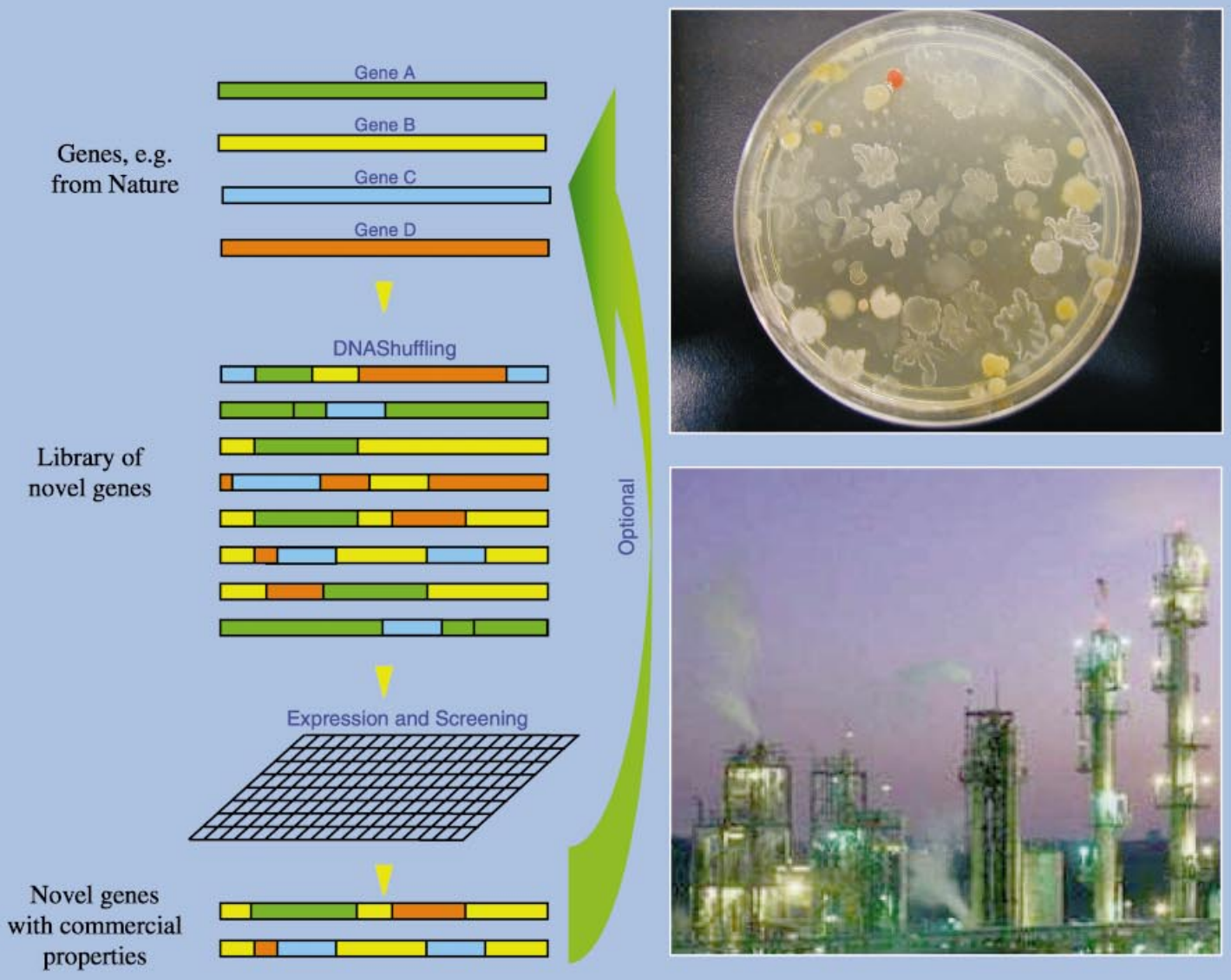

Directed Evolution and Biocatalysis

Keith A. Powell,* Sandra W. Ramer, Stephen B. del Cardayré, Willem P. C. Stemmer, Matthew B. Tobin, Pascal F. Longchamp, and Gjalt W. Huisman

This review describes the current state of biocatalysis in the chemical industry. Although we recognize the advantages of chemical approaches, we suggest that the use of biological catalysis is about to expand dramatically because of the recent developments in the artificial evolution of genes that code for enzymes. For the first time it is possible to consider the rapid development of an enzyme that is designed for a specific chemical reaction. This technology offers the opportunity to adapt the enzyme to the needs of the process. We describe herein the development of enzyme evolution technology and particularly DNA shuffling. We also consider several classes of enzymes, their current applications, and the limitations that should be addressed. In a review of this length it is impossible to describe all the enzymes with potential for industrial exploitation; there are other classes, which given appropriate activity, selectivity, and robustness, could become useful tools for the industrial chemist. This is an exciting era for biocatalysis and we expect great progress in the future.

Keywords: DNA shuffling · directed evolution · enzyme catalysis · enzyme libraries · proteins

1. Introduction

Most reviews on biocatalysis start with the assumption that chemistry is waiting for inherently better biocatalytic processes. On the other hand, chemistry has effectively delivered compounds from drugs to domestic cleaning products without the help of biocatalysts for 200 years. Thus there is no reason to believe that chemistry should not be as successful for centuries to come. Since the great advantages of chemistry are simplicity, speed (increased by the use of heat and pressure), and low cost, why should biocatalysts be of interest? These advantages are irrelevant when the product required needs regio- or stereospecific reactions in which the starting material or product is labile, or in which side products are problematic contaminants. Biocatalysts are attractive because of their exquisite chemo-, regio-, and stereoselectivity, their impressive catalytic efficiency, and their reactivity in aqueous media. Although these characteristics can be useful, they can also limit the exploitation of biocatalysts. The last twenty years have seen significant commercial advances in biocatalysis (Figure 1). Some of these processes are spectacularly successful on a large scale. What is it that makes these processes successful?

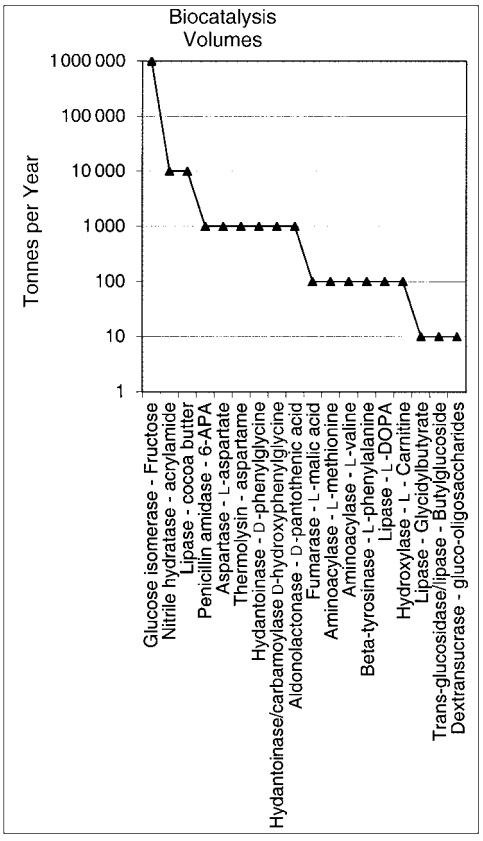

Figure 1. Commercial compounds currently produced in biocatalytic processes.

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- The enzyme existed in a natural isolate with an activity that is close to that required for a commercial process.
- The enzyme could be immobilized with retention of activity, or the activity was such that a single use was still economically viable.
- No cofactors were required for enzyme activity, or whole cells could be used to provide the activity without any significant side reactions.

If some reactions can be run commercially with enzymes, then what limits the broader use of biocatalysis (Table 1)?

Table 1. Current limitations of biocatalysis that need to be addressed.

Limited substrate range
Limited stability to temperature, pH, solvent
Limited enantioselectivity
Limited lifetime
Limited turnover number

- The enzyme may not exist in nature or it may be difficult to procure or produce.
- The properties of the enzyme may be unsuitable for chemical synthesis.
- The activity may be insufficient.
- The enzyme may be inhibited by the substrate or the product, it may not work well under suitable operating temperatures or pH values, or it may simply be too labile for effective industrial use.
- The reaction may require an expensive cofactor that cannot be effectively recycled.

What, then, is the future of enzyme-based chemistry?

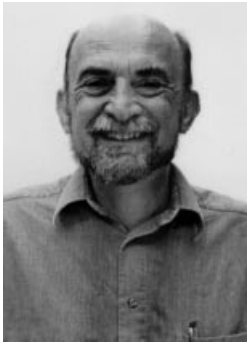
There are now examples of enzymes that have been isolated and then modified into effective biocatalysts.[1–3] Recent advances have shown that directed evolution techniques can be used to develop enzymes that are modified to fit the demands of the chemical process. Whereas in the past commercial enzymes were often tested and, when appropriate, rejected for specific reactions, they can now be selected from a library of related enzymes with a range of properties: stability over a temperature range, increased activity, enantioselectivity, novel substrate activity, or compatibility under different physical and chemical conditions. The combination of flexibility in enzyme characteristics and a rapid approach to the generation of this flexibility has given rise to a new paradigm in the development of biocatalytic processes.

In this review we will describe some of the advances in enzyme improvement technology, as well as some classes of biocatalysts, the current state of the art, and the future potential for improved biocatalysis. We focus primarily on hydrolytic and oxidative enzymes. The very successful application of other enzyme classes will not be discussed. However these biocatalysts are also prime targets for improvement through directed evolution.

2. Genetic Approaches to Improved Biocatalysis

All the beautifully complex biological structures and sequences that Nature has provided us with have been designed by a single process, that of natural evolution. However, natural evolution has selected the most suitable enzymes that ensure the survival of the organism in its natural environment. No matter how extreme that natural environment, the enzyme is unlikely to be ideal for a chemical process. Enzymes have been successfully improved for many years by mutation and selection, but because of the long and slow nature of this process, it has not been possible to rapidly develop catalysts with novel properties in the timescale acceptable to process chemists. Many improvements have been made to this technology, and a recent review describes the resulting biocatalytic processes.[4] Existing approaches to improve single proteins or parts thereof include modeling-based point mutagenesis, cassette library mutagenesis (including random, biased, saturation, and codon-based cassette mutagenesis), and random point mutagenesis (including error-prone polymerase chain reaction (PCR), mutator strains, and UV or chemical mutagenesis), which introduce (partially) random mutations either into a selected small region of a gene or throughout a gene.

Molecular biologists have been using a fundamentally different approach to manipulate these same molecules—rational design by molecular modeling. Although molecular modeling can be powerful, it has limitations and cannot be used for proteins that have not yet been crystallized, for determining protein–environment interactions, or for predicting the effect of mutations beyond protein structure. Although rational design is a beautiful approach and will be extremely valuable in the future, a process is currently required to develop poorly understood proteins into useful



Keith Powell started his career with a degree in Microbiology and Chemistry at the University of Reading. After a PhD in the genetics of heme synthesis, he joined Glaxo to improve the yield in the synthesis of vitamin B12. Finding a need to do some more dangerous research, he moved to the University of Newcastle where he became involved in research into the mechanism of DNA repair. Later, he joined ICI to develop the genetic system for Methylophilus methylotrophus*, the organism used in single-cell protein synthesis. After studying the synthesis of biopolymers and various enzymes, he joined ICI Plant Protection to develop biological methods for crop protection. Unable to keep away from chemicals, he joined Zeneca Agrochemicals to discover new chemical entities and later managed groups in biotechnology, environmental science, and even patents. The lure of biocatalysis drew him to Maxygen in 1999, where he is currently vice-president of Chemicals and Applied Technology.*

catalysts without the need for a fundamental understanding of their structure and function.

A logical approach to adapt natural sequences further is to apply the same proven evolutionary tools that created these sequences in the first place. The green revolution clearly demonstrated that complex, multigenic traits of whole organisms can be optimized without any sequence or structural information of the underlying genes. Breeding is a conceptually simple, recursive process that involves homologous DNA recombination between related genomes, followed by screening of the progeny for clones with improved properties. If whole genomes could readily be improved by classical breeding, then a variation of this approach should be useful for the improvement of the much shorter sequences used by molecular biologists. We have adopted the term "MolecularBreeding" (directed molecular evolution) for the concept of rapid breeding of subgenomic sequences by using recursive recombination and functional selection. In contrast to classical breeding, which is a proven but slow method for optimizing whole, typically eukaryotic genomes, MolecularBreeding comprises a variety of recombination and screening techniques that together allow the rapid evolution of single genes, pathways, episomes, viruses, and bacterial and eukaryotic genomes. An aim of MolecularBreeding is to reduce the generation time to only a few days, typically by performing the screening process in bacteria or in single cells. The selection is focused on commercially relevant properties.

MolecularBreeding has the greatest advantage over rational design, cassette mutagenesis, and random point mutagenesis when the target is complex, for example, a multicomponent protein with complex interactions, when structural information is not yet available, or when the principal target does not involve a protein structure.[5] MolecularBreeding involves the recombination of multiple DNA sequences to create a library of chimeras. A preferred format for MolecularBreeding, also called DNA shuffling, involves the random fragmentation of a pool of homologous DNA sequences with DNase I.[6] After denaturation of the fragments, homologous sequences from different templates hybridize and prime each other, and the resulting crossovers are locked in by polymerase extension. Multiple cycles of this fragment reassembly result in a library of full-length, homologously recombined chimeric sequences caused by a variable number of crossovers. After insertion of this library of DNA sequences into an expression vector and transfer into a host cell for expression, the clones are screened for new or improved properties. The screening identifies the best combinations of the permutated sequence diversity. As in classical breeding, a pool of the best clones is then used as the input for the next round of breeding (Figure 2). Important variables for MolecularBreeding are the length of the shuffled DNA, the DNA homology, the quality and conservative nature of the sequence diversity (natural proven diversity versus random point mutations), the crossover number (determined by fragment size), as well as the number and molar ratio of the parents. Several other formats for in vitro and in vivo recombination have been developed.

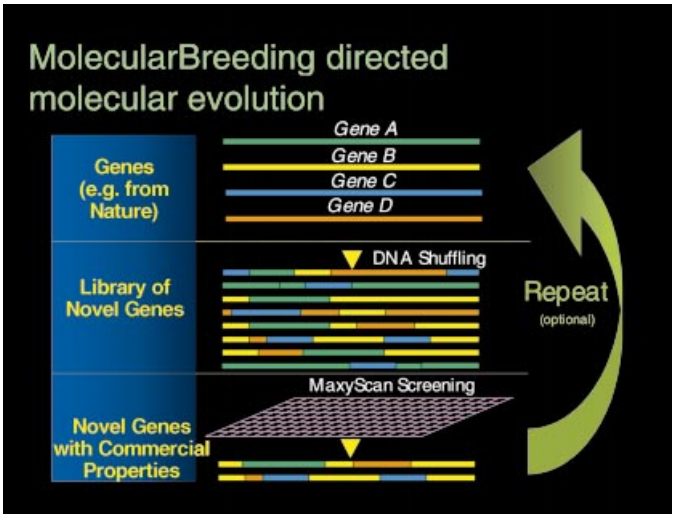


Figure 2. The MolecularBreeding directed molecular evolution process.

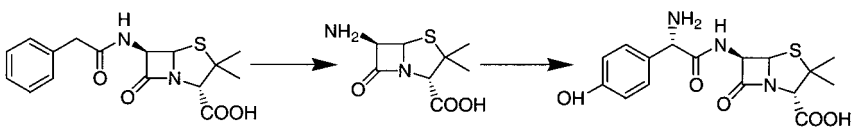
MolecularBreeding has been applied to a variety of enzymes to improve the substrate selectivity, catalytic efficiency, stability, pH optima, and function under diverse physical and chemical conditions. We believe that directed evolution is the key to realizing the potential of biocatalysis in the near future. In the next chapter we describe a variety of important chemical reactions that currently take advantage of biocatalysis, as well as some that may soon benefit as a result of directed evolution technology.

3. Enzymes as Biocatalysts

3.1. Acylation Reactions

Acylation chemistry is integral to the synthesis of numerous antibacterial, antifungal, and small-molecule pharmaceuticals. Despite the robust nature of acylation chemistry, biocatalysts play important roles in the synthesis and hydrolysis of a variety of commercial acyl compounds. Historically, the penicillin acylases (penicillin amidohydrolase E.C. 3.5.1.11) have received considerable attention for their role in the manufacture of semisynthetic penicillins and cephalosporins. The natural acyl side chains of penicillin G (**1**), penicillin V, and cephalosporin C are hydrolytically removed, and the resulting nucleus is reacylated to produce a variety of commercial derivatives, such as amoxicillin (**3**), carbenicillin, piperacillin, cephalexin, cefaclor, and cefotaxime (Scheme 1). This industry now has annual sales of more than $1 billion.

The natural side chains of penicillin G, penicillin V, and cephalosporin C are phenylacetyl-, phenoxyacetyl-, and aminoadipyl-, respectively. Once hydrolyzed, the resulting 6-ami-



1 penicillin G **2** penicillic acid **3** amoxicillin

Scheme 1. Reactions that are catalyzed by penicillin acylase.

nopenicillic acid (6-APA) and 7-aminocephalosporic acid (7-ACA) form the nucleus for modification. The commercial hydrolysis of the natural aromatic penicillins was originally an inefficient three-step process in CH_2Cl_2 at $-40\,^{\circ}C$, but is now carried out by means of a single enzymatic reaction with penicillin G acylase in water at $37\,^{\circ}C$. An efficient cephalosporin C acylase has not yet been described; however, other approaches to the biocatalytic production of the cephem nuclei, 7-ACA and 7-aminodeacetoxycephalosporic acid (7-ADCA), have been developed.

The penicillin acylases catalyze both the addition and the hydrolysis of the acyl side chain. Unfortunately, penicillin G acylase is specific for the phenylacetyl side chain of penicillin G. This prevents the use of this enzyme for the enzymatic acylation of 6-APA and 7-ADCA with different acyl donors. An evolved enzyme with a broad substrate specificity for such donors could potentially provide a biocatalytic route to the semisynthetic β-lactams. Broadened specificity, as well as increased specific activity, altered pH and temperature optima, and overall stability are desired traits for these enzymes. Activity at low pH values is particularly desired since synthesis is favored over hydrolysis under these conditions.

In semisynthetic β-lactam-manufacturing processes, the competing acylation and hydrolysis reactions are either thermodynamically or kinetically controlled. In a thermodynamically controlled process, the reaction equilibrium is established by controlling pH, temperature, ionic strength, and solvent composition. Enzyme variants that exhibit optimal activity under thermodynamically favorable conditions[7] are expected to support the most efficient process. In kinetically controlled reactions, the substrate is converted into the product before equilibrium is reached. To obtain superior product yields, enzyme variants that have optimal kinetic constants for the substrate are desired.

Rational protein engineering of penicillin acylases from *Kluyvera citrophila*, *Escherichia coli*, and *Pseudomonas* has been used to obtain insight into the function[8–11] and the maturation pathway of this enzyme.[11, 12] A hypermutagenic *E. coli* strain was used to expand the substrate specificity of the *K. citrophila* acylase.[13] Ishii et al. employed site-directed mutagenesis based on data from chemical modification experiments to improve the activity of a cephalosporin C acylase from *Pseudomonas*.[14] Typically, small improvements in enzyme activity were observed.

Most industrial enzymes are produced heterologously. The heterologous production of the acylase enzyme is not trivial, as most acylases are modified post-translationally. Early site-directed mutagenesis revealed some of the critical residues for this processing.[11, 12] Subsequently, host–vector systems were developed for improved acylase production, but these resulted in the formation of inclusion bodies.[15] While problems that are encountered in transcription and translation may be approached rationally,[16] it is expected that the expression of foreign proteins can have deleterious effects on the viability of the host and on the quality of the expressed protein. Directed evolution and screening for cells that produce high levels of the desired catalyst is a simple means to address the complex interaction of the target protein and the host environment. For example, DNA shuffling has been employed to improve recombinant protein production by increasing protein solubility.[17]

Acylases have also been used for the deacylation of natural products. The isolation of microbial strains that produce acylases for the hydrolysis of the acyl side chain of a cyclic lipopeptide with antifungal activity is described in patent US6146872.[18] Other examples are listed in Table 2.

In analogy to the β-lactams, it can be expected that with the identification of more and more bioactive natural products, the need will increase for acylases that hydrolyze the existing amide bonds and catalyze the acylation in the manufacture of semisynthetic derivatives. Because of significant economic investments, the development of industrial processes for these new compounds will only be successful if the modifying enzymes are promptly available. We foresee that DNA shuffling will play an important role in the commercialization of these novel bioactive molecules.

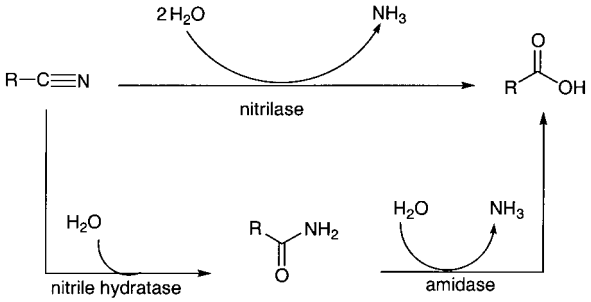
3.2. Nitrile Conversion

Nitriles are convenient and synthetically important chemical building blocks because they are readily generated and can be converted into useful amides and carboxylic acids. Chemically, this conversion requires hydrolysis at high temperatures in the presence of strong acids or bases. Enzymatically, the conversion of a nitrile into a carboxylic acid can be accomplished in a single step by the action of a nitrilase with the incorporation of two mole equivalents of water and the liberation of a mole equivalent of ammonia, or

Table 2. Examples of industrial applications of acylase enzymes from the patent literature.

Patent	Title	Ref.
US5316944	Enzymatic resolution of a racemic mixture of gamma-amino acids using penicillin acylase	[85]
US5068189	4″-*O*-Isovaleryl acylase for the production of novel antibiotic compounds	[86]
US4877734	α-Acetylamino cinnamic acid acylase for enzymatic conversions which run via the intermediary stage α-imino-β-phenylpropionic acid or phenylpyruvic acid	[87]
US4699879	Streptomyces or Streptoverticillium acylase for 3-(3,4-dihydroxyphenyl)serine manufacture	[88]
US5916774	D-aminoacylase from *Amycolatopsis* for producing D-amino acids	[89]
US5733754	Acylase from *Alcaligenes denitrificans* for preparing (*S*)-pipecolic acid	[90]
US5637768	Penicillin acylase for making (2*S*,5*S*)-5-fluoromethylornithine	[91]
US5212069	*N*-Acetyl-2,3-didehydroleucine acylase for the preparation of D- or L-tryptophyl glycine, D- or L-tryptophyl-D-methionine or L-tryptophyl-D-cysteine	[92]
US5219741	*N*-Acyl-L-proline acylase for making L-proline, L-pipecolic acid and L-thiazolidine-4-carboxylic acid	[93]
US5057607	Penicillin G amidase(acylase) for enantiomerically selective acylation of racemic 3-amino azetidinone intermediates	[94]

in a two-step process by the sequential actions of a nitrile hydratase to generate an amide and its subsequent hydrolysis by an amidase to yield the carboxylic acid (Scheme 2). There is considerable industrial interest in the enzymatic conversion of nitriles owing to the desirability of conducting such conversions under mild reaction conditions that would not alter other labile reactive groups. Additionally, the existence of nitrile-hydrolyzing enzymes that show enantioselectivity and/or regioselectivity offers synthetic possibilities that are difficult or impossible to achieve by conventional catalytic approaches.



Scheme 2. Two pathways for the enzymatic conversion of nitriles into carboxylic acids.

The first and most successful industrial application of nitrile-hydrolyzing enzymes on a commercial scale is the Nitto process for the production of acrylamide from acrylonitrile.[19] The process, which originally used whole cells of *Pseudomonas chlororaphis* B23 or *Rhodococcus* sp. N-774, currently uses *Rhodococcus rhodochrous* J1, which is 10-fold more productive. This strain produces two different nitrile hydratase enzymes as well as a nitrilase and an amidase. The reaction is run at 2–4 °C, at which temperature the formation of contaminating acrylic acid is barely detectable, and the productivity of the reaction is >7 kg of acrylamide per gram of cells with a conversion of 99.7%. The strain is tolerant of concentrations of acrylamide up to 50%, but is used only for a single batch conversion. This process is currently run on an annual scale of 40000 tons. *Rhodococcus rhodochrous* J1 is also used by Lonza in its commercial process for the conversion of 3-cyanopyridine into nicotinamide, a vitamin used in animal feed supplements.[20] Annually, 3000 tons are produced by using immobilized whole cells, and no nicotinic acid, a common by-product in the chemical synthesis of nicotinamide, is found.

The isolation and use of nitrile-hydrolyzing enzymes, especially for the production of specific chiral synthetic intermediates, has been described (Table 3). To our knowledge, there is no industrial process that currently uses these enzymes for such production. Enantioselective enzymes have been described that preferentially hydrolyze either the D- or L- form of a variety of nitrile-containing substrates.[21–29] In general, the nitrilases show more enantioselectivity than the nitrile hydratases, and the enantioselectivity is highly substrate dependent. In the case of the nitrile hydratases, these nonspecific enzymes are often co-expressed with highly enantioselective amidases.

Although nitrile-hydrolyzing enzymes offer attractive mild reaction conditions, their routine industrial use has been hampered by other factors. The half-lives of the enzymes that have been purified and studied to date range from 7.5 minutes at 45 °C for a *Corynebacterium* sp. to 58 minutes at 60 °C for the *Rhodococcus rhodochrous* J1 nitrile hydratase.[30–32] Both the acrylamide and nicotinamide processes require *Rhodococcus* whole cells, partly because the purified nitrile hydratase is too unstable. Although systems that involve whole cells avoid expensive enzyme purifications, they are in general undesirable, as contaminating side products can result from the reactions of other enzymes within the cell. There is currently no commercial source for purified nitrilase or nitrile hydratase. Although Novo previously sold a combination of nitrile hydratase and amidase from *Rhodococcus* sp. CH5, this product is no longer available. For reactions in which regio- or stereoselectivity is desired, it has been found that the degree, extent, and even absolute configuration of the final product are highly dependent on the substrate. Thus it is difficult to predict which, if any, of the known nitrilases or nitrile hydratases will efficiently convert a given substrate into the desired product.

There have been no published reports of successful attempts to utilize directed evolution to improve the characteristics of the nitrile-hydrolyzing enzymes or to adapt them to industrial use. Classical mutagenesis and screening were used to improve the characteristics of the original acrylamide-producing strains, making them less mucoid, easier to separate by centrifugation, and less inhibited by substrate concentration,[33] but these improvements were largely superceded by the isolation of *Rhodococcus rhodochrous* strain J1. Directed evolution continues to deliver enzymes with improved

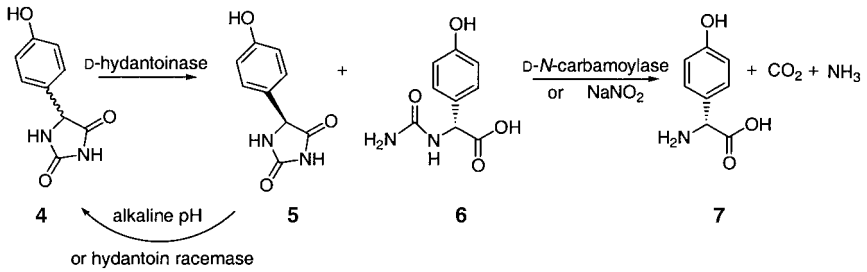
Table 3. Patents on the isolation and use of nitrile-hydrolyzing enzymes.

Patent	Title	Ref.
US05811286	Nucleic acid fragments encoding stereospecific nitrile hydratase and amidase enzymes and recombinant organisms expressing those enzymes useful for the production of chiral amides and acids	[95]
US06133421	Polypeptides and polypeptide subunits of a stereospecific nitrile hydratase enzyme	[96]
US05888785	Method for using hydratase or a hydratase–amidase fusion for stereospecifically bioconverting certain racemic nitriles to the corresponding enantiomeric *R*- or *S*-amide or *S*-carboxylic acid	[97]
US05814497	Enzymatic hydrolysis of racemic α-substituted 4-methylthiobutyronitriles using a nitrilase from *Alcaligenes faecalis*, *Gordona terrae*, or *Rhodococcus* sp.	[98]
US05443973	Method of producing α-hydroxyisobutyramide from acetone cyanohydrin by nitrile hydratase	[99]
JP08131188	Production of optically active alpha-hydroxyacid or alpha-hydroxyamide	[100]
US05206158	Process for the preparation of difluorobenzamide	[101]

substrate specificity, stability, and activity under a variety of physical and chemical conditions;[34] thus, the evolution of stable nitrile-hydrolyzing enzymes that are active under optimal process conditions and that regio- or stereoselectively hydrolyze commercial nitrile-containing substrates may be achievable. Such enzymes would be a valuable addition to the biocatalytic toolbox currently available to the synthetic chemist.

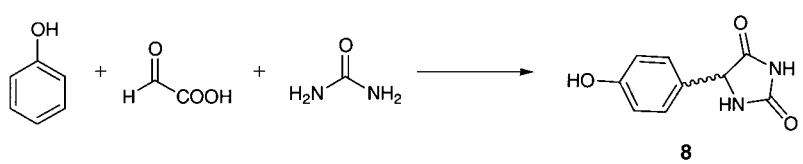
3.3. Hydantoinases

Hydantoinases are used commercially in the production of non-natural amino acids on a scale of several thousand tons per year. The most significant processes are the production of D-phenylglycine and D-*p*-hydroxyphenylglycine, which are important building blocks for semisynthetic β-lactams such as ampicillin, amoxicillin, and cephalexin.[35] They are used also to make D-tryptophan, D-phenylalanine, D-valine, D-alanine, D-methionine, and a variety of substituted amino acids.[36] Hydantoinases catalyze the stereospecific hydrolysis of 5-monosubstituted hydantoins **4** to give *N*-carbamoyl α-amino acids **6**. The *N*-carbamoyl α-amino acids are then hydrolyzed either in the presence of a carbamoylase (in the Recordati process) or in a solution of sodium nitrite (in a process commercialized by Kanegafuchi) to yield the amino acid **7**, carbon dioxide, and ammonia (Scheme 3).[37] Most hydantoinases identified to date are specific for the D form of the hydantoin.



Scheme 3. Enantioselective preparation of **7** from **4** by using D-selective hydantoinase and D-selective *N*-carbamoylase.

Racemic hydantoins **8** are generally synthesized in the Mannich condensation of phenol derivatives, glyoxylic acid, and urea (Scheme 4). Many 5-substituted hydantoins racemize readily as a result of their acidic hydrogen atom through keto-enol tautomerism, thus allowing a dynamic kinetic resolution and a 100% theoretical yield of the desired product. In practice, however, the actual rate of racemization is highly dependent on the nature of the substitution at C5. For example, the $\tau_{1/2}$ for phenylhydantoin racemization is 0.3 h, whereas that for isopropylhydantoin is 56 h.[38] Furthermore, many hydantoins are sparingly soluble to insoluble, so in practice the substrate is often added to the reaction mixture as a suspension, and the dissolution of the substrate can be the rate-limiting step. Currently, the commercially available hydantoinases require Mn^{2+} as a cofactor and are irreversibly inactivated if the Mn^{2+} center is oxidized. Therefore, the reaction must be run anaerobically, which typically requires that the oxygen be replaced with nitrogen each time a new cycle is started. Thus, although native hydantoinases are currently being used commercially, the properties of both the enzyme and the substrate suggest that the process could be improved through directed evolution. A catalyst that functions at elevated temperatures would allow a process in which the substrate is more soluble and racemization is enhanced. If the evolution of the enzyme removed or altered the Mn^{2+} requirement, the process could then be carried out aerobically.

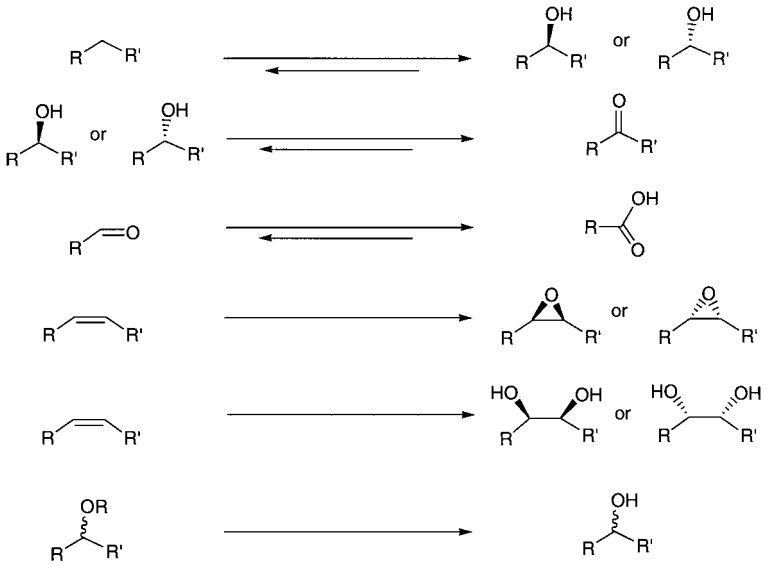


Scheme 4. Preparation of racemic **8** by means of a Mannich condensation.

In contrast to many industrially promising enzymes, the hydantoinases have been subjected to intensive directed evolution studies. Early mutagenesis efforts have been followed by increasingly sophisticated strategies. Hybrid enzymes that pair the amino-terminal half of a hydantoinase from *Bacillus stearothermophilus* with the carboxy-terminal half of a hydantoinase from *Bacillus thermocatenulatus* showed altered substrate specificities relative to both parent enzymes.[39] Kim et al. fused a hydantoinase and a carbamoylase in an attempt to circumvent unequal expression, inclusion body formation, and substrate transport problems, which are inherent in traditional co-expression systems.[40] The resulting *N*-carbamoylase-D-hydantoinase fusion enzyme proved to be as active and catalytically as efficient as the co-expressed wildtype enzymes. However, the fusion enzyme was unstable as a result of extensive proteolysis, and DNA shuffling was used subsequently to enhance the stability of the fusion enzyme. An evolved enzyme was identified that was six times as active as the parent fusion enzyme in converting the hydantoin into the D-amino acid.[41] A direct comparison of the performance of the evolved fusion enzyme with the best available co-expression system has yet to be reported. Finally, in a striking example of the utility of directed molecular evolution, May et al. have overcome a key drawback to hydantoinase-mediated amino acid production: the rigid stereoselectivity of known hydantoinases. By using gene shuffling, this group inverted the stereospecificity of an *Arthrobacter* hydantoinase from the D to the L enantiomer of methionine hydantoin.[3] Roche now employs this evolved enzyme in a commercial process for the production of L-methionine. There are no reports to date of efforts to alter the thermal activity profile or to reduce/replace the cofactor requirement of hydantoinases, two properties that might translate directly to process improvements.

3.4. Oxidoreductases

Oxidoreductases (EC1) catalyze a myriad of chemo-, stereo-, and regioselective reactions (Scheme 5), and currently are used for the production of a variety of pharmaceutical



Scheme 5. Reactions that are catalyzed by oxidases and oxidoreductases.

and chemical products (Table 4). Oxidoreductases are generally employed in fermentation-based processes, or for whole cell biotransformations, which, as mentioned earlier, can be undesirable. To better exploit the industrial potential of oxidoreductases, processes that utilize isolated enzymes

Table 4. Examples of biocatalytic processes with oxidoreductases.

Reaction Product	Enzyme	Ref.
L-*tert*-leucine	Leucine dehydrogenase	[102]
6-hydroxynicotinic acid	*Achromobacter xylosoxidans*	[103]
5-methylpyrazine-2-carboxylic acid	*Pseudomonas putida*	[103]
(*R*)-2-(hydroxyphenoxy)propionic acid	*Beauvaria bassiana*	[103]
Steroids	Several	[104]

(preferably immobilized) would be desirable. However, oxidoreductases are not practical for use in vitro since they require expensive cofactors (e.g. NAD(H)or NADP(H)), and are often membrane associated. As with most enzymes, improvements in stability, turnover, and specificity would benefit many applications.

There have been several attempts to overcome these challenges. For example, several NAD(P)H-regeneration systems have been developed to make NAD(P)H a cofactor (catalytic) rather than a substrate in the reaction. Process-based solutions include chemical and electrochemical regeneration techniques, whereas host-cell-based solutions involve the overproduction of NAD(P)H-regeneration enzymes.[42] More appropriate to the use of purified or immobilized enzyme catalysis are gene fusions between an enzyme of interest and a gene that codes for a redox partner.[43, 44] Although these NAD(P)H-regeneration systems are all important technical milestones, the need for NAD(P)H in more than catalytic concentrations is still a serious issue for cell-free formats.

Directed evolution offers hope that the NAD(P)H requirement may be addressable. In particular, Arnold and co-workers have successfully produced mutants of bacterial cytochrome P450 enzymes that catalyze the hydroxylation of naphthalene approximately 20 times better than the wildtype, and use a hydrogen peroxide shunt pathway that does not require NAD(P)H.[45] The same group also developed a high-throughput digital imaging screen for arene-specific oxygenases by coupling them to horseradish peroxidase.[46] The peroxidase catalyzes the oxidative coupling of hydroxylated aromatics that are produced by the oxygenases, resulting in the creation of fluorescent compounds. Different substrate hydroxylation patterns result in compounds that have different fluorescent spectra. Arnold and co-workers used this system to identify evolved oxidases that have new hydroxylation specificities. Such screens should assist the development of mono- and dioxygenases as useful biocatalysts for the production of hydroxylated aromatic compounds.

Of the oxidoreductases, the ubiquitous cytochrome P450 enzymes, of which several hundred have been characterized, are particularly intriguing. Structural and mutational studies have shown that minor alterations in the P450 protein structure can lead to changes in chemo-, regio-, and stereoselectivity[47]. The directed evolution of these enzymes has also begun to produce biocatalysts that are useful for the selective oxidation of a variety of interesting substrates. For example, directed evolution was used to create an indole hydroxylase from cytochrome P450 BM-3, a fatty acid hydroxylase that is not active on this substrate.[48] Arnold and co-workers also used directed evolution to improve the solubility of a mammalian cytochrome P450 that is normally membrane associated.[49] Hybrid cytochrome P450 genes were created from a membrane-associated human cytochrome P450 and the heme domain of a bacterial cytochrome P450 by using SHIPREC (sequence homology-independent protein recombination). Screening for the activity of the mammalian gene identified two functional hybrids that were more soluble in the bacterial system.

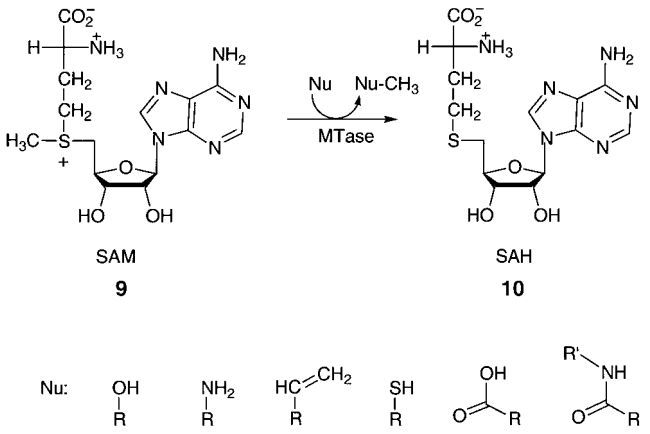
Other oxidoreductases have also been manipulated by evolutionary approaches. For example, directed evolution was used to increase the peroxidase activity of horse heart myoglobin, an effort that enabled the direct enzymatic high-throughput screening of peroxidase-producing *E. coli* strains.[50] A more thermostable catechol 2,3-dioxygenase was created by DNA shuffling of *nahH* and *xylE* 2,3-dioxygenases.[51, 52] By using a combination of rational and evolutionary methods, Cherry et al. improved the oxidative and thermal stability of a heme peroxidase for use as a laundry additive.[53] Horseradish peroxidase has also been evolved to express better in a soluble form in *E. coli*.[54] Ultimately, it is possible that the stability of cytochrome P450 and other heme-dependent enzymes may be limited by the instability of

the porphyrin structure under oxidative conditions.[55] It has been suggested that monooxygenases that are not dependent on heme and that catalyze similar reactions as the cytochrome P450 enzymes might also be excellent targets of directed evolution.[56]

3.5. Methyl Transfer

We have discussed a variety of important chemical reactions that have been addressed by using biocatalysis. There are other chemical reactions that might be suitable for biocatalysis and that have not yet been fully explored, for example, methylation. Methylation is particularly useful because it is so subtle. Methylation is generally effected by using a methyl halide such as MeI. The reaction is promiscuous, although regioselective methylation can be accomplished if the reactivity of competing nucleophiles within a molecule can be differentiated by the manipulation of solvent, solute, and physical parameters or by selective protection. Selectivity is generally not complete and purification of the desired isomer is required. A biocatalytic route to regio- and stereospecific methylation could greatly improve yields and avoid costly downstream processing.

S-Adenosylmethionine (SAM; **9**) is a methyl donor in enzymatic methylation reactions, that is, SAM is the biological equivalent of methyl halides. The active group of SAM is a methyl sulfonium cation. Enzyme-catalyzed nucleophilic attack at the methyl group displaces *S*-adenosylhomocysteine (SAH; **10**) (Scheme 6). The loss of the positive charge on the sulfur atom is thermodynamically favorable and drives the reaction to completion. Biological methylation of most biological nucleophiles is documented. Unlike chemical methylation, enzymatic methylation is regio- and enantioselective, as expected from biological catalysts.



Scheme 6. *S*-adenosylmethionine (SAM) acts as a methyl donor in enzyme-catalyzed methyl-transfer reactions. Methyltransferases (MTase) catalyze the methylation of a diverse set of biological nucleophiles.

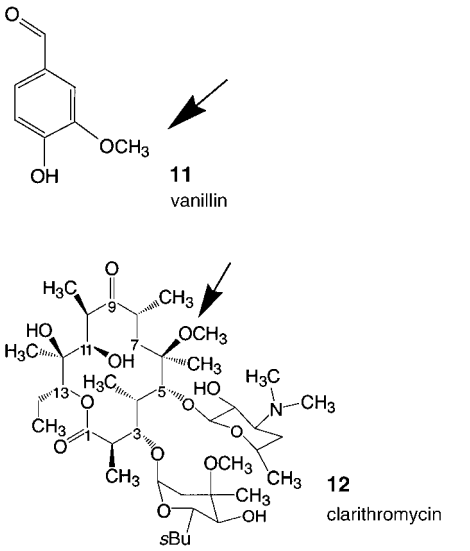
The use of methyltransferases for biocatalysis is limited. SAM is expensive and methyltransferases react notoriously slowly ($k_{cat} \approx 1\ s^{-1}$) and are often membrane associated. Working in vitro requires stoichiometric amounts of SAM, since no alternative methyl donors or SAM regeneration systems have been described. Increased solubility, stability, selectivity, and catalytic efficiency would be desirable for useful industrial processes. These all represent opportunities for enabling biocatalytic methyl transfer through directed evolution, and there is precedence for the tailoring of methyltransferase specificity by rational design. Trautner et al. produced chimeric DNA methyltransferases that are specific for different DNA sequence combinations.[57] Methyltransferases have also been discussed for combinatorial biosynthesis.[58] However, cited examples of biocatalytic exploitation of methyltransferases are difficult to find.

In vivo there are presently a variety of useful targets for the application of methyltransferases. In the natural products arena, methyltransferases are commonly involved in the terminal step of the synthesis of small molecule antibiotics such as erythromycin, tylosin, rapamycin, and daunomycin.[59, 60] For example, macrocin methyltransferase, which is encoded by the *tylF* gene in *Streptomyces fradiae*, is rate limiting in the production of the commercial antibiotic tylosin. Additional copies of the *tylF* gene result in measurable increased levels of tylosin in fermentations of *S. fradiae*.[61] DNA shuffling of *tylF* for improved catalytic efficiency could further increase the yield and productivity of tylosin fermentations. Li and Frost described a method for the biocatalytic methylation of the 3-hydroxy group of protocatachuate by catechol OMTase (COMTase).[62] Unfortunately, COMTase methylates both the 3- and the 4-hydroxy groups of protocatachuate. Directed evolution of COMTase to be specific for the 3 position would provide an improved biocatalytic route to vanillic acid; subsequent reduction by aryl aldehyde reductase would provide a complete route to natural vanillin (**11**; Scheme 7).

The specific methylation of the 6-hydroxy group of erythromycin A has become something of a holy grail. Such an enzyme would provide a single step synthesis of clarithromycin (**12**; Scheme 7), a billion dollar drug that presently requires a seven-step chemical methylation.[63] Methyltransferases that are specific for tertiary alcohols are rare, perhaps because they are more sterically hindered than primary or secondary alcohols. One such enzyme is EryG, which methylates the tertiary alcohol group of the mycarose moiety of erythromycin C.[59] Thus tertiary O-methylation is not unprecedented, and the evolution of an erythromycin 6-O-methyltransferase may be quite feasible.

3.6. Hydrolytic Enzymes: Lipases, Esterases and Proteases

Hydrolytic enzymes (EC 1) have been used as catalysts in organic synthesis more than any other class of enzyme. Hydrolases catalyze the selective hydrolysis of esters, amides, halides, peptides, and glycosides. Many of these enzymes have been adapted to function in environments with low water content in which they catalyze the thermodynamically more favorable synthetic reaction. Lipases, which naturally function on hydrophobic compounds, in particular have shown the ability to catalyze ester and lactone synthesis in organic solvents.[64, 65] Commercial hydrolase-mediated processes in-



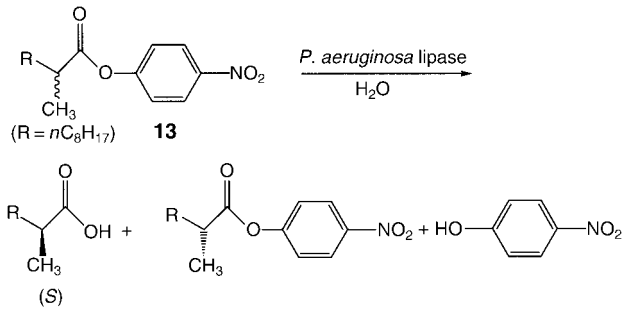
Scheme 7. Molecular targets for evolved methyltransferases. An erythromycin 6-O-methyltransferase would provide a biocatalytic route to clarithromycin (**12**). A catechol 3-O-methyltransferase would provide a biocatalytic route to natural vanillin (**11**). The arrows indicate the position of the desired addition of the methyl group.

clude the production of intermediates for pharmaceuticals, pesticides, and drugs, the production of chiral synthons for asymmetric synthesis, as well as applications in the food industry, such as the production of high-fructose syrup, low-lactose milk, and aspartame. Current examples have been compiled and discussed previously.[37, 66, 67]

As is the case with most enzymes, natural hydrolases are generally not optimal biocatalysts. Multiple parameters could be improved by directed evolution to enhance their performance. Hydrolase enzymes are by nature active on a broad range of substrates but paradoxically maintain a high degree of chemo-, regio-, and stereoselectivity. Directed evolution provides a means to tune this specificity for desired processes. To this end, improvements in substrate specificity,[68] enantioselectivity,[1, 69] specific activity, thermostability,[70–74] solvent tolerance,[70, 75] activity over a broad pH range, and combinations thereof[34] have been described.

3.6.1. Lipases and Esterases

Lipases and esterases both catalyze the hydrolysis of esters. Lipases prefer less soluble esters, and have a broader substrate specificity and a higher enantioselectivity than esterases. Lipases have been used extensively to produce optically active alcohols, acids, esters, and lactones by kinetic resolution. Such applications are of great importance since industry generally demands compounds with an optical purity $\geq 95\,\%$. For this reason, Reetz et al. improved the enantioselectivity of a *Pseudomonas aeruginosa* lipase by directed evolution.[69] The native lipase catalyzes the hydrolysis of racemic **13** with an *ee* of 2% (*S*) (Scheme 8). Four cycles of mutagenesis and screening produced an evolved lipase with an improved *ee* of 81%. Additional rounds of error-prone PCR combined with saturated mutagenesis resulted in an *ee* of more than 90%.[1] Modeling of the evolved lipase structure



Scheme 8. Kinetic resolution of **13** by lipase-catalyzed hydrolysis.

revealed that the amino acid substitutions might increase the conformational flexibility of the enzyme. In a similar fashion, Bornsheuer and co-workers increased the *ee* of 3-hydroxyethyl ester resolution by means of the directed evolution of an esterase.[68, 76, 77] Increased catalytic activity and stability in the presence of an organic solvent is of particular interest,[78] as these conditions are often necessary to dissolve the substrate or to favor reaction thermodynamics. For this reason, Moore and Arnold improved the activity of a *Bacillus subtilis* esterase 150-fold in 15% DMF by using directed evolution.[79, 80]

3.6.2. Proteases

Proteases catalyze the selective hydrolysis of peptides, amides, and esters in a manner mechanistically similar to lipases and esterases. They are primarily used in organic synthesis for the enantioselective hydrolysis of α-amino acid esters and carboxylic acid esters. As is the case with esterases, they also catalyze transamination, transesterification, and synthetic reactions under the appropriate conditions. Commercial proteases such as subtilisin are primarily used in laundry detergents and have been engineered extensively for improved stability and activity at high temperatures and pH values. Despite 30 years of rational improvements, only recently did gene shuffling of subtilisin with 25 homologues result in impressive improvements in activity under a variety of chemical and physical extremes.[34] In a different study, the specific activity of subtilisin E was improved 470-fold.[81] The temperature optimum of this enzyme was also broadened and its half-life at 65 °C was extended 200-fold.[82, 83] Taguchi et al. evolved subtilisin BPN′ and thus doubled the activity at 10 °C,[84] which showed that the activity could be improved at relatively low temperatures.

Clearly, directed evolution is expanding biocatalytic options for the organic chemist. Hopefully, the development of additional hydrolytic enzyme families such as dehalogenases and epoxide hydrolases will result in further applications of commercial biocatalysis.

4. Summary and Outlook

We stand on the edge of a new era for industrial biocatalysis. The capacity to improve enzymes provides the opportunity to effect regio- and stereospecific chemical

reactions with selected enzymes as biocatalysts. The potential for cooperation between chemists and biologists to design and implement these catalysts will become clear in the near future. The potential for libraries of enzymes that are rapidly available and the further development of targeted and specific catalysts for a desired reaction are intriguing and exciting developments for the future of chemical synthesis.

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