented shortly as a result of dedicated efforts within the scope of protein engineering. These have led to amylases made available commercially with tailor-improved features, so as to fit in advantageously in the production processes. One such enzyme, LpHera, is an α -amylase with intermediate acid stability, hence allowing for starch liquefaction to be carried out within pH 4.5–4.8, unlike the usual pH of 5.0–6.5 required due to the range of operation of current commercial α -amylases. Typically, pH has to be lowered downstream to 4–5, for the saccharification step, to comply with the range of activity of glucoamylases (Eshra et al., 2014). The range of operation of this α -amylase results in a lower demand of chemicals for pH adjustment and of ion-exchange resins. Moreover, the final yield in dextrose from starch processing is increased by 0.2%, in the whole leading to a more cost-effective process. Within the scope of rendering well-established industrial processes more cost-effective, a microbial β -amylase, Secura, with enhanced thermal stability over plant-derived counterparts, has also been recently made commercially available. Also within the scope of making starch processing more cost-effective, variants of glucoamylase were obtained by oligo-directed mutagenesis that display high catalytic activity at pH 5.0 and about 65°C, and moreover, displaying high retention of activity within 60–75°C. Such features enable the combined use of the variant glucoamylases with α -amylases, therefore allowing the liquefaction and saccharification of starch in a single step (Jing et al., 2014).

19.2.6.1.2 Other Sweeteners and Prebiotics from Fructose and Glucose

Invertase is used for the hydrolysis of sucrose into glucose and fructose. The resulting invert sugar syrup is sweeter than sucrose and is widely used in

confectionery, bakery, and pastries, as it features enhanced moisture-preserving properties and is less prone to crystallization.

High fructose syrups can also be obtained in a single step process from inulin, a polyfructan with a terminal glucose residue, used for energy storage by several plants, for example, agave, chicory, and Jerusalem artichoke. Full inulin hydrolysis can be obtained through the action of exoinulinase, or in combination with endoinulinase, that hydrolyzes randomly the inner linkages releasing fructooligosaccharides (FOS) with 1–9 units of fructose. These long chain FOS are known to play a prebiotic role, as they stimulate the growth of intestinal bifidobacteria, with a concomitant positive health effect in the intestines (Singh and Singh, 2010; Chi et al., 2011). Short chain fructooligosaccharides, scFOS, with up to 4 fructose units and a single glucose unit, are obtained from sucrose by transfructosylation through the action of fructosyltransferase. Again these scFOS have also a well-identified prebiotic role and their longer chain counterparts are used as ingredients in functional food, such as baking and dairy products, breakfast cereals, frozen desserts, infant formulae, fruit preparations, dietetic products, and sweeteners (Nobre et al., 2015). Oligosaccharides composed mostly of glucose units, glucooligosaccharides (GLOS), have also gained the interest of consumers, again as an outcome of the prebiotic role. Hence their production for incorporation in functional foods has also been focused. GLOS synthesis typically relies on the use of sucrose as glycosyl donor and a suitable acceptor, such as lactose, maltose, or even sucrose. The relatively broad range of acceptors allows for a wide diversity of products. GLOS synthesis is catalyzed by glucansucrases (alternansucrases, dextranases, mutansucrases, reuteransucrases) that promote successive transfers of glycosyl units onto the oligosaccharide (Daudé et al., 2014).

Also within the scope of the growing public interest on functional foods and low caloric sweeteners, alternatives to traditional sweeteners have been sought. A promising candidate is stevioside, yet this diterpene glycoside features an after-taste bitterness that hampers its application. Such drawbacks can be eliminated through transglycosylation with glucanotransferases, such as β -cyclodextrin glucanotransferase, pullulanase or β -galactosidase, and a suitable donor, that is, starch, pullulan, or lactose, so that carbohydrates are attached into proper positions of the stevioside molecule (Magomet et al., 2010).

19.2.6.2 Dairy

Enzymatic processing of milk has a long tradition (Law, 2010). One of the most significant applications involves the use of rennet, a preparation of proteases with milk-clotting activity for a specific form of coagulation, essential in the production of cheese—specifically rennet hydrolyze k-casein, which releases its terminal hydrophilic region, caseinomacropeptide (CMP). Hydrolysis proceeds concomitantly with a decrease in milk viscosity, until the micelles aggregate and eventually network into a gel, at which point almost all CMP has been released

into solution (Dalgleish and Corredig, 2012). The gel thus formed undergoes a series of operations where whey is released, ultimately enhancing by 10-fold the concentration of casein, fat, and calcium phosphate, and leading to a curd with high dry matter. The enzymatic-driven coagulation process typically occurs at pH within 6.4–6.6 and at temperatures within 30–35°C in order to have adequate control over curd firmness, a feature of interest considering downstream operations in cheesemaking (Harboe et al., 2010). Depending on the source, the composition of rennet varies. Traditionally, rennet was obtained from the stomach of young calves, where chymosin is vastly dominant over pepsin, in a ratio of 9:1. This was until quite recently the standard against which all formulations were matched with. Extracts from older animals have an increased amount of pepsin, so that their ratio may be of up to 1:1. Both enzymes are aspartic proteases, but chymosin is much more specific in action, hence formulations rich in pepsin have an overall higher proteolytic activity. Moreover pepsins are more sensitive to pH than chymosins. Extracts from sheep, goats, and pigs also provide rennet but these are far from ideal for clotting milk from cows. Extracts from the macerated and dried stomachs of suckling calves, lambs, or kid-caprine may be used as a source of rennet. This particular rennet is furthermore enriched with lipase, therefore adding a piquancy to the flavor of the cheese, and is thus suited for the production of some particular cheeses. Throughout the years, the demand for rennet far exceeded the offering provided by young calves, hence alternative sources were looked up. These include plants and microbial sources, which were shown to produce coagulants with a set of features that allow their use as rennet alternatives, namely: the ability to clot milk without excessive proteolysis, in a manner akin to chymosin; low proteolytic specificity toward β -casein to prevent bitter taste development in cheese; and a cost comparable or lower than that of traditional rennet (Mistry, 2012). Extracts from *Cynara cardunculus* (L.) cardoon plant have been traditionally used in artisan cheesemaking in Portugal, a pattern that currently endures and allows the production of cheeses like Serra and Serpa. Suitable clotting activity is due to cyprosin and cardosin, two aspartic proteases, the former of which has been awarded GRAS status (<http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-foods-gen/documents/document/ucm434979.pdf>, assessed May 31, 2015). Additionally, extracts from *Withania coagulans* have also been used in India to produce soft cheeses. Among the wide variety of proteolytic enzymes from microbial sources, only a very few aspartic proteases, particularly those from *Rhizomucor miehei* and from *Cryphonectria parasitica* have proved suitable for cheesemaking, particularly the former, with a broader range of application as it presents relatively high milk-clotting to proteolytic activity and is heat labile. The features of the latter of high overall proteolytic activity, low pH dependency, and heat thermostability makes it suitable for the manufacture of cheeses cooked at high temperatures. Otherwise, the high proteolytic activity may result in off-flavors and bitter taste, resulting from high levels of hydrophobic peptides from the C-terminal region of β -casein, if long maturation periods are considered. Still, these fungal proteases

are relatively cheap and can comply with requirements of some relevant niches of the market, that is, Kosher, Halal, GMO-free products, and vegetarian. Therefore, and in order to capitalize on some of these advantages, chemical and genetic engineering methods have been implemented in order to decrease thermal stability and increase clotting to proteolysis ratio. Some of these successfully engineered proteases are currently commercially available, with a particular highlight being variants termed XL, more heat sensitive and with lower proteolytic activity than the native enzyme, and purified forms of variant XL, obtained by chromatography, free of lipase and amylase activity (Harboe et al., 2010; Feijoo-Siota et al., 2014). Still, expression of chymosin in genetically modified microorganisms, that is, *K. lactis* and *A. niger* and concomitant production in large-scale fermentation, so-called fermentation-produced chymosin, is currently the most widely disseminated alternative for calf rennet. These heterologous chymosine formulations commercially available are from bovine and camelus sources, the latter being considered to display the highest clotting to proteolytic ratio (Harboe et al., 2010; Feijoo-Siota et al., 2014). Other enzymes are used in cheesemaking. Thus, lipases are used to accelerate cheese ripening and modify flavor, as a result of their lipolytic action that releases fatty acids. Excessive lipolytic action may however lead to unwanted odors. Lipases also affect the structure of cheese, eventually allowing for a product with softer texture (Law, 2010). During ripening of some cheeses formation of gas can occur ("late-blowing"), as a result of contamination with spores of the Gram-positive bacteria *Clostridium tyrobutyricum*. These are able to grow at between 1°C and 45°C and colonize pasteurized goods, producing butyric acid, hence the gas effect. Antimicrobial action is implemented through the action of egg white lysozyme, an enzyme relatively stable in cheese and whey, which hydrolyzes the carbohydrate polymers of alternating *N*-acetyl-glucosamine and *N*-acetyl-muramic acid. Such polymers, combined with peptide chains, constitute the mucopolysaccharide walls of Gram-positive bacteria. Hence lysozyme promotes the lysis of the cell wall (Law, 2010).

Lactase or β -galactosidase is another relevant enzyme within the dairy industry. Typical sources of this enzyme are *A. oryzae*, *A. niger*, and *K. lactis*. Lactase promotes the hydrolysis of lactose in milk to its monosaccharides, glucose, and galactose. Thus it allows the extension of the market of milk-based products to those suffering from lactose intolerance or lactose maldigestion. Moreover, as a result of lactose hydrolysis, the sweetness of milk is increased as both monosaccharides are sweeter than lactose. The need for the addition of sugars in the manufacture of flavored milk drinks is therefore minimized, if not avoided. Additionally, lactase is used in the processing of whey, the watery part of the milk resulting from the formation of curd. Disposal of whey, formed in large amounts, was traditionally carried out by using it as feed for animals or fertilizers, or simply dumping it in sewers or watercourses, a serious environmental hazard. Besides current environmental constraints, and in order to make production processes more profitable, there is a growing interest in turning this

by-product into a commercially interesting product. The hydrolysis of lactose present in whey results in the formation of sweet syrup, which provides a source of sugar and is used as such in confectionery, dairy desserts, ice creams, as well as in feedstuff (Panesar et al., 2010b; Shukla et al. 2013). The sweetness of sweet syrup can be further enhanced by processing with immobilized glucose isomerase (Weetall and Yaverbaum, 1974). Besides lactose hydrolysis, lactases can also promote the synthesis of galactooligosaccharides (GOS), nondigestible oligosaccharides typically composed of 2–10 galactose units and a terminal glucose unit. GOS have a prebiotic role. GOS are also currently added to infant food formula in order to mimic human milk oligosaccharides (HMOs). Several studies suggest that blends of GOS with FOS stimulate the intestinal flora of formula-fed infants leading to looser fecal stools. Moreover FAO supports the use of GOS/FOS blends in infant formula for infants aged five months and older (Ackerberg et al., 2012). GOS synthesis is carried out through a transgalactosylation reaction, where lactose (or other carbohydrate in the mixture) serve as galactosyl acceptor. The reaction is influenced by several environmental parameters, namely lactose concentration, pH, temperature, but under optimal conditions GOS yields of about 40% are achievable. For initial lactose concentrations above 30% (w/v) the influence of this parameter in GOS yield clearly decreases. To overcome the relatively low solubility of lactose, reactions are carried out at temperatures of 40°C and above. GOS production largely uses whey permeate as raw material, where lactose concentration can be adequately adjusted, rather than milk, due to the relatively low concentration of lactose in the latter, 5% (w/v) for cow milk (Torres et al., 2010; Diez-Municio et al., 2014). Still, the production of GOS-enriched milk in a concentration close to that of HMO in human milk and with a low titer in lactose has been successfully performed (Rodriguez-Colinas et al., 2014).

Lactose is also the substrate for the production of lactulose ($4-O-\beta-D\text{-galactopyranosyl-D-fructose}$). The traditional role of lactulose had been of a laxative, however its prebiotic action has been identified and its production is thus receiving increased attention. Enzymatic production of this disaccharide has been implemented as an alternative to the chemical alkaline isomerization of lactose, to avoid costly and cumbersome downstream processing. Again the process involves transglycosylation from lactose and fructose, using either β -galactosidases or β -glycosidases. Product yields, up to 45%, are however lower than those obtained through chemical synthesis of about 87% (Wang et al., 2013, Guerrero et al., 2015). An alternative synthetic method, where yields are similar to those obtained chemically, involves the use cellobiose 2-epimerase from *Caldicellulosiruptor saccharolyticus*. Given the isomerization ability for the glucose moiety of cellobiose, the enzyme is considered to display a functionality to convert directly lactose ($4-O-\beta-D\text{-galactopyranosyl-D-glucose}$) into lactulose ($4-O-\beta-D\text{-galactopyranosyl-D-fructose}$), thus doing away with the need for fructose addition (Kim et al., 2013; Wang et al., 2013). In the quest for novel prebiotics with improved properties, the synthesis of lactulose-based

GOS has been implemented, involving either the enzymatic route, based on transglycosylation activity of β -galactosidases, or chemo-enzymatic methods, where the enzymatic activity is combined with a chemical catalyst that promotes the isomerization of either lactose to lactulose in whey permeate, or of the transgalactosylated cheese whey into the lactulose oligosaccharides (Wang et al., 2013; Padilla et al., 2015).

The role played by lipases in cheesemaking is extended to milk fats, as these enzymes promote the partial hydrolysis (lipolysis) of triacylglycerols, about 96% of the milk fat, to release fatty acids among those that are located at given positions on the triacylglycerol backbone and produce lipolyzed milk fat (LMF). Typically most saturated long-chain fatty acids are located in the sn-2 position, whereas short-chain fatty acids are located in the sn-3 position contains. Hence the region-selective nature of lipases has to be considered for the intended goal. Moreover, the extent of reaction conveys significantly different flavors to the resulting product. Therefore, lipolysis is carefully controlled and the reaction allowed to proceed until either the intended flavor/fragrance or acid degree value is achieved. LMF is incorporated in a wide variety of goods, that is, butter, bakery, and snacks. On the other hand lipases can also perform esterification reactions (*inter-* and *trans*), acidolysis and alcoholysis, that improve the physical and chemical properties of milk. In recent years these efforts have focused on the production of triacylglycerols with structures similar to those in human milk fat (Ferreira-Dias and Tecelão, 2014).

Proteases are also used to produce bioactive peptides from milk and whey proteins. The use of digestive enzymes such as chymotrypsin, elastase, pepsin, and trypsin has been reported, yet microbial proteases such as alcalase, subtilisin, and thermolysin are also effective. Moreover, blends from commercial enzyme preparation are also of interest as they allow the production of a larger array of peptides compared to pure preparations.

Transaminases promote cross-linking both within a protein molecule and between molecules' different proteins. Thus these enzymes are used for conditioning the strength and texture of several products, for example, cheese, particularly by increasing the yield in curd and yoghurt (Kieliszek and Misiewicz, 2014).

19.2.6.3 Bakery

The bread making process typically involves the synergistic use of α - and β -amylases. As α -amylases hydrolyze starch to dextrans, β -amylases promotes further hydrolysis to maltose. Thus, while the former releases low molecular chain dextrans from starch, the latter hydrolyzes them to maltose, which can be used as a fermentable sugar by yeast. Several advantages result from the amylase action, namely enhanced bread volume and crumb texture and lower dough viscosity. Moreover, the reducing sugars formed allow for enhanced Maillard reactions, which are accountable for the browning of the crust and intensification of pleasant flavor (Miguel et al., 2013). The combined action of amylases, namely, maltogenic amylases and glucoamylase, can be advantageously used to minimize

staling of bread. Staling of baked goods is a chemical and physical process that reduces their palatability, and is often mistaken for a simple drying-out process due to water evaporation. Staling is noticeable through the increase of the firmness of the crumb, decreased elasticity of the crumb, and tough and leathery appearance of the crust. Staling partly results from the retrogradation of starch. This results from the realignment of amylose and amylopectin molecules upon migration of moisture from the starch granules into the interstitial spaces. Retrogradation starts immediately after baking, during which gelatinization of starch occurs and large amounts of water are absorbed. Amylose retrogrades faster than amylopectin. The partial hydrolysis resulting from enzyme action, preferably performed after gelatinization, significantly alters the structure of starch, as the fragments resulting from hydrolysis are too small to retrograde ([Else et al., 2013](#)).

Lipases are used to enhance handling, machinability, and strength of dough, and to improve bread oven spring and enhance volume and crumb structure of white bread, through their action on flour lipids or added fat. Thus lipases hydrolyze triacylglycerols into mono- and diacylglycerols, glycerol, and free fatty acids. Besides chemical modifications, the surface active nature of the reaction products is accountable for the positive action on bread observed. Different generations of lipases have been presented, allowing for using an increasingly wider range of substrates, for example, diacylgalactolipids and phospholipids and concomitantly, a larger array of products ([van Oort, 2010](#); [Miguel et al., 2013](#); [Gerits et al., 2014](#)).

Lipoxygenases catalyze the oxidation of polyunsaturated fatty acids (PUFA) containing a *cis, cis*-1,4-pentadiene moiety to form fatty acid hydroperoxides. Lipoxygenases can be isolated from animal and plant sources. The selectivity depends on the origin of the enzyme, as wheat lipoxygenases act on PUFA in free or monoacyl glycerol form, while enzyme from soybean also act on PUFA in triacylglycerol form. As oxidation occurs hydroxyl radicals are formed, which react with the yellow carotenoid present in wheat flour and with peptides/proteins present in the dough, with concomitant formation of hydroxyacids. This results in a reduction of the yellow color and thus in a whiter crumb. Besides this bleaching effect, the oxidation of thiol of gluten proteins results in the rearrangement of disulfide bonds and cross-linking of tyrosine residues, ultimately leading to enhanced loaf volume. Several oxidases have been used in bread making as an alternative to the use of chemical oxidants, such as potassium bromate or potassium iodate in order to enhance dough strength and handling properties, and improve texture and appearance of the baked product. Often referred to within this type of enzymes are glucose oxidase and hexose oxidase. The use of the former is somehow conditioned given the low amount of glucose in dough from cereal flours, the latter with a broader range of substrates being more appealing. Yet the mechanism of action of oxidases is not yet fully established ([van Oort, 2010](#); [Miguel et al., 2013](#)). Overdosage of oxidase can result in excessive cross-linking, tamper with gas retention, and thus handling of dough, leading to a product of poor quality ([Bonet et al., 2006](#)).

Proteases have been traditionally used in the production of bread and baked goods. The proteolytic activity of these enzymes is used advantageously on both gluten and dough. As an outcome, mixing times are reduced, dough consistency is decreased and becomes more uniform. Alongside this, controlled hydrolysis helps to regulate gluten strength, and makes pulling and kneading easier. Most of these effects are promoted by endopeptidases, since their action has a more noticeable impact in the gluten network and in dough rheology. The action of exopeptidases is more noticeable in flavor and color, as a result of Maillard reactions involving amino acids released and sugars present. Given their effective action and environmentally friendly nature, proteases have gradually replaced sodium metabisulfite in dough conditioning (Miguel et al., 2013; Hassan et al., 2014).

Transglutaminases are also used in baking, as its cross-linking action over gluten proteins improves the stability and volume of dough, as well as its elasticity and resilience. Transglutaminases promote the formation of an isopeptide bond between the group of γ -carboxamides of glutamine residues (donor) and the primary ϵ -amine groups of proteins/peptides, acceptors of an acyl residue (Miguel et al., 2013; Kieliszek and Misiewicz, 2014).

Xylanases are used to break down hemicelluloses, namely arabinoxylans, as the insoluble nature of the latter hampers the formation of the gluten network. As a result of enzyme action, handling of dough is improved. In addition, the concentration of arabino xylo-oligosaccharides in bread increases, with a positive impact on human health, given their prebiotic nature (Broekaert et al., 2011).

19.2.6.4 Beer Making

The efficiency of the malting process, where a fermentable extract for later yeast action has to be obtained, depends on the addition of exogenous amylolytic (hemi)cellulolytic and proteolytic enzymes in a controlled and quantifiable manner. Poor malting ultimately leads to defective fermentation, low alcohol titer, hampered filtration, and low quality and stability of the final product. Thus, glucanases are required for the breakdown of cell walls of the grains of cereal (ie, barley, whose cell wall is composed of about 70% of glucans). Xylanases are also included in the process, so as to contribute to the degradation of nonstarch polysaccharides, namely arabinoxylans, also significantly present in the cell; proteases (endo- and exopeptidases) to hydrolyze the large chain protein molecules of the cereal, ease the access of amylolytic enzymes to starch, and provide amino acids and small peptides for fermentation, ultimately also having an influence in the flavors produced during fermentation. Excess proteolysis tampers with the foam stability of the final beer by reducing the level of foam positive proteins, while deficient proteolysis will also tamper the colloidal stability of beer; α - and β -amylases, amyloglucosidases, pullulanases, and α -glucosidases are required for the process of starch hydrolysis to glucose units (Souppe and Beudeker, 2002; Lalor and Goode, 2010; Blanco et al., 2014).

Another key enzyme in the process of beer making is α -acetolactate decarboxylase, as it allows the decarboxylation of acetolactate to acetoin. Acetolactate is one of the many flavor compounds produced by yeast during fermentation, but in excess gives beer a butterscotch taste. Acetoin is rather tasteless and enzyme addition allows the speeding up of the maturation process (Dulieu et al., 2000).

19.2.6.5 Juices

Enzymes used in the juice industry help in the separation of juice from the fruit/vegetable cells, and in the clarification of the juice by degrading pectin and naturally occurring starches that contribute to undesired viscosity, hamper filtration, and give the final product a cloudy appearance. Pectin is a generic name for complex structural polysaccharides in fruits and plants, with a backbone of galacturonic acid residues linked by α -1,4 bonds. The side chains of the pectin molecule are composed of sugar residues, that is, arabinose, galactose, and xylose. On the other hand, the carboxyl groups of galacturonic acid are partially esterified by methyl groups. The degradation involves the use of pectinases, a broad designation that encompasses several enzymes, endo- and exopolygalacturonases, that promote the hydrolysis of galacturonans in a random or terminal action pattern, respectively, the former decreasing viscosity, the latter releasing galacturonic acid; pectinmethyl esterase, which hydrolyzes the carboxyl ester bond; endoarabinases, that promote the endohydrolysis of α -1,5-arabinofuranosidic linkages in 1,5-arabinans, preventing haze formation; endopectinlyase, that eliminates cleavage of α -1,4-D-galacturonan methyl ester, decreasing viscosity. Polygalacturonases and pectin methyl esterases are also included in the process of peeling of citrus fruits. Amylases can also be used to promote the hydrolysis of starch. Cellulases and hemicellulases (xylanases) are used to disintegrate the cell wall (Cautela et al., 2010; Grassin and Coutel, 2010; Tapre and Jain, 2014).

In order to avoid the bitter taste of citrus juices, these can be processed with naringinase, an enzyme complex composed of α -rhamnosidase and β -glycosidase. Naringinase hydrolyzes naringin, the molecule that conveys the bitter taste: first to prunin (α -L-rhamnosidase) that is then hydrolyzed to naringenin, (β -D-glycosidase) (Puri, 2012).

19.2.6.6 Processing of Meat, Fish, and Seafood

Proteases are used to obtain uniform tender meat, by decreasing the amount of connective tissue while retaining myofibrillar proteins. Bromelain, ficin, and papain, proteases from plants are typically used for such a goal, however they present some drawbacks, namely poor selectivity and relatively high thermal stability, preventing their full denaturation during cooking. Microbial proteases, for example, subtilisin and neutral protease, are more selective, tend to display higher activity at relatively low temperature, particularly cold-active proteases, and denature at cooking temperatures (Bekhit et al., 2014).

The cross-linking action of transglutaminases is widely used in meat, fish, and seafood areas, particularly in the manufacture of restructured meat, as it allows to improve the texture, cohesiveness, and shelf life of goods, for example, sausages, fish protein paste, and other fish raw materials, and moreover without the need of phosphate addition, with a positive impact on health (López and García-Carreno, 2000). Application of *trans*-glutaminases in meat processing enables the use of lower quality materials, that is, collagen, in the production of highly nutritive goods, provided suitable amino acids are added (Ashie and Lanier, 2000; Kieliszek and Misiewicz, 2014).

Proteases, along with carbohydrases and lipases, are used in the preparation of fish protein hydrolysates. Given the limited availability and cost of animal and plant proteases, bacterial proteases are mostly used, such as neutral protease and Alcalase, a commercial preparation developed for the detergent industry. Enzymatic extraction of protein hydrolysates from cod, capelin, salmon and tuna, among others, has been reported (Ghaly et al., 2013). Lipases have been used in the hydrolysis of fish oils to promote enrichment of PUFA (Yan et al., 2012).

19.2.6.7 Wine Making

Wine results from the maceration of grapes and processing of the resulting juice, hence enzymes used for juice processing, for example, pectinases, (hemi) cellulases, are also used, aiming at the same goals. The concerted action of exoglycosidases and β -glycosidases enables the release of molecules accountable for aroma compounds, by cleaving the intersugar linkage of glycosides, thus liberating sugars and β -glucosides, thereby allowing for β -glycosidase to release glucose and aromatic aglycone. Care has to be taken with the use of this exogenous activity in the case of red wines, as it may hamper color stability. Urease may also be required to avoid the formation of ethyl carbamate, a potential mild carcinogen, formed by the spontaneous reaction of urea and ethanol, as the enzyme hydrolyzes urea to ammonia and carbon dioxide. Lysozyme can also be used to counter unwanted malolactic fermentation (Gómez-Plaza et al., 2010). Laccases provide an effective alternative to traditional approaches for the removal of polyphenols in white wines, such as the use of polyvinylpyrrolidone and high doses of sulfur dioxide. The enzymatic approach is more selective and thus prevents the loss of given organoleptic characteristics due to indiscriminate removal of polyphenols (Kunamneni et al., 2008).

19.3 CONCLUDING REMARKS

The use of enzymes has a long tradition within the scope of the food industry. Nevertheless, dedicated efforts are continuously being made in order to improve existing production processes, implementing new ones, and introducing new products. Such efforts are necessary to tackle the need for improved cost-effective production processes in an increasingly competitive market; the

public demand for new and healthier goods; and stringent safety regulations. Concomitantly the use of enzymes in traditional areas has been significantly improved, but novel applications have been implemented. These result from the increased ability for screening in the quest for suitable biocatalysts and by manipulating the biomolecule in order to improve key properties or create novel functionalities; increased understanding of the mechanisms of enzyme action; and development of suitable enzyme formulation and design of operational conditions, so that biocatalysts can be used in the most advantageous manner. Such outcomes have been made possible since a multidisciplinary approach has been increasingly implemented, a trend that is to be stressed. Together with the growing public demand for safer and high quality foods, alongside sustainable and environmentally friendly production processes, a set of conditions has gathered for exciting developments related to the use of enzymes in the food sector.

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Chapter 20

Productive Chain of Biofuels and Industrial Biocatalysis: Two Important Opportunities for Brazilian Sustainable Development

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20.1 INTRODUCTION

Brazil is well known for its biofuel production and replacement of fossil fuels. The country was a pioneer in the 1970s in the production of ethanol from sugarcane juice, also known as first generation ethanol (1G), which was used at that time in blends of up to 20% or at 100% in cars equipped with special engines. The current Brazilian law states that the government can fix the ethanol blend in gasoline from 18% to 27.5%. The percentage is determined by the government according to economic and market factors. In early 2015, this percentage was fixed at 27%. Currently, more than 90% of new cars sold in Brazil are flex-fuel, which can run on any mixture of ethanol and gasoline. These vehicles now make up approximately 60% of the country's entire light vehicles fleet. More recently, biodiesel has been included in blends with diesel, starting with mandatory mixtures of 2% (called B2) in 2008 and reaching a 7% blend in 2014, with the expectation of further enhancement in the ratio. The challenge now is the establishment of a cellulosic ethanol industry, also known as second-generation

ethanol (2G), where lignocellulosic materials, abundant in Brazil not only from the sugarcane sector but also from other agroindustrial activities, should be processed to obtain ethanol and other products of chemical industry interest. This new industrial approach, biorefinery from sugars, offers a wide range of possibilities. At this point, the abundance of options may increase the challenge of its implementation. Although it is very clear that the industrial chain of 1G and 2G ethanol opens a number of opportunities for innovation worldwide, some economic aspects and the low price of oil have introduced uncertainty into the sector and slowed down the rhythm of investment. The low cost of oil reduces the competitiveness of biomass ethanol, which is a technology under development with steps that still need cost optimization. Enzymatic hydrolysis, for example, is still a bottleneck, although its contribution to ethanol's final price decreased from 50% in 2010 to 15–25% in 2015, according to the Dyadic Company ([Brooks & Tchelet, 2014](#)). For biodiesel, the innovation lies in the transition to enzyme-catalyzed processes and the use of oils derived from microalgae. Additionally, by-products from the biodiesel industry, such as glycerin, pies from oil extraction, and other liquid wastes, have huge potential for the production of other products of interest through biotechnological processing. Last but not least, some opportunities are presented in the production of methane and hydrogen, highlighting the use of different agroindustrial wastes to obtain energy and minimize environmental pollution. It is important to mention that the biofuel production sector offers opportunities not only for the development of biotechnological processes and industrial biocatalysis but also for chemical procedures. [Fig. 20.1](#) summarizes some opportunities considering sugar and oil biorefinery integration that this chapter will cover, with a focus on microbial and enzymatic approaches.

20.2 BIOCATALYSIS AND BIOMASS PROCESSING FOR FUELS AND CHEMICALS PRODUCTION

20.2.1 Brazilian Potential for Processing Lignocellulosic Biomass

Brazil is the world's largest producer of sugarcane, with an estimated production in the 2015/16 harvest of 655 million tons, which was used for the production of approximately 29 billion liters of ethanol and 35 million tons of sugar ([CONAB, 2015](#)).

In Brazil, approximately 600 million tons of agricultural residues are produced annually, including crop residues from sugarcane, corn, rice, cassava, wheat, citrus fruits, coconut, and grasses, with the residues from sugarcane cultivation being the most representative, as large amounts of lignocellulosic residues (bagasse and straw) are generated during the production of ethanol and sugar ([Ferreira-Leitão et al., 2010](#)).

Bagasse is the fibrous material obtained after sugarcane processing by crushing and extraction for the recovery of the sugar juice ([Deepchand, 1986](#),

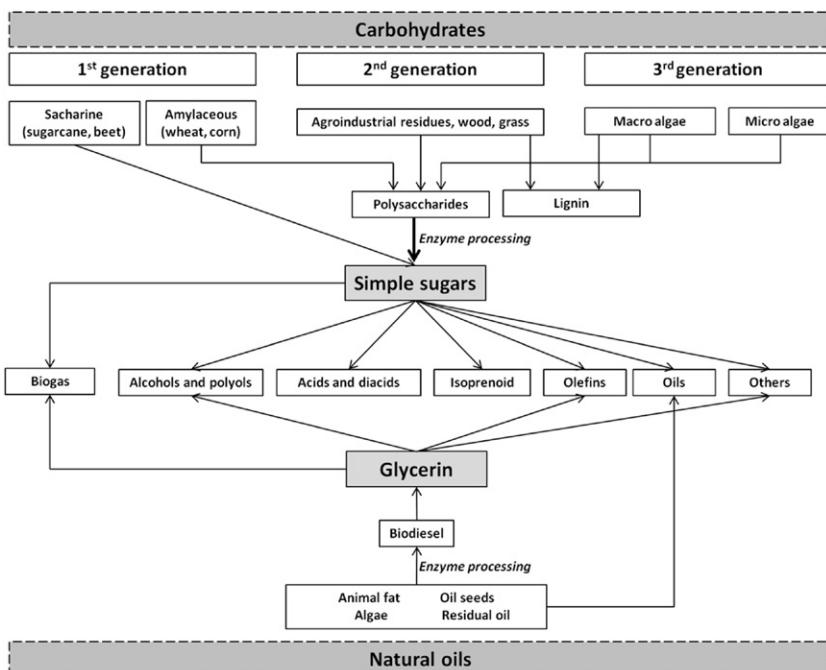


FIGURE 20.1 Sugar and oil biorefinery integration.

while straw, also known as trash, is represented by the mixture of dry and green leaves and cane tops that remains on the field after mechanical harvest of the cane stem (Pippo et al., 2011). Most commercial sugarcane varieties have an average bagasse yield of 140 kg per ton of milled sugarcane (dry basis) (Pippo et al., 2011), while it is reported that for each ton of processed cane stem, 140 kg of straw are left in the fields (Hassuani et al. 2005). Thus, considering these numbers, it is estimated that approximately 185 million tons of bagasse and straw (dry weight) will be produced in the 2015/16 harvest in Brazil.

In the current production plant operation systems, 88–92% of the bagasse is used to produce steam and electricity (cogeneration process) (Coelho et al., 2006). However, it is expected that the gradual replacement of low-pressure boilers (22 bar) for more efficient combined heat and power systems will increase the availability of residual biomass in sugar mills (Bozell and Peterson, 2010). Straw is a residue that is not currently used industrially, although approximately 50% of the straw should remain in the field to ensure soil quality and pest control (Macrelli et al., 2012). Thus, with the prospect of expanding the cultivation of sugarcane, the advancement of cogeneration technologies and harvest mechanization, an increase is expected in the bagasse and straw surplus, which would be available for processing to produce cellulosic ethanol and chemicals. In addition to its availability in industrial quantities, these materials are located near

the plant, minimizing the cost for biomass transportation, which puts Brazil in a very favorable situation to deploy this technology.

20.2.2 Perspectives for the Production of Cellulosic Ethanol

Cellulosic ethanol has the potential not only to partially replace fossil fuels but also to add value to agricultural residues. In addition to fuel production, biomass can be processed to produce fermentable sugars that can be used as platform molecules for the synthesis of different chemical feedstocks in a concept of biorefinery (Soccol et al., 2011).

However, it is a major challenge to degrade the highly recalcitrant lignocellulosic biomass into simple carbohydrates at the industrial scale, due to its heterogeneous and complex structure. Among the options available for biomass conversion into simple sugars, the one relying on the use of enzymes has been opted for by most of the companies that are building commercial plants for the production of cellulosic ethanol (Schwab et al., 2015). In nature, biomass carbohydrates (cellulose and hemicellulose) are degraded by specific enzymes produced by microorganisms, such as fungi, bacteria, and protozoans, but also by plants and animals (Bhat and Bhat, 1997). The use of enzymes for the industrial conversion of lignocellulosic biomass is advantageous because it provides high specificity and leads to low energy and chemical consumption and the reduced generation of toxic wastes (Binod et al., 2011).

Nonetheless, the enzymatic conversion of untreated biomass results in very low product yields and reaction rates and thus is unfeasible. Before enzymatic hydrolysis can be conducted, the key obstacle of biomass recalcitrance needs to be addressed by performing a pretreatment step to improve the accessibility of cellulose to hydrolytic enzymes. Enzyme production and pretreatment are the critical technology steps that have been the focus of research over the past years, aiming to develop better and more cost-effective technologies.

In order to compete with other economically viable alternatives such as sugarcane and corn ethanol, fossil fuels and recently established technologies, such as shale gas, it is of supreme importance to obtain cheap fermentable sugars from biomass.

Cellulosic ethanol technology has been under development for many years, but it was only in the last 10 years that this technology evolved to another level with the launch of new and potent enzymes and the construction of the first pilot, demo, and commercial facilities (Mohr and Raman, 2013; Schwab et al., 2015; Larsen et al., 2012). All these developments occurred in a scenario where macro- and microenvironmental factors contributed to trigger the technology establishment, where oil prices were rising, the world was facing a financial crisis, and the climate change consensus was pressuring governments to seek sustainable energy options.

More recently, the cellulosic ethanol industry has faced new challenges due to the dramatic drop in oil prices in the second half of 2014 and uncertainty

about the political support for biofuels. In the United States, much attention has been given to shale gas, while in Brazil, many investments have shifted to the pre-salt oil exploration; if the price of oil goes under \$70 per barrel, even those initiatives are threatened. Therefore, considering the fluctuation of oil prices and the instability of the geopolitical scenario, it is important for the success of the cellulosic ethanol technology that significant information be captured from the first commercial-scale plants and that companies continue to invest in R&D to improve yields and production economics. Effective communication of the benefits and lessons learned to the public and policy makers will be essential for the full development of this growing industry.

The high cost of enzymes for biomass hydrolysis has long hindered the implementation of commercial facilities for cellulosic ethanol production. Therefore, in the early 2000s, Genencor and Novozymes, two major enzyme manufacturers, received substantial funds from the US Department of Energy to reduce the cost of cellulases production (Born scheuer et al., 2014). Since then, many enzyme producers have made significant progress in reducing the production cost of enzymes to an estimated cost of \$0.30–0.50 per gallon of ethanol in 2014 (Brooks and Tchelet, 2014). However, compared to the cost of enzymes used for the production of corn-based ethanol, of \$0.03 per gallon, biomass-degrading enzymes are still 10–15 times more expensive (Christian, 2015). Current estimates suggest that the cost of producing cellulosic ethanol is \$2.00–\$2.30 per gallon (Fletcher, 2014), so a major emphasis on research will continue to reduce the cost of hydrolytic enzymes and improve efficiency.

20.2.2.1 Lignocellulosic Biomass Components and Biomass-Degrading Enzymes

20.2.2.1.1 Composition of Lignocellulosic Materials

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin but can also contain small amounts of pectin, proteins, extractives, and ash. The full energy and technological potential of this raw material has not been fully explored, partly due to the formation of a global supply chain based on the production of feedstock based in the petrochemical industry. Other factors are related to the recalcitrant nature of biomass and the difficulty of modifying the properties of their constituents, making the processing costly.

Typically, the distribution of biomass components is 40–50% cellulose, 20–40% hemicellulose, and 18–35% lignin (Sun et al., 2011).

Lignin is an aromatic macromolecule present in all vascular plants. Lignin is not evenly distributed in the cell wall; it is absent in the primary cell wall and found in high amounts in the middle lamella (Gellerstedt and Henriksson, 2008). The presence of lignin in the plant cell wall provides the plant tissue with stiffness, impermeability, and resistance to microbial and mechanical attacks (Grabber, 2005). As a consequence, lignin imposes one of the main limiting factors on the enzymatic attack of biomass carbohydrates (cellulose and hemicellulose) by

hindering the access of enzymes to the substrates (Zhu et al., 2008). Lignin is formed by the polymerization of phenyl-propane alcohols, namely *p*-coumarilic, coniferilic, and synapilic alcohols, which differ in structure depending on the type of plant. In coniferous (softwood) trees, lignin consists almost exclusively of coniferilic alcohol, with small amounts of *p*-coumarilic alcohol. In hardwoods, both coniferilic and synapilic alcohols are present, while in monocots, such as sugarcane, all three alcohols are present as lignin precursors (Fengel and Wegener, 1984; Sjöström, 1993; Sakakibara and Sano, 2001).

The hemicelluloses are branched heteropolymers with low molecular weight, having a degree of polymerization of 80–200 (Peng et al., 2012). Hemicellulose may include pentoses (xylose and arabinose), hexoses (mannose, glucose, galactose), and/or uronic acids (glucuronic and galacturonic acids), which are connected to each other mainly through β -(1→4) glycosidic linkages but also by β -(1→3), β -(1→6), α -(1→2), α -(1→3), and α -(1→6) glycosidic bonds in branches. Other sugars such as rhamnose and fucose may also be present in small amounts, and the sugars' hydroxyl groups may be partially replaced by acetyl groups (Gírio et al., 2010). The variety of linkages and branching, as well as the presence of different monomer units, contribute to the structural complexity of hemicellulose, which thus requires a complex enzyme system for its degradation. However, as the hemicelluloses are amorphous, their hydrolysis is easier than the hydrolysis of cellulose.

Cellulose is the most abundant organic polymer in nature and the major constituent of plant cell walls, with an annual production estimated at 1.5×10^{12} tons (Klemm et al., 2005). Cellulose is defined as a linear polymer consisting of glucose residues connected by type β -(1,4) linkages. The formation of β -(1,4) glycosidic bonds requires that adjacent residues be positioned 180° relative one to another, forming cellobiose units (O'Sullivan, 1997). The degree of polymerization of plant cellulose ranges from 500 to 15,000 D-glucose residues, depending on its location in the primary or secondary cell wall (Albersheim et al., 2011). The linear character observed in cellulose chains allows adjacent chains to be positioned close to each other (Dufrene, 2012). Thus, cellulose chains are aligned in strands forming organized fibrils.

Cellulose microfibrils contain crystalline and amorphous regions. The crystalline regions consist of highly ordered cellulose molecules derived from the organization of cellulose chains linked by hydroxyl groups to form intra- and intermolecular hydrogen bonds in different arrangements, while the molecules are less ordered in the amorphous regions (Park et al., 2010). The crystalline regions are more recalcitrant to enzymatic attack, while the amorphous regions are more readily hydrolyzed (Cao and Tan, 2005).

20.2.2.1.2 Cellulose-Degrading Enzymes

The typical reported enzyme system for complete cellulose degradation into glucose comprises three main activities: cellobiohydrolases (CBHs),

endoglucanases (EGs), and β -glucosidases (BGLs) (Chandel et al., 2012; Cao and Tan, 2002). EGs randomly hydrolyze internal glycosidic bonds in amorphous regions of cellulose, creating new chain ends and releasing oligosaccharides. CBHs can act on the amorphous or crystalline part of cellulose, removing cellobiose units from reducing and nonreducing free chain ends. EGs and CBHs act synergistically, as EGs create new chain ends for CBH action and CBHs create more substrate for EGs by disrupting the crystalline substrate and/or exposing previously inaccessible less ordered substrates (Al-Zuhair, 2008). The cellobiose released by CBHs is hydrolyzed by BGLs to glucose. BGLs can also, to a lesser extent, hydrolyze other small cello-oligosaccharides to glucose (Kostylev and Wilson, 2012). As BGLs are inhibited by their end product, that is, glucose, new improved commercial cellulase preparations, such as Novozymes' Cellic series and Dupont's Accelerase, present engineered BGLs for increased glucan conversion and reduced product inhibition (Cannella and Jorgensen, 2014).

In addition to these hydrolytic enzymes, many cellulolytic microorganisms produce enzymes that can degrade crystalline cellulose through an oxidative mechanism of action (Zifcakova and Baldrian, 2012). Although studies on cellulose degradation date to the early 1950s, it was only in 2010 that studies demonstrated the ability of metallo-enzymes, now known as lytic polysaccharides monooxygenases (LPMOs), to disrupt crystalline cellulose. It has been shown that LPMOs are copper-dependent monooxygenases that oxidize polysaccharides at C1 and/or C4, starting a chain breakage (Harris et al., 2010; Vaaje-Kolstad et al., 2010; Hemsworth et al., 2013). LMPOs were incorporated into commercial cellulases, such as Cellic CTec 2 and CTec3 (Novozymes), within a few years after their discovery, as they were proven to boost cellulose degradation (Horn et al., 2012). The addition of LMPOs to commercial enzyme cocktails adds a new variable to cellulose hydrolysis, as those enzymes require oxygen. To benefit from LMPOs' boosting activities, processes must be designed to avoid any competition with dissolved oxygen, preferably by conducting the hydrolysis step separately from fermentation (Cannella and Jorgensen, 2014).

20.2.2.1.3 Hemicellulose-Degrading Enzymes

Due to the structural complexity of hemicellulose, its complete breakdown requires several enzymes with distinct specificities. Different plants have different types of hemicellulose, and hence different hemicellulases are needed for the conversion of each type of biomass. Grasses, such as sugarcane, have arabinoxylan as a major hemicellulose component (Sweeney and Xu, 2012).

The prerequisite for the conversion of hemicellulose to value-added chemicals is its complete depolymerization into monosaccharides. The complete depolymerization of hemicellulose requires the synergistic action of a spectrum of hemicellulase enzymes, including endoxylanase, β -xylosidase, α -L-arabinofuranosidase, α -glucuronidase, acetyl xylan esterase, and feruloyl xylan esterase (Juturu and Wu, 2012).

The requirement of hemicellulases for lignocellulosic biomass hydrolysis is strongly dependent on the type of pretreatment used. As some pretreatments remove the hemicellulose content almost completely, the need for hemicellulases is reduced in those cases. However, the trend in new commercial plants for cellulosic ethanol production is to use low-severity pretreatments to reduce capital cost and toxic waste generation (Harris et al., 2014). Pretreatments conducted in lower-severity conditions tend to leave more hemicellulose in the biomass material, thus requiring the incorporation of hemicellulase in commercial enzymatic pools. Commercial enzymes for the hydrolysis of lignocellulosic materials, such as the Cellic enzymes series, offer the option to mix cellulases and hemicellulases for a better hydrolysis response. Although the application sheet of Cellic CTec2 describes the product as a mixture of aggressive cellulases, high levels of BGL, and hemicellulases, the manufacturer also offers the option to mix Cellic CTec2 with HTec2, which is described as a product rich in endoxylanases with high specificity to soluble hemicellulose, with a cellulase background. It is recommended to perform a dose response test by the addition of Cellic HTec2 to CTec2 in comparison to CTec2-only dose, when pretreatment results in a feedstock that would benefit from additional hemicellulose degradation.

Cellic HTec3, which is the new generation enzyme, is described as a hemicellulase complex that contains endoxylanase and β -xylosidase activities for effective hemicellulose conversion. The manufacturer describes this product as highly effective for the hydrolysis of liquid process streams rich in xylan-oligomers to xylose and for the conversion of soluble and insoluble hemicellulose in pretreated biomass slurries.

Other commercial preparations, for example, Accelerase TRIO from Dupont, include a combination of multiple enzyme activities, with cellulases and hemicellulases in the same blend.

20.2.2.2 Enzyme-Based Initiatives for Cellulosic Ethanol Production at Commercial Scale

Brazilian initiatives to stimulate cellulosic ethanol production from sugarcane biomass have been made available from various funding agencies (Funding Authority for Studies and Projects (FINEP), Ministry of Science Technology and Innovation (MCTI), National Counsel of Technological and Scientific Development (CNPq) and Brazilian Development Bank (BNDES)). In 2011 and 2012, the PAISS Program, from the BNDES and FINEP, financed the sugarcane industry sector, including cellulosic ethanol with US\$ 560 million and US\$ 2.2 billion, respectively. Two commercial-scale facilities for the production of ethanol from sugarcane biomass were in the process of installation in Brazil. GranBio built its first unit in Alagoas with an initial production capacity of 82 million liters of ethanol per year. The technology is based on the pretreatment developed by PROESA of the BetaRenewables (company Group M&G), the enzymes are from Novozymes, and the yeasts were developed from DSM (GranBio, 2014). GranBio started operation in September 2014. The technology used at the GRANBIO unit was firstly

tested in Crescentino, Italy, where an industrial plant for cellulosic ethanol began production in 2013 with a capacity of 20 million gallons of ethanol per year.

Raizen, a company based on the merger of Shell and Cosan, in partnership with Iogen Corporation, began operation in November 2014 of its biomass-to-ethanol facility in Piracicaba, São Paulo, using Iogen Energy's technology ([RAIZEN, 2014; IOGEN, 2015](#)). The 2G ethanol unit has a capacity of 40 million liters of ethanol per year. The company also signed an agreement with Novozymes regarding the development of enzyme technology for 2G ethanol production ([UNICA, 2014](#)).

Of the total transport fuel consumption in the United States in 2012, 13.8 billion gallons, biofuels accounted for approximately 7%. Starch ethanol is the most relevant biofuel in the country, accounting for 94% of all biofuel produced in this year. According to [Coyle \(2010\)](#), in 2010, there were 38 2G biofuel companies in United States, which included not only ethanol but also biobutanol and biocrude. Recent data from the USDA, released in December 2014, register the contribution or projection of six main companies: Poet, Abengoa, DuPont, INEOS Bio, GranBio, and Edeniq ([USDA, 2015](#))

20.2.3 Perspectives on Biomass Processing for Chemicals Production

Biochemical routes comprise enzymatic or microbial conversions, which can be biobased or not. A very important example of enzyme-catalyzed commodity production is the hydration of acrylonitrile into acrylamide ([Ashina and Suto, 1993; Straathof, 2013](#)). In this case, the substrate is not a renewable material, which demonstrates the versatility of enzymes. However, the focus here is not only on biochemical routes but also on biobased products, in other words, the use of renewable substrates: specifically lignocellulosic biomass or oleaginous materials, for biorefinery integration considering the biofuels chain in Brazil. Therefore, the main available substrates include sucrose, cellulose, hemicellulose, triglyceride, glycerol, and all by-products and residues generated during the biofuel product processes.

Each available substrate from both main processes mentioned here can be observed as a platform molecule (C6-sugars from sucrose and cellulose, C5-sugars from hemicellulose, triglycerides from oleaginous materials, and glycerol as coproduct from biodiesel production), and a great number of derived products are possible, simultaneously offering a great opportunity and an enormous challenge. Although cellulose and hemicelluloses should be previously hydrolyzed, C5 and C6 sugars are versatile substrates. Many sources of triglycerides are available in Brazil; however, the biodiesel industry is mainly based on soybean oil. Specialty products and other products of industrial interest can be obtained from triglycerides: fatty acids, fatty alcohols, glycerin, amines, amides, esters, sulfates, alkoxylates, and others. These compounds are applied as polymers, lubricants, surfactants, emollients, soaps, detergents, and food additives.

Biocatalyzed processes for oleochemicals production have received increasing attention as a cleaner process production, due to some important applications of these products, such as personal care, cosmetics, and food additives ([Ansorge-Schumacher and Thum, 2013](#); [Bozell and Peterson, 2010](#); [Straathof, 2013](#)). Glycerin can be considered as a mini-sugar and consequently used in different fermentative processes. Currently, research is concentrated on 1,3-propanediol production ([Bozell and Petersen, 2010](#); [Straathof, 2013](#)).

According to Straathof ([Straathof, 2013](#)), there are at least 22 types of industrial or pilot-scale processes of biobased chemicals production by enzyme or cell catalysis, including the synthesis of different classes of compounds: hydrocarbons, alcohols, carbohydrates, carboxylic acids, esters, amines, and amino acids. [Tables 20.1 and 20.2](#) show some examples of platform molecules, products, applications, and stage of development.

TABLE 20.1 Examples of Products from C6-Sugars, Their Main Applications and Stage of Development

Product	Application	Development stage
Methanol	Antifreeze agent, solvent	Pilot
Isoprene	Synthetic rubber and thermoplastic elastomer	Pilot
Farnesene	Solvents, surfactants, resins, adhesives	Research
Styrene	Synthesis of polymers	Research
Ethanol	Fuel, solvent, beverages	Industrial
1-propanol	Synthesis of <i>n</i> -propyl acetate	Research
Isopropanol	Solvent and cleaning fluid	Research
1-Butanol	Drop-in fuel	Industrial
2-Butanol	Precursor of amines and esters	Research
Isobutanol	Mobile phase in thin layer chromatography	Pilot
1-Hexanol	Fragrances, plasticizers	Research
1,2-Propanediol	Heat-transfer fluid, cosmetics	Research
1,3-Propanediol	Monomer for polyester with terephthalic acid	Research
1,4-Butanediol	Synthetic rubber	Pilot
Erythritol	Sweetener	Industrial

(Continued)

TABLE 20.1 Examples of Products from C6-Sugars, Their Main Applications and Stage of Development (*Continued*)

Product	Application	Development stage
Sorbitol	Vitamin C synthesis	Industrial
D-Mannitol	Sweetener	Research
Phenol	Polycarbonates and resins	Industrial
Epoxyethane	Production of ethylene glycol	Research
Acetone	Solvent for many plastics	Industrial
Butanone	Used in paint and glues	Research
Formaldehyde	Resins	Research
Acetaldehyde	Chemical intermediate for various compounds	Research
Propanal	Chemical intermediate for various compounds	Research
Isobutyraldehyde	Chemical intermediate for various compounds	Research
Formic acid	Silaging	Research
Acetic acid	Vinyl acetate, acetic anhydride, acetate salts	Pilot
Glycolic acid	Cosmetics	Research
Propionic acid	Food preservation	Pilot
Lactic acid	Cosmetics, leather industry	Industrial
3-Hydroxypropionic acid	Precursor of acrylic acid	Pilot
Pyruvic acid	Fine chemical	Research
Butyric acid	Cellulose acetate butyrate plastics	Pilot
3-Hydroxybutyric acid	Monomer of polyhydroxybutyrate	Research
Succinic acid	Building block for polymers	Industrial
Malic acid	Acidulant	Research
Fumaric acid	Acidulant	Discontinued by industry
Itaconic acid	Synthesis of resins and chemicals	Industrial
Hexanoic acid	Fine chemical	Research
Adipic acid	Production of nylon 6,6	Research

(Continued)

TABLE 20.1 Examples of Products from C6-Sugars, Their Main Applications and Stage of Development (*Continued*)

Product	Application	Development stage
Citric acid	Food/beverage industry	Research
D-Gluconic acid	Solvent for multivalent cations	Industrial
Ascorbic acid	Production of Vitamin C	Industrial
1-Butyrolactone	Solvent	Research
2-Aminoethanol	Anticorrosive, detergent, gas sweetening	Research
1,4-Diaminobutane	Production of nylon 4,6	Research
1,5-Diaminopentane	Production of polyamides	Pilot
L-Threonine	Chemical commodity	Research
L-Valine	Chemical commodity	Research
L-Aspartic acid/L-Arginine	Chemical commodity	Research
L-Glutamic acid	Nylon 6	Industrial
6-Aminohexanoic acid	Silicones	Research
Methyl chloride	Synthesis of polymers	Research

Source: From Ansorge-Schumacher and Thum (2013); Bozell and Peterson (2010); Straathof (2013).

20.2.4 Perspectives on the Use of Macro- and Microalgae as Sources of Fermentable Sugars

Studies on the use of macro- and microalgae as sources of biomolecules for fuel production have been increasing in recent decades. Historically, the idea of using algae as an energetic feedstock dates back to the end of the 1950s and was furthered by the 1970s oil crisis (Chen et al., 2009). From 1978 to 1996, the US Department of Energy invested US\$ 25 million in a program on the study of algal fuels, achieving great advances in this field (Waltz, 2009).

As some species of microalgae, when cultivated under metabolic stress, are able to accumulate higher amounts of lipids as an energy reserve (Mata et al., 2010), they have been mostly studied for biodiesel production. However, the carbohydrate-rich algae cell wall has also been considered a potential source of fermentable sugars for ethanol production. Indeed, as observed for lipid accumulation, certain algae species, under metabolic stress, can increase the carbohydrate content of the cell wall, allowing a high ethanol yield. Table 20.3 compares the ethanol yield of several plant feedstocks and of microalgae, showing its high

TABLE 20.2 Examples of Products from C5-Sugars, Fatty Acids, Fatty Alcohols, and Glycerol, their Main Applications and Stage of Development

Platform molecule	Products	Application	Development stage
C5	Ethene	Polyethylene, ethylene oxide, vinyl chloride	Industrial/Research
	Isobutene	Synthetic resins, adhesive resins, vitamins	Research
	Ethylene glycol	Antifreeze agent, production of polyester	Research
	Xylitol	Sweetener	Industrial
	Acetic acid	Vinyl acetate, acetic anhydride, acetate salts	Research
	Glycolic acid	Cosmetics	Research
Fatty acids	Terminal alkenes	Surface-active agents	Research
	FAME	Biodiesel	Industrial
	1-Hexanol	Fragrances	Research
Fatty alcohols	Butadiene	Synthetic rubbers and plastics	Research
Glycerol	1-propanol	Synthesis of <i>n</i> -propyl acetate	Research
	1,2 propanediol	Heat-transfer fluid, cosmetics	Research
	1,3-propanediol	Solvent, additive, monomer for polyester	Research
	3-hydroxypropionic acid	Precursor of acrylic acid	Pilot

FAME, Fatty acid methyl esters

Source: From [Ansorge-Schumacher and Thum \(2013\)](#); [Bozell and Peterson \(2010\)](#); [Straathof \(2013\)](#).

potential as a source of fermentable sugars for ethanol production. Common algae, such as species belonging to the *Chlorella* genus, can accumulate between 40% and 70% of carbohydrates depending on the cultivation conditions ([Brennan and Owende, 2010](#)).

The polysaccharides of microalgae biomass are more easily hydrolyzed by enzymes than the ones in plant biomass. This difference may be due to the

TABLE 20.3 Ethanol Yield from Different Sources

Source	Ethanol yield (L/ha)
Wheat	2590
Cassava	3310
Sweet sorghum	3050–4070
Corn	3460–4020
Sugar beet	5010–6680
Sugarcane	6190–7500
Microalgae	46,760–140,290

Source: Adapted from *Mussatto et al. (2010)*.

absence of lignin in the algal cell wall, as this molecule in the plant cell wall prevents microbial attacks. Furthermore, microalgae present cellulose in its triclinic crystalline form, which forms weaker hydrogen bonds and more isolated cellulose chains than the monoclinic form found in higher plants, which allows better packing of the fibers (Atalla and Van der Hart, 1948; Hayashi et al., 1997). The triclinic configuration allows better access of cellulases to the cellulose molecule, making the hydrolysis step easier. However, it has been reported that some cellulases show higher affinity for the monoclinic configuration of cellulose (Corgie et al., 2011), and therefore, the enzymatic cocktail for the hydrolysis of the algal biomass must be selected specifically for this purpose.

In addition to cellulose and hemicellulose, which is also present in a lesser extent in some microalgae cell walls, several microalgae can accumulate high amounts of starch as a reserve polymer (Branyikova et al., 2011), which can also be used for ethanol production.

The enzymatic hydrolysis of the algae biomass is usually conducted using cellulases for the cell wall cellulose hydrolysis and amylases for the hydrolysis of the internal starch, although there are some studies using other enzymes, such as pectinases and proteases. The glucose yields for the enzymatic hydrolysis of several species of microalgae with the use of cellulases vary from 50% to 70% (Harun and Danquah, 2011; Hung et al., 2011; Rodrigues et al., 2015); however, a pretreatment step may be used to increase these yields. Kim et al. (2014) used pectinase to hydrolyze pretreated *Chlorella vulgaris* biomass, increasing glucose yields from 45% to 76% when using bead-milling as a pretreatment. When hydrolyzing starch-rich microalgae using amylases, higher glucose yields are achieved, with values between 80% and 95% (Choi et al., 2010; Ho et al., 2013) after a pretreatment step.

A recent study by Rodrigues et al. (2015) found that to hydrolyze the biomass of a carbohydrate-rich *Chlorella homosphaera* it is only necessary to

use an enzymatic pool rich in BGL and amylase with a small amount of EG. This pool showed no significant activity of exoglucanase, a key enzyme in the hydrolysis of plant biomass crystalline cellulose, showing that the algal cellulose is more susceptible to enzymatic hydrolysis, requiring a much less complex cellulase pool.

Considering now the industrial use of microalgae, the US company Algenol, which was founded in 2006, is engaged in building the first commercial facility for ethanol production from microalgae. The company already has a pilot-scale facility in Florida where they cultivate and process microalgae for ethanol, jet fuel, diesel, and gasoline production. Their process is not based on the enzymatic hydrolysis of the algal carbohydrates; instead, their engineered cyanobacteria are capable of producing ethanol directly through metabolic pathways and secreting it to the cultivation medium. The residual biomass obtained after harvesting is processed for production of the other three fuels.

In Brazil, the company Algae, founded in 2010, uses microalgae technology focusing on the production of biofuels, biofixation of CO₂, animal nutrition, and effluent treatment. The activities of the company regarding biofuel production are still on the developmental scale, aiming at strain selection and the improvement of algae cultivation and harvesting methods, as well as increasing knowledge on conversion methods.

Macroalgae, which are phylogenetically closer to higher plants in comparison to microalgae, present a cell wall with high carbohydrate content regardless of the cultivation conditions, which is advantageous in comparison to the microalgae stress metabolic needs regarding the high accumulation of lipids and/or carbohydrates. Macroalgal structural carbohydrates can account for nearly 60% of their dry weight, reaching 80% in certain species (Kim et al., 2011; Jang et al., 2012a,b). Some species also present starch as a reserve polymer (Kraan, 2013).

Although some macroalgae have lignin in their composition, its concentration is lower than in higher plants, which makes them more susceptible to enzymatic processing (John, 2011). However, a pretreatment step is necessary to improve hydrolysis yields and make the process more economically viable, although it seems that more moderate conditions are required than for lignocelulosic biomass.

Schultz-Jensen et al. (2013) tested five different pretreatments for the hydrolysis of a green macroalga, including thermal and mechanical methods. All tested pretreatments improved the algae susceptibility to enzymatic hydrolysis, as the ethanol yields obtained in a simultaneous saccharification and fermentation process were higher for all pretreated materials. Ball-milling, although an energy-intensive pretreatment, was the preferred method, allowing the highest ethanol yields with lower biomass loss.

Another study by Trivedi et al. (2013) reported that, when using different pretreatment methods, the yield of reducing sugars varied from approximately 30 to 200 mg/g of biomass, with autoclaving being the preferred method.

Macroalgal pretreatment is still in its infancy, and comparative studies with different macroalgae species are still needed regarding the technology of choice. In the aforementioned work, the authors compared three commercial cellulase preparations and found a threefold span in the hydrolysis yields, showing the importance of a customized, well-balanced enzymatic cocktail for the hydrolysis of the macroalgal biomass.

In the industrial use of macroalgae for ethanol production, the reported enterprises in Chile, Australia, and Vietnam have not moved to the industrial scale, as the production costs are still too high for a commodity product such as fuel.

Regarding the Brazilian prospectus for macro- and microalgae technology, Brazil has a high global solar irradiation in any region of its territory of 4200–6700 kWh/m², which is higher than for most countries in the European Union, at 900–1850 kWh/m². Moreover, as a great part of its territory is located near the equator, Brazil has an even distribution of sunlight throughout the year (Pereira et al., 2006). Recent studies have identified more than 3000 algae species in Brazil, with two genera and 52 species endemic (Bicudo and Menezes, 2010). Therefore, Brazil has an enormous potential for growing macro- and microalgae, a potential thus far hardly explored, particularly for the production of biofuels.

20.3 TECHNICAL AND ECONOMIC PROSPECTS OF USING LIPASES IN BIODIESEL PRODUCTION

20.3.1 Biodiesel and Lipases: History and Prospects

Biodiesel, a mixture of mono alkyl esters produced from vegetable oils or animal fats, is a much sought-after alternative in recent years and has already been implemented in several countries (Robles-Medina et al., 2009). In Brazil, its production and use have become mandatory since the foundation of the National Program for Use and Production of Biodiesel (PNPB) in 2005, establishing a minimum percentage of biodiesel blended with petrodiesel (2%, named B2) in January 2008, which has increased over time (Bergmann et al., 2013). Currently, Brazil is using B7; however, it is expected that this production will increase further. Brazil is the fourth highest world producer of biodiesel, with 58 authorized plants in operation and a monthly production of approximately 300,000 m³, with approximately 75% obtained by the alkaline transesterification of soybean oil with methanol (ANP, 2015). However, this current technology production presents a number of difficulties, such as the production of large amounts of highly alkaline wastewater, causing an environmental threat and imposing additional handling costs, and the difficulty of glycerol (by-product) and catalyst recovery. Moreover, the alkaline route requires high-quality and expensive raw materials (oil acidity of less than 0.5%), and the prices of these refined oils correspond to 70–80% of the total biodiesel production costs, hindering the

economic competitiveness of this biofuel (Jegannathan et al., 2011; Fjerbaek et al., 2009; Talukder et al., 2009; Robles-Medina et al., 2009; Al-Zuhair, 2007).

To circumvent these drawbacks, several studies have been conducted focusing on the development of new technologies in biodiesel production to increase its competitiveness and viability in the international fuel market. Currently, particular attention is paid to the biocatalysis in biodiesel synthesis, which can be achieved by enzymatic processes (using lipases as biocatalysts) and, more recently, microbial processes (using whole cells as catalysts). The use of lipases or whole cells in biodiesel production requires relatively simple downstream processing steps for the purification of biodiesel and the by-product glycerol (without catalyst contamination), which has a better quality (higher value). Furthermore, enzymatic processes allow the use of cheaper raw materials with high FFA (free fatty acids) and water content, as lipases can catalyze both esterification and transesterification reactions (Fjerbaek et al., 2009). This option offers a great advantage over the conventional transesterification process, which generates soaps in the presence of FFA, making it difficult to separate the biodiesel from the glycerol and reducing the yield of these plants (Ranganathan et al., 2008).

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of esters, particularly long chain triglycerides, releasing FFAs, di- and monoglycerides, and glycerol. Additionally, in organic media, these enzymes also catalyze synthetic reactions, including esterification, alcoholysis, acidolysis, and interesterifications, with high selectivity and high specificity under mild conditions (Villeneuve et al., 2000; Ranganathan et al., 2008). Lipases are found in many species of animals, plants, bacteria, and fungi (Hasan et al., 2006). The extreme versatility and ease of mass production of microbial lipases make them part of the most widely used group of biocatalysts for biotechnological applications, including detergent formulation, food processing, leather, textile and paper manufacturing, cosmetics, biocatalytic resolution of intermediates for pharmaceuticals and chemicals, waste treatment, and diagnostics (Sharma et al., 2001; Jaeger and Reetz, 1998; Kapoor and Gupta, 2012; Hasan et al., 2006; Horchani et al., 2012). Moreover, these enzymes have been shown to be viable for the transesterification of vegetable oils and other oils into fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE) (biodiesel). Due to their versatility, lipases are the fifth most frequently used group of enzymes in the industrial sector worldwide. Suppliers of lipases include DuPont, Novozymes, Roche, Amano, and Verenium (Freedonia, 2014). World lipase demand was \$255 million dollars in 2012. However, it is expected that this demand will increase by 6.2% annually to \$460 million in 2022, driven by a strong growth in both industrial and specialty markets. The increased investment in research and biotechnology and the improvements in enzyme production based on directed evolution and other molecular biology techniques will allow the introduction of new lipases with greater stability and robustness under a wide range of reaction conditions (Freedonia, 2014).

20.3.2 State of the Art of Biodiesel Production by Enzymatic Routes

Enzymatic biodiesel production has been conducted by several research groups using commercial lipases (free or immobilized) and conventional raw materials (refined vegetable oils) (Zhao et al., 2007; Chen et al., 2008; Hernández-Martin and Otero, 2008; Köse et al., 2002; Lee et al., 2006; Royon et al., 2007). The immobilized commercial lipases most widely applied in biodiesel synthesis are Novozym 435 (lipase B from *Candida antarctica*), Lipozyme RM IM (lipase from *Rhizomucor miehei*), and Lipozyme TL IM (lipase from *Thermomyces lanuginosus*).

Alternative feedstocks with high FFA content and lower cost, such as crude vegetable oils (Talukder et al., 2009; Jang et al., 2012a,b), waste cooking oils, and wastes from vegetable oil refining (Wang et al., 2006; Halim and Kamaruddin, 2008; Watanabe et al., 2002; Kojima et al., 2004), as well as nonedible oils obtained from many plant species potentially available in a local area (Carvalho et al., 2013; Shah and Gupta, 2007; Aguiéiras et al., 2014), have also been used for enzymatic biodiesel production, with conversion above 90% in batch reactors and packed-bed reactors.

The use of these alternative raw materials that do not rely on food crops is an interesting approach to combat negative issues related to the use of edible vegetable oils for biodiesel production and is an important aspect of the economic competitiveness of biodiesel industries. Moreover, in Brazil, due to its wide territorial dimension and availability of local biomass, it is expected that alternative vegetable nonedible oil crops, with higher oil yield than soybeans, will contribute increasingly as feedstock for biodiesel production (Bergmann et al., 2013; Hama and Kondo, 2013). Examples of nonedible oils that can serve as promising feedstocks for biodiesel production in Brazil include the physic nut (*Jatropha curcas* L.), macauba (*Acrocomia aculeata*), andiroba (*Carapa guianensis*), and babassu (*Orbignya* sp.). The use of these nonedible oils allows a diversification of the oil crops used for biodiesel production, contributing to the best use of regional resources, and provides the social seal to biodiesel producers that buy this oil from family farmers. Moreover, the seed cake after oil expelling can be used as fertilizer for soil enrichment or as a substrate for the production of enzymes and other valuable products by solid state fermentation (SSF). It is important to note that most of these oilseeds and fruits are obtained from nondomesticated crops, and methods for collecting and processing the fruits and seeds are still not technologically developed. However, the growing global demand for vegetable oils, destined mainly for food and energy, has contributed to a change in this scenario (Silva and Andrade, 2013). The cultivation of these crops, once established, together with government policies can increase investment in the use of these oils as alternative raw materials for biodiesel production.

Microalgae are another alternative lipid source being considered for biodiesel production. The lipid content of microalgae varies from 1% to 70% and can be as high as 90% depending on the species and the cultivation conditions. More

common algae, including the genus *Chlorella*, present lipid contents between 20% and 50% (Chisti, 2007; Mata et al., 2010). One factor that drove attention to microalgae as a feedstock for biodiesel production is their high oil yield per cultivated area. Palm, the most productive oleaginous land plant, has a yield of 5950L of oil per hectare, while a microalga with 30% lipid content presents a yield of 58,700L per hectare (Chisti, 2007). Promising results were obtained using immobilized lipases with oils extracted from *Chlorella* species, with enzymatic transesterification yields of 98% (Li et al., 2007; Tran et al., 2012).

Despite the advantages of the enzymatic route, the use of lipases in biodiesel synthesis still faces some obstacles, such as lower reaction rates, enzyme inactivation caused by alcohol and glycerol, and the high cost of such enzymes (Gog et al., 2012; Freire et al., 2011). The commercial lipases available on the market are imported and expensive, hindering the use of this biocatalyst for the production of commodities such as biodiesel in Brazil. Thus, several efforts have focused on minimizing the production costs of lipases and developing more stable and active biocatalysts to obtain low-cost enzyme preparations and to maximize their reutilization (Fernandez-Lafuente, 2010). These studies are essential for reducing the final price of biodiesel obtained by the enzymatic route, contributing to its economic competitiveness with biodiesel attained by the conventional process. In this context, the development of “new” biocatalysts can be approached by two promising technologies that comprise the biorefinery concept in the productive chain of biodiesel and industrial biocatalysis: whole cells and solid enzymatic preparations (SEPs).

The development of whole-cell processes, which involve the use of the entire microorganism containing the enzyme as a biocatalyst, has been described as a new approach to reduce production cost and simplify the operation (Yan et al., 2014a). The three different types of whole cells used in biodiesel synthesis include filamentous fungi, mainly *Rhizopus* sp. and *Aspergillus* sp., which produce intracellular or cell-bound lipases (Qin et al., 2008; Hama et al., 2007); recombinant yeast cells such as *Pichia pastoris*, *Yarrowia lipolytica*, and *Saccharomyces cerevisiae*, which express the lipase on the microbial cell surface (surface display technology) (Liu et al., 2014a; Huang et al., 2012; Matsumoto et al., 2002); and recombinant *Escherichia coli* or yeast cells or *Aspergillus oryzae* strains, which overexpress intracellular lipases from different microorganisms (Yan et al., 2012a, 2014b). Although in most studies with whole cells, high yields (>80%) have only been attained after long reaction times (>72 h) (Arumugam and Ponnusami, 2014; Huang et al., 2012; Yan et al., 2014b; Adachi et al., 2013); in the work of Jin et al. (2013), ester yields above 90% were attained in 10 h of methanolysis of vegetable oils catalyzed by *P. pastoris* whole cells with surface display of *R. miehei* lipase and lipase B from *C. antarctica*. Moreover, lipase B from *C. antarctica* and lipase from *T. lanuginosus* were codisplayed on the surface of *P. pastoris* surface, and the biocatalyst was able to produce 95% of esters in 13 h (Yan et al., 2012b). The use of liquid residues such as crude glycerin (by-product

from biodiesel industry) and molasses (by-product from sugar and alcohol industry) as substitutes for expensive components in the culture medium can further contribute to reducing the production costs of the biocatalysts and the environmental problems of disposal of large amounts of these liquid residues (Ramachandran et al., 2007).

Another approach recently studied to reduce the final costs of the biocatalyst is the use of agroindustrial solid residues, such as oil cakes, bran, husk, and bagasse, as culture media for microorganism growth in SSF. The use of agroindustrial residues from the productive chain of biofuels for the production of lipases is an interesting alternative to add value to these residues, especially in countries such as Brazil, due to the central role played by agricultural and extractive activities in their economy and their large capacity to produce renewable resources (Gutarra et al., 2009; Ramachandran et al., 2004). After the reaction, the biocatalyst could still be used as manure (if GRAS (generally recognized as safe) microorganisms are used) or burned to generate energy, which represents an alternative to the full use of the solid residue. To reduce the costs of extraction and immobilization, the SEP obtained after fermentation that have lipase activity in the fermented matrix can be directly used as biocatalysts in biodiesel production. Some works have recognized the potential of using SEPs obtained in different agroindustrial residues as biocatalysts for biodiesel production by the esterification of FFA (Fernandes et al., 2007; Soares et al., 2013; Aguiarias et al., 2014) as well as transesterification (Salum et al., 2010; Liu et al., 2013, 2014b). In the work of Aguiarias et al. (2014), the SEP obtained by SSF of *R. miehei* in babassu cake was used as a biocatalyst in the esterification reactions of the FFAs obtained after the enzymatic hydrolysis of macauba acid oil. A conversion of 91% at 8 h was attained, and the fuel properties were in accordance with important Brazilian Standards. This low-cost biocatalyst was used to reduce the FFA content in acid oils, allowing their use in the alkaline transesterification route (Castro et al., 2014a). Enzymatic hydrolysis followed by enzymatic esterification using SEP as a biocatalyst in both steps is also described in a patent (Castro et al., 2014b).

As described above, these promising approaches can be considered interesting opportunities to integrate, in the same industrial unit, processes for the production of biodiesel and low-cost biocatalysts produced by technologies that use residues and by-products from the productive chain of this biofuel.

Moreover, as can be seen in Fig. 20.2, the use of these agroindustrial residues represents an approach of processes integration in productive chain of biodiesel, with use of oil cakes to obtain other several products of industrial interest.

20.3.3 Commercial Initiatives and Economic Evaluation of Enzymatic Biodiesel Production in Brazil and Worldwide

Regarding the future prospects of enzymatic biodiesel production, the development of pilot-scale operations and the data from their operation are fundamental

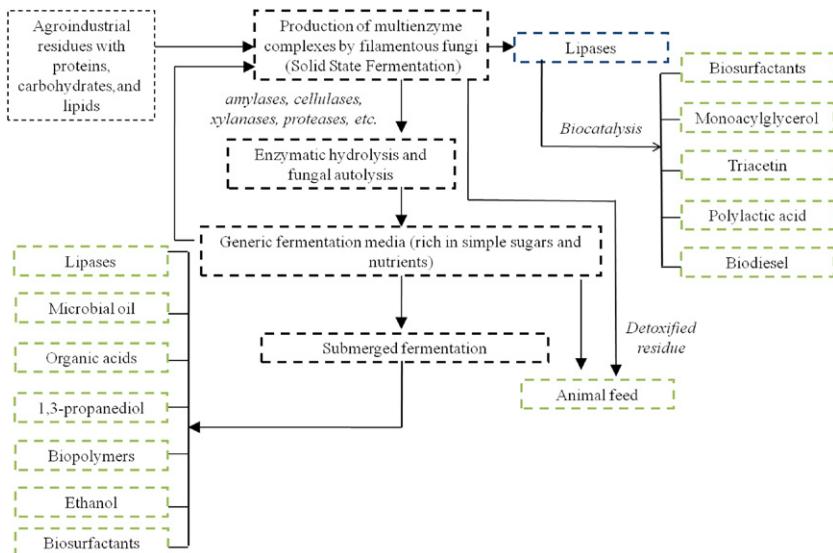


FIGURE 20.2 Examples of products of industrial interest that can be obtained using agroindustrial residues from the productive chain of biodiesel.

to support future techno-economic analyses of enzymatic technology. A pilot-scale facility (50 L) for enzymatic biodiesel production was developed by Park et al. (2008) using activated bleaching earth from the oil refining industry as raw material, *C. rugosa* lipase as the biocatalyst and diesel as cosolvent. A conversion of 97% was attained in a batch stirred tank reactor after 12 h. In their review, Christopher et al. (2014) cited the operation of two industrial-scale enzymatic biodiesel production plants. The Chinese company Hainabachuan Co. Ltd. established the production of enzymatic biodiesel on a 20,000 tons per year scale in 2006, which was doubled to 40,000 tons per year in 2008 (Christopher et al., 2014). A biodiesel production plant (10,000 ton capacity) using immobilized *Candida* sp. lipase as catalyst and waste cooking oil as feedstock was developed in 2007 by another Chinese company, Lvming Co. Ltd. Yields of FAMEs of 90% were achieved in a stirred tank reactor (Christopher et al., 2014). In 2010, a company in North Carolina (Piedmont Biofuels, 2015), in cooperation with Novozymes, developed a technology for continuous biodiesel production using immobilized or soluble enzyme (<http://www.biofuels.coop/enzymatic-biodiesel>). In 2014, the American Blue Sun Energy Company inaugurated a commercial-scale plant with a production capacity of 135,500 m³ per year of biodiesel using enzymatic biodiesel technology developed by Novozymes (Novozymes, 2014).

Moreover, there have been few reports of economic analyses of simulated enzymatic biodiesel plants, and these studies provide information on

the practical applications of enzymatic technology for biodiesel production and can contribute to making the enzymatic synthesis of biodiesel a sustainable prospect in the future. It should be noted that enzymatic and chemical biodiesel plants are considerably different from each other, and an economic analysis based solely on the catalyst prices is not sufficient to compare the processes. [Sotoft et al. \(2010\)](#) performed an economic analysis of enzymatic biodiesel plants using refined rapeseed oil (0.61 euro/kg) as raw material. The plants could be operated using *tert*-butanol as a solvent or without solvent. According to the authors, the cost of the raw materials and the sale price of the by-product (glycerol) were the factors that most impacted production costs in all scenarios evaluated. The results showed that for an enzyme price of 762.7 euro/kg, only the solvent-free process would be economically viable, with a payback period of 3 months and a biodiesel production cost of 0.73 euro/kg. The plant using solvent was economically unfeasible due to the costs of separation and solvent recovery units. [Al-Zuhair et al. \(2011\)](#) simulated an enzymatic biodiesel plant with a production capacity of 1 ton/h, using waste oil (0.02 dollars/kg) as raw material, Novozym 435 as catalyst and *tert*-butanol as solvent. The economic study indicated a payback period of 4 years, with a selling price of biodiesel of 0.86 dollars/kg. The economic feasibility of an enzymatic biodiesel produced from cooking oil (0.25 euro/kg), using supercritical carbon dioxide as solvent and lipase Lipozyme TL IM (800 euro/kg), resulted in a biodiesel cost of 1.64 euro/L, which is not economically viable ([Lisboa et al., 2014](#)). Estimating enzyme reuse for 100 days, the biodiesel cost can be reduced to 0.75 euro/L.

[Fjerbaek et al. \(2009\)](#) calculated productivities from different studies in the literature and compared the results with the productivities attained using alkaline catalyst (NaOH concentration of 1 wt% based on weight of oil), which presents a yield of 100 kg biodiesel per kg of catalyst. A productivity 74 times higher can be achieved using Novozym 435, considering lipase reuse (100 times) and the use of a by-product acid from the refining of oil vegetables as raw material. Considering the purchase prices of Novozym 435 (1000 dollars/kg) and sodium hydroxide (0.62 dollars/kg) and their respective yields, a final cost of 0.14 dollars/kg ester is obtained for the enzymatic process against 0.006 dollars/kg ester for NaOH. If the cost of lipase was reduced to 44 dollars/kg, or if the enzyme could be reused for at least 6 years, the use of the biocatalyst could become economically viable. According to [Nielsen et al. \(2008\)](#), the maximum cost of the biocatalyst should be the same as a chemical catalyst (25 dollars/ton biodiesel). Thus, an enzyme cost of 12–185 dollars/kg could be feasible, depending on the productivity. [Jin et al. \(2013\)](#) assessed the production costs of *P. pastoris* cells with heterologous expression of lipase B from *C. antarctica* and *R. miehei* lipase, obtaining a value of 27 dollars/kg. This result is interesting, considering the high price of commercial lipases Novozym 435 (2510 dollars/kg), Lipozyme RM IM (847 dollars/kg), and Lipozyme TL IM (141 dollars/kg).

20.3.4 Perspectives on Enzymatic Biodiesel Production

In conclusion, there are still technical and economic limitations for the large-scale production and commercialization of enzymatic biodiesel, and economic data show that enzymatic processes for biodiesel production currently have higher costs than the conventional route. However, a number of technologies have been developed in this area, and there is a great potential for growth in this market due to the advantages of the enzymatic route over the current chemical process. The production of cheaper and more resistant lipases, the development of bioreactors that allow the continuous use of the enzymes, and the exploitation of cheaper potential feedstocks are important keys to the integration of biotechnology with cost-effective industrial processes.

20.4 BIOGAS/BIOMETHANE PRODUCTION

The process of anaerobic fermentation (AF) has been considered as a viable technology for the treatment of organic waste materials and for bioenergy production. This process generates intertwined agricultural and environmental benefits, such as renewable energy production, environmentally friendly organic waste treatment, greenhouse gas emission reduction, pathogen reduction through sanitation, and improved fertilization efficiency (Holm-Nielsen et al., 2009; Mao et al., 2015). For these reasons, the AF of organic waste has received great attention worldwide in recent years.

AF is a degradation process of organic material into biogas by microorganisms, in the absence of oxygen. AF offers significant advantages, such as low power consumption, low nutrient requirements, low sludge production, and high efficiency in the reduction of organic load and generation of biogas (Khalid et al., 2011; Rajagopal et al., 2013). A variety of organic materials have been used as feedstocks for AF, for example, lignocellulosic biomass (Arreola-Vargas et al., 2015; Li et al., 2015; Sambusiti et al., 2013), municipal solid waste (Beevi et al., 2015; Luo et al., 2014), animal manure (Babaei et al., 2013; Dareioti and Kornaros, 2015), and food processing waste (Browne and Murphy, 2013; Zhang et al., 2014, 2015), among others. These feedstocks are usually available in small-scale biogas plants, which avoid additional transportation costs and thereby render biogas production economically feasible (Naik et al., 2014; Yang et al., 2014). In addition to biogas production, AF also makes it possible to obtain an effluent called digestate, which can be used as a biofertilizer. The quality of the digestate is essential for its acceptance as a replacement for mineral fertilizers in crop production. Parameters that allow high-quality digestate are appropriate pH, nutrient and chemical content, with no inorganic impurities and no pathological contamination (Hamawand, 2015).

Several factors can affect the productivity and stability of the anaerobic fermentative system for biogas production, such as temperature, pH,

carbon-to-nitrogen mass ratio (C:N ratio), redox potential, organic loading rate (OLR), and retention time. Temperature is one of the main factors affecting AF, as it directly influences the CH₄ yield. In general, CH₄ production increases with increasing temperature (Zhang et al., 2014). The growth rate of microorganisms is significantly affected by pH. For example, the growth rate of methanogenic archaea is greatly reduced at pH below 6.0 and above 8.0 (Mao et al., 2015). The C:N ratio affects the performance of the AF, as the anaerobic bacteria require a balanced nutritional medium for their growth and maintenance of a stable environment. According to the literature, a C:N range of 20–30 was considered to be the optimum condition for AF (Puyuelo et al., 2011; Zhang et al., 2014). The redox potential can be used as an indicator of the AF, as the growth of methanogenic archaea requires a low redox potential. This redox potential has been reported to range from –200 to –400 mV (Naik et al., 2014). The stability of the AF is dependent on the OLR and hydraulic retention time (HRT). When the OLR is high, the fermentative system may become unbalanced due to excessive production of volatile acids, leading to inhibition of the process. The same behavior is observed at short HRT. Thus, a low OLR and a long HRT provide the best strategy for achieving constant and maximal methane yields (Naik et al., 2014; Mao et al., 2015).

The conversion of organic material into biogas is conducted by a consortium of microorganisms through a series of metabolic phases, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The first phase involves the hydrolysis of complex organic materials into simple organic materials such as sugars, amino acids, and fatty acids. In the acidogenic phase, the soluble products from the previous step are converted into volatile organic acids, alcohols, CO₂, H₂, and new bacterial cells. The acetogenic bacteria are responsible for oxidation of the products generated in the acidogenic phase into suitable substrates (H₂ and acetic acid) for methanogenic archaea. In the last step, the methanogenic archaea convert the H₂ and acetic acid into CH₄ and CO₂ (Sá et al., 2014; Christy et al., 2014).

The composition of biogas varies with the type of feedstock used in the process and the operating conditions of the digester. In general, biogas consists of 50–75% methane (CH₄) and 25–50% carbon dioxide (CO₂) with small amounts of water vapor (H₂O), hydrogen sulfide (H₂S), and ammonia (NH₃), among others (Surendra et al., 2014). CH₄ is the component of biogas responsible for heating value. In general, 1 m³ of biogas containing 60% CH₄ has a heating value of 21.5 MJ, which corresponds to approximately 6 kWh of electricity (Hamawand, 2015; Surendra et al., 2014). In addition to electricity generation, biogas also allows heat generation in a combined heat and power unit (Yang et al., 2014).

20.4.1 Generation and Use of Biogas/Biomethane in Brazil

Biogas produced from the AF process has been presented as an efficient alternative in the production of bioenergy. Biogas production in the IEA Bioenergy Task 37 member countries is clearly dominated by Germany, with more than

10,000 biogas plants. None of the other member countries (Austria, Brazil, Denmark, Finland, France, Norway, Ireland, Korea, Sweden, Switzerland, Netherlands, and the United Kingdom) have more than 1000 biogas plants apiece. The annual biogas production is approximately 80 TWh in Germany, 20 TWh in the United Kingdom, 4 TWh in both the Netherlands and France, and between 0.5 and 2 TWh in remaining countries. In countries such as the United Kingdom, Brazil, and South Korea, biogas produced in landfills is the largest source, while landfill gas is only a minor contributor in countries such as Germany, Switzerland, and Denmark. The biogas produced is mainly used for the generation of heat and electricity in most countries, with the exception of Sweden, where approximately half of the produced biogas is used as vehicle fuel. Many countries, such as Denmark, Germany, and South Korea, among others, show initiatives and interest in increasing the share of biogas to be used as a vehicle fuel in the near future ([IEA, 2015](#)).

The Brazilian potential for biogas production is great because of the amount of wastewater and organic waste generated, as well as the disposal of waste in landfills. According to Applied Economic Research Institute (IPEA) data, in 2009, Brazil produced 291 million tons of wastes from the agroindustry. If all these residues can be used for energy production, it could represent an energy potential of up to 23 GW/year, which is equivalent to 191,398 GWh/year. The wastes with the highest potential for energy production, approximately 69% of the total estimated for the sector, are bagasse and filter cake from sugarcane, generated mostly in Brazil's Southeast region. In addition, this region has a high amount of waste generated by dairy cattle (106 million tons/year) and laying hens (4.3 million tons/year). The South Brazil region already generates a large amount of waste through the creation of broilers (7.5 million tons/year—not considering the poultry litter) and pig manure (9.8 million tons/year) ([IPEA, 2012](#)). The projection of the biogas/biomethane production potential is approximately 12 billion cubic meters per year in the sugar and alcohol sector and 8 billion cubic meters per year in the food agroindustry sector ([Bley Jr., 2015](#)). However, the energy use of biogas for electrical systems in Brazil remains insufficient. The majority of the biogas plants are located on agricultural properties to process residues and on landfills ([IEA, 2015](#)). According to the Bank of Generation Information of the Brazilian Electricity Regulatory Agency (ANEEL), there are 403 thermoelectric plants fueled by biomass in operation in the country. Of the total related plants, only 24 are driven by biogas, totaling approximately 66.1 MW of installed capacity, which represents little more than 0.5% of the electricity production by biomass ([ANEEL, 2015](#)).

The purification of biogas, through the removal of CO₂, H₂O, H₂S, NH₃ and other impurities, makes it possible to obtain biomethane, which can be used as a substitute for natural gas and as a transportation fuel ([Holm-Nielsen et al., 2009; Pöschl et al., 2010](#)). This approach allows an efficient integration of biogas into the energy sector, and it is also observed that the industries are strongly interested in this product, not only in Brazil but in Africa, Europe and throughout the

Americas (Bley Jr., 2015). Applications of gaseous fuels developed from shale gas in the United States have been tendered competitively around the world. In Brazil, impacts are already observed on the use of engines relying on 100% natural gas (perfectly replaceable by biomethane), including heavy loads, trucks and buses. Shale gas has accelerated the arrival of the “Age of gas” in the world energy matrix and in Brazil (Bley Jr., 2015).

Currently, the use of biogas as a vehicle fuel is rare. However, one project developed by ITAIPU Binacional, the Itaipu Technology Park Foundation, Scania, Haacke Farm, and the International Center on Renewable Energy-Biogas/CIBiogás-ER has demonstrated the viability to use biomethane as a vehicle fuel (IEA, 2015). A recent initiative is the creation of legislation (Resolution No. 8, Jan 30, 2015) that will allow the development of the biomethane market in Brazil. This legislation was developed by the government’s National Agency of Petroleum, Natural Gas and Biofuels (ANP) and applied to biomethane produced from biodegradable materials originating from agroforestry and organic waste and intended for nationwide use as a fuel for vehicles, in commercial shipping and for residential use. The standard includes obligations regarding quality control to be met by the various economic agents who trade biomethane throughout Brazil (IEA, 2015).

20.4.2 Biohydrogen Production via AF

Another strategy that has been extensively studied is H₂ production from the AF process. In particular, hydrogen has attracted great interest due to its high energy content (143 kJ·g⁻¹) and clean burning. H₂ production via AF can be performed by mixed microbial cultures derived from natural environments or pure cultures selected from H₂-producing bacteria. The use of mixed cultures for large-scale processes is considered favorable due to the control and operation of the process being facilitated by the use of nonsterile media, reducing the overall cost. In this approach, H₂-consuming microorganisms are inhibited and/or eliminated, allowing the selection of H₂-producing microorganisms. This effect is obtained by pretreatment of the inoculum. During pretreatment, the H₂-producing-bacteria, such as *Clostridium*, can form endospores as a result of bacterial stress when in hostile environmental conditions (high temperature, nutrient limitation, extreme acidity and alkalinity), while methanogenic archaea (H₂-consuming microorganisms) cannot resist these conditions (Sá et al., 2013). Different chemical (acid, alkali, or organic compounds) and physical (heat, aeration, ultraviolet, ultrasonic, and freezing/thawing) methods of inoculum pretreatment have been reported in the literature to favor H₂ production (Cui and Shen, 2012; Dong et al., 2010; Wang and Wan, 2008; Wang et al., 2011).

Studies have shown that microorganisms of the genus *Clostridium* are primarily responsible for H₂ production in inocula with different methods of pretreatment (Lee et al., 2009; Liu et al., 2009; Ren et al., 2008). These microorganisms produce hydrogenase enzymes that catalyze the reversible reaction

of hydrogen oxidation ($2\text{H}^+ + 2\text{e}^- \leftrightarrow \text{H}_2$) (Kirtay, 2011). Sá et al. (2013, 2011) have used the level of hydrogenase gene (*hyd*) expression as an indicator of H_2 production in different systems.

A wide variety of materials rich in carbohydrates, lipids, and/or proteins can be used as substrates in the production of H_2 by AF. Carbohydrates, such as sucrose and glucose, are easily assimilated by fermentative bacteria. However, sources of pure carbohydrates represent expensive substrates for H_2 production in large scale. In this context, the use of waste materials as potential substrates for H_2 production has attracted great interest (Lin et al., 2012). Different waste materials have been used for H_2 production, such as food waste (Yong et al., 2015; Gadhe et al., 2014), sugarcane vinasse from ethanol production (Fernandes et al., 2010; Santos et al., 2014), dairy industry wastewater (Karadag et al., 2014), lignocellulose hydrolysates (Chen et al., 2013; Nissila et al., 2014), glycerin from biodiesel processing (Fernandes et al., 2010), and palm oil mill effluent (Chong et al., 2009a,b), among others.

20.4.3 Sequential Production of H_2 and CH_4

The use of waste materials for H_2 production has been gaining importance to support environmental sustainability. However, most of the organic fraction remains soluble at the end of the fermentation process (Peixoto et al., 2012). A two-stage process for sequential production of H_2 and CH_4 has been considered as an alternative to improve the viability of soluble organic fraction treatment. This system includes the separation of acidogenic and methanogenic processes for the production of H_2 and CH_4 , respectively. In the first stage (acidogenic process), organic matter is degraded to organic acids and H_2 , and in the second stage (methanogenic process), organic acids are metabolized to CH_4 and CO_2 (Kiran et al., 2014).

The purpose of using a two-stage system for the production of H_2 and CH_4 is to optimize each process separately. In addition, previous studies showed that the two-stage process for the production of H_2 and CH_4 allows the production of energy with higher efficiency than a single-stage process for CH_4 production (Luo et al., 2011; Liu et al., 2006). A recent review showed that the sequential production of H_2 and CH_4 has higher energy potential than the production of CH_4 in a single-stage process. The authors have shown through calculations of the theoretical energy production that the two-stage process using sucrose, glucose or fructose as a substrate presented approximately 9% more energy than the CH_4 production process in a single stage. Values of approximately 14% and 11% were obtained for xylose and glycerol, respectively (Sá et al., 2014).

20.5 CONCLUDING REMARKS

A sustainable means of industrial production is an unavoidable choice. A biorefinery can integrate, in the same industrial unit, processes for the production of biofuels, chemicals, electricity, and heat and is thus considered the most

promising approach to the creation of new industries based on renewable raw materials. Although in the current biorefinery context, industrial investments are largely directed towards the production of low-added-value biofuels, such as biodiesel and ethanol, the production of high-added-value products, such as green chemicals and polymeric resins, from residues and by-products derived from biomass processing may improve the material value two- or fourfold. Consequently, six to eight times more jobs could be created as well, and the amount of residues could be highly reduced. Economic and regional aspects influence the establishment of a renewable industry for fuel and chemical production and also control the speed of its implementation. However, it is important to maintain investments on this strategic field to take advantage of the opportunities, which includes, with a high degree of protagonism, enzymatic and microbial processes to support a sustainable industry.

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BIOTECHNOLOGY OF MICROBIAL ENZYMES

PRODUCTION, BIOCATALYSIS AND INDUSTRIAL APPLICATIONS

Edited by

GOUTAM BRAHMACHARI

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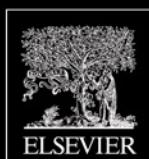
ARNOLD L. DEMAIN • JOSE L. ADRIO

Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications provides a complete survey of the latest innovations on microbial enzymes, highlighting biotechnological advances in their production and purification along with information on successful applications as biocatalysts in several chemical and industrial processes under mild and green conditions.

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