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Cascade Biocatalysis

Integrating Stereoselective and Environmentally Friendly Reactions
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Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at <http://dnb.d-nb.de>.

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Print ISBN: 978-3-527-33522-0
ePDF ISBN: 978-3-527-68248-5
Mobi ISBN: 978-3-527-68250-8
oBook ISBN: 978-3-527-68249-2

Cover Designer Adam-Design, Weinheim, Germany
Typesetting Laserwords Private Limited, Chennai, India
Printing and Binding Markono Print Media Pte Ltd, Singapore

Printed on acid-free paper
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Preface

Sustainability is one of the key issues to enhance, or at least maintain, the quality of life in our modern society. As it has been codified in 1987 in an official UN document, a “sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs”. Applied to chemical processes, sustainability has generated the concept of Green Chemistry, for which guidelines have been summarized as the well-known Twelve Principles of Green Chemistry.

In Europe, this effort has been recognized at the institutional level: the European Technology Platform for Sustainable Chemistry (SusChem, http://www.suschem.org) was created in 2004 with the main objective to revitalize and inspire the European chemistry research, development, and innovation in a sustainable way. Industrial Biotechnology, also known as White Biotechnology, is one of the three pillars that support sustainable chemistry nowadays and that are expected to support it even more profoundly in the future. It is defined as “the use of enzymes and micro-organisms to make efficient and sustainable products in sectors as diverse as chemicals, plastics, food and feed, detergents, paper and pulp, textiles or bioenergy.”

Although long and reiterating, this introduction is meant to raise the awareness that the roots and the branches of biocatalysis – as well as its fruits! – are deeply embedded in modern synthetic chemistry. In fact, the majority of the above-mentioned Principles of Green Chemistry (PGC) fit perfectly with the peculiar properties and synthetic application of enzymes, which are Nature’s catalysts. The contributions collected in this book offer a convincing testimony that biocatalysis is highly qualified to contribute to the development of future sustainable technologies. Enzymes are highly efficient catalysts offering superior selectivity (PGC #9), thereby meeting criteria for atom economy by maximizing the incorporation of starting materials into the final product (PGC #2) while avoiding unnecessary and unproductive derivatization, such as the use of temporary protection groups (PGC #8). Such steps are unavoidable when using conventional synthetic chemistry approaches and require additional reagents and generate waste materials, particularly when utilizing multifunctionalized, bio-based renewable feedstocks.

Inherently, enzymes are biodegradable (PGC #10) and innocuous to the environment (PGC #3), not the least because they operate in water as a safe solvent (PGC #5) at ambient temperature and pressure, which minimizes energy consumption (PGC #6).

Cascade Biocatalysis is an effort to imitate the style of chemical conversions occurring in living beings, which are totally different from the traditional use of single enzymes by synthetic chemists in the laboratory for catalyzing isolated transformations. Instead, cells apply multistep synthetic strategies, catalyzed by several enzymes acting sequentially along a pathway, in which a product formed in one reaction \textit{in situ} becomes the substrate of the next catalyst. This is possible because of the very similar mild reaction conditions under which most enzymes operate, which facilitates their combination and allows effective strategies of reaction engineering, for example, to shift unproductive equilibria by coupling to thermodynamically favored processes for overall high conversion and economic efficiency.

This concept has recently been recognized as the major focus for a series of international symposia on \textit{Multistep Enzyme-Catalyzed Processes}, the last symposium having just been celebrated in Madrid in April 2014. Research in this area has also been coordinated within the activities of the European Union funded COST network CM0701 entitled \textit{Cascade Chemoenzymatic Processes – New Synergies Between Chemistry and Biochemistry} (2008–2012; http://www.cost-cascat.polimi.it). This handbook brings together contributions from scientists deeply involved in the activities of this COST action as well as complementary chapters on related research from additional authors, who are well known for their seminal work in this contemporary research field. The topics covered in the chapters span from examples related to integrated applications of cofactor-dependent oxidoreductases to the exploitation of transferases; from the multistep modification of the nitrile functional group to the synthesis of complex carbohydrates; and from developments of new dynamic kinetic resolution processes to intricate examples of chemoenzymatic multistep one-pot procedures.

We would like to thank all the authors who, despite their busy schedules, have participated in this project to share their expertise with the future readers of this book. Thanks are also due to Elke Maase and Stefanie Volk at Wiley-VCH Publishers, for their careful editorial support and for their continuous goad in order to meet assigned deadlines.

Finally, we hope that our readers will find this volume useful as a stimulating source of ideas for their own research and/or teaching activities.

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1 Directed Evolution of Ligninolytic Oxidoreductases: from Functional Expression to Stabilization and Beyond

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1.1 Introduction

The ligninolytic enzymatic consortium, formed mainly by nonspecific oxidoreductases (laccases, peroxidases, and \( \text{H}_2\text{O}_2 \)-supplying oxidases), is a potentially powerful multipurpose tool for industrial and environmental biotechnology. In nature, these enzymes are typically produced by basidiomycete white-rot fungi that are involved in lignin decay. Thanks to their broad substrate specificity, high redox potential, and minimal requirements, these enzymes have many potential applications in the field of green chemistry, including the production of biofuels, bioremediation, organic syntheses, pulp biobleaching, food and textile industries, and the design of bionanodevices. The implementation of this enzymatic armoury in different biotechnological sectors has been hampered by the lack of appropriate molecular instruments (including heterologous hosts for directed evolution) with which to improve their properties. Over the last 10 years, a wealth of directed evolution strategies in combination with hybrid approaches has emerged in order to adapt these oxidoreductases to the drastic conditions associated with many biotechnological settings (e.g., high temperatures, the presence of organic co-solvents, extreme pHs, the presence of inhibitors). This chapter summarizes all efforts and endeavors to convert these ligninolytic enzymes into useful biocatalysts by means of directed evolution: from functional expression to stabilization and beyond.

1.2 Directed Molecular Evolution

Enzymes are versatile biomolecules that exhibit a large repertory of functions acquired over millions of years of natural evolution. Indeed, they are the fastest known catalysts (accelerating chemical reactions as much as \( 10^{19} \)-fold) and are environmentally friendly molecules, working efficiently at mild temperatures, in water, and releasing few by-products. Moreover, they can exhibit high enantioselectivity and chemoselectivity. Nonetheless, when an enzyme is removed...
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from its natural environment and introduced into a specific biotechnological location (e.g., the transformation of a hydrophobic compound in the presence of co-solvents or at high temperatures), its molecular structure may not tolerate the extreme operational conditions and may unfold becoming inactive. Unfortunately, the enzymes that cells use to regulate strict metabolic pathways and that promote fitness and survival in nature are not always applicable to the harsh requirements of many industrial processes.

The development of the polymerase chain reaction (PCR) in the early 1980s heralded a biotechnological revolution for protein engineers, allowing us for the first time to manipulate and design enzymes by site-directed mutagenesis supported by known protein structures: the so-called rational design. However, further advances were frustrated owing to the limited understanding of protein function and the lack of protein structures available at the time. Nevertheless, the following decade saw a second biotechnological revolution with the development of directed molecular evolution. This powerful protein engineering tool does not require prior knowledge of protein structure to enhance the known features or to generate novel enzymatic functions, which are not generally required in natural environments. The key events of natural evolution (random mutation, DNA recombination, and selection) are recreated in the laboratory, permitting

![Figure 1.1 Directed molecular evolution.](image)

The basic premises to carry out a successful directed evolution experiment are (i) a robust heterologous expression system (typically *S. cerevisiae* or *E. coli*); (ii) a reliable high-throughput (HT)-screening assay; and (iii) the use of different molecular tools for the generation of DNA diversity.
1.3 The Ligninolytic Enzymatic Consortium

Lignin is the most abundant natural aromatic polymer and the second most abundant component of plant biomass after cellulose. As a structural part of the plant cell wall, lignin forms a complex matrix that protects cellulose and hemicellulose chains from microbial attack and hence from enzymatic hydrolysis. This recalcitrant and highly heterogeneous biopolymer is synthesized by the dehydrogenative polymerization of three precursors belonging to the \( p \)-hydroxycinnamyl alcohol group: \( p \)-coumaryl, coniferyl, and sinapyl alcohols [9]. As one-third of the carbon fixed as lignocellulose is lignin, its degradation is considered a key step in the recycling of carbon in the biosphere and in the use of the plant biomass for biotechnological purposes [10, 11]. Lignin is modified and degraded to different extents by a limited number of microorganisms, mainly filamentous fungi and bacteria. Lignin degradation by bacteria is somewhat limited and much slower than that mediated by filamentous fungi [12, 13]. Accordingly, the only organisms capable of completing the mineralization of lignin are the white-rot fungi, which produce a white-colored material upon delignification because of the enrichment in cellulose [14, 15].

Through fungal genome reconstructions, recent studies have linked the formation of coal deposits during the Permo-Carboniferous period (~260 million years ago) with the nascent and evolution of white-rot fungi and their lignin-degrading enzymes [16]. Lignin combustion by white-rot fungi involves a very complex extracellular oxidative system that includes high-redox potential laccases (HRPLs), peroxidases and unspecific peroxygenases (UPOs), \( \text{H}_2\text{O}_2 \)-supplying oxidases and auxiliary enzymes, as well as radicals of aromatic compounds and oxidized metal ions that act as both diffusible oxidants and electron carriers [12, 13, 15, 17]. Although the role of each component of the consortium has been studied extensively, many factors remain to be elucidated (Figure 1.2).

Laccases typically oxidize the phenolic units of lignin. Lignin peroxidases (LiPs) oxidize both nonphenolic lignin structures and veratryl alcohol (VA), a metabolite synthesized by fungi that helps LiP to avoid inactivation by \( \text{H}_2\text{O}_2 \) and whose radical...
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cation may act as a redox mediator [20]. Manganese peroxidases (MnPs) generate Mn$^{3+}$, which upon chelation with organic acids (e.g., oxalate synthesized by fungi) attacks phenolic lignin structures; in addition, MnP can also oxidize nonphenolic compounds via lipid peroxidation [21]. Versatile peroxidases (VPs) combine the catalytic activities of LiP, MnP, and generic peroxidases to oxidize phenolic and nonphenolic lignin units [22]. Some fungal oxidases produce the H$_2$O$_2$ necessary for the activity of peroxidases. Among them, aryl-alcohol oxidase (AAO) transforms benzyl alcohols to the corresponding aldehydes; glyoxal oxidase (GLX) oxidizes glyoxal producing oxalate, which in turn chelates Mn$^{3+}$; and then methanol oxidase (MOX) converts methanol into formaldehyde; all the above oxidations are coupled with O$_2$ reduction of H$_2$O$_2$. Other enzymes such as cellobiose dehydrogenase (CDH) have been indirectly implicated in lignin degradation. This is because of CDH ability to reduce both ferric iron and O$_2$-generating hydroxyl radicals via Fenton reaction. These radicals are strong oxidizers that act as redox mediators playing a fundamental role during the initial stages of lignin polymer decay, when the small pore size of the plant cell wall prevents the access of fungal enzymes [23]. The same is true for laccases, whose substrate spectrum can be broadened in the presence of natural mediators to act on nonphenolic parts of lignin [24].

High-redox potential laccases and peroxidases/peroxygenases are of great biotechnological interest [25, 26]. With minimal requirements and high redox potentials (up to +790 mV for laccases and over +1000 mV for peroxidases), these enzymes can oxidize a wide range of substrates, finding potential applications in a variety of areas, which are as follows:

**Figure 1.2** General view of the plant cell wall and the action of the ligninolytic enzymatic consortium. The lignin polymer is oxidized by white-rot fungi laccases and peroxidases, producing nonphenolic aromatic radicals (1) and phenoxy radicals (2). Nonphenolic aromatic radicals can suffer nonenzymatic modifications such as aromatic ring cleavage (3), ether breakdown (4), C$_a$–C$_b$ cleavage (5), and demethoxylation (6). The phenoxy radicals (2) can repolymerize on the lignin polymer (7) or be reduced to phenolic compounds by AAO (8) (concomitantly with aryl alcohol oxidation). These phenolic compounds can be re-oxidized by fungal enzymes (9). In addition, phenoxy radicals can undergo C$_a$–C$_b$ cleavage to produce p-quinones (10). Quinones promote the production of superoxide radicals via redox cycling reactions involving QR, laccases, and peroxidases (11, 12). The aromatic aldehydes released from C$_a$–C$_b$ cleavage, or synthesized by fungi, are involved in the production of H$_2$O$_2$ via another redox cycling reaction involving AAD and AAO (13, 14). Methanol resulting from demethoxylation of aromatic radicals (6) is oxidized by MOX to produce formaldehyde (15). Fungi also synthesize glyoxal, which is oxidized by GLX to produce H$_2$O$_2$ and oxalate (16), which in turn chelate Mn$^{3+}$ ions produced by MnP (17). The Mn$^{3+}$ chelated with organic acids acts as a diffusible oxidant for the oxidation of phenolic compounds (2). The reduction of ferric ions present in wood is mediated by the superoxide radical (18) and they are re-oxidized by the Fenton reaction (19) to produce hydroxyl radicals, which are very strong oxidizers that can attack the lignin polymer (20). AAO, aryl-alcohol oxidase; AAD, aryl-alcohol dehydrogenase; GLX, glyoxal oxidase; LiP, lignin peroxidase; MnP, manganese peroxidase; MOX, methanol oxidase; QR, quinone reductase; VP, versatile peroxidase. (Figure adapted from [18, 19].) (Source: Bidlack, J.M. et al. 1992 [18], Fig. 1, p. 1. Reproduced with permission of the Oklahoma Academy of Science.)
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- The use of lignocellulosic materials (e.g., agricultural wastes) in the production of second-generation biofuels (bioethanol, biobutanol) or the manufacture of new cellulose-derived and lignin-derived value-added products.
- The organic synthesis of drugs and antibiotics, cosmetics and complex polymers, and building blocks.
- In nanobiotechnology as (i) biosensors (for phenols, oxygen, hydroperoxides, azides, morphine, codeine, catecholamines, or flavonoids) for clinical and environmental applications; and (ii) biofuel cells for biomedical applications.
- In bioremediation: oxidation of polycyclic aromatic hydrocarbons (PAHs), dioxins, halogenated compounds, phenolic compounds, benzene derivatives, nitroaromatic compounds, and synthetic organic dyes.
- The food industry: drink processing and bakery products.
- The paper industry: pulp biobleaching, pitch control, manufacture of mechanical pulps with low energy cost, and effluent treatment.
- The textile industry: remediation of dyes in effluents, textile bleaching (e.g., jeans), modification of dyes and fabrics, detergents.

A few years ago, the engineering and improvement of ligninolytic oxidoreductases was significantly hampered by the lack of suitable heterologous hosts to carry out directed evolution studies. Fortunately, things have changed and several reliable platforms for the directed evolution of ligninolytic peroxidases, peroxygenases, and several medium-redox potential laccases and high-redox potential laccases (HRPLs) have been developed using the budding yeast *Saccharomyces cerevisiae*. These advances have allowed us, for the first time, to specifically tailor ligninolytic oxidoreductases to address new challenges.

1.4 Directed Evolution of Laccases

Laccases (EC 1.10.3.2) are extracellular glycoproteins that belong to the blue multicopper oxidase family (along with ascorbate oxidase, ceruloplasmin, nitrite reductase, bilirubin oxidase, and ferroxidase). Widely distributed in nature, they are present in plants, fungi, bacteria, and insects [27, 28]. Laccases are *green* catalysts, which are capable of oxidizing dozens of compounds using O$_2$ from air and releasing H$_2$O as their sole by-product [29–31]. These enzymes harbor one type I copper (T1), at which the oxidation of the substrates takes place, and a trinuclear copper cluster (T2/T3) formed by three additional coppers, one T2 and two T3s, at which O$_2$ is reduced to H$_2$O. The reaction mechanism resembles a battery, storing electrons from the four monovalent oxidation reactions of the reducing substrate required to reduce one molecule of oxygen to two molecules of H$_2$O. Laccases catalyze the transformation of a wide variety of aromatic compounds, including ortho- and para-diphenols, methoxy-substituted phenols, aromatic amines, benzenothiols, and hydroxyindols. Inorganic/organic metal compounds are also substrates of laccases, and it has been reported that Mn$^{2+}$ is oxidized by laccase to form Mn$^{3+}$, and organometallic compounds such