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Strategies for Improving Enzymes for Efficient Biocatalysis

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Summary

Biocatalytic processes are finding increasingly widespread application not only in academia, but also in industry. This is particularly true in the pharmaceutical and agrochemical industries where the need for optically pure molecules is critical. Biocatalysis is also receiving a major thrust from the generation of new and novel biocatalysts via microbial screening, developments in the biocatalytic processes themselves (*e.g.* use of nonaqueous solvents for synthetic purposes) and improvements in the activation of the enzymes by numerous mechanisms (solvent and enzyme modifications), including protein engineering. The most dramatic results have been achieved by targeting the gene encoding a particular enzyme in order to undertake protein engineering by rational and non-rational methods. This review discusses various means of improving enzyme properties or creating new activities.

Key words: biocatalysis, biocatalyst engineering, directed evolution, enzyme, medium engineering

Introduction

Biocatalysis has reached a particularly exciting time. Now we can dispel most of the old thinking about enzymes, because they are not 'very expensive, highly sensitive and difficult to handle, stable and active only in dilute aqueous solutions'. Although all the problems concerning enzyme utilization have not yet been solved, we currently have a wealth of new information and effective tools to solve numerous problems. Biocatalysts are being used extensively in the industrial production of bulk chemicals, pharmaceutical and agrochemical intermediates, active pharmaceuticals and food ingredients (*1,2*). Over 300 processes to date have been implemented in industry (*3*), demand for complex chiral drugs is high (*4*), and environmentally clean processes (*5,6*) are increasingly required.

Once it was realized that enzymes can also act within organic solvents containing little or no water (*7*), biocatalysis received a further boost (*8–10*). In fact, most synthetic uses of enzymes to date (*1,11–19*) have involved the use of hydrolases in such systems. However, this scenario is changing rapidly as more and more reports on the use of other important enzymes (including the co-factor dependent ones) are appearing.

Aqueous solutions are undeniably the natural milieu for enzyme action. However, it is often beneficial to switch to nonaqueous media when employing enzymes as practical catalysts in organic chemistry or biotechnology. There are numerous reasons that underlie such a switch, including the insolubility of many commercially relevant compounds in water, various side reactions

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promoted by water, the unfavorable thermodynamic equilibria of numerous processes in water and the difficulties of product recovery from aqueous solutions (*20*). When placed in this highly unnatural milieu, enzymes not only retain their activities but also exhibit new, novel and potentially valuable properties, including greater stability, markedly altered selectivity that can be readily controlled by the solvent, and molecular memory.

There were, and still are, numerous misconceptions regarding nonaqueous enzymology including the idea that this technology is limited to lipases or to a few hydrolases, but there is neither factual nor mechanistic basis for this view. Recently, several researchers have addressed these myths and have succeeded to a large extent in dispelling at least some of them (*2,21–24*).

Certain realizations have emerged from the progress made in nonaqueous enzymology by numerous researchers. The major ones being:

(i) Solvent nature

Hydrophobic solvents typically afford higher enzymatic activity than their hydrophilic counterparts (*25*). This is due to the fact that a few clusters of water molecules (presumably bound to charged groups on the enzyme surface) are required for enzymatic function, and hydrophilic solvents tend to strip some of this essential water, thereby lowering the catalytic activity. This effect can be reversed with restored enzymatic activity, by adding small quantities of exogenous water to the solvent (*26*).

(ii) The pH memory and effect of enzyme treatment

Higher catalytic activities in organic solvents are also achieved if the enzyme is lyophilized from an aqueous solution of optimal pH for enzymatic activity, and if that aqueous solution contains a ligand for the enzyme or a lyoprotectant (*7*). The former phenomenon manifests the 'pH memory' of enzymes and stems from the optimal ionization state of the enzyme, preserved on lyophilization and on subsequent placement in an organic solvent. Ligands and lyoprotectants can also preserve the catalytically active conformation of the enzyme through different mechanisms (*27,28*). Both effects can be dramatic. Lyophilization of soybean peroxidase from an aqueous solution containing phenolic substrates resulted in an enzyme that was hundreds of times more active in 99.5 % acetone than a preparation lyophilized without a ligand (*28*).

(iii) Water activity

Water activity, a_w, plays an important role in enzyme-catalyzed reactions. Halling (*29*) suggests the use of water activity instead of water content to describe the effect of water in an organic solvent, and demonstrates its usefulness in understanding the impact of water on enzyme performance. The water activity describes the effect of mass action of water on the equilibrium and is therefore a better measure of the effect of water than the water content. A polar solvent requires a higher water content to reach a certain water activity than does a non-polar solvent. Consequently, in order to compare enzyme reactions in different media it is of fundamental importance to do this at an equal and constant water activity. There are many ways to control water activity, such as the use of salt hydrates, saturated salt solutions, and others (*30–32*). Controlling a_w has been shown to influence enzyme activity in organic solvents as well as ionic liquids (*33–37*).

Strategies to Achieve Efficient Biocatalysis

In spite of the impressive number of applications (Table 1) (*4,13,22,38–51*), enzymes do not exhibit satisfying performance or characteristics in terms of activity, stability and above all selectivity. Researchers have overcome some of these shortcomings by altering substrates (substrate engineering), by modifying reaction system (medium engineering) or by enzyme engineering. Enzyme engineering is based either on physical modifications of the protein or on modifications at the genetic (DNA) level. Microbial screening is a simple and frequently used method for finding new biocatalysts with required properties (*52–54*). These days it utilizes high-throughput-screening (*54–59*) and metagenome analysis of uncultured microorganisms (*60–63*), with these and related approaches enabling researchers to take full advantage of the microbial diversity that exists (*64,65*). Also, extremozymes isolated from extremophiles are very attractive for industrial applications (*66*).

Recently, it has been shown that small changes in the active site of enzymes enable new possibilities for catalyzing novel reactions. Bornscheuer and Kazlauskas

Table 1. Properties and possibilities of enzyme applications

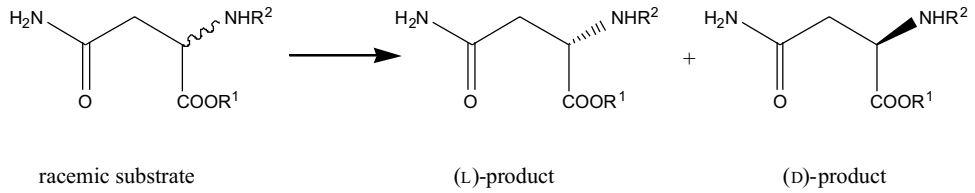
Enzyme	Main industrial application	Reference
Lipases	Interesterification of triacylglycerols, chiral synthesis	*(17,69–71)*
Glutaminase	Flavor-enhancing enzyme	*(72)*
Glycosynthases	Oligosaccharide synthesis	*(73,74)*
Epoxide hydrolases	Resolution of aryl- and substituted alicyclic epoxides Enantioselective catalyst	*(75,76)*
Monooxygenases, oxidizing enzymes	Oxidation, Baeyer-Villiger oxidation	*(77–80)*
Amylases	Pharmaceutical and fine-chemical industries	*(81)*
Oxidoreductases	Oxidation/reduction reactions	*(82)*
Lipoxygenases	Biosynthesis of inflammatory mediators	*(83,84)*
Nitrilase family	Different reactions	*(85)*

(*67*) described these properties in an excellent review about catalytic promiscuity. Todd *et al.* (*68*) described this phenomenon in terms of the plasticity of enzyme active sites.

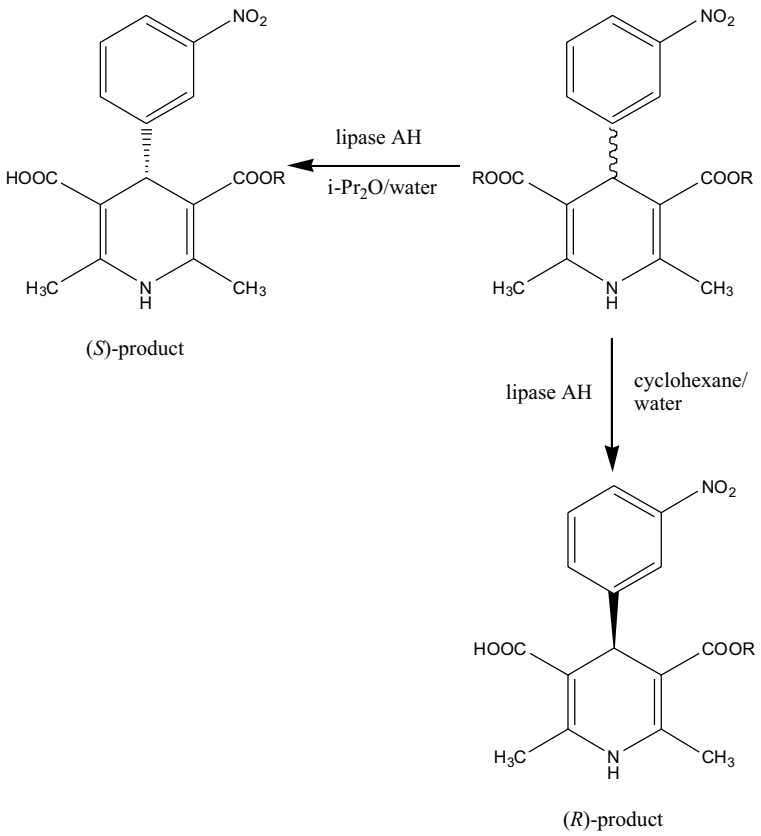
Medium engineering

During the last two decades variations in solvent systems have been used to influence the performance of enzymatic transformations. Enzymes in nonaqueous solvents can be active provided that the essential water layer around them is not stripped off. Medium engineering in the context of biocatalysis in nonaqueous media involves the modification of the immediate vicinity of the biocatalyst. Non-polar solvents are superior to polar ones since the former provide a better microenvironment for the enzyme, however, the solvent effects cannot be generalized too far. There are various exceptions, of which lipases are a particular case. For instance, porcine pancreatic lipase is active in anhydrous pyridine (*86*), suggesting that it can retain the bound water even in a water-miscible solvent.

Despite the fact that the effect of an organic solvent or an additive cannot be reliably predicted, addition of such solvents or additives has formed a useful approach for improving enzyme properties. For the *Candida rugosa* lipase-catalyzed hydrolysis of various substituted phenoxypropionates, the addition of 30–70 % dimethyl sulfoxide (DMSO) has resulted in increased enantioselectivity, (E = 4 to >100) (*87*). Similar effects were also observed for the same reaction by adding aqueous sodium dodecyl sulfate (SDS) (E = 3.8 to >100) (*88*) or by chemically modifying the lipase (*89*). Recently, Iding *et al.* (*90*) described the chemo-enzymatic preparation of chiral N-protected 3-aminopyrrolidine derivatives at preparative scale, based on resolution of N-protected D,L-asparagine esters with proteases as a key step (Scheme 1). The enzyme inhibition that occurred at high substrate levels in this process was overcome by the addition of co-solvents.



Scheme 1. Resolution of N-protected D,L-asparagine esters (*90*)



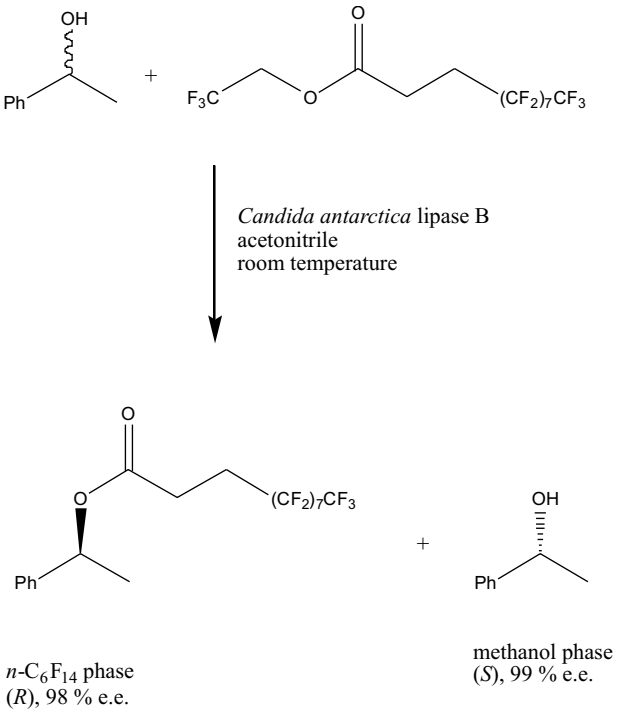
Scheme 2. Complete solvent-dependent reversal of enantioselectivity in hydrolysis by *Candida rugosa* lipase in organic solvent saturated with water (*91*). Lipase AH: *C. rugosa* lipase; i-Pr_2O: diisopropyl ether

In a classical example, Hirose *et al.* (*91*) described the reversal of enantioselectivity with a change of solvent (Scheme 2).

Several aspects have to be considered in choosing an appropriate solvent for a given reaction. These include compatibility with the selected reaction (substrates and products), inertness, low density to minimize mass transfer limitations, and other properties that are suitable (*e.g.* surface tension, toxicity, flammability, waste disposal and cost). Halling (*29,92*) presented a detailed account of predictions that can be made to elucidate the influence of solvent selection on the equilibrium. Reetz (*93*) reviewed various medium engineering successes with particular reference to lipase catalysis.

Most recently, the Sheldon group (*94*) discovered that enzyme-catalyzed processes can also be performed in ionic liquids (ILs) and reported similar performance of *Candida antarctica* lipase B in these liquids compared to organic solvents, this being true for various different reactions. Furthermore, the properties of ILs can be modulated using different cations or anions, resulting in water miscible and immiscible systems (*95*). The enantioselectivity of lipases was maintained or substantially improved in the presence of ILs during the resolution of secondary alcohols (*96*). Kragl *et al.* (*97*) comprehensively reviewed various aspects of biocatalysis in ILs.

In recent years, ultrasonic- (20–100 MHz and beyond) and microwave- (0.3–300 GHz) assisted enzymatic reactions have been added to the repertoire of enzymology (*98*). A lot of attention has also been paid to biocatalysis in supercritical fluids (*99*) or at high pressure (*100,101*).



Scheme 3. Use of fluorinated acyl donors combined with extraction using perfluorinated solvents resulting in efficient resolution and easy separation. Downstream processing involves filtering lipase, evaporating solvent, redissolving in methanol and extracting 6 times with n-C_6F_{14} (*102*)

Substrate engineering

Use of modified substrates has been investigated to improve efficiency of enzymatic reactions. Easy separation of the substrate and the product of a lipase-catalyzed acylation was achieved by using fluorinated acyl donors (Scheme 3) in combination with an extraction step with perfluorinated solvents (*102*). This process has the advantages of easy scalability, a slightly higher enantioselectivity compared to the reaction performed with vinyl acetate as an acyl donor and a simple flash chromatographic separation. The same concept was also used for the resolution of 1-(2-naphthyl)ethanol in a triphasic separative reaction (*103*).

Creating variations in the substrate structure has also been tried in order to achieve improvements in biocatalysis. Conversion rates and yields, enantioselectivity, and biocatalyst stability were enhanced by using vinyl esters of chiral carboxylic acids instead of commonly used ethyl esters (*104–106*). The use of various substrate-engineering strategies in biohydroxylation has recently been reviewed (*107*).

Biocatalyst engineering

Enzymes are versatile biocatalysts and find increasing application in many areas, including organic syntheses (*1,12,44,49,69,108–119*). However, even after an enzyme has been identified as being useful for a certain reaction, its application is often hampered by the lack of long-term stability under process conditions and also by difficulties in recovery and recycling. These problems can be overcome by modifying enzymes (*e.g.* immobilization). Enzymologists have always strived to identify methods by which they can link an enzyme to a carrier or modify the enzymes to suit them for the required application (*98,120*).

A variety of forms and formulations of enzymes have been employed in nonaqueous systems: native enzymes, suspended enzyme powder, solid enzyme adsorbed on support, polyethylene glycol-modified enzymes soluble in organic solvents (*121*), enzyme entrapped within a gel or microemulsion or reversed micelle (*122,123*) and immobilized enzyme (*111,124,125*). The most frequently used immobilization techniques fall into four categories: (*i*) non-covalent adsorption or deposition, (*ii*) covalent attachment, (*iii*) entrapment in a polymeric gel, membrane or capsule, and (*iv*) cross-linking of an enzyme. Most of these approaches are a compromise between retaining high catalytic activity while achieving the advantages of immobilization. These techniques have resulted in biocatalysts exhibiting significantly higher stability than the native enzyme. Recent trends are focused on the use of new reagents/carriers and approaches that make use of the knowledge of enzyme structure and mechanism. These aspects have been explicitly dealt with very recently in an elegant review (*126*). However, a general and broadly applicable method for enzyme immobilization is still elusive and needs to be discovered. There are also no general guidelines for choosing the most appropriate form of the enzyme for a specific purpose.

Most often, the major criteria for enzyme immobilization are the price of the final immobilized enzyme and the simplicity of the immobilization procedure. Re-

cently, Novozymes A/S presented a simple and cost-efficient procedure for *Thermomyces lanuginosus* lipase immobilization using silica granulation technology (*127*). This cheap immobilized lipase can be used for industrial interesterification reactions (*128*).

Various developments have taken place in this area, including the generation of active and stable homogeneous (soluble) biocatalysts by covalent or noncovalent modification of the native enzyme. Covalent techniques are well described in the literature (*e.g.* the attachment of polyethylene glycol chains to enzymes). Noncovalent modifications tend to be less common, although they are able to provide highly active and soluble enzyme forms (*129*). These developments have been well documented in various reviews (*130–134*).

Enzymes are usually prepared from an aqueous/buffer solution via lyophilization and this results in undesirable changes in the protein's secondary structure, which could lead to denaturation of about 40 % of the active sites. The addition of specific small molecules (excipients) in the freeze-drying stage often improves catalytic activity. This formed the basis of molecular imprinting that involves formation of a complex between a macromolecule and a low-molecular-weight ligand in solution, followed by drying and washing with a selective solvent that removes the ligand. The protein retains the ligand-induced conformation even after the removal of the ligand (*135*). Enzyme activation was achieved by adding crown ethers (*136,137*), lyoprotectants (*138*), transition-state analogues (*139*) and substrate or substrate mimics or competitive inhibitors (*135*). Non-ligand lyoprotectants, such as sorbitol, sugars and PEG, also enhanced enzyme activity in organic solvents when added during lyophilization. On the other hand, the addition of excipients to suspensions of native enzymes in organic solvents had no appreciable effect, indicating that the interaction of the excipient with soluble enzyme is essential to alleviate enzyme denaturation during lyophilization (*140*). Addition of surfactants or hydrophobic sol-gel materials before lyophilization enhanced the lipase activity in organic solvents by up to 100-fold (*141*).

More dramatic was the activation of enzymes caused by adding an inorganic salt (*142,143*). Ion pairing of biocatalysts in the presence of very low concentrations of ionic surfactants resulted in remarkably active ion-paired enzymes. Subtilisin and α-chymotrypsin with >1000-fold higher activities over native enzymes were generated (*144*).

Activated biocatalysts have already found application in the pharmaceutical industry. Salt-activated thermolysin (a bacterial protease) is used to acrylate taxol selectively in *t*-amyl alcohol (*44*). Commercially, Roche (Mannheim, Germany) and Novozymes A/S (Bagsvaerd, Denmark) market several adsorption-immobilized enzymes. Altus Biologics (Cambridge, MA, USA) offers various cross-linked enzyme crystals (CLECs) of different enzymes. Fluka (Buchs, Switzerland) supplies sol-gel entrapped enzymes.

Biocatalyst discovery and optimization

With the onset of genome sequencing projects, bioinformatics is widely used to identify genes from different organisms and genome-wide comparisons form a major research area. With the modern tools of screening, high throughput screens, and availability of protein sequence databases and bioinformatics (*145*), screening for novel biocatalysts is pursued with increased vigor (*146, 147*). Mathematical and data-mining tools have now been applied to analyze sequence-activity relationships of peptides and proteins and to assist in the design of proteins and peptides with specified properties (*148*). Decreasing costs of DNA sequencing and methods to synthesize statistically representative sets of proteins allow modern heuristic statistics to be applied to protein engineering (*149*). This also provides an alternative approach to expensive assays or unreliable high-throughput surrogate screens. Biocatalysts and drugs from non-cultured microorganisms can also be developed from genomic information (*150*). The fact that only a fraction of the microbial flora in the environment can be cultured by standard techniques has led to the development of metagenomics, which is the genomic analysis of uncultured microorganisms. A function-driven approach or a sequence-driven approach is undertaken for this purpose and the results of such studies are already apparent from the discoveries of new antibiotics and novel enzymes (*61,62*).

Strategies to Alter the Protein Properties at Genetic (DNA) Level

Protein engineering has become an increasingly important strategy for improving enzymes despite intense competition from chemical modifications and related techniques (*151–154*). The application of protein engineering continues to grow at a very significant rate. Current strategies of protein engineering include rational design and directed evolution.

Rational design

Rational protein design was the earliest approach to engineering enzymes and is still widely employed to introduce the desired characteristics into a target protein. Crystallographers have already solved many enzyme structures (*155,156*) and have paved the way to use computer modeling. The growing knowledge base on how to engineer certain basic enzyme properties (*e.g.* stability, activity and surface properties) is beginning to make rational design more efficient. Advances in rational design depend on the progress made in structure determination, improved modeling protocols and significant new insights into structure-function relationships (*157*). Advances in modeling of free energy perturbation methods and molecular dynamics calculations also influence this area (*158*).

There are numerous impressive cases of successful rational design. The literature on the rational engineering of biocatalyst stability has recently been reviewed (*159*). The enantioselectivity of the *Candida antarctica* lipase-catalyzed resolution of 1-chloro-2-octanol was improved (E = 14 to 28) by a single amino acid exchange (Ser-47→Ala), as predicted by molecular modeling (*160*). In another example (*161*), a transition-state stabilizing threonine near the active site was replaced by valine through site-directed mutagenesis, resulting in activity

loss and the lost activity was restored by using chiral substrate (2-hydroxy-propanoate) containing the missing functional –OH group, which resulted in improved enantioselectivity (E = 1.6 to 22). Creating tailored enzymes with opposite enantiopreference may also be possible with this approach.

Although numerous research groups are using modeling to predict enantioselectivity, much of the available data is still empirical and is not easily interpreted at the molecular level (*162*). This is largely due to ambiguity in identifying the parameters responsible for enzyme-substrate interactions. In addition, properties like activity, specificity and stability are controlled by multiple sites on proteins. Substantial progress is essential in establishing these features, which would aid application of rational design in a truly directed manner (*163*). The importance of this exciting area of research was aptly reflected in a special issue of *Biochimica et Biophysica Acta*: *Protein Science and Molecular Enzymology* that was devoted to protein engineering, spanning the advances achieved with various enzymes (*164*).

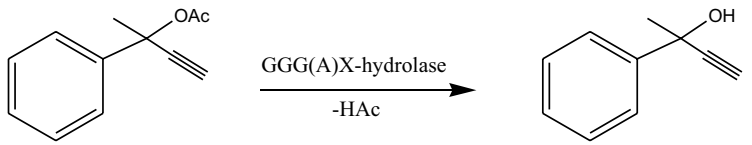
There is another striking example of protein engineering success in relation to the activity of hydrolases on tertiary alcohol ester substrates (an important class of compounds that have not been accepted by numerous enzymes). Pleiss *et al.* (*165*) pointed out that lipases and esterases differ in a certain motif (located in the active site), which is responsible for the stabilization of the tetrahedral intermediate during catalysis. The motif could be either GX or GGGX (in single letter amino acid code; G is glycine, X is any amino acid; in a few cases the GGAX motif is the equivalent of the GGGX motif, where A is alanine) and is located in the oxyanion-binding pocket. Until recently this motif has only been described in a phenomenological manner. Bornscheuer's group was the first to identify the role of this motif in the activity towards tertiary alcohols (*166*). Only two lipases (*Candida rugosa* and *C. antarctica* lipase A) show activity on tertiary alcohol substrates. Both *C. rugosa* and *C. antarctica* lipases, which contain the GGGX or the GGAX motif, and also numerous other GGGX-motif-containing hydrolases have been shown to be effective in hydrolyzing tertiary alcohol esters. *Bacillus subtilis p*-nitrobenzyl esterase (*p*NBE) was one of the GGGX motif-containing enzymes that showed moderate enantioselectivity. Through computer modeling, Gly-105 was predicted to be crucial for improving enantioselectivity and a mutant (Gly-105→Ala) developed based on the modeling showed significant improvement (E = 6 to 19) in the resolution of (*R*,*S*)-2-phenyl-but-3-yn-2-yl acetate (Scheme 4). GX-motif hydrolases were all inactive towards the tertiary alcohols.

In an extension of the study of this motif, Krishna *et al.* (*167*) reported the dramatic resolution of the same substrate through transesterification. The reaction shown in Scheme 4 also occurred to a certain extent by autohydrolysis, which circumvented high enantioselectivity. Therefore, the authors undertook transesterification of 2-phenyl-but-3-yn-2-ol (Scheme 5) to avoid autohydrolysis and optimized reaction conditions for the same to achieve high enantioselectivity (E = 65). This report also serves as an example of the improvement of enzyme efficiency by medium engineering. Starting with a conversion of 9 % (E = 22) in *n*-hexane, the authors showed the dramatic effect of the solvent by obtaining 21 % conversion (E = 49) in isooctane. By optimizing other parameters, the yield and enantioselectivity were further improved.

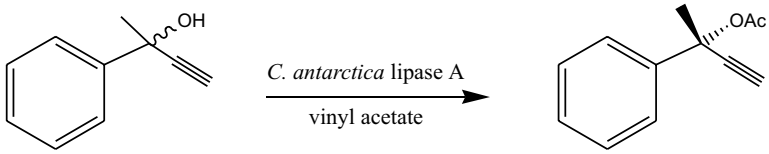
Directed evolution

While site-directed mutagenesis and rational protein design are widely practiced, an alternate method, directed evolution, is gaining increased attention from academic and industrial laboratories to modify and improve important biocatalysts (*168–171*); and to achieve various other objectives (*172,173*). This strategy combines random mutagenesis of the target gene with screening or selection for the desired property (*174–180*). Thermostability is difficult to improve rationally and hence is a good target for directed evolution. Random mutagenesis and screening have resulted in more thermostable subtilisin and lipase (*181*). Contrary to these successes, a rational approach to increase the thermostability of *Penicillium camembertii* lipase by introducing a disulfide link was a failure (*182*). Tao and Cornish (*183*) reviewed the milestones in directed evolution of enzymes.

Numerous choices are available for creating DNA libraries, including error-prone PCR, combinatorial oli-



Scheme 4. Activity of hydrolases towards tertiary alcohol substrates is determined by the amino acid motif: GGGX or GGAX. Numerous GX motif containing hydrolases did not catalyze the reaction (*166*)



Scheme 5. Transesterification of 2-phenyl-but-3-yn-2-ol with vinyl acetate in isooctane to achieve high enantioselectivity (E = 65) compared to less selective hydrolysis reaction (reversal of the shown reaction) (*167*)

gonucleotide mutagenesis, DNA shuffling, exon shuffling, random-priming recombination, random chimeragenesis on transient templates (RACHITT), staggered extension process (StEP recombination), heteroduplex recombination, incremental truncation for the creation of hybrids (ITCHY), recombined extension on truncated templates (RETT), degenerate oligonucleotide gene shuffling (DOGS) and *in vivo* recombination. Lutz and Patrick (*153*) discussed recently developed methods for directed evolution, including MAX randomization, which is an oligonucleotide-directed randomization method for creating molecular diversity in zinc-finger protein (*184*), and sequence saturation mutagenesis (SeSaM) (*185*).

situations. Many reviews on various aspects of directed evolution, citing various examples, have appeared (*187, 199–204*). Several developments have been reported for the high-throughput screening of enzyme libraries created by directed evolution (*141,146,205–207*). Also, several algorithms and computational models have been proposed to improve the efficiency of directed evolution (*208*). Table 2 illustrates some examples of important biocatalysts that have been improved by directed evolution. Improvements of enzymes by directed evolution include modifications of stability, activity and specificity. Bornscheuer (*209*) subdivided examples for novel substrate specificity by directed evolution into three groups:

Table 2. Examples of enzymes modified by directed evolution

Enzyme	Improved properties	Reference
Specificity improvement		
Pseudomonas aeruginosa lipase	Enantioselectivity towards 2-methyldecanoate	*(186,187)*
Hydantoinase	Enantioselectivity towards D- or L-form	*(188)*
D-2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase	Enantioselectivity towards both D- and L-glyceraldehyde	*(189)*
β-galactosidase	Changing activity to β-galactosidase	*(190)*
Staphylococcus aureus lipase	Changing activity to phospholipase	*(191)*
Pseudomonas fluorescens esterase	Activity on sterically hindered substrate	*(192–194)*
Bacillus thermocatenulatus lipase	Increase in phospholipase activity	*(195)*
Activity and stability improvement		
Homologous lipases from *Candida antarctica, Hyphozyma* sp., *Cryptococcus tsukubaensis*	Chimeric lipases more stable and active toward 3-(3′,4′-dichlorophenyl)glutarate	*(196)*
D-amino acid amidase	Enhanced thermostability and activity	*(197)*
N-carboamyl-D-amino acid amidohydrolase from *Agrobacterium tumefaciens*	Improvement of oxidative stability and thermostability	*(198)*

The mutant genes obtained from any method of directed evolution are transformed and expressed in a suitable host. Enzyme libraries are selected and screened for a range of selection properties. Improved genes obtained in the first round may be used as templates for the subsequent evolution cycles. For sexual evolution, a pool of homologous genes (or genes generated using asexual methods) is partially digested with DNase I and recombined by PCR. Alternatively, homologous recombination can also be achieved *in vivo* based on the transformation of *Saccharomyces cerevisiae* or *Hansenula polymorpha*. The linearized fragments possessing overlapping DNA sequences are cotransformed into the yeast. These fragments undergo homologous recombination *in vivo*, resulting in the restoration of the plasmid's circular topology. The plasmid repair is an efficient process and recombination between overlapping sequences as short as 30 bp can be readily achieved. *In vivo* recombination offers some unique advantages: efficient cloning of the resulting hybrid sequences in a large yeast expression vector and constitution of a second round of DNA shuffling without using PCR-based techniques.

Whether specific regions or the whole gene should be targeted for mutation and methods of identifying improved variants (screening vs. selection) are the issues of debate. There is no agreement between different research groups as to a single best approach and there may never be one, since they all address different needs and (*i*) conversion of substrates which in principle should be accepted by the enzyme under investigation, but do not react due to steric hindrance or electronic effects; (*ii*) conversion of substrates which are usually converted by another enzyme; (*iii*) conversion of compounds that are already converted by the enzyme of interest, but at very low activity compared to the well accepted substrates.

Synthesized oligonucleotides have been used to create suitably diverse gene libraries and to allow researchers to tailor the diversity of a library with higher precision (*210,211*). There are numerous success stories of directed evolution to list (*212–215*). DeSantis *et al.* (*216*) developed a new technique called gene site saturation mutagenesis (GSSM), whereby each amino acid of the protein is replaced with each of the other 19 naturally occurring amino acids to create highly enantioselective nitrilase mutants. Although GSSM has led to the identification of novel mutants, it may not be a feasible technique for academic laboratories owing to the limited manpower and availability of instrumentation for each research area.

Many of the protein design efforts carried out to date have focused on using only a single method. While rational design is well suited to optimize direct interactions, it is not suitable for identifying distant mutations. Directed evolution, although good for optimizing an existing activity, may not be suitable for introducing a completely novel activity. Therefore, it is beneficial to

combine the powers of individual techniques. Recently there have been attempts to combine rational design and directed evolution to improve biocatalysts (*201,208*). Also, directed evolution has been applied for metabolic pathway engineering (*217,218*). The ability of directed evolution to transfer a large number of genes simultaneously has also formed the basis of the emerging area of combinatorial biocatalysis, which employs iterative reactions catalyzed by isolated enzymes or whole cells, in a natural or unnatural environment, on substrates in solution or on a solid phase and harnesses the natural diversity of enzymatic reactions to generate libraries of organic compounds (*219*).

Very recently, researchers have had a breakthrough success in stringing together artificial DNA bases, which could aid the creation of altered genetic material for applications in medicine and technology. The tricky part is stitching these artificial bases into a chain, and getting the chains to replicate as natural DNA does, with the help of DNA polymerase, which works its way along a template strand of DNA, reading each base and collecting a matching base to create a new, copied strand. However, the enzyme fails to copy artificial bases. All natural DNA is made up of just four bases, but researchers have created non-natural bases, which can be used to make forms of DNA that are more robust than the natural kind and do not break apart when exposed to high temperatures. Such super-DNA could be useful in a wide range of medical and technological applications. Romesberg and co-workers (*220*) have harnessed directed evolution to find an enzyme capable of assembling non-standard DNA. After four rounds of selection, they found several mutants capable of doing the job. One was particularly impressive. Interestingly, in a different study, Benner and colleagues (*221*) found an enzyme made by HIV, the virus that causes AIDS, to do the same job. Benner's team created a more successful polymerase by starting with a different enzyme altogether – a reverse transcriptase (RT) that is made by the HIV-1 form of the virus that causes AIDS. This RT mutates very efficiently when the virus is hit with anti-HIV drugs. Benner's group checked mutated forms to find a modified RT capable of stitching two non-standard bases into strings of DNA. They fine-tuned this enzyme using rational design to create the capability of making multiple copies of non-natural DNA, opening up the possibility that the code could eventually evolve on its own.

The success of selection or screening procedures of mutant libraries obtained using the directed evolution technique continues to be one of the main concerns (*222*). Phage display is a powerful *in vitro* tool that extends the range of modern combinatorial selection techniques, allowing discovery and characterization of proteins that interact with a desired target (http://cfac.uk/phrmy/PCB/PagePhageDisplay.htm) (*223*). In phage display technology the proteins displayed a range from short amino acid sequences to antibody fragments, cDNA, hormones and enzymes. A selection process of enzyme mutants using phage display can handle a large library ($>10^7$ clones). The most often used vectors are the filamentous bacteriophages, fd, M13, or related phagemids (*224*). Another approach for library creation is *in-vitro* (cell free) compartmentalization (IVC) (*225*). Unfortunately, both these techniques are difficult to use and a range of enzyme selections has not been presented.

Recently, Moore *et al.* (*226*) have presented surface-enhanced resonance Raman scattering (SERRS) to measure lipase activity and selectivity on silver nanoparticles. The method enables detection of ultra-low levels of hydrolase activity and hence is useful to detect *in vivo* levels of enzyme concentrations. In its current format it can detect the activity of 500 enzyme molecules. Bornscheuer (*227*) discussed the merits of SERRS and identified the need for specific instrumentation, the need to adapt the system for microtiter plate format, and the necessity of further work to design substrate dyes as bottlenecks.

Concluding Remarks

Biocatalysis has not yet achieved the status of a first-line alternative, being used only as a last resort when other possible synthetic schemes fail. Nevertheless, there are numerous examples of industrial biocatalytic processes. Although the enzymatic reactions are no longer restricted to aqueous solutions, only a fraction of the current industrial processes utilize organic solvents as media. Protein engineering is emerging as a major thrust area in improving enzyme activities and in finding novel applications. Continuing improvements and a better understanding of biocatalysis are expected to greatly influence the production of fine chemicals.

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Poboljšanje enzima za djelotvornu biokatalizu

Sažetak

Biokatalitički procesi imaju veliku primjenu ne samo u znanosti nego i industriji. To je posebno izraženo u farmaceutskoj i agrokemijskoj industriji gdje je osobita potreba za optički čistim molekulama. Proces biokatalize sve se više razvija generiranjem novih i inovativnih biokatalizatora dobivenih mikrobnim skriningom, razvojem unutar samih biokatalitičkih procesa (npr. korištenje nevodenih otapala za sintezu) te poboljšanjem aktivnosti enzima različitim mehanizmima (mijenjanjem otapala i modifikacijom enzima) uključujući proteinsko inženjerstvo. Najbolji rezultati dobiveni su utvrđujući gen koji kodira odgovarajući enzim kako bi se provelo proteinsko inženjerstvo racionalnim i neracionalnim postupcima. U radu su razmatrani različiti postupci za poboljšanje svojstava enzima ili pri kreiranju novih aktivnosti.