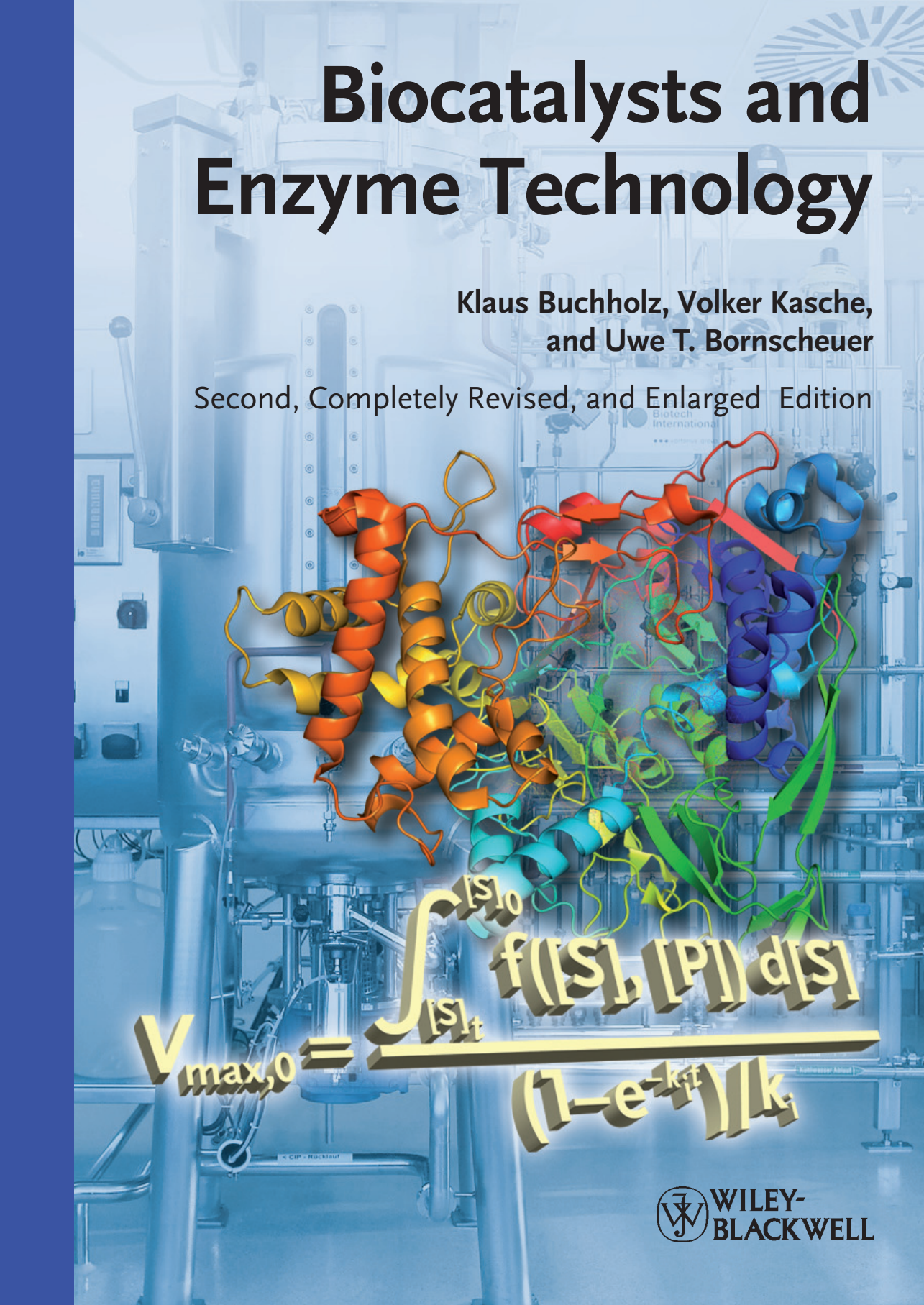
Biocatalysts and Enzyme Technology

Klaus Buchholz, Volker Kasche,
and Uwe T. Bornscheuer

Second, Completely Revised, and Enlarged Edition



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For
Diana, Helene, Melanie, and Peter
Karin, Maria, Anna, Andreas, Magdalena, Johann, and Richard
Tanja and Annika

Contents

Preface to the Second Edition

We have been very pleased by the success of the first English edition of our book. We are especially grateful that it serves as primary source for teaching courses in *Biocatalysis and Enzyme Technology* at many universities around the world. We would also like to thank the readers who pointed out corrections and to those who made useful suggestions for this second edition.

More than 7 years have passed since the first English edition was published and we have observed substantial and exciting developments in all areas of biocatalysis. Hence, we did not simply update the first edition with new references and add singular sentences, but substantially expanded and reorganized the book. For instance, the importance of enzyme discovery and protein engineering is now treated in a separate new section (Chapter 3). Although biocatalysis primarily refers to the use of isolated (and immobilized) enzymes, we decided to cover also the use of designed whole cells for biotransformations in the new Chapter 5 to allow the reader to get a glimpse on the emerging field of metabolic engineering, where the understanding and biochemical characterization of enzymes is of course an important aspect. Furthermore, we have included several new case studies in Chapter 12, to exemplify how biocatalysis can be performed on the large scale and which criteria are important to establish a novel process. These include starch processing and glucose isomerization, biofuels from biomass, and the production of 7-ACA by direct hydrolysis of cephalosporin C as examples for current as well as potential industrial processes. In addition, enzymatic routes for the synthesis of advanced pharmaceutical intermediates for the drug Lipitor are covered.

Furthermore, we have added a few new sections on topics of current interest: process sustainability and ecological considerations, process integration, biofilm catalysis, microbial fuel cells, and regulations that influence the production and use of enzymes.

We have also expanded and updated the exercises and decided that also the solutions be directly given in the book – in Appendix B – so that the students can first try to answer the questions by themselves and then look up the solutions.

Finally, we would like to thank Ulrich Behrendt, Sonja Berensmeier, Matthias Höhne, Zoya Ignatova, Hans-Joachim Jördening, Burghard König, Sven Pedersen,

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June 2012

Klaus Buchholz/Braunschweig
Volker Kasche/Bremen
Uwe T. Bornscheuer/Greifswald

Preface

To the First German Edition

Biotechnology is the technical application of biological systems or parts thereof to provide products and services to meet human needs. It can, besides other techniques, contribute towards doing this in a sustainable manner. Since, in the majority of cases, renewable raw materials and biological systems are used in biotechnological processes, these processes can – and should – be performed practically without waste, as all of the byproducts can be recycled.

The development of natural and engineering science fundamentals for the design of such processes remains a challenge to biotechnology – a field that originated from the overlapping areas of biology, chemistry, and process engineering.

The requisite education for a career in biotechnology consists, in addition to a basic knowledge of each of these fields, of further biotechnological aspects which must provide an overview over the entire field and a deeper insight into different areas of biotechnology. The biotechnological production of various materials is performed either in fermenters using living cells (technical microbiology), or with enzymes – either in an isolated form or contained in cells – as biocatalysts. Indeed, the latter aspect has developed during recent years to form that area of biotechnology known as enzyme technology, or applied biocatalysis.

The aim of the present textbook is to provide a deeper insight into the fundamentals of enzyme technology and applied biocatalysis. It especially stresses the following inter-relationships: A thorough understanding of enzymes as biocatalysts and the integration of knowledge of the natural sciences of biology (especially biochemistry), cell and molecular biology; physico-chemical aspects of catalysis and molecular interactions in solutions; heterogeneous systems and interphase boundaries; and the physics of mass transfer processes. The same applies to the inter-relations between enzyme technology and chemical and process engineering, which are based on the above natural sciences.

In less than a century since the start of industrial enzyme production, enzyme technology and its products have steadily gained increasing importance. In the industrial production of materials to meet the demands of everyday life, enzymes play an important role – and one which is often barely recognized. Their application ranges from the production of processed foods such as bread, cheese, juice and beer,

to pharmaceuticals and fine chemicals, to the processing of leather and textiles, as process aids in detergents, and also in environmental engineering.

Meeting the demand for these new products – which increasingly include newly developed and/or sterically pure pharmaceuticals and fine chemicals – has become an important incentive for the further development of biocatalysts and enzyme technology. Of similar importance is the development of new sustainable production processes for existing products, and this is detailed in Chapter 1, which forms an Introduction.

Enzymes as catalysts are of key importance in biotechnology, similar to the role of nucleic acids as carriers of genetic information. Their application as isolated catalysts justifies detailed examination of the fundamentals of enzymes as biocatalysts, and this topic is covered in Chapter 2. Enzymes can also be analyzed on a molecular level, and their kinetics described mathematically. This is essential for an analytical description and the rational design of enzyme processes. Enzymes can also catalyze a reaction in both directions – a property which may be applied in enzyme technology to achieve a reaction end-point both rapidly and with a high product yield. The thermodynamics of the catalyzed reaction must also be considered, as well as the properties of the enzyme. The amount of enzyme required for a given conversion of substrate per unit time must be calculated in order to estimate enzyme costs, and in turn the economic feasibility of a process. Thus, the quantitative treatment of biocatalysis is also highlighted in Chapter 2.

When the enzyme costs are too high, they can be reduced by improving the production of enzymes, and this subject is reviewed in Chapter 3 (*Chapter 4 in the present book*).

In Chapter 4 (*here Chapter* 5), applied biocatalysis with free enzymes is described, together with examples of relevant enzyme processes. When single enzyme use is economically unfavorable, the enzymes can be either reused or used for continuous processes in membrane reactors (Chapter 4; *here Chapter 5*) or by immobilization (Chapters 5 and 6; *here Chapters 6 and* 7). The immobilization of isolated enzymes is described in detail in Chapter 5, while the immobilization of microorganisms and cells, with special reference to environmental technology, is detailed in Chapter 6.

In order to describe analytically the processes associated with immobilized biocatalysts that are required for rational process design, the coupling of reaction and diffusion in these systems must be considered. To characterize immobilized biocatalysts, methods which were developed previously for analogous biological and process engineering (heterogeneous catalysis) systems can be used (Chapter 7; *here Chapter 8*).

Details of reactors and process engineering techniques in enzyme technology are provided in Chapter 8 (*here Chapter 9*), while the analytical applications of free and immobilized enzymes is treated in Chapter 10 (*not covered in the present book*).

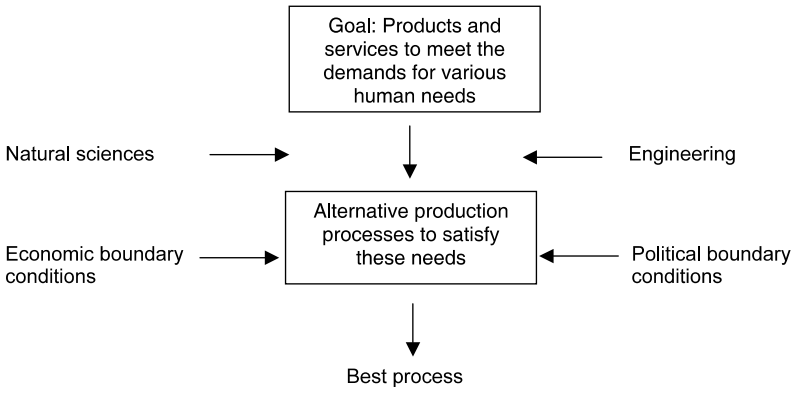
Within each chapter an introductory survey is provided, together with exercises and references to more general literature and original papers citing or relating the content of that chapter.

This textbook is designed to address both advanced and graduate students in biology, chemistry and biochemical, chemical and process engineering, as well as

scientists in industry, research institutes and universities. It should provide a solid foundation that covers all relevant aspects of research and development in applied biocatalysis/enzyme technology. It should be remembered that these topics are not of equal importance in all cases, and therefore selective use of the book – depending on the individual reader's requirements – might be the best approach to its use.

In addition to a balanced methodological basis, we have also tried to present extensive data and examples of new processes, in order to stress the relevance of these in industrial practice.

From our point of view it is also important to stress the interactions, which exist beyond the scientific and engineering context within our society and environment. The importance and necessity of these interactions for a sustainable development has been realized during the past two decades, and this has resulted in new economic and political boundary conditions for scientific and engineering development. Problems such as allergic responses to enzymes in detergents and, more recently, to enzymes produced in recombinant organisms, have direct influences on enzyme technology/applied biocatalysis. Therefore, an integrated process design must also consider its environmental impact, from the supply and efficient use of the raw materials to the minimization and recycling of the byproducts and waste. Political boundary conditions derived from the concept of sustainability, when expressed in laws and other regulations, necessitates due consideration in research and development. The design of sustainable processes is therefore an important challenge for applied biocatalysis/enzyme technology. Ethical aspects must also be considered when gene technology is applied, and this is an increasing consideration in the production of technical and pharmaceutical enzymes. The many interactions between research and development and economic and political boundary conditions must be considered for all applications of natural and engineering sciences. Most importantly, this must be appreciated during the early phases of any development, with subsequent evaluation and selection of the best alternative production processes to meet a variety of human needs, as is illustrated in the following scheme:



This book has been developed from our lecture notes and materials, and we also thank all those who provided valuable help and recommendations for the book's production. In particular, we thank Dipl.-Ing. Klaus Gollembiewsky, Dr. Lieker, Dr. Noll-Borchers, and Dipl. Chem. André Rieks.

Klaus Buchholz
Volker Kasche

To the First English Edition

The basic philosophy of the previous German edition is retained, but the contents have been revised and updated to account for the considerable development in enzyme technology/applied biocatalysis since the German edition was prepared some 10 years ago. Hence, a new chapter (Chapter 3) has been added to account for the increasing importance of enzymes as biocatalysts in organic chemistry. Recent progress in protein design (by rational means and directed evolution) has been considerably expanded in Chapter 2. The final chapter has been amended with more detailed case studies to illustrate the problems that must be solved in the design of enzyme processes. An appendix on information retrieval using library and internet resources has also been added, and we thank Thomas Hapke (Subject Librarian for Chemical Engineering at the Library of the Technical University Hamburg-Harburg) for help in the preparation of this material. The chapter on enzymes for analytical purposes has been removed in this English edition as it now is beyond the scope of this textbook.

We thank Prof. Dr. L. Jaenicke and Prof. Dr. J.K.P. Weder for their very constructive suggestions for corrections and improvements of the German edition.

The authors of this edition thank Prof. Dr. Andreas Bommarius, Dr. Aurelio Hidalgo, Dr. Janne Kerovuo, Dr. Tanja Kummer, Dr. Dieter Krämer, Dr. Brian Morgan, Sven Pedersen, Poul Poulsen, Prof. Dr. Peter Reilly, Dr. Klaus Sauber, Dr. Wilhelm Tischer, and Dr. David Weiner for valuable discussions, revisions and suggestions while preparing this book.

January 2005

Klaus Buchholz
Volker Kasche
Uwe T. Bornscheuer

1
Introduction to Enzyme Technology

1.1
Introduction

Biotechnology offers an increasing potential for the production of goods to meet various human needs. In enzyme technology – a subfield of biotechnology – new processes have been and are being developed to manufacture both bulk and high added-value products utilizing enzymes as biocatalysts, in order to meet needs such as food (e.g., bread, cheese, beer, vinegar), fine chemicals (e.g., amino acids, vitamins), and pharmaceuticals. Enzymes are also used to provide services, as in washing and environmental processes, or for analytical and diagnostic purposes. The driving force in the development of enzyme technology, both in academia and in industry, has been and will continue to be

- the development of new and better products, processes, and services to meet these needs, and/or
- the improvement of processes to produce existing products from new raw materials such as biomass.

The goal of these approaches is to design innovative products and processes that not only are competitive but also meet criteria of sustainability. The concept of sustainability was introduced by the World Commission on Environment and Development (WCED, 1987) with the aim to promote a necessary ". . . development that meets the needs of the present without compromising the ability of future generations to meet their own needs." This definition is now part of the *Cartagena Protocol on Biosafety to the Convention on Biological Diversity*, an international treaty governing the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another. It was adopted on January 29, 2000 as a supplementary agreement to the Convention on Biological Diversity and entered into force on September 11, 2003 (http://bch.cbd.int/protocol/text/). It has now been ratified by 160 states. To determine the sustainability of a process, criteria that evaluate its economic, environmental, and social impact must be used (Gram *et al.*, 2001; Raven, 2002; Clark and Dickson, 2003). A positive effect in all these three fields is required for a sustainable process. Criteria for the quantitative evaluation

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of the economic and environmental impact are in contrast with the criteria for the social impact, easy to formulate. In order to be economically and environmentally more sustainable than an existing process, a new process must be designed not only to reduce the consumption of resources (e.g., raw materials, energy, air, water), waste production, and environmental impact, but also to increase the recycling of waste per kilogram of product (Heinzle, Biwer, and Cooney, 2006).

1.1.1
What are Biocatalysts?

Biocatalysts either are proteins (*enzymes*) or, in a few cases, may be nucleic acids (*ribozymes*; some RNA molecules can catalyze the hydrolysis of RNA). These ribozymes were detected in the 1980s and will not be dealt with here (Cech, 1993). Today, we know that enzymes are necessary in all living systems, to catalyze all chemical reactions required for their survival and reproduction – rapidly, selectively, and efficiently. Isolated enzymes can also catalyze these reactions. In the case of enzymes, however, the question whether they can also act as catalysts outside living systems had been a point of controversy among biochemists in the beginning of the twentieth century. It was shown at an early stage, however, that enzymes could indeed be used as catalysts outside living cells, and several processes in which they were applied as biocatalysts have been patented (see Section 1.3).

These excellent properties of enzymes are utilized in enzyme technology. For example, they can be used as biocatalysts, either as isolated enzymes or as enzyme systems in living cells, to catalyze chemical reactions on an industrial scale in a sustainable manner. Their application covers the production of desired products for all human material needs (e.g., food, animal feed, pharmaceuticals, bulk and fine chemicals, detergents, fibers for clothing, hygiene, and environmental technology), as well as for a wide range of analytical purposes, especially in diagnostics. In fact, during the past 50 years the rapid increase in our knowledge of enzymes – as well as their biosynthesis and molecular biology – now allows their rational use as biocatalysts in many processes, and in addition their modification and optimization for new synthetic schemes and the solution of analytical problems.

This introductory chapter outlines the technical and economic potential of enzyme technology as part of biotechnology. Briefly, it describes the historical background of enzymes, as well as their advantages and disadvantages, and compares these to alternative production processes. In addition, the current and potential importance and the problems to consider in the rational design of enzyme processes are also outlined.

1.1.2
Bio- and Chemocatalysts – Similarities and Differences

Berzelius, in 1835, conceived the pioneering concept of catalysis, including both chemo- and biocatalysis, by inorganic acids, metals such as platinum, and enzymes

(Berzelius, 1835). It was based on experimental studies on both bio- and chemocatalytic reactions. The biocatalytic system he studied was starch hydrolysis by diastase (a mixture of amylases). In both systems, the catalyst accelerates the reaction, but is not consumed. Thus, bio- and chemocatalysis have phenomenological similarities. The main differences are the sources and characteristics of these catalysts. Chemocatalysts are designed and synthesized by chemists, and are in general low molecular weight substances, metal catalysts, complexes of metals with low molecular weight organic ligands, such as Ziegler-Natta and metallocene catalysts, and organocatalysts (Fonseca and List, 2004). In contrast, biocatalysts are selected by evolution and synthesized in living systems. Furthermore, enzymes (including ribonucleic acid-based biocatalysts) are macromolecules, their highly sophisticated structure being essential for their function, and notably for their regio-, chemo-, and enantioselectivity.

Due to development of gene and recombinant technologies in the past 40 years, enzymes that previously only could be obtained in limited amounts from microorganisms and tissues can now be synthesized in nearly unlimited quantities in suitable microorganisms. Further, based on the development in biochemistry, bioinformatics, and micro- and molecular biology, new tools have been developed to improve the properties of enzymes for their use in biocatalytic processes. They are rational protein design and *in vitro* evolution in combination with high-throughput screening tools. Very recently, also the *de novo* computational design of enzymes was described, but so far these show little activity in the same range as catalytic antibodies (Jiang *et al.*, 2008; Röthlisberger *et al.*, 2008).

Until the first oil crisis of 1973, the development and application of bio- and chemocatalysis occurred in – at that time – nonoverlapping fields. Biocatalysis was mainly studied by biochemists, biochemical engineers, microbiologists, physiologists, and some physical organic chemists (Jencks, 1969). It was mainly applied in the food, fine chemical, and pharmaceutical industries and medicine (see Section 1.3). Chemocatalysis was mainly studied by chemical engineers and chemists. It was applied in the production of bulk chemicals such as acids and bases, and products derived from coal and oil (fuel, plastics, etc.). This resulted for a long time in a small exchange of fundamental results between those who studied and applied bio- and chemocatalysis. The analytical description of heterogeneous catalysis, where the catalyst is located only in a part of the system, was first developed and verified experimentally for living systems in the 1920s by biochemists. Contrary to homogeneous catalysis, this description involves the coupling of the reaction with mass transfer. This applies also for heterogeneous chemocatalysis. The same description as for living systems was derived independently by chemical engineers in the end of the 1930s (see Chapter 10).

The detailed mechanism of the catalyzed reactions has now been determined for many bio- and chemocatalysts. This knowledge that is continuously increasing yields information that can be used to design improved bio- and chemocatalysts. This, however, requires a closer cooperation of those working with these catalysts. Fortunately, due to the increasing use of enzymes by organic chemists in the past decades, this cooperation has increased markedly.

1.2
Goals and Potential of Biotechnological Production Processes

Biomass – that is, renewable raw materials – has been and will continue to be a sustainable resource that is required to meet a variety of human material needs. In developed countries such as Germany, biomass covers ≈30% of the raw material need – equivalent to ~7000 kg per person per year. The consumption of biomass for different human demands is shown schematically in Figure 1.1. This distribution of the consumption is representative for a developed country in the regions that have a high energy consumption during the winter. However, the consumption of energy (expressed as tons of oil equivalent per capita in 2007) showed a wide range, from 8 in the United States to 4 in Germany and the United Kingdom, 1.5 in China, and 0.5 in India (IEA, 2010).

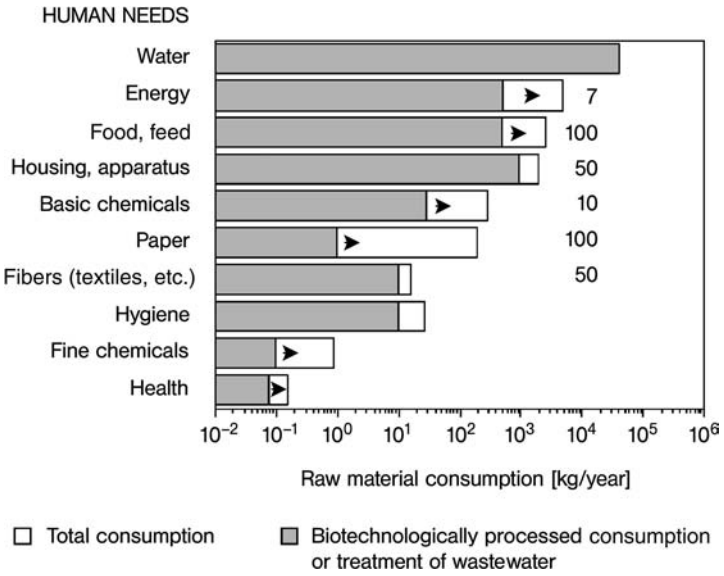

Figure 1.1 Consumption of raw materials for various human needs per person and year in Germany 1992. The water consumption is only for household use. These numbers are still valid. The energy consumption per capita has hardly changed since then. However, now (2010) 11% is derived from renewable resources (biomass, solar, water, wind) (AGEB, 2010). The arrowheads indicate the current increase in biotechnological processing of the products for different demands. For food and animal feed, only renewable raw materials (biomass) can be used; the figures to the right give the percentage for biomass of the raw materials currently used for the production. They can, especially for energy, only increase when they do not interfere with the biomass demand for food and feed. Due to the low material demands for hygiene, fine chemicals, and health products, 0–100% of the raw materials can be biomass, depending on the product. After the use of the products, the unavoidable waste must be recycled in a sustainable manner. Besides wastewater, this results in about 1000 kg of solid waste per year (soil, building materials, plastics, sludge, etc.). Energy is measured in coal equivalents.

This is mainly due to differences in energy use for housing, transport, and the production of other material needs. In less-developed countries, although the fraction of biomass as raw material to meet human demands is higher than that in the developed countries, the total consumption is smaller.

Biomass – in contrast to nonrenewable raw materials such as metals, coal, and oil – is renewable in a sustainable manner when the following criteria are fulfilled:

- the C, N, O, and salt cycles in the biosphere are conserved, and
- the conditions for a sustainable biomass production through photosynthesis and biological turnover of biomass in soil and aqueous systems are conserved (Beringer, Lucht, and Schaphoff, 2011).

Currently, these criteria are not fulfilled on a global level, one example being the imbalance between the CO_2 production to meet energy requirements and its consumption by photosynthesis in the presently decreasing areas of rain forests. This leads to global warming and other consequences that further violate these criteria. International treaties – for example, the Kyoto Convention and the Convention on Biological Diversity – have been introduced in an attempt to counteract these developments and to reach a goal that fulfills the above criteria (see Section 1.1).

Only when the above sustainability criteria are fulfilled, biomass can be used as raw material to meet the human demands illustrated in Figure 1.1. The needs for human food and animal feed must be met completely by biomass, though when these needs of highest priority are met, biomass can be used to fulfill the other demands shown in Figure 1.1. This applies especially to those areas with lower total raw material consumption than for food. From this point, it also follows that a large consumption of biomass to meet energy demands is only possible in countries with a low population density and a high biomass production.

By definition, biotechnological processes are especially suited to the production of compounds from biomass as the raw material (Figure 1.2). The amount produced in, and economic importance of, such processes is detailed in Table 1.1.

This also involves the development of suitable concepts, methods, and equipment to obtain more sustainable processes. From the information provided in

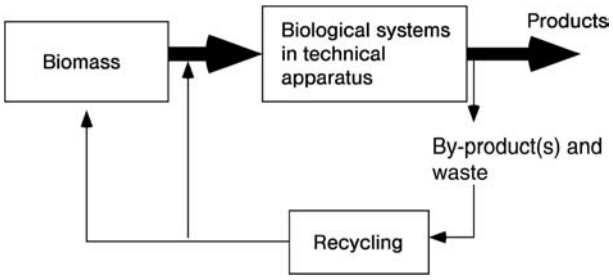

Figure 1.2 Schematic view of an ideal sustainable biotechnological production process. Biomass as a regenerable resource is converted into desired products with minimal waste and by-product production. The waste and by-products must be completely recycled.

Table 1.1 Yearly production and value of biotechnologically produced products to meet human needs.

Human need	Product (year)	World production (t year^{-1})	Value ($\times 10^9$ euros year^{-1})	Production method	
				Biotechnological	Chemical
Food and feed	Beer/wine (2009)	195 000 000 (a)	≈300	F, E	
	Cheese (2009)	19 400 000 (a)	≈100	F, E	
	Baker's yeast (1992)	1 800 000	?	F	
	Vegetable oils (partly used for biodiesel) (2009)	107 000 000 (a)	?	E	+
	Vinegar (10% acetic acid)	>1 500 000*		F	
Fine chemicals including feed and food additives	Amino acids (2006)	3 000 000 (c)	3 (c)	F, E	+
	Glucose–fructose syrup	12 000 000 (g)	5	E	
	Vitamin C (2006)	80 000 (c)	1 (c)	F, E	+
	Aspartame (dipeptide) (2006)	15 000 (c)	?	F, E	+
	Citric acid (2006)	1 500 000 (c)	1.2 (c)	F	
	Herbicides, insecticides	>2 200 000*	?	E	+
	Enzymes (2010)	>10 000	2.6 (d)	F	
Basic chemicals	Products from biomass				
	Biodiesel from vegetable oils (2007)	9 000 000 m^3 (e)			
	Bioethanol (2007)	50 000 000 m^3 (e)		E, F	
	Acrylamide	>150 000*	?	E	+
	1,3-Propanediol	>100 000*		F	
	Polylactic acid	140 000 (g)		F	+
	Acetic acid (2003)	3 400 000 (b)			+

Fibers for textiles	Cotton (2008)	25 000 000 (a)	30 (a)	E	+
	Wool (2008)	2 000 000 (a)	3.5 (a)	E	+
	Linen (2008)	600 000 (a)	0.3 (a)	F, E	+
Paper	All forms (2009)	370 000 000 (a) (50% recycled)		E	+
Hygienics/detergents	Biotensides	?	?	E, F	+
	Washing powder (2003)	24 000 000 (b)		F	+
Therapeuticals	Antibiotics	>60 000*	≈35 (h)	F, E	+
	Insulin (2010)	>10	10 (f)	F, E	
	Recombinant proteins (factor VIII, interferons, tPA, hormones, growth factors, etc.) (2008)	?	50 (g)	F, E	
	Monoclonal antibodies (2008)	?	20 (g)	F	
Diagnostics (estimated figures)	Monoclonal antibodies	?	>1	F, E	+
	DNA/protein chips	?	?	F, E	+
	Enzyme-based	<1	>1	F, E	+
Environment	Clean water (only Germany)	13 000 000 000	13	F	+
	Clean air/soil (soil remediation)	?	?	F	+
Comparison					
Chemical industry	All products (2009)	>1 000 000 000*	≈1900 (i)		
	Chemical catalysts (2009)	?	12 (j)		

All production data are from 2003 to 2010; values are only given where sources give the present data or when they can be estimated, based on prices in the European Union (EU). F = fermentation; E = enzyme technology.

Sources: (a) FAOSTAT (http://faostat.fao.org/site/339/default.aspx); (b) UN (2003); (c) Soetaert and Vandamme (2010); (d) Novozymes (2011); (e) FAO (2008); (f) Novo Nordisk (2011); (g) Buchholz and Collins (2010); (h) Hamad (2010); (i) CEFIC (2010); (j) Bryant (2010).
* Estimated as newer data are not available for open access.

Figure 1.1, it also follows that biotechnology has a major potential in the development of sustainable processes to meet all human needs.

Enzyme technology is a part of biotechnology that is defined in the internationally accepted Cartagena Convention (see Section 1.1) as follows:

"*Biotechnology means any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use.*"

with the following amendment:

"*The use must be sustainable, this means the use of components of biological diversity in a way and at a rate that does not lead to long-term decline of biological diversity, thereby maintaining its potential to meet the needs and aspirations of present and future generations.*"

This requires that traditional classical – as well as new biotechnological – processes must be improved and/or developed in order to be sustainable (Figure 1.2). The fundamentals needed for the development of such processes in the interdisciplinary field of biotechnology require the close cooperation of biologists, chemists, and biochemical and chemical engineers.

1.3 Historical Highlights of Enzyme Technology/Applied Biocatalysis

1.3.1 Early Developments

Applied biocatalysis has its roots in the ancient manufacture and preservation of food and alcoholic drinks, as can be seen in old Egyptian pictures. Cheese making has always involved the use of enzymes, and as far back as about 400 BC, Homer's Iliad mentions the use of a kid's stomach for making cheese.

With the development of modern natural science during the eighteenth and nineteenth centuries, applied biocatalysis began to develop a more scientific basis. In 1833, Payen and Persoz investigated the action of extracts of germinating barley in the hydrolysis of starch to yield dextrin and sugar, and went on to formulate some basic principles of enzyme action (Payen and Persoz, 1833):

- small amounts of the preparation were able to liquefy large amounts of starch,
- the material was thermolabile, and
- the active substance could be precipitated from aqueous solution by alcohol, and thus be concentrated and purified. This active substance was called *diastase* (a mixture of amylases).