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Young Je Yoo
School of Chemical and Biological Engineering
Seoul National University
Seoul, Korea

Yan Feng
College of Life Science and Biotechnology
Shanghai JiaoTong University
Shanghai, China

Yong Hwan Kim
School of Energy and Chemical Engineering
Ulsan National Institute of Science and Technology
Ulsan, Korea

Camila Flor J. Yagonia
School of Chemical Engineering
University of San Carlos
Cebu City, Philippines

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Preface

Biotechnology has become one of the most important technologies in twenty-first century contributing every aspect of our life and industry. Biotechnology can contribute to the problems of our human culture and civilization from health, food, energy, environment, and to materials issues. Biotechnology is therefore playing a key role in pharmaceutical, medical, chemical, electronics, energy, and environment industries.

For the development of biotechnology, deep understanding and fusions in biology, chemistry, enzymology and engineering are required. One fundamental area of biotechnology is enzyme engineering which covers enzymology, enzyme technology, and engineering of enzymes. Enzymes have been used for food preparations such as cheese and alcohols from long time ago. In 1970s, immobilized enzymes have accelerated the development of enzyme engineering. In 1980s, the understanding of enzyme reaction in organic solvent has created a new area in enzyme engineering. Also with the energy and environment crisis, bio-based chemicals and bioenergy have opened a new area in enzyme engineering. With systems biology and metabolic engineering, enzymes for bio-based chemicals including polymers have become more and more important nowadays. Recently with the knowledge on molecular dynamics and quantum mechanics, computational tools have become stronger, which will contribute to the design of novel enzyme in the long run. Cheaper enzymes and more stable enzymes as well as more applications are required for their wide commercial use.

Even though enzymes are becoming more and more important, it is not easy to find a good textbook for the students to study the role of enzymes in biotechnology area, except for handbook style books or books dealing with current issues and specific topics, which gave us an idea to start to write the book, Fundamentals of Enzyme Engineering.

This book is written mainly for senior level or graduate students in biotechnology. However, this book can be also used as a guidebook for an overview of enzyme engineering working in relevant industries. This book consists of four

parts with 15 chapters and deals with fundamentals of enzyme chemistry, classical enzyme reaction engineering, recent molecular level understanding of enzyme, and various applications of enzymes.

Even though there are so many research results and industrial experiences reported so far, fundamentals and basic concepts with some cases are introduced and emphasized in the text instead of a knowledge-oriented description of every case of enzyme engineering. For the details or for specific cases, reading and discussion using related references are desirable. Some of which are introduced in the text as case studies and examples. For industry, searching for the patents and discussion based on the patents are also desirable for understanding of the technology and for further development. Since our knowledge and understanding of enzymes is still not enough and many challenges are waiting, further discussions on these issues are therefore presented at the end of each chapter.

For students who are familiar with basic biotechnology including biochemistry and biology, it is recommended to emphasize advanced level and recent advancement of enzyme engineering including molecular understanding and applications which are the integration of diverse principles. Reactor design and optimization which is generally required in industry are not described in detail in this text, which can be supported using the texts on reactor design.

We would like to express our gratitude to many colleagues, friends, students who gave tips, comments, and assistance during the development of this book as well as to the publisher who gave us a chance to publish this book.

We hope this book Fundamentals of Enzyme Engineering can be useful for the students in academia as well as the engineers in industry for the future development of enzyme engineering and biotechnology.

Seoul, Korea — Young Je Yoo
Shanghai, China — Yan Feng
Ulsan, Korea — Yong Hwan Kim
Cebu City, Philippines — Camila Flor J. Yagonia

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Introduction to Enzyme Engineering

Chapter 1
Introduction

1.1 Brief History of Enzyme Engineering

The microorganisms were widely used among ancient people. The manufacture of cheeses, breads, alcoholic beverages, and many other applications depends upon microorganisms which were found from ancient text of Babylon, Greece, Egypt, China, and India. Enzymes were the main components in microorganisms for the food manufacturing and other applications. However, ancient applications were relied upon observations and repeated experiences rather than scientific and technological. From the early of nineteenth centuries, enzymes have been investigated by scientists in a more systematic way. Tables 1.1 and 1.2 show the milestone and some Nobel Prize winners during this period. Many books relating to enzyme technology and engineering have been introduced (Wang *et al.* 1979; Dordick 1991; Fersht 1999; Bommarius and Riebel 2000) which can be good references in this area.

1.1.1 Brief History

The steroid biotransformations in the 1950s replaced the chemical reactions required to introduce a hydroxyl group at the 11α position and this marks the first bioconversion of industrial importance. Peterson *et al.* (1952) described this steroid modification using *Rhizopus nigricans*. Two other steroid modifications were also reported about the same time using *Curvularia lunata* and *Corynebacterium simplex* (Shull *et al.* 1953; Nobile *et al.* 1955).

There have been historically four steps of technology advances which brought a big impact for enzyme as alternative catalysts on bioconversions namely: enzyme isolation, enzyme immobilization, enzymes in non-conventional media, and recombinant DNA technology (Lilly 1994).

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Table 1.1 History and trends of enzyme engineering

Year	Significant event/discovery
1890	"Lock-and-Key" model was proposed (Fisher)
1893	Definition of the term "catalyst" (Ostwald) was introduced
1897	Explained that enzymes do not require a cell (Buchner)
1926	Enzyme was proved to be a protein (Sumner)
1952	Steroid biotransformation was performed
1958	"Induced-fit" model was proposed (Koshland)
1963	Amino acid sequence of ribonuclease was identified
1965	"Allosteric model" of enzyme was proposed (Monod)
1970	Immobilized enzymes were used for the production of HFCS
1980	Enzymatic synthesis of chiral compounds and polymers in organic solvent were reported
1990	Protein engineering including directed evolution was used
2000	Computational design of enzyme was introduced
2010	Enzymes for metabolic engineering become popular

Table 1.2 Nobel prize winners on enzymes/proteins

Year	Prize winner	Scientific discovery
1907	Eduard Buchner	Alcoholic respiration with cell-free extract
1909	Wilhelm Ostwald	Definition of the word "catalyst"
1946	James B. Sumner	Urease enzyme crystallized from jack beans
1958	Frederick Sanger	Structure of insulin
1972	Stanford Moore and William Stein	Connection between chemical structure and catalytic activity of ribonuclease
1993	Michael Smith	Site-directed mutagenesis for enzyme sequence change
	Kary B. Mullis	Invention of polymerase chain reaction (PCR)
2002	John B. Fenn, Koichi Tanaka and Kurt Wüthrich	Tools (Mass Spec) for identification and structure analysis of biomacromolecules
2008	Martin Chalfie, Roger Y. Tsien and Osamu Shimomura	Green fluorescent protein (GFP)
2013	Martin Karplus, Michael Levitt and Arieh Warshel	Multiscale models for complex chemical systems (Quantum mechanics/molecular mechanics)

The technology development for the separation of enzymes from microorganisms is one of the important technological advancements. The disruption of microorganisms by mechanical means (e.g., high-pressure homogenizer) allowed the isolation of intracellular enzyme. However, isolation of these intracellular enzymes was quite expensive; thus their applications had been limited. Engineering them for repeated use, higher activity and stability has allowed more feasible processes.

Table 1.3 Industrial bioconversions of substrates which are poorly soluble in water

Process	Enzyme	Company	Operating since
Fat interesterification	Lipase	Fuji Oil	1979
Ester hydrolysis	Lipase	Sumitomo	1988
Transesterification	Lipase	Unilever	1990
Aspartame synthesis	Thermolysin	DSM	1992
Acylation	Lipase	BASF	1996

Enzyme immobilization is a technique that converts a water-soluble enzyme into an insoluble form which can be easily recovered and reused. If properly designed, immobilization can enhance the applications of enzyme in many ways such as production of chemicals and pharmaceuticals, enzyme biosensor, etc. In the 1960s, several industrial technologies have been developed using immobilized enzymes, one example is the enzymatic isomerization of glucose to fructose for the production of high fructose corn syrup (HFCS). Clinton Corn Processing initiated the commercial production of HFCS in 1974 using glucose isomerase immobilized on an ion-exchange resin (Antrim *et al.* 1979). In 1976, the first continuous production process for HFCS was performed in Japan.

Generally, industrial enzymatic reactions before mid-1970s use substrates and products which are soluble in aqueous solutions. The production of cholestenone from cholesterol by *Nocardia* (NCIB 10554) in high proportions of water-immiscible solvent performed by Buckland *et al.* (1975) led to the third technological advance. Klibanov (1986) showed that enzymes can also function in organic solvents. Since then, various enzymatic processes using organic solvents have been industrially set up (Table 1.3).

The next technological advancement is recombinant DNA technology. This technology together with protein engineering allows new and better enzyme variants to be quickly produced. Since around 1990, directed evolution has been used as a powerful tool for enzyme engineering. This method contributed a lot in the development of enzymatic processes for industrial applications to harness the capability of naturally occurring enzyme since in most cases, natural enzymes are not optimized for industrial reaction conditions.

Current issues and recent advances in enzyme engineering is the computational design. The computational design of enzyme is another method that has been developed around year 2000. This is accomplished using computer models to suggest sequences and structures that can work for the desired properties of the enzyme. Understanding the mechanism of enzymes in detail and the structure of functional enzyme can make enzyme technology jump one more step. At present, the study of enzymes is still one of the important issues to the scientific community and to the industry sector in general. Artificial enzymes, catalytic antibody are examples of current issues in enzyme engineering. Recently, synthesis of ammonia through enzymes was reported (Brown *et al.* 2016), which can be a breakthrough of enzyme engineering in chemistry and chemical industry. Enzymes are

continuously utilized for many industrial applications including their recent usages in chemicals production as well as their traditional roles. Many challenging issues are still waiting.

1.1.2 Enzyme Technology and Engineering

Enzyme technology is fundamental in biotechnology that the European Federation of Biotechnology defined: "Biotechnology is the integration of natural sciences and engineering sciences in order to achieve the application of organisms, cells, parts thereof and molecular analogs for products and services. In enzyme technology, many products such as food, fine chemicals and pharmaceuticals have been and are being manufactured utilizing enzymes as biocatalysts. Aside from that, enzymes are applied for analytical and diagnostic purposes. They are also used in many fields including environmental remediation. In recent years, enzymes for chemical synthesis replacing petrochemicals and for carbon dioxide utilization are new topics in enzyme engineering society."

Modern enzyme technology was started when it was shown that sugars can be obtained from starch using an alcohol precipitate of malt extract. The compound in the precipitate which can yield dextrins from starch was later called diastase. By the mid-nineteenth century, more enzymes were discovered including pepsin, invertase, and peroxidase.

After enzyme technology became established, enzymes as catalyst for industrial use were widely investigated. Taka-Diastase was patented for industrial application: amylolytic enzyme produced by *Aspergillus oryzae*. Now enzymes are being utilized for various applications from pharmaceuticals to diagnostics.

Biotechnological processes use one or more enzymes as biocatalysts depending on the required process condition. Compared to fermentation processes, enzymatic catalysis with isolated or immobilized enzymes has the following advantages: (1) by-product formation is minimized by other enzymes in the cells, (2) no need for complex nutrients medium (e.g., carbon, nitrogen, and other nutrient sources essential for cell growth), and (3) smaller reactors can be used since higher productivity can be obtained than with living cells. However, fermentation using living cells are still suitable than isolated enzymes when several enzymes in series and cofactor regeneration are involved.

Enzymes are more selective than conventional chemical catalyst. High selectivity is one of the main advantages including reduced side reactions and thus easier separations. In addition, enzymatic catalysis can be carried out under mild reaction conditions and it has, in many cases, high turnover numbers compared to the conventional chemical catalysis. Chemical catalysis is typically operated at much higher temperature and pressure.

However, there are also several issues associated with enzymatic catalysis for chemical synthesis: one of these is the difficulty for enzyme isolation. Enzymes

Table 1.4 Advantages and disadvantages of enzyme technology compared to chemical processes

Advantages	Disadvantages
High degree of selectivity Reactions at mild conditions	Expensive
Environmentally friendly	Unstable
Catalyze broad spectrum of reactions	Low productivities
Less by-products	
Non-toxic and non-flammable	

are inhibited by relatively low concentrations of end products, high temperature, extreme pH conditions, some metal ions, and solvents. In addition, some enzymes are still very expensive for industrial use and may require expensive cofactors for catalysis. Enzymatic catalysis tends to be too specific for general applicability. Table 1.4 summarizes the pros and cons of enzyme technology. Enzyme engineering is also widely used terminology covering traditional enzyme-related technology and engineering of enzymes which is very critically important nowadays to enhance the applicability of enzymes.

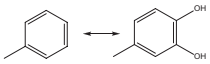
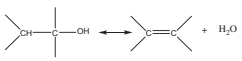
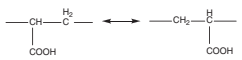
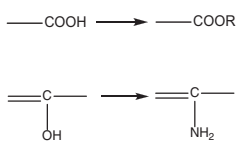
1.1.3 Classification of Enzymes

Approximately 5000 enzymes have been characterized so far, while more than 300 enzymes are commercially available and supplied from enzyme manufactures. Depending on the reactions they catalyze, enzymes are grouped according to the report of the Nomenclature Committee of the International Union of Biochemistry (1984). The six distinct classes are shown in Table 1.5.

Enzymes are named by adding the suffix—*ase* to the name of their substrate. However, there are also enzymes that have been given names that do not denote their substrates such as pepsin and trypsin. To avoid ambiguities, International Union of Biochemistry (IUB) assigned each enzyme a name and a four-level number. The Enzyme Commission (EC) numbers divide enzymes into six main groups depending on the reactions they catalyze as shown in Table 1.5. For this EC number system, the first, second, third, and fourth number refers to the class of enzyme, subclass by the type of substrate or the bond cleaved, subclass by the electron acceptor of the type of group removed and serial number of enzyme found, respectively. For example, as shown in Fig. 1.1, glucose isomerase is EC 5.3.1.5, also called xylose isomerase.

Some chemical reactions that are also catalyzed by enzymes are shown in Table 1.6. Alcohol dehydrogenase is also called aldehyde reductase. This enzyme acts on primary or secondary alcohols or hemi-acetals. Lipases can catalyze transesterification and other reactions in organic solvent system including esterification, amino lysis, acyl exchange, thiotransesterification, and oximolysis.

Table 1.5 Classification of enzymes

No.	Class	Representative subclasses	Type of reaction	Example
1	Oxido-reductases	Oxidases, oxygenases, peroxidases, dehydro-genases	Transfer of elec-trons (oxidation and reduction)	$-CH_2OH \leftrightarrow -CH{=}O$ $-SH \leftrightarrow -S-S-$ $-CH_2NO_2 \leftrightarrow -CH = O$ 
2	Transferases	Glycosyltransferases, methyltransferases, transaldolases, tran-sketolases, acultrans-ferases, alkyltrans-ferases, transaminases, sulfotransferases, phosphotransferases, nucleotidyltransferases	Group-transfer reaction	$-CH_3$ $-CH_2OH$ $-CHO$
3	Hydrolases	Esterases, lipases, glycosidases, proteases, sulfatases, phos-phalases, aminoac-ylases, endo- and exo-nucleases, halohy-drolases	Hydrolysis reac-tions	$-COOR \leftrightarrow$ $-COOH + ROH$ $-COSR \leftrightarrow$ $-COOH + RSH$ $-CONH_2 \leftrightarrow$ $-COOH + NH_3$
4	Lyases	Decarbosylases, aldolases, ketolases, hydratases, dehy-dratases, polysaccharide lyases, ammonia lyases	Addition of groups to double bonds	
5	Isomerases	Racemases, epimerases, isomerases	Transfer of groups within molecules to isomeric forms	Glucose ↔ fructose 
6	Ligases	Synthetases, carboxy-lases	Formation of C–C, C–S, C–O and C–N bonds	

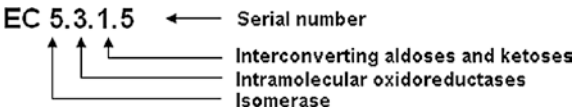


Fig. 1.1 EC number system for glucose isomerase

Table 1.6 Examples of enzyme-catalyzed reactions

Reaction	EC number	Enzyme
Meerwein-Ponndorf-Verley reduction	1.1.1.1	Alcohol dehydrogenase
Baeyer-Villiger oxidation	1.14.13.22	BV monooxidase
Ether cleavage	1.14.16.5	Glyceryl etherase
Disproportionation	1.15.1.1	Superoxide dismutase
Etherification	2.1.1.6	COMT[a]
Transamination	2.6.1.x	Transaminase
Oximolysis	3.1.1.3	Lipase
Aldol reaction	4.1.2.x	Aldolase
Racemization	5.1.2.2	Mandelate racemase
Claisen rearrangement	5.4.99.5	Chorismate mutase

[a]*COMT* Catechol-*o*-methyltransferase

The quantification of enzymes is often difficult to determine in absolute terms such as grams, since the activity changes due to conformations and the environments such as temperature and pH. More relevant parameter is to express the *enzyme activity* in terms of the activity unit (U), which is defined as the amount which will catalyze the transformation of 1 μmole of the substrate per minute under standard conditions or optimum pH and temperature and in the presence of specific chemicals if required. Another parameter of interest is the specific activity (e.g., U kg^{-1}) having some utility as an index of the enzyme purity.

1.2 Industrial Application of Enzymes

Enzymes have numerous applications in food, medical, chemical, and pharmaceutical industries. The industries have grown rapidly over the past decades and are expected to continue their growth. Table 1.7 shows the applications of biocatalysts and its production scale. For this enzymatic processes, various enzymes have been applied.

Table 1.7 Industrial application of enzymes and its production scale

Production scale	Product	Enzyme	Company
>1,000,000	High-fructose corn syrup (HFSC)	Glucose isomerase	Various
>100,000	Lactose-free milk	Lactase	Various
>10,000	Acrylamide	Nitrilase	Nitto Co.
	Cocoa butter	Lipase (CRL)	Fuji oil
>1000	Aspartame ®	Thermolysin	Tosoh/DSM
	Nicotinamide	Nitrilase	Lonza
>100	Ampicillin	Penicillin amidase	DSM-Gist Brocades
	(S)-methoxyisopropylamine	Lipase	BASF

Biocatalysts in industries are generally used to produce their natural products and derivatives. Carbohydrates and fatty acid derivatives are mostly used in food industry, while other types of compounds are mostly applied in pharmaceutical and agro industries. Pharmaceutical sector especially dominates applications of biocatalysts (Straanthof *et al.* 2002).

As discussed above, enzymes are being applied in various fields from food, pharmaceuticals, chemicals, and other industrial applications. At present, more than 5000 enzymes are known. Approximately more than 200 microbial-origin enzymes are used commercially. Commercial enzyme production has grown during the past decades in response to increasing demands and application for enzymes. Table 1.8 shows the leading enzyme manufacturers. The enzyme manufacture is relatively concentrated on a few countries such as Denmark, Switzerland, Germany, Netherlands, USA, Japan, Russia, and Korea.

For more applications

Enzymes have been used from old days which resulted in more understanding in enzymes, increasing demand and applications. However, many researchers in academia and industry are still looking for more applications and better technologies. The relationship between structure and function has been extensively investigated, but still remains as one of the hottest current issues in enzyme engineering. Recently, CRISPER (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas system is widely investigated as a novel and powerful tool for studying gene regulation, gene expression, genome-wide screening after the introduction of DNA lyases long time ago and requires molecular understanding of the system to improve the specificity and other properties for further applications. With these backgrounds, activity and stability versus structure issues can find a solution for novel functions and applications. Since metabolic engineering can also contribute to the bio-based chemicals synthesis, enzyme engineering, and technology will play an important role as a key technology in metabolic engineering and systems biology. Also novel applications of current enzymes are also being considered as important issue. For example, in cosmetics industry, more natural and nontoxic ingredients are especially required, which can be obtained from natural products or enzymatic synthesis instead of chemical synthesis where by-products are in many cases formed.

Case Study: Enzymes in detergent industry

Enzymes have been used in detergent formulations since 1960s to overcome the eutrophication of water caused by phosphorus detergents. Proteases, amylases, lipases, and cellulases have been used to degrade protein, carbohydrate, and lipid stains on clothes. Using enzymes in detergent industry, energy can be saved by washing at lower temperatures with comparable efficiency with that at high temperature and using traditional surfactants. In summary, enzymes in detergent industry have many advantages as follows:

– Lower cost since used at low detergent concentrations,
– Acceptable to environment: biodegradability and no harmful impact on sewage treatment processes;

Table 1.8 Leading enzyme manufacturers

Company	Location	Established year	Major products
Novozymes	Bagsvaerd, Denmark	1921	Household care, food and beverage, bioenergy, feed and biopharmaceuticals
Dupont (Genencor)	Delaware, USA	1982	Biofuels, food ingredients, animal nutrition, textiles and detergent
DSM	Delft, the Netherlands	1952	Animal nutrition, food ingredients, personal care, pharmaceutical
Roche	Grenzacherstrabe, Switzerland	1896	Diagnostics, pharmaceuticals
Amano	Nagoya, Japan	1899	Pharmaceuticals, dietary supplement, biotransformation, diagnostics, food processing
BASF	Luwigshafen, Germany	1865	Feed additives, pharmaceuticals, detergents
KAO	Tokyo, Japan	1882	Beauty care, health care, home care
AB Enzymes	Feldbergstrasse, Germany	1907	Feed additives, food, textile, detergent, pulp and paper, biofuels
Verenium	San Diego, USA	2007	Animal health and nutrition, grain processing, oilfield services
Iogen	Ontario, Canada	1970s	Biofuels, pulp and paper, textile, grain processing and brewing, animal feed
Dyadic	Florida, USA	1979	Food, brewing and animal feed enzymes, biofuels, pulp and paper, textile enzymes
Meiji	Tokyo, Japan	1916	Food
Enmex	Tlalnepantla, Mexico	1961	Alpha-amylase, alkaline protease
Nagase	Osaka, Japan	1832	Pharmaceuticals, food, agriculture, household, textiles
Amicogen	Jinju, Korea	2003	Functional food ingredients
InnoTech MSU	Moscow, Russia	2009	Peroxidases, formate dehydrogenase, D-amino acid oxidase
SibEnzyme	Novosibirsk, Russia	1991	Restriction enzymes, ligases, polymerases

– Higher efficiency in stain removal,
– Less use of pollutants such as phosphate, bleach, and caustic.

The enzymes related to detergent have been traditionally isolated from nature, but nowadays are being engineered to provide better properties to meet the formulation conditions and conditions of washing processes where high temperature and high pH conditions are sometimes required.

Further Discussion

1. What are the motivation and limit for the enzyme to be used in industry? How we can overcome the disadvantages of enzyme reaction compared to chemical catalysis and fermentation process?
2. Search the backgrounds and history of novel enzyme discovery such as enzyme for PCR and novel applications of the enzyme such as glucose isomerase.
3. What enzymes are being produced in leading enzyme producers? What are the main application areas for major enzymes produced by leading enzyme producers?
4. What properties are required for the enzyme to be used as detergent? Washings are being performed at low temperature or mild temperature depending upon the country and the pH for washing is not neutral.

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