# BIOCATALYSIS FOR THE PHARMACEUTICAL INDUSTRY
## Discovery, Development, and Manufacturing

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# Contents

# Preface

Biocatalysis is evolving to be a transformational technology as a result of a confluence of factors, which include (1) large scale and ever increasingly cost-efficient DNA sequencing technologies; (2) exponential growth in GenBank; (3) powerful directed enzyme evolution and high-throughput screening technologies; (4) robust expression systems for enzyme production; (5) deep understanding of the logic of natural product biosynthesis; (6) industrial successes of metabolic engineering and pathway engineering.

Consequently, many successful stories and a number of reviews have been reported recently in developing biocatalysis for the pharmaceutical industry, across drug discovery, development, and manufacturing. The book is dedicated to these advances, and divided into four parts:

- Chapters 1–4 serve as an introduction to emerging biocatalysts, modern expression hosts, state of the art of directed evolution, high-throughput screening, and bioprocess engineering for industrial applications.
- Chapters 5–8 are directed to emerging enzymes, which include oxynitrilases, aldolases, ketoreductases, oxidases, nitrile hydratases, and nitrilases, and their recent applications especially in synthesis of chiral drugs and intermediates.
- Chapters 9 and 10 focus on synthesis of drug metabolites and intermediates catalyzed by P450s or whole cells.
- Chapters 11–13 are devoted to combinatorial biosynthesis, metabolic engineering, and autonomous enzymes for the synthesis and development of complex medicinal molecules.
- Chapter 14 discusses the recent impact of biocatalysis in green chemistry and chemical development.

Our main goal is to come up with a concise but comprehensive, practical but insightful book covering the topics discussed above. We hope you enjoy reading this book. Any suggestions and comments are welcome.

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# 1

# Enzymes and Their Synthetic Applications: An Overview

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## 1.1 Introduction

Whole-cell biocatalysis has been exploited for thousands of years; for example, in preparing barley for beer brewing. While the chemical, economic and social advantages of biocatalysis over traditional chemical approaches were recognized a long time ago, their applications for the drug industry have been largely underexplored until the recent technological breakthroughs in large-scale DNA sequencing, robust protein expression systems, metabolic engineering and directed evolution. In this chapter, emphasis will be directed to the discussion of those isolated enzymes which are uniquely suited for the synthesis of small-molecule pharmaceutical ingredients.

## 1.2 Enzyme Families

Based on reactions they catalyze, enzymes can be broadly classified into six major categories (Table 1.1) [1]. It was estimated that about 60% of biotransformations currently rely on the use of hydrolases, followed by 20% of oxidoreductases [2]. On the other hand, some of the C−C bond-forming and oxygenation enzymes catalyze reactions with very high reaction efficiency and very low waste generation, underlining the potential of emerging enzymes.

*Biocatalysis for the Pharmaceutical Industry* edited by Junhua Tao, Guo-Qiang Lin, and Andreas Liese

**Table 1.1** Enzyme classes

| Enzyme class | Examples | Reaction catalyzed |
|---|---|---|
| Hydrolases | lipase, protease, esterase<br>nitrilase, nitrile hydratase<br>glycosidase, phosphatase | hydrolysis reactions in $H_2O$ |
| Oxidoreductases | dehydrogenase, oxidase<br>oxygenase, peroxidase | oxidation or reduction |
| Transferases | transaminase, glycosyltransferase<br>transaldolase | transfer of a group from one molecule to another |
| Lyases | decarboxylase, dehydratase,<br>deoxyribose-phosphate aldolase | nonhydrolytic bond cleavage |
| Isomerases | racemase, mutase | intramolecular rearrangement |
| Ligases | DNA ligase | bond formation requiring triphosphate |

## 1.3 Enzyme Discovery and Optimization

Traditionally, enzymes are discovered through screening of environmental samples and culture enrichment. As a result of recent technological breakthroughs in large-scale DNA sequencing and high-throughput screening, both the metagenomic approach and sequence-based discovery have drastically shortened the cycle of enzyme discovery.

In the metagenomic approach, DNA was directly extracted from uncultured samples followed by cloning and expression [3]. For example, by combination of directed evolution with the metagenome approach, an $\alpha$-amylase mutant with optimal activity at pH 4.5 and optimal thermostability at 105 °C was discovered for starch liquefaction and EtOH production [4].

Sequence-based discovery (genome hunting) is increasingly attractive, as the public sequence bank is growing rapidly. In this approach, known sequences encoding an enzyme of interest are used to search gene databases to uncover enzymes of homologous sequences. For example, using this method, a library of deoxyribose-phosphate aldolases (DERAs) were rapidly constructed and from them a novel DERA was identified to catalyze a sequential aldol reaction of a nonnative substrate with high throughput and excellent stereoselectivity for the synthesis of statin side chains [5].

Since most synthetic applications require enzymes catalyzing nonnatural substrates, their properties often have to be improved. One way to achieve this is to optimize reaction conditions such as pH, temperature, solvents, additives, etc. [6–9]. Another way is to modulate the substrates without compromising the synthetic efficiency of the overall reaction [10]. In most cases for commercial manufacturing, however, the protein sequences have to be altered to enhance reactivity, stereoselectivity and stability. It was estimated that over 30 commercial enzymes worldwide have been engineered for industrial applications [11]. Precise prediction of which amino acids to mutate is difficult to achieve. Since the mid 1990s, directed evolution

has been demonstrated to be a powerful and robust technology to improve the desired properties [12,13]. Among them, the error-prone PCR method is probably the most popular to create random mutants by changing polymerization reaction conditions [14]. Alternatively, recombination of homologous sequences or DNA-shuffling methods can be used to introduce mutants with improved properties [15]. The major challenge in directed evolution is not generation of mutant libraries; rather, it is the availability of high-throughput assays [16]. In most cases, it requires screening of tens of thousands of mutants, which is usually tedious and time consuming. As more and more protein structures are available from the protein database, focused directed evolution or semi-rational protein design is becoming more and more popular [17]. In this approach, the three-dimensional (3-D) structure of a suboptimal enzyme is constructed by a computer algorithm from a homologous enzyme with known 3-D structure. Docking studies are then applied to search potential 'hot spots', which are then swapped with other amino acids by site-saturation mutagenesis. In this way, there are generally less than a few thousand mutants to be screened, significantly shortening the cycle of enzyme development. The fact that most beneficial mutations are proved to be near the active site makes this approach even more attractive [18].

## 1.4 Enzyme Production

Although some enzymes are still extracted from animal or plant tissue, most of them are now produced from microorganisms by fermentation. Bacteria and fungi are the most popular hosts for producing industrial enzymes, due to easy handling and high productivity. They can also be readily genetically engineered to improve their performance; for example, by incorporating secretion systems to facilitate enzyme isolation and purification. Some of the most popular expression hosts are *Escherichia coli*, *Pichia pastoris*, *Pseudomonas fluorescens*, *Aspergillus* sp. and *Bacillus* sp. Mammalian or plant cells are used in special cases [19–21]. By regulation, the production host should have GRAS status (Generally Regarded as Safe Status).

In a typical enzyme production procedure, cells containing genes encoding desired enzymes are grown in an Erlenmeyer flask. At large scale, a computer-controlled fermenter or bioreactor is required to maintain an appropriate control of pH, $O_2$, $NH_3$ and $CO_2$ to maximize cell density. The cells are harvested by centrifugation in a batch or continuous fashion. Alternatively, they can be collected through membrane filtration devices. The cell membranes are then disrupted by an ultrasonicator or French press at small scale. At a scale of over 5–10 L, a homogenizer is usually used. After centrifugation to remove cell debris, the crude enzymes remain in the supernatant and can be concentrated through precipitation by adding either inorganic salts (e.g. ammonium sulfate) or organic solvents (e.g. acetone). The crude enzymes are then purified by dialysis or a variety of chromatographic methods. The dry powder is usually obtained after lyophilization under freeze-drying conditions [22,23].

## 1.5 Enzymes and Synthetic Applications

Historically, the most popular enzymes used for chemical synthesis are lipases, esterases, proteases, acylases and amidases, among others. Recently, a number of recombinant biocatalysts have been discovered and isolated, significantly expanding the toolbox for biotransformations. In this section, the focus will be on these new enzymes.

### 1.5.1 Ketoreductases (EC 1.1.1.2)

Ketoreductases (KREDs) catalyze the conversion of a wide range of ketones and some aldehydes to chiral alcohols regio- and stereo-selectively in the presence of NADH or NADPH (Figure 1.1) [24,25]. This powerful transformation has been demonstrated in a number of industrial transformations using either isolated enzymes or whole cells. The use of isolated enzymes is often preferred because of a higher volumetric productivity and the absence of side reactions. A key to its success is the availability of efficient and cost-effective cofactor regeneration methods by using a formate dehydrogenase to recycle $NAD^+$ or a glucose dehydrogenase to recycle $NADP^+$ (Figure 1.1) [26,27]. It shall be noted that some alcohol dehydrogenases are also able to catalyze the oxidation of alcohols to ketones or aldehydes [28].

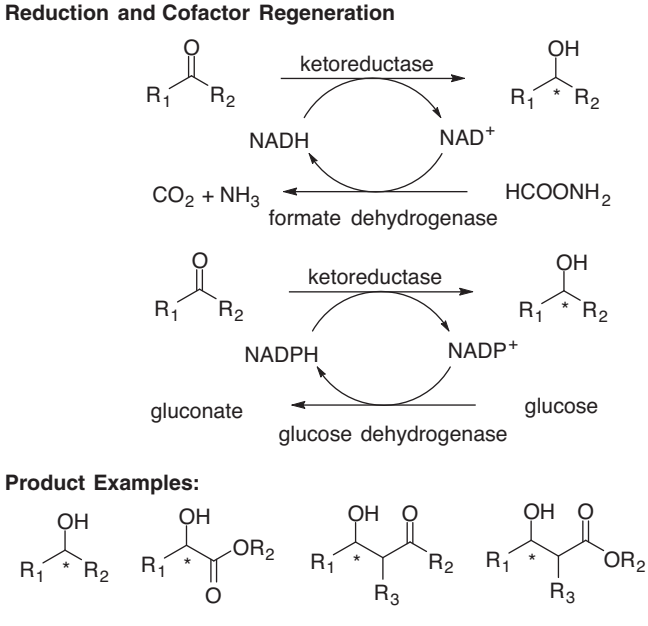


**Figure 1.1**

### 1.5.2 Enoate Reductases or Ene Reductases (EC 1.3.1.16)

Enoate reductase (ER) catalyzes NAD(P)H-dependent reduction of carbon–carbon double bonds of nonactivated enoates, as well as of $\alpha,\beta$-unsaturated aldehydes, ketones, nitros, and nitriles (Figure 1.2). For example, the ER from *Clostridium tyrobutyricum* shows high stereospecificity and regioselectivity and broad substrate specificity [29]. Alkanes with up to two chiral centers can be directly produced by asymmetric reduction of electron-deficient alkene enzymes from the 'old yellow enzyme' family at the expense of NAD(P)H. The cofactor can be regenerated *in vitro* using a formate dehydrogenase or glucose dehydrogenase. Alternatively, a whole-cell system can be used to co-express ERs with redox enzymes for NAD(P)H recycling [30].

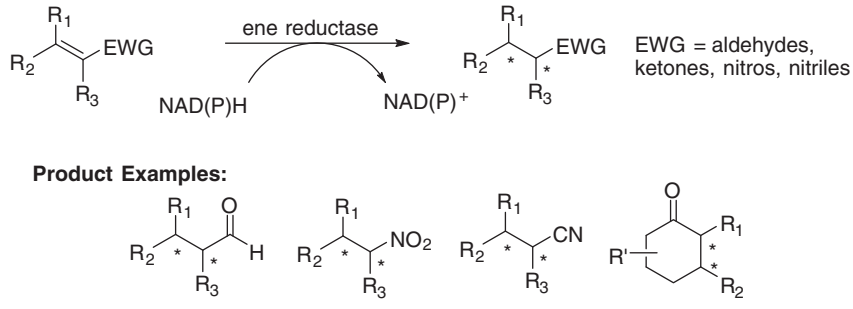


**Figure 1.2**

## *1.5.3 Oxygenases (EC. xxxx)*

Oxygenases catalyze direct incorporation of molecular oxygen into substrates to produce oxygenated molecules [31,32]. They are categorized as either monooxygenases (MOs) or dioxygenases, depending upon whether one or both atoms of dioxygen are inserted into a substrate. The metal-dependent MOs, such as P450s, catalyze a wide range of reactions via metal oxo species (e.g. hydroxylation of alkanes and aromatics; epoxidation of alkenes (Figure 1.3)), while flavin adenine dinucleotide (FAD)-dependent MOs are found to catalyze oxidation of heteroatoms (S, N, Se, P) and Baeyer–Villiger reactions via FAD-hydroperoxide (FAD-OOH) species (Figure 1.4).

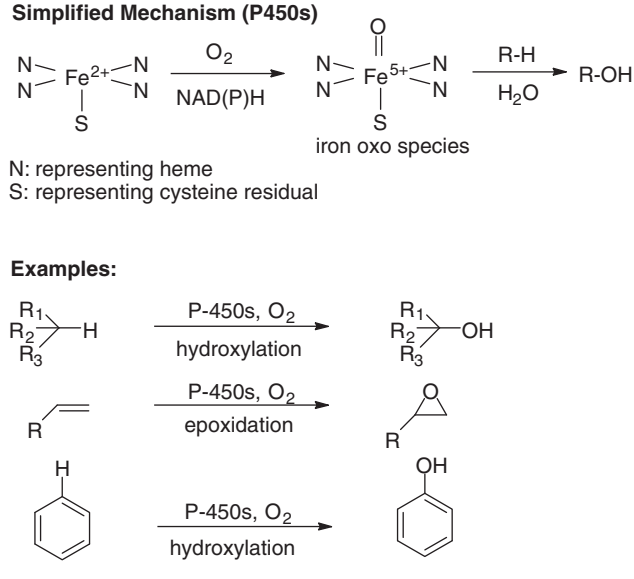


**Figure 1.3**

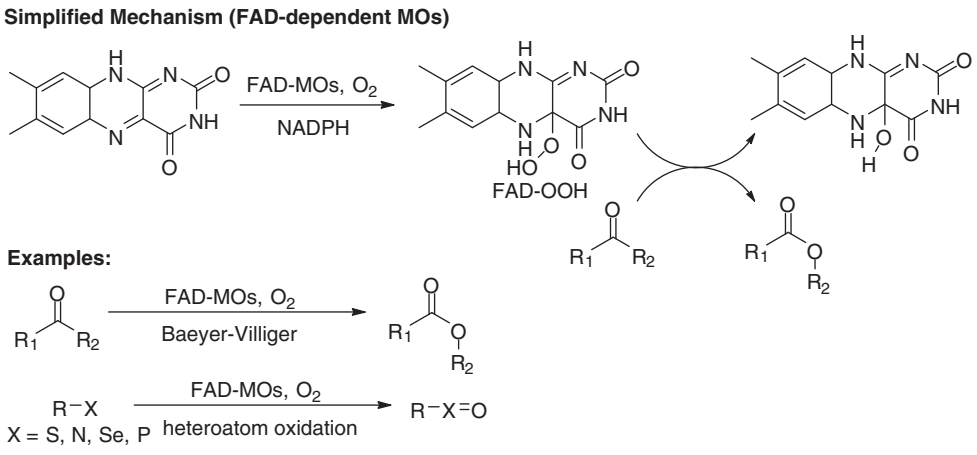


**Figure 1.4**

Dioxygenases usually contain a tightly bound iron atom and catalyze hydroperoxidation of allylic molecules or carboxylic acids, and dihydroxylation of aromatics (Figure 1.5) [33].

Currently, these oxygenation reactions are usually carried out in whole cells, the outcome of which is often unpredictable. The discovery of novel oxygenases and efficient hosts for protein expression remain keys to further expanding the applications of these enzymes in chemical synthesis and drug metabolism studies [34–37].

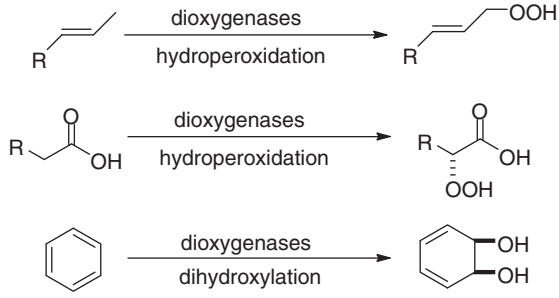


**Figure 1.5**

## *1.5.4 Alcohol Oxidases (EC 1.1.3.X)*

Alcohol oxidases (AOs) catalyze oxidation of alcohols to aldehydes or ketones in the presence of molecular oxygen, with hydrogen peroxide being the usual by-product (Figure 1.6). Some of the most well-studied AOs are cholesterol oxidases, short-chain aliphatic alcohol oxidases, aromatic alcohol oxidases, pyranose oxidases, glycolate oxidases, glucose oxidases, galactose oxidases and nucleoside oxidases [38–40]. Cholesterol oxidases catalyze oxidation of allylic alcohol in the cholesterol scaffolds [41]. The regeneration of cofactor FAD is relatively easier, as it is tightly bound. Although these enzymes use oxygen, they can also be deactivated by oxygen or the hydrogen peroxide by-product. It is to be noted that peroxidases and cholorperoxidases can also catalyze the oxidation of alcohols using hydrogen peroxide ($H_2O_2$).

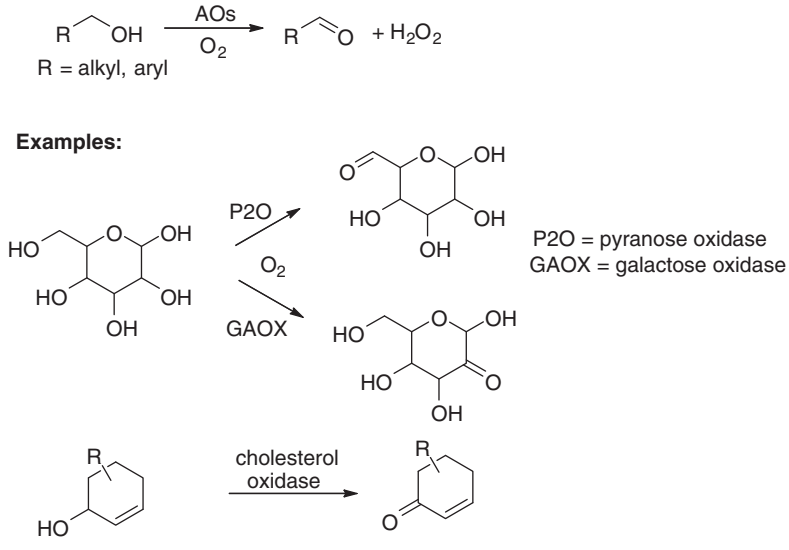


**Figure 1.6**

## *1.5.5 Peroxidases (EC 1.11.1.X)*

Peroxidases utilize $H_2O_2$ as the oxidant (Figure 1.7). The active site of peroxidases may involve a heme unit (horseradish peroxidase), selenium (glutathione peroxidase), vanadium (bromoperoxidase) and manganese (manganese peroxidase). These enzymes catalyze a wide range of oxidations, including hydroxylation of arenes, oligomerization of phenols and aromatic amines, epoxidation and halogenation of olefins, oxygenation of heteroatoms and reduction of hydroperoxides [42–44].

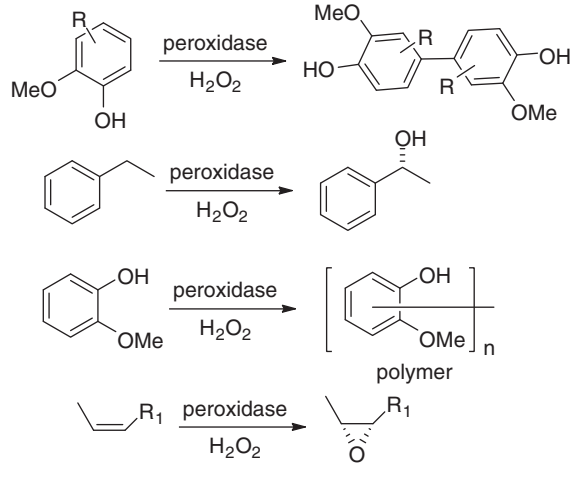


**Figure 1.7**

### *1.5.6 Halogenases (EC. xxxx)*

Halogenases catalyze regio- and stereo-selective halogenation (Figure 1.8) [45,46]. For electron-rich substrates, nature often uses flavin-dependent halogenases for chlorination, bromination or iodination via FADH-OX (X = halide) as the halogenation agent (Figure 1.9). For electron-deficient molecules such as alkanes, mononuclear iron halogenases are utilized through a radical mechanism (Figure 1.9). Fluorinases adopt an $S_N2$ nucleophilic substitution

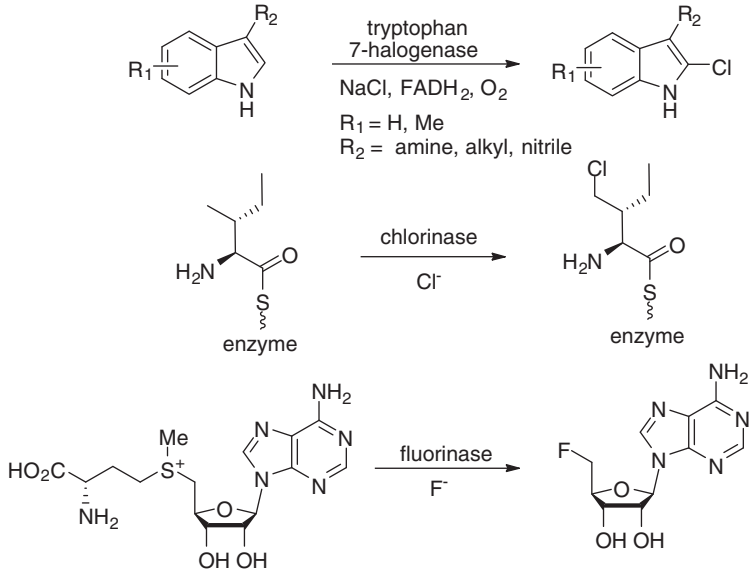


**Figure 1.8**

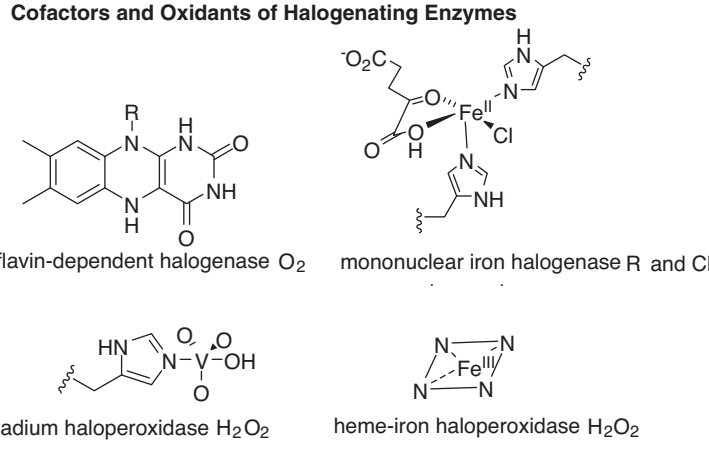


**Figure 1.9**

mechanism in the presence of $F^-$ to introduce a fluorine atom [47]. While it is still in its infancy, the use of enzymatic halogenation has shown great promise, especially in whole-cell systems (Figure 1.9). For example, tryptophan 7-halogenase was able to catalyze regioselective halogenation of a wide range of indole derivatives and aromatic heterocycles [48]. It is to be noted that halogenation can also be catalyzed by haloperoxidases, which often gives poor regio- and stereo-selectivity, since the activated halogen source, (e.g. hypohalous acids) is freely diffusible within and away from enzymes (Figure 1.8) [49].

## *1.5.7 Nitrilases (EC 3.5.5.1)*

Nitrilases convert nitriles to the corresponding carboxylic acids and $NH_3$ through a cysteine residue in the active site [50]. Because of their high enantio- and regio-selectivity, nitrilases are attractive as 'green' catalysts for the synthesis of a variety of carboxylic acids and derivatives (Figure 1.10) [51,52]. Recently, a number of recombinant nitrilases have been cloned and characterized heterologously for synthetic applications [50,53,54].

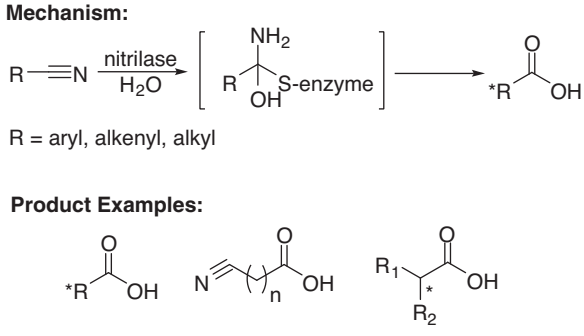


**Figure 1.10**

## *1.5.8 Nitrile Hydratases (EC 4.2.1.84)*

Nitrile hydratase (NHase) catalyzes the hydration of nitriles to amides (Figure 1.11) and has been used for production of acrylamide and nicotinamide at large scale. NHases are roughly

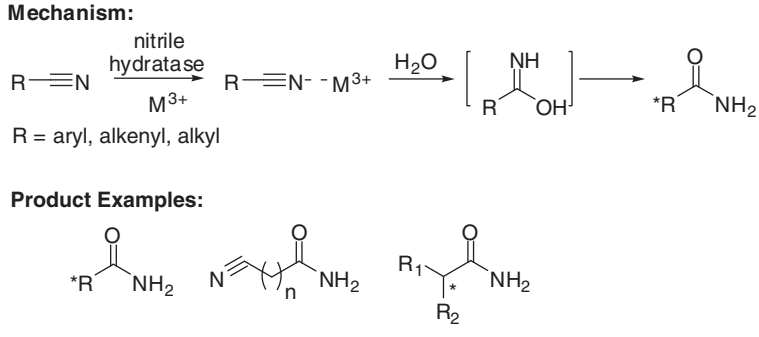


**Figure 1.11**

classified into iron and cobalt types according to the metal involved in the active site [55,56]. Recent elucidation of the catalytic mechanism and characterization of a number of NHases have led to a wide range of applications in both biotransformation and bioremediation [53].

### 1.5.9 Epoxide Hydrolases (EC 3.3.2.X)

Epoxide hydrolases (EHs) catalyze the hydrolysis of a wide range of epoxides (Figure 1.12). They are cofactor independent and robust for the synthesis of enantiopure epoxides, diols and their derivatives [57–59]. There are over 100 epoxide hydrolase gene sequences, and the X-ray structures are available for fungal, bacterial and mammalian epoxide hydrolases [60]. Heterologous expression in *E. coli* and other hosts has also been successful. In addition, several efficient high-throughput screening methods have been developed, allowing the improvement of EHs through site-directed mutagenesis and directed evolution [61,62]. EHs also play important roles in the detoxification of genotoxic compounds and drug metabolism [63].

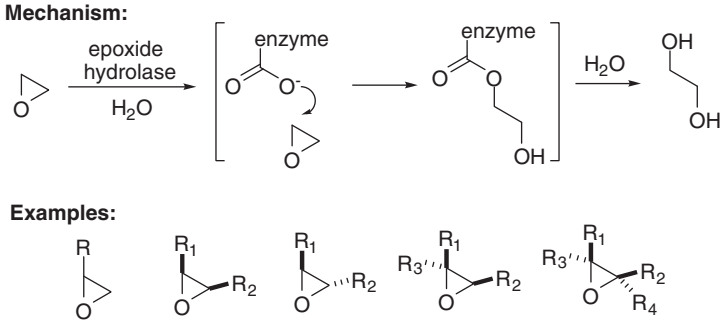


**Figure 1.12**

### 1.5.10 ω-Transaminases (EC 2.6.1.X)

ω-Transaminases catalyze the conversion of a ketone group to an amine in the presence of the cofactor pyridoxal-5′-phosphate (PLP), which is tightly bound to the active site. The catalysis starts with the formation of an imine between the cofactor and an amine donor (Figure 1.13) [64]. Since this is a one-step method to prepare (*R*)- or (*S*)-amines from ketones, it has huge potential (Figure 1.14) [65,66]. For example, a high-throughput biocatalytic process to (*S*)-methoxyisopropylamine, a moiety common to the two important chloroacetamide herbicides metolachlor and dimethenamid, has been reported by enzymatic transamination of methoxyacetone using isopropylamine as the donor [67].

### 1.5.11 Hydroxynitrile Lyases (EC 4.1.2.X)

Hydroxynitrile lyases (HNLs or oxynitrilases) catalyze C−C bond-forming reactions between an aldehyde or ketone and cyanide to form enantiopure cyanohydrins (Figure 1.15), which are versatile building blocks for the chiral synthesis of amino acids, hydroxy ketones, hydroxy acids, amines and so on [68]. Screening of natural sources has led to the discovery of both

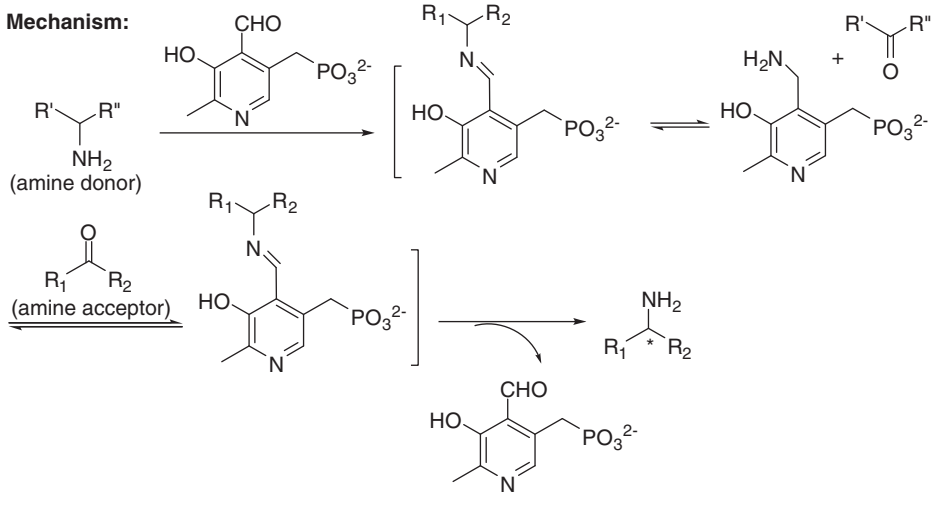


**Figure 1.13**

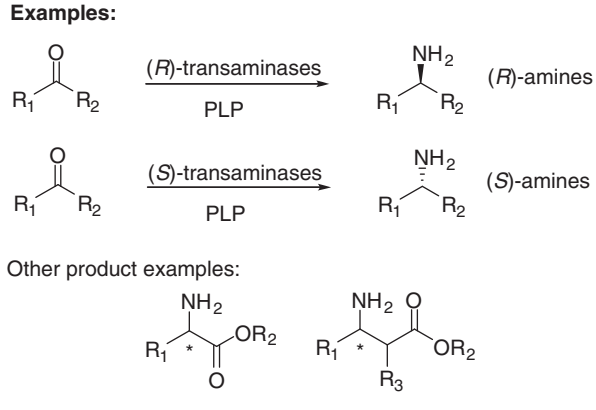


**Figure 1.14**

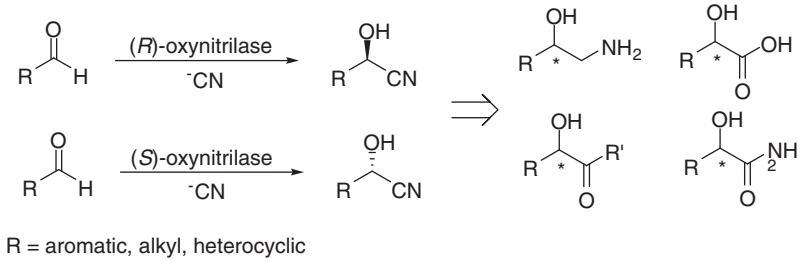


**Figure 1.15**

(*R*)- and (*S*)-selective HNLs. A number of recombinant HNLs have also been expressed in *E. coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris*. Recently, protein engineering has been successfully applied to the development of a tailor-made HNL for large-scale production of specific cyanohydrins [69,70].

## *1.5.12 Aldolases (EC. xxxx)*

Aldolases catalyze asymmetric aldol reactions via either Schiff base formation (type I aldolase) or activation by $Zn^{2+}$ (type II aldolase) (Figure 1.16). The most common natural donors of aldoalses are dihydroxyacetone phosphate (DHAP), pyruvate/phosphoenolpyruvate (PEP), acetaldehyde and glycine (Figure 1.17) [71]. When acetaldehyde is used as the donor, 2-deoxyribose-5-phosphate aldolases (DERAs) are able to catalyze a sequential aldol reaction to form 2,4-didexoyhexoses [72,73]. Aldolases have been used to synthesize a variety of carbohydrates and derivatives, such as azasugars, cyclitols and densely functionalized chiral linear or cyclic molecules [74,75].

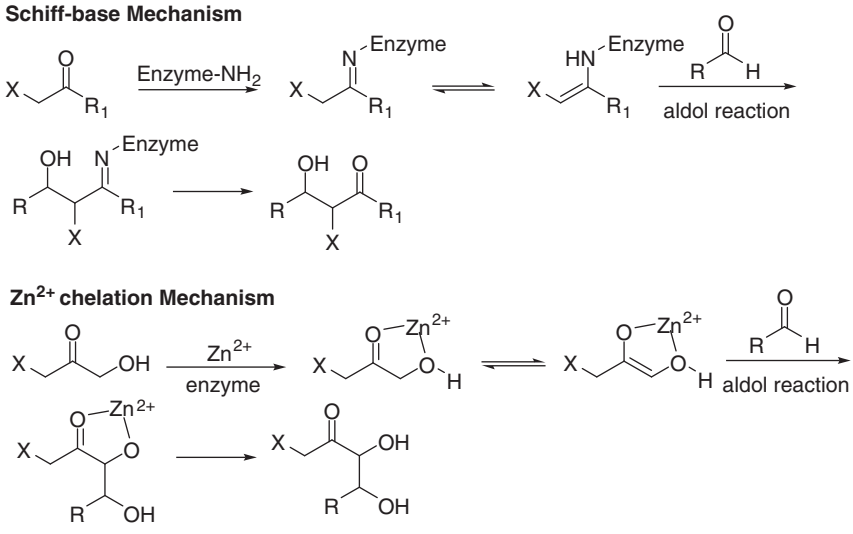


**Figure 1.16**

## *1.5.13 Glycosidases (EC. xxxx)*

Glycosidases catalyze the hydrolysis of glycosidic linkages via an oxonium intermediate or transition state similarly to acid-catalyzed hydrolysis of glycosides under either a retention of the configuration at the anomeric center (Mechanism, Figure 1.18) or less common inversion. The oxonium is presumably stabilized by a carboxylate group such as glutamic acid, a common structural motif in the active site of glycosidases [76]. Glycosidase-catalyzed synthesis of glycosides can be achieved under either equilibrium-controlled conditions or kinetic-controlled conditions [77–79]. In the former case, the reaction is established to shift the equilibrium toward a product; for example, by adding organic solvents. In the latter case, activated glycosyl donors are

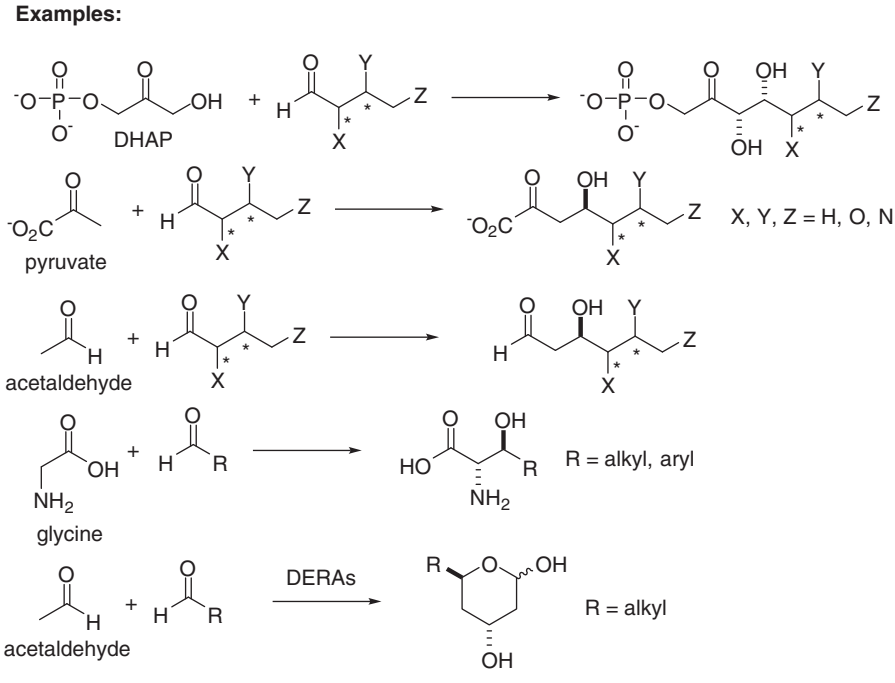


**Figure 1.17**

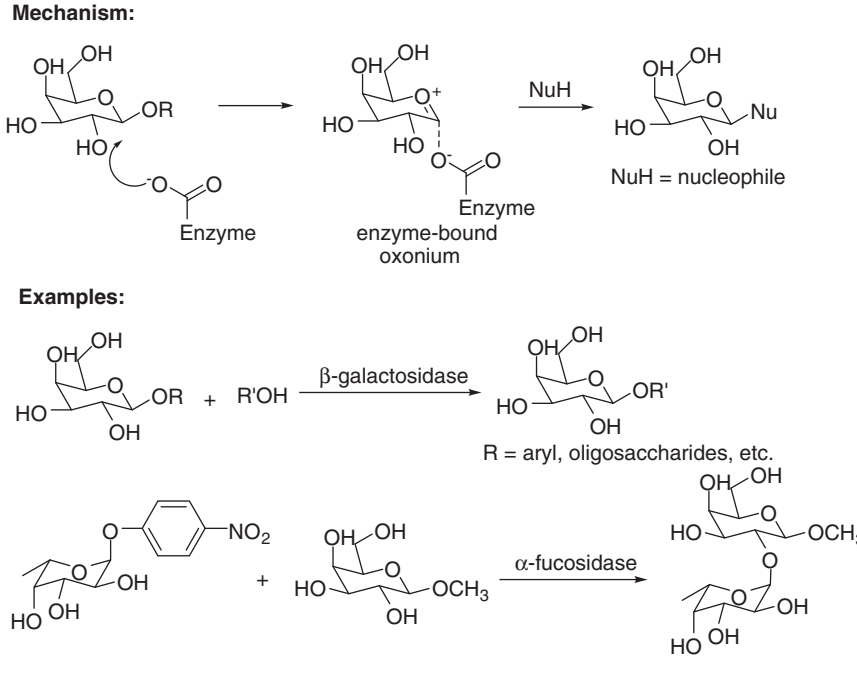


**Figure 1.18**

used, which include di- or oligo-saccharides, aryl glycosides, glycosyl fluorides and so on [80]. Owing to greater promiscuity toward donors and wide availability, these enzymes were found to have a wide range of applications in preparation of carbohydrates and derivatives [81–83].

### *1.5.14 Glycosyltransferase (EC. xxxx)*

Glycosyltransferases (Gtfs) accept activated sugars such as uridine diphosphate (UDP) nucleotide sugars or glycosyl phosphates as monosaccharide donors under either a retention or inversion of the configuration at the anomeric center (Mechanism, Figure 1.19; only inversion scenario shown). Recently, a number of Gtfs have been discovered to be quite promiscuous, and used to synthesize many oligosaccharides, their derivatives or glycosylated natural products, which are otherwise difficult to obtain [84–87]. For large-scale applications, the main issue to be overcome is to recycle released nucleotide monophosphate (NMP) or nucleotide diphosphate (NDP), which are expensive. Several methods have been reported [88,89], and one utilizes sugar nucleotide pyrophosphorylase, which transfers the sugar moiety of a sugar phosphate to a free uridine triphosphate (UTP) to regenerate the desired UTP-sugar (Figure 1.19) [90]. The availability of a wide range of Gtfs and sugar donors also provides a general strategy to synthesize glycosylated molecules *in vivo* through pathway engineering and combinatorial biosynthesis. For example, novel macrocyclic polyketides have been produced by applying a promiscuous Gtf from picromycin biosynthesis, which accepts a wide range of sugar donors (Figure 1.19) [91,92].

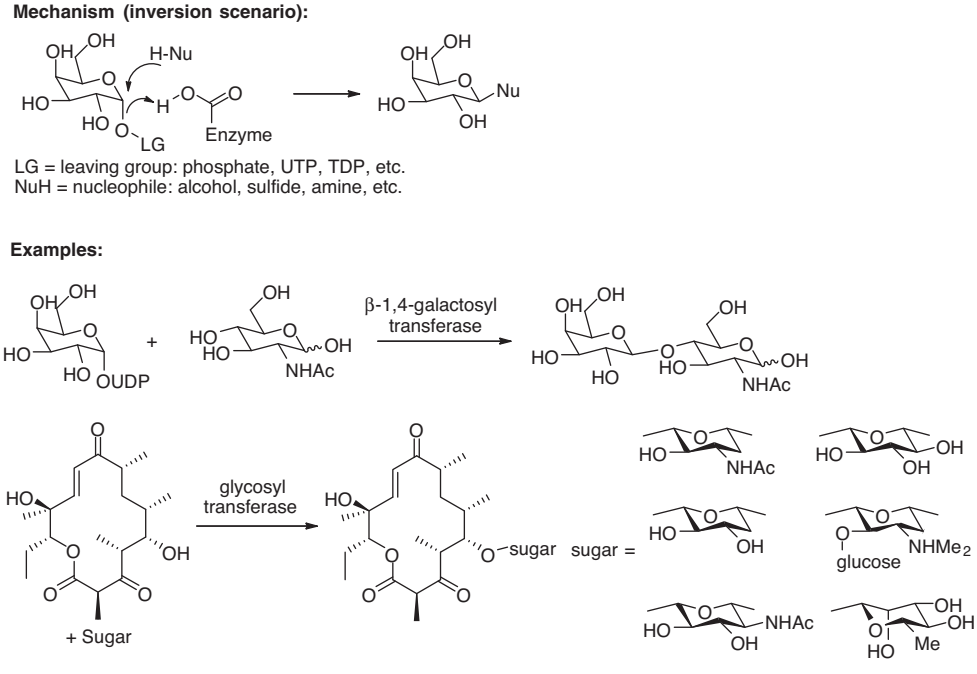


**Figure 1.19**

## 1.6 Conclusions

Biocatalysis has been practiced historically mostly by whole-cell systems, which limits its applications due to low throughput and complex *in vivo* pathways. As a result of recent advances in genomics and high-throughput screening, more and more diverse recombinant enzymes are available in catalogs. Subsequently, a number of them have been successfully applied to the commercial production of nonnatural molecules. More recently, biocatalysis is emerging to be one of the greenest technologies for chemical synthesis [93,94]. Specifically, biocatalysis can prevent waste generation by using catalytic processes with high stereo- and region-selectivity, prevent or limit the use of hazardous organic reagents by using water as the green solvent, design processes with higher energy efficiency and safer chemistry by conducting reactions at room temperature under ambient atmosphere, and increase atom economy by avoiding extensive protection and deprotection to maximize the use of renewable feedstock designed for degradation.

Enzymes can catalyze transformations which are difficult to achieve by traditional chemical methods. To truly realize the promise of this emerging technology for the pharmaceutical industry, it is essential to integrate biocatalysis into drug discovery, development and manufacturing. For drug production, the key relies on integration of enzymatic transformations with modern chemical research and development at the retrosynthetic level to deliver efficient and practical synthetic sequences with fewer synthetic steps and significantly reduced waste streams [95,96].

## References


[1] Tipton, K. and Boyce, S. (2000) History of the enzyme nomenclature system. *Bioinformatics*, **16**, 34–40.

[2] Straathof, A.J., Panke, S. and Schmid, A. (2002) The production of fine chemicals by biotransformations. *Current Opinion in Biotechnology*, **13**, 548–556.

[3] Langer, M., Gabor, E.M., Liebeton, K. *et al.* (2006) Metagenomics: an inexhaustible access to nature's diversity. *Biotechnology Journal*, **1**, 815–821.

[4] Mathur, E.J., Toledo, G., Green, B.D. *et al.* (2005) A biodiversity-based approach to development of performance enzymes: applied metagenomics and directed evolution. *Industrial Biotechnology*, **1**, 283–287.

[5] Hu, S., Tao, J. and Xie, Z. Chemoenzymic synthesis of atorvastatin. WO2006134482.

[6] Liu, Y.-Y., Xu, J.-H., Xu, Q.-G. and Hu, Y. (1999) Significant enhancement of lipase enantioselectivity toward (*S*)-ketoprofen ester at pH 2. *Biotechnology Letters*, **21**, 143–146.

[7] Xu, T.-W., Xu, J.-H., Yu, W. and Zhong, J.-H. (2006) Investigating pH and Cu(II) effects on lipase activity and enantioselectivity via kinetic and spectroscopic methods. *Biotechnology Journal*, **1**, 1293–1301.

[8] Liu, Y.-Y., Xu, J.-H. and Hu, Y. (2000) Enhancing effect of Tween-80 on lipase performance in enantioselective hydrolysis of ketoprofen ester. *Journal of Molecular Catalysis B: Enzymatic*, **10**, 523–529.

[9] Gong, P.-F., Xu, J.-H, Tang, Y.-F. and Wu, H.-Y. (2003) Improved catalytic performance of *Bacillus megaterium* epoxide hydrolase in a medium containing Tween-80. *Biotechnology Progress*, **19**, 652–654.

[10] Tao, J., Zhao, L. and Ran, N. (2007) Recent advances in developing chemoenzymatic processes for active pharmaceutical ingredients. *Organic Process Research Development*, **11**, 259–267.

[11] Eggleston, G. and Vercellotti, J.R. (eds) (2007) *Industrial Applications of Enzymes on Carbohydrate-based Materials*, ACS Symposium Series 6, Oxford University Press.

[12] Bloom, J.D., Meyer, M.M., Meinhold, P. *et al.* (2005) Evolving strategies for enzyme engineering. *Current Opinion in Structural Biology*, **15**, 447–452.

[13] Reetz, M.T. (2007) Directed evolution of enzymes for asymmetric syntheses, in *Asymmetric Synthesis* (eds Christmann M. and Braese S.), Wiley–VCH, pp. 207–211.

[14] Eggert, T., Jaeger, K.-E. and Reetz, M.T. (2004) Directed evolution of random mutagenesis: a critical evaluation, in *Enzyme Functionality* (ed. A. Svendsen), Marcel Dekker, Inc., New York, pp. 375–390.

[15] Kagami, O., Baik, S.-H. and Harayama, S. (2004) Effective DNA shuffling methods for enzyme evolution, in *Enzyme Functionality* (ed. A. Svendsen), Marcel Dekker, Inc., New York, pp. 425–441.
[16] Arnold, F.H. and Georgiou, G. (eds) (2003) *Directed Enzyme Evolution: Screening and Selection Methods*, Methods in Molecular Biology, vol. **230**, Humana Press, Inc., Clifton, NJ.
[17] Lutz, S. and Patrick, W.M. (2004) Novel methods for directed evolution of enzymes: quality, not quantity. *Current Opinion in Biotechnology*, **15**, 291–297.
[18] Morley, K.L. and Kazlauskas, R.J. (2005) Improving enzyme properties: when are closer mutations better? *Trends in Biotechnology*, **23**, 231–237.
[19] Macauley-Patrick, S., Fazenda, M.L., McNeil, B. and Harvey, L.M. (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast*, **22**, 249–270.
[20] Sorensen, H.P. and Mortensen, K.K. (2005) Advanced genetic strategies for recombinant protein expression in Escherichia coli. *Journal of Biotechnology*, **115**, 113–128.
[21] Nohr, J., Kristiansen, K. and Krogsdam, A.-M. (2003) *Expression of Recombinant Proteins: An Introduction, in Methods in Molecular Biology*, vol. **232**, Humana Press, Inc., Clifton, NJ, pp. 93–101.
[22] Gellissen, G. (ed.) (2005) *Production of Recombinant Proteins*, Wiley–VCH.
[23] Scopes, R.K. (ed.) (1994) *Protein Purification*, 3rd edn, Springer-Verlag.
[24] Goldberg, K., Schroer, K., Luetz, S. and Liese, A. (2007) Biocatalytic ketone reduction – a powerful tool for the production of chiral alcohols – part I: processes with isolated enzymes. *Applied Microbiology and Biotechnology*, **76**, 237–248.
[25] Yang, W., Xu, J.-H., Xie, Y. *et al.* (2006) Asymmetric reduction of ketones by employing *Rhodotorula* sp. AS2.2241 and synthesis of the β-blocker (*R*)-nifenalol. *Tetrahedron: Asymmetry*, **17**, 1769–1774.
[26] Husain, M. and Husain, Q. (2008) Applications of redox mediators in the treatment of organic pollutants by using oxidoreductive enzymes: a review. *Critical Reviews in Environmental Science and Technology*, **38**, 1–42.
[27] Goldberg, K., Schroer, K., Luetz, S. and Liese, A. (2007) Biocatalytic ketone reduction – a powerful tool for the production of chiral alcohols – part II: whole-cell reductions. *Applied Microbiology and Biotechnology*, **76**, 249–255.
[28] Kroutil, W., Mang, H., Edegger, K. and Faber, K. (2004) Biocatalytic oxidation of primary and secondary alcohols. *Advanced Synthesis & Catalysis*, **346**, 125–142.
[29] Steinbacher, S., Stumpf, M., Weinkauf, S. *et al.* (2002) Enoate reductase family. In Flavins and Flavoproteins (2002), Proceedings of the 14th International Symposium, Cambridge, United Kingdom, July 14–18, pp. 941–949.
[30] Stuermer, R., Hauer, B., Hall, M. and Faber, K. (2007) Asymmetric bioreduction of activated C:C bonds using enoate reductases from the old yellow enzyme family. *Current Opinion in Chemical Biology*, **11**, 203–213.
[31] Holland H.L. (1999) C−H activation. *Current Opinion in Chemical Biology*, **3**, 22–27.
[32] Que, L. Jr. (2000) One motif–many different reactions. *Nature Structural Biology*, **7**, 182–184.
[33] Emerson, J.P., Farquhar, E.R. and Que, L. Jr. (2007) Structural 'snapshots' along reaction pathways of non-heme iron enzymes. *Angewandte Chemie, International Edition*, **46**, 8553–8556.
[34] Yun, C.-H., Yim, S.-K., Kim, D.-H. and Ahn, T. (2006) Functional expression of human cytochrome P450 enzymes in *Escherichia coli*. *Current Drug Metabolism*, **7**, 411–429.
[35] Celik, A., Flitsch, S.L. and Turner, N.J. (2005) Efficient terpene hydroxylation catalysts based upon P450 enzymes derived from actinomycetes. *Organic & Biomolecular Chemistry*, **3**, 2930–2934.
[36] Fujita, K.-I. and Kamataki, T. (2002) Genetically engineered bacterial cells co-expressing human cytochrome P450 with NADPH-cytochrome P450 reductase: prediction of metabolism and toxicity of drugs in humans. *Drug Metabolism and Pharmacokinetics*, **17**, 1–22.
[37] Uno, T., Nakao, A., Masuda, S. *et al.* (2006) Modification of small molecules by using cytochrome P450 expressed in *Escherichia coli*. *Journal of Industrial Microbiology & Biotechnology*, **33**, 1043–1050.
[38] Kroutil, W., Mang, H., Edegger, K. and Faber, K. (2004) Biocatalytic oxidation of primary and secondary alcohols. *Advanced Synthesis & Catalysis*, **346**, 125–142.
[39] Kumar, A.K. and Goswami, P. (2006) Functional characterization of alcohol oxidases from *Aspergillus terreus* MTCC 6324. *Applied Microbiology and Biotechnology*, **72**, 906–911.
[40] Overhage, J., Steinbuechel, A. and Priefert, H. (2006) Harnessing eugenol as a substrate for production of aromatic compounds with recombinant strains of *Amycolatopsis* sp. HR167. *Journal of Biotechnology*, **125**, 369–376.