Biocatalysis

A. S. Bommarius, B. R. Riebel

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Preface

The field of biocatalysis is at a crossroads. On one hand, the frontier of research races ahead, propelled by advances in the database-supported analysis of sequences and structures as well as the designability of genes and proteins. Moreover, the "design rules" for biocatalysts have emerged from vague images on the horizon, to come into much clearer view. On the other hand, experienced practitioners from other areas as well as more and more students entering this field search for ways to obtain the level of knowledge in biocatalysis that advances their own agenda. However, both groups find a rapidly growing field with too little *guidance* towards the research front and too little *structure* in its guiding principles. In this situation, this book seeks to fill the gap between the research front and the area beyond basic courses in biochemistry, organic synthesis, molecular biology, kinetics, and reaction engineering. Students and practitioners alike are often left alone to bridge the gulf between basic textbooks and original research articles; this book seeks to cover this intermediate area.

Another challenge this book strives to address results from the interdisciplinary nature of the field of biocatalysis. Biocatalysis is a synthesis of chemistry, biology, chemical engineering, and bioengineering, but most students and practitioners enter this field with preparation essentially limited to one of the major contributing areas, or at best two. The essence of biocatalysis, as well as most of its current research, however, is captured in the interdisciplinary overlap between individual areas. Therefore, this work seeks to help readers to combine their prior knowledge with the contents and the methods in this book to make an integrated whole.

The book is divided into three parts:

- Chapters 1 through 7 cover *basic tools*. Many readers have probably encountered the contents of some chapters before; nevertheless, we hope to offer an update and a fresh view.
- Chapters 8 through 14 expand on *advanced tools*. While command of such advanced concepts is indispensable in order to follow, much less to lead, today's developments in biocatalysis, the mastering of such concepts and tools cannot necessarily be expected of all practitioners in the field, especially if their major course of study often did not even touch on such topics.
- Chapters 15 through 20 treat *applications* of all the tools covered in previous chapters. "Applications" here encompass not just industrial-scale realization of bio-

catalysis but also new intellectual frontiers in biological catalysis that are possible with today's technologies, such as rapidly expanding DNA databases or comprehensive coverage of three-dimensional structure analysis for many enzymes.

In the early part of the book, several chapters have a fairly clear emphasis on chemistry, biology, or chemical engineering. Chapters on the isolation of microorganisms (Chapter 3), molecular biology tools (Chapter 4), protein engineering (Chapter 10), or directed evolution (Chapter 11) have a distinct biological flavor. Chemistry is the main topic in the chapters on applications of enzymes as products (Chapter 6), in bulk and fine chemicals (Chapter 7), and in pharmaceuticals (Chapter 13). Chemical engineering concepts predominate in the chapters on biocatalytic reaction engineering (Chapter 5) or on processing steps for enzyme manufacture (Chapter 8). Other chapters contribute a perspective from biochemistry/enzymology, such as characterization of biocatalysts (Chapter 2) and methods of studying proteins (Chapter 9), or from informatics, most notably bioinformatics (Chapter 14).

Finally, a word on the history of this book: the idea for the present work originated during a lectureship of one of us (A.S.B.) as an adjunct faculty member at the Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen in Aachen, Germany, for nine years while he was working at Degussa in Wolfgang, Germany. Time and time again, students enjoyed the interdisciplinary nature and coverage of biocatalysis but lacked adequate preparation in those basic tools that were not provided during their courses for their respective major, be it chemistry, biology, or chemical engineering. Similar observations were made when teaching biocatalysis or related subjects at the Georgia Institute of Technology in Atlanta/GA, USA. One of the aims of this book is to take readers back to scientific fundamentals often long forgotten, to let them to participate in the joy of discovery and understanding stemming from a multi-faceted picture of nature. While scientific fundamentals are a source of immense satisfaction, applications with an impact in the day-to-day world are just as important. Two of the biggest challenges facing mankind today (and not exclusively the industrial societies) are maintenance and improvement of *human health*, and maintenance and improvement of *the environment*. Biocatalysis aids the first of these goals through its selectivity in generating ever more complex pharmaceutically active molecules, and the second goal by opening new routes to both basic and performance chemicals with the aim of achieving sustainable development.

We hope that you enjoy reading this book. We encourage you to contact us to voice your opinion, gripe, laud, discuss aspects of the book, point out errors or ambiguities, make suggestions for improvements, or just to let us know what you think. The easiest way to do this is via email at bommariu@bellsouth.net or andreas.bommarius@alum.mit.edu.

We wish you pleasant reading.

Andreas S. Bommarius and Bettina R. Riebel
Atlanta/GA, USA
December 2003

Acknowledgments

For more than a decade, one of us (A.S.B.) had the good fortune to be associated with Degussa, one of the early players, and currently still strong, in the area of biocatalysis, in its R&D center in Wolfgang, Germany. While several factors were responsible for Degussa's venture into biocatalysis, certainly the most influential was the steadfast support of biocatalysis by Degussa's former board member and Head of Research, Professor Heribert Offermanns. His unconventional and far-sighted way of thinking remains an example and A.S.B. thanks him warmly for his attitude and encouragement. A.S.B. is also grateful to Professor Karlheinz Drauz, himself an accomplished author with Wiley-VCH, for sustained support and also for supporting biocatalysis at Degussa during difficult times. A.S.B. also fondly remembers co-workers at Degussa and its many subsidiaries. He thanks Wolfgang Leuchtenberger, his predecessor and representing a group too numerous to acknowledge individually, and encourages Harald Gröger, his successor.

The origin of this book stems from a biweekly lectureship that A.S.B. held at the RWTH Aachen (in Aachen, Germany) from 1991 to 2000, first at the Institute of Biotechnology under the late Harald Voss, then in the Institute of Technical Chemistry and Petroleum Chemistry under Wilhelm Keim. A.S.B. expressly thanks Wilhelm Keim for his continued support and advice, not just with the lectureship but also during his habilitation.

Both of us have several reasons to thank Professors Maria-Regina Kula at the University of Düsseldorf, Germany, and Christian Wandrey at the Research Center Jülich, Germany. While both of them have left a huge impact on the field of biocatalysis in general (acknowledged, among other honors, by the German Technology Transfer Prize in 1983 and the Enzyme Engineering Award in 1995 to both of them), they influenced each of us markedly. One of us (B.R.R.) thanks her advisor Maria-Regina Kula and, specifically, her direct mentor, Werner Hummel, for sustained support and interest during her formative thesis years and beyond. A.S.B. gladly acknowledges both of them and Christian Wandrey for many years of fruitful collaboration. The impact of their views on both of us is evident in many parts of this book.

One of us (A.S.B.) gratefully acknowledges the support from Georgia Tech, from the higher administration to the laboratory group, for getting his own research group started. As representatives for a much more numerous group, A.S.B. thanks Dr. Ronald Rousseau, his School Chair, himself an author of one of the most influ-

ential textbooks on chemical engineering, for his trust and his support of the area of biocatalysis in chemical engineering, as well as Dr. Phillip Gibbs, his first postdoctoral associate, for countless discussions on the research front in the field.

We thank our publisher, Wiley-VCH, in Weinheim, Germany, for their continual support and enthusiasm. The publishing team, including Karin Dembowsky, Andrea Pillmann, Eva Wille, Karin Proff, and Hans-Jochen Schmitt, had to put up with quite a scheduling challenge, not to mention the pain resulting from the need for both authors to relocate to Atlanta/GA, USA, and establish their careers there. Both of us thank the publishers for exemplary support and the high quality of workmanship reflected in the layout of this book.

Last but not least, we could write this book because we enjoyed countless interactions with other scientists and engineers who shaped our view of the field of biocatalysis. A representative, but certainly not exhaustive, list of these individuals, besides those already mentioned above, includes Frances Arnold, Uwe Bornscheuer, Stefan Buchholz, Mark Burk, Robert DiCosimo, David Dodds, Franz Effenberger, Uwe Eichhorn, Wolfgang Estler, Andreas Fischer, Tomas Hudlicky, Hans-Dieter Jakubke, Andreas Karau, Alexander Klibanov, Andreas Liese, Oliver May, Jeffrey Moore, Rainer Müller, Mark Nelson, David Rozzell, Roger Sheldon, Christoph Syldatk, Stefan Verseck, and George Whitesides. We thank all of them for their contribution to our view of the field.

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Atlanta/GA, USA
December 2003

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Introduction to Biocatalysis

Summary

Over the last 20 years, many reservations with respect to biocatalysis have been voiced, contending that: (i) enzymes only feature limited substrate specificity; (ii) there is only limited availability of enzymes; (iii) only a limited number of enzymes exist; (iv) protein catalyst stability is limited; (v) enzyme reactions are saddled with limited space–time yield; and (vi) enzymes require complicated co-substrates such as cofactors.

Driven by the discovery of many novel enzymes, by recombinant DNA technology which allows both more efficient production and targeted or combinatorial alterations of individual enzymes, and by process development towards higher stability and volumetric productivity, synthesis routes in which one or all of the steps are biocatalytic have advanced dramatically in recent years. Design rules for improved biocatalysts are increasingly precise and easy to use.

Biocatalysts do not operate by different scientific principles from organic catalysts. The existence of a multitude of enzyme models including oligopeptidic or polypeptidic catalysts proves that all enzyme action can be explained by rational chemical and physical principles. However, enzymes can create unusual and superior reaction conditions such as extremely low pK_a values or a high positive potential for a redox metal ion. Enzymes increasingly have been found to catalyze almost any reaction of organic chemistry.

Biotechnology and biocatalysis differ from conventional processes not only by featuring a different type of catalyst; they also constitute a new technology base. The *raw materials base* of a biologically-based process is built on sugar, lignin, or animal or plant wastes; in biotechnology, unit operations such as membrane processes, chromatography, or biocatalysis are prevalent, and the product range of biotechnological processes often encompasses chiral molecules or biopolymers such as proteins, nucleic acids or carbohydrates.

Cost and margin pressure from less expensive competitors and operation with emphasis on a clean (or less polluted) environment are two major developments. Fewer processing steps, with higher yields at each step, lower material and energy costs, and less waste are the goals. Biotechnology and biocatalysis often offer unique technology options and solutions, they act as *enabling technologies*; in other cases, biocatalysis has to outperform competing technologies to gain access. In the phar-

maceutical industry, the reason for the drive for enantiomeric purity is that the vast majority of novel drugs are chiral targets, favoring biocatalysis as the technology with the best selectivity performance.

Biocatalytic processes increasingly penetrate the chemical industry. In a recent study, 134 industrial-scale biotransformations, on a scale of > 100 kg with whole cells or enzymes starting from a precursor other than a C-source, were analyzed. Hydrolases (44%), followed by oxido-reductases (30%), dominate industrial biocatalytic applications. Average performance data for fine chemicals (not pharmaceuticals) applications are 78% yield, a final product concentration of 108 g L^{-1}, and a volumetric productivity of 372 g $(L \cdot d)^{-1}$.

1.1 Overview: The Status of Biocatalysis at the Turn of the 21st Century

1.1.1 State of Acceptance of Biocatalysis

Over the last 20 years, many reservations with respect to biocatalysis have been voiced. The critics, often focusing on the drawbacks, have contended that

- enzymes only feature limited substrate specificity,
- there is only limited availability of enzymes,
- only a limited number of enzymes exist,
- protein catalyst stability is limited,
- enzyme reactions are saddled with limited space–time yield, and
- enzymes require complicated co-substrates such as cofactors.

The renaissance of biocatalysis, supported by the advent of recombinant DNA, is only about 20 years old. Recently, several publications have appeared which deal specifically with the attitudes listed above (Rozzell, 1999; Bommarius, 2001; Rasor, 2001). Most of the points above can either be refuted or they can be levied against any novel catalytic technology; the situation with some competing technologies such as chiral homogeneous catalysts is similar to that with enzymes (Chapters 18 and 20).

- *Enzymes only feature limited substrate specificity*. Often, enzymes designed to convert small molecules such as hydrogen peroxide, urea, fumaric acid, or L-aspartic acid feature extremely narrow substrate specificity; the corresponding enzymes catalase, urease, fumarase or aspartase, and L-aspartate decarboxylase take either few other substrates, such as alkyl peroxides in the case of catalase, or no other substrate, such as urease, which only converts urea. On the other hand, very large enzymes acting as multi-enzyme complexes such as nonribosomal peptide synthetases (NRPSs) (Kleinkauf, 1996) are often highly specific. Ordinary-sized enzymes working on medium-sized substrates, however, in most cases feature broad substrate specificity, a fact already noted by Rasor and Voss (Rasor, 2001).

- *There is only limited availability of enzymes.* Until very recently, limited availability of enzymes was indeed a major problem. About ten years ago, with 3196 different enzymes already listed in Enzyme Nomenclature (Moss, 1992), only about 50 enzymes were fully characterized and only about a dozen enzymes available commercially on a regular basis. However, recombinant DNA technology, discovered in 1978 by Cohen and Boyer (Stanford University, Palo Alto, CA, USA), over the next 20 years allowed enzymes to be produced much more efficiently, in higher purity, and more inexpensively (Baneyx, 1999), so that today a multitude of enzymes are available not only from established suppliers such as Sigma–Aldrich–Fluka (Milwaukee, WI, USA), E. Merck (Darmstadt, Germany), Mercian (Tokyo, Japan), or Roche Diagnostics (Mannheim, Germany) but increasingly also from smaller, more focused suppliers such as Biocatalytics (Pasadena, CA, USA) or Jülich Fine Chemicals (Jülich, Germany). The argument of unavailability or scarcity will be less and less justified in the future.

- *Only a limited number of enzymes exist.* This criticism, while depending on the observer's position, is indeed a drawback at the moment. Although enzymes have been found for every conceivable organic chemical reaction except the hetero-Cope rearrangement (Table 1.4, below), there are enzymes sought for many more reactions than there are enzymes available. If enzymes were inferior catalysts this situation would not arise, of course. In fact, enzymes are often superior catalysts (see the next section), so superior is fact, that the community seeks plenty more of them. Chapter 3 treats the discovery of novel enzymes, whereas Chapters 10 and 11 cover improvement of existing enzymes through rational (protein engineering) and combinatorial random mutagenesis (directed evolution).

- *Protein catalyst stability is limited.* This is one of major drawbacks of enzymes. They commonly require temperatures around ambient to perform (15–50°C), pH values around neutral (pH 5–9), and aqueous media. In addition, any number of system components or features such as salts, inhibitors, liquid–gas or liquid–solid interfaces, or mechanical stress can slow down or deactivate enzymes. Under almost any condition, native proteins, with their Gibbs free enthalpy of stability of just a few kilojoules per mole, are never far away from instability. In this book, we cover inhibitors (Chapter 5, Section 5.3) or impeding system parameters (Chapter 17) and successful attempts at broadening the choice of solvents (Chapter 12).

- *Enzyme reactions are saddled with limited space–time yield.* The notion that biocatalysts are slow catalysts is false. Slow catalysts, applied at low concentrations, certainly lead to low space–time yields. However, optimized syntheses not only produce very good selectivities or total turnover numbers but also satisfactory to excellent space–time yields. Examples with such good s.t.y. values are
  – in commodity biochemicals, the synthesis of L-aspartate from fumaric acid and ammonia with space–time-yields of up to 60 kg $(L \cdot d)^{-1}$ (Rozzell, 1999), and

– in advanced pharmaceutical intermediates, kinetically-controlled peptide synthesis to kyotorphin (Tyr-Arg) catalyzed by α-chymotrypsin from maleyl-L-Tyr-OEt and Arg-OEt, employing a highly soluble protecting group at the electrophile (Fischer, 1994). Space–time-yields of 1.34 kg $(L \cdot d)^{-1}$ have been achieved.

The question of high volumetric productivity is coupled to the solubility of substrates. High space–time-yields have been demonstrated to be correlated with high solubilities of substrates (Bommarius, 2001).

- *Enzymes require complicated co-substrates such as cofactors.* Much has been made of the requirement of some enzymes for cofactors, such as nicotinamide-containing compounds, NAD(P)(H), for dehydrogenases; flavin compounds, FMN or FAD, for oxidases; pyridoxylphosphate, PLP, for transaminases and decarboxylases; thiamine pyrophosphate, TPP, for carboligases, and vitamin B12 for glycerate dehydratase, among others. The scale-up of L-aspartate decarboxylation to L-alanine with the help of PLP-requiring L-aspartate decarboxylase, or of reductive amination of trimethylpyruvate to L-tert-leucine with the help of NADH-requiring leucine dehydrogenase demonstrates the feasibility of industrial processing with cofactor-requiring enzymes. The implementation also gives credence to the suggestion that cofactors are no longer the dominating cost component, as was believed until recently. Requirements for cofactors constitute a technological challenge but one that has been met successfully and so should not be regarded as impeding the use of biocatalysts in processing.

1.1.2 Current Advantages and Drawbacks of Biocatalysis

1.1.2.1 Advantages of Biocatalysts

The biggest advantage of enzymes is their often unsurpassed selectivity. While enzymes are used beneficially to increase chemical selectivity or regioselectivity of a reaction, their biggest advantage lies in the differentiation between enantiomeric substrates, a pair of substrates with Gibbs free enthalpy differences between the *R*- and the *S*-enantiomer ΔG_{RS} of around 1–3 kJ mol^{-1}. With enzymes, enantioselectivities of > 99% e.e. can be achieved routinely, although by no means in every case. This fact becomes increasingly important for using biocatalysts in the synthesis of advanced pharmaceutical intermediates, as regulatory agencies require separate toxicological studies for every impurity comprising above 1% of the content (Chapter 13, Section 13.1.4) (Crossley, 1995).

The fact that enzymes are active mostly at mild, near-ambient conditions of temperature and pH and preferentially in aqueous media is often regarded as an advantage rather than a drawback nowadays. Goals for industrial processing such as "sustainable development", "green chemistry", or "environmentally benign manufacturing", an increasingly important boundary condition for industrial activity in a large part of the world, would be much harder to attain without the availability of biocatalysts which tolerate and require such conditions.