Biocatalysis and industry have not always been associated. For much of chemical antiquity, most chemists did not accept the idea of using enzymes as catalysts for industrial applications. Among the reasons cited were that enzymes are too inflexible in their substrate choice and chemistry performed, they are unstable at high temperatures, they function poorly in organic solvents, and they are too costly to develop. However, with help from recent advances in genetic engineering and bioprocess engineering, the development of biocatalysts for industrial applications is one of the fastest growing segments in the chemistry arena. Biocatalysts are making their mark on numerous industrial processes (1–4).

Two impressive examples include the use of enzymes by Bristol-Myers Squibb in the synthesis of Omapatrilat, 1, a vasopeptidase inhibitor (Figure 1) for the treatment of hypertension (5) and the use of nitrile hydratase by Nitto Chemical Company of Japan to produce acrylamide, 3, from acrylonitrile, 2, on a 40,000 ton per year scale (Scheme I; ref 2).

One of the greatest rewards of biocatalysis in industry is the reduction of waste. With each passing year more and more legal restrictions on waste storage and disposal are employed in this country and throughout the world. It is getting harder and more costly for companies to capriciously create waste. Enzymes often represent almost zero waste for companies as they can be reused, and once they reach the end of their service life they can be discarded through conventional waste streams. The chemical catalysts that enzymes often replace are heavy metals, which are tightly regulated and in many cases hazardous to the environment and human health. One further reduction of waste is that enzymes operate in ambient conditions without the necessity for organic solvents, high temperature, or high pressure. Nature has adequately evolved enzymes into the cleanest catalysts.

Perhaps eclipsing the reduction of waste is the incredible selectivity provided by enzymes. There are numerous stereoselective enzymes that either utilize or create only one stereomer of substrate or product, respectively. The importance of stereoselectivity in chemical synthesis has clearly been shown by a particularly distasteful example. Thalidomide, 4, was commonly given to pregnant women as a sedative to combat morning sickness in the late 1950s and early 1960s (Figure 2). This drug was marketed as a racemic mixture of (R)- and (S)-enantiomers. Only later was it discovered that (S)-thalidomide causes birth defects such as blindness, deafness, and internal disabilities, while (R)-thalidomide is a safe and effective medication (6, 7).

The huge disparity between the biological actions of enantiomers has resulted in the U.S. Food and Drug Administration requiring each enantiomer of a racemic drug to pass clinical testing individually. Only then will a drug receive approval for widespread distribution. Since the cost of bringing a new drug to market is on the order of hundreds of millions of dollars, it is advantageous for the pharmaceutical industry to produce enantiopure compounds for clinical trials, which account for approximately
one-third of that total drug development cost. Similarly, enantiopure compounds are also highly desirable in the agricultural industry because often only one stereomer of the pesticides is the active agent.

In addition to being stereoselective, enzymes are also regioselective; that is, enzymes may distinguish between identical or similar functional groups that are chemically situated in different regions of the same substrate molecule. For example, in a peptide all of the amide bonds are identical, but there are proteases that recognize and hydrolyze specific amide bonds based on the surrounding protein sequence. This type of selectivity is very desirable in chemical synthesis because it eliminates the need for protecting groups and therefore shortens and simplifies syntheses significantly. Finally, enzymes also create few side products, reducing the need to remove them and simplifying purification in general, while often performing more sophisticated chemistry than current inorganic and organic catalysts.

Biocatalyst Discovery and Optimization

Despite all of the above-mentioned advantageous features, the naturally occurring enzymes often cannot meet the stringent requirements for a specific industrial setting since they are optimized for very controlled and specific reactions under the constraints of homeostatic life. So how is industry getting around the problems such as low substrate flexibility, poor stability, and high development and operation costs? In the past decades, several powerful molecular approaches have been developed, including rational design, bioprospecting, and directed evolution.

Rational design is an approach in which site-directed mutagenesis is used to introduce site-specific changes into the protein of interest. Although it has been widely used, this method has only resulted in limited success for several reasons. First, one must have a detailed understanding of the protein of interest. For example, the minimum knowledge required for rational design must contain some structural information, the reaction catalyzed, the mechanism of catalysis, and a short list of amino acid residues likely to influence the protein function of interest. Second, even when a protein is exhaustively studied it can still be very difficult to identify the amino acid residues that are responsible for existing protein functions and the amino acid substitutions that might give rise to new functions. In fact, protein engineers frequently have been surprised by the range of effects caused by single mutations designed to change only one specific and simple property of an enzyme. This situation becomes even more complicated now that many directed evolution experiments have shown that residues far away from the active site can significantly affect activity (8, 9).

Prospecting natural diversity is a proven approach for discovering new industrial enzymes. Traditionally, enzymes used in industry have come from a small number of well-classified microorganisms, such as genera Bacillus and Pseudomonas, while the vast majority of microorganisms remain untapped (10). Several companies including Oxford GlycoSciences, Diversa, and GlaxoSmithKline have taken advantage of this unexplored diversity for prospecting proteins that might better meet the requirements for industrial processes and to discover novel enzymes (11). In the past few years, numerous new enzymes have been isolated from cultivated and uncultivated microorganisms using novel molecular approaches such as expression cloning, molecular screening, and database mining using bioinformatic tools (10).

Directed evolution, as illustrated in Figure 3, has recently emerged as a powerful alternative approach for engineering biocatalysts (8, 12–15). This in vitro method mimics the Darwinian evolution and involves creation of genetic diversity followed by screening or selection for those enzymes with the desired features. Unlike the natural evolution of species, which may take millions of years, directed evolution can be carried out within weeks or months and with unlimited number of parents. Also, unlike rational design, directed evolution does not need any structural or mechanistic information of the protein of interest. As a result, directed evolution is a fast and inexpensive way of developing biocatalysts.

Directed Evolution

For centuries, people have been using a process of selective breeding (classical breeding) to produce new varieties of plants and animals, such as dogs, crops, and flowers. By Darwin’s own accounts, “The key (of breeding) is man’s power of accumulative selection: nature gives successive variations; man adds them up in certain directions useful to them.” (16)

As shown in Figure 3, the first step in directed evolution is to create genetic diversity starting from a target gene or a family of related genes. This is usually achieved by random mutagenesis or gene recombination. The resulting gene library is inserted into an expression vector and transformed into a laboratory microorganism for protein expression. The expressed proteins are then sorted for the desired function(s) by screening or selection and those genes, whose encoding improved proteins, are used as the parents for the next round.

![Figure 3. Schematic representation of directed evolution. As with Darwinian natural evolution, genetic diversity is introduced into a target gene through random mutagenesis or gene recombination. Functionally improved mutants are first identified by a selection or high throughput screening method and then used as the parents for next round of evolution. The process will be repeated until the goal is achieved.](image-url)
of evolution. This process of improvement is repeated until the goal is achieved.

Random mutagenesis at the nucleotide level is the simplest way to introduce genetic diversity. Effective methods include chemical mutagens, UV radiation, mutator strains, and error-prone polymerase chain reaction (EP-PCR) (17). Among these methods, EP-PCR utilizes a low-fidelity polymerase such as Thermus aquaticus (Taq) polymerase in combination with reaction conditions that further decrease the fidelity of the polymerase including unequal concentrations of nucleotide bases and varying concentrations of Mg$^{2+}$ and Mn$^{2+}$. As a result, the products of EP-PCR are gene variants that contain random point mutations along the full-length genes. It should be noted that because of codon degeneracy, not every nucleotide substitution results in an amino acid change.

Gene recombination represents an alternative approach for creating genetic diversity that is based on the reassortment of genetic information among a number of parental genes. Compared to random mutagenesis, the key advantage of gene recombination is its ability to accumulate beneficial mutations while simultaneously removing deleterious mutations. Many gene-recombination methods have been developed, such as DNA shuffling, staggered extension process (StEP) recombination, random priming recombination (RPR), random chimeragenesis on transient templates (RACHITT), incremental truncation for the creation of hybrid enzymes (ITCHY), and sequence homology-independent protein recombination (SHIPREC) (17). Among these methods, DNA shuffling was the first developed and most widely used method for in vitro gene recombination (18). In this method, parental genes from a pool of selected variants are first randomly fragmented and the purified fragments are then reassembled into full-length gene products by repeated cycles of overlap extension reaction. Recombinogenic events occur when fragments derived from different parental genes prime one another. The end products of gene recombination are chimeric genes containing pieces of DNA from different parental genes. The process of gene recombination closely resembles the sexual recombination of classical breeding. In essence, gene recombination is classical breeding at the DNA level, potentially between many parents.

Given a thoughtful approach to creating genetic diversity, the development of an efficient and sensitive method to search for the desired protein function is probably the most challenging step. There are two categories of searching methods: screening and selection. Screening is the process in which every library member is assayed individually by using biochemical or biophysical analysis. This is the most commonly done in either petri dishes or microtiter plates (usually 96-well plates or 384-well plates) using a colored or otherwise easily detected substrate or product. These high throughput screens can be done with whole cells, cell lysates, or partially purified enzymes. However, these screens must be performed in a short time frame and with as many variants at a time as possible if a sufficient portion of the library is to be assessed. Often screening limits the number of library members assessed to $10^4$–$10^6$, even with the use of robotic hardware. On the other hand, screening is very flexible and versatile since experimental conditions can be easily tailored to meet the applied reaction constraints, such as nonnatural environments or substrates. In some cases, nonnatural substrates cannot be directly screened by a colorimetric method, and surrogate substrates are not available to produce the desired effect on the enzyme. As a result, fast analytic methods such as high-pressure liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) might be utilized for performing an efficient screen of a nonnatural substrate.

In a selection, the enzyme is linked to the survival or growth of the host organism such that only the organisms possessing the desired property will grow or grow faster. This is often achieved by genetic complementation of host organisms that are deficient in a certain pathway or activity. Using selection, large libraries of enzyme variants can be assessed and the size of the library is limited only by the cell transformation efficiency. Unfortunately, devising a selection method for a given enzyme is very difficult in most cases since the desired enzyme feature is often nonnatural and cannot be coupled to the growth and survival of the host organism. Even when a selection is available, because of the redundancy and complexity of genetic regulatory networks, host organisms can often create solutions that are not related to the targeted enzyme feature. Thus, extra care must be taken to ensure the positives are indeed the result of the mutations in the targeted enzyme.

Successful Applications of Directed Evolution

During the past few years, directed evolution has blossomed in both academia and industry. It has been successfully utilized to engineer numerous DNA, RNA, or protein based biological products or processes, as well as to decipher the intimate relationship of protein structure and function. A few selected, recent examples of applying directed evolution to redesign enzymes in an isolated form or in the context of pathways are briefly described below. Collectively, these examples have demonstrated that directed evolution is an extremely powerful tool for biocatalyst development.

Enzyme Engineering

A common problem in biocatalysis is that many of the desired substrates have very low solubility in water, and the catalytic activity for most enzymes is significantly reduced by the addition of even small quantities of organic solvents. Traditionally this has limited the use of biocatalysts, but now several examples have shown that biocatalysts could be evolved to function in nonaqueous solvents allowing their use with these water-insoluble substrates (19–21). One example is the directed evolution of p-nitrobenzyl esterase for enhanced activity in aqueous organic solvents (20). This enzyme can hydrolyze an ester bond and release the desired substrate or product. These high throughput screens can be done with whole cells, cell lysates, or partially purified enzymes. However, the screens must be performed in a short time frame and with as many variants at a time as possible if a sufficient portion of the library is to be assessed. Often screening limits the number of library members assessed to $10^4$–$10^6$, even with the use of robotic hardware. On the other hand, screening is very flexible and versatile since experimental conditions can be easily tailored to meet the applied reaction constraints, such as nonnatural environments or substrates. In some cases, nonnatural substrates cannot be directly screened by a colorimetric method, and surrogate substrates are not available to produce the desired effect on the enzyme. As a result, fast analytic methods such as high-pressure liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) might be utilized for performing an efficient screen of a nonnatural substrate.

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(DMF) rapidly deactivated the wild-type enzyme. Thus, directed evolution was used to create a $p$-nitrobenzyl esterase mutant that maintains high activity in nonaqueous solvents. To find the desired mutant from a large library of enzyme mutants, a colorimetric screening assay was devised using a slightly modified substrate that formed a $p$-nitrophenol (yellow color) upon hydrolysis of the ester bond (Scheme III). EP-PCR was utilized to create a library of esterase genes, which were then cloned into an expression plasmid and transformed into E. coli. Colonies harboring different esterase mutants were screened for enzyme activity in 96-well plates in the presence of aqueous DMF using a 96-well plate reader. The colonies that were more active than the parent from the screening were assayed again using purified enzymes and the real substrate. The confirmed best mutant was then used as the parent for next round of EP-PCR. The $p$-nitrobenzyl esterase mutant that resulted from four rounds of directed evolution had a 24-fold increase in catalytic efficiency ($k_{\text{cat}}/K_M$) in 15% DMF. When the sequences of the mutants were compared to that of the wild-type enzyme, it was found that none of the substitutions yielding greater activity actually contacted the substrate. In fact, some of the mutations involved residues greater than 20 Å away from the nearest active site residue (Figure 4; ref. 22). For example, the distance between the side chain of mutation H322R and the side chain of the active site residue S189 is 21.8 Å. In addition, it was difficult for the amino acid substitutions yielding greater activity to be rationally explained, highlighting the power of directed evolution to circumvent preconceived notions.

Another typical problem with using enzymes as biocatalysts is that the available enzymes simply do not make the desired product because of their poor or undesired enantio-
selectivity and substrate preference. There are a few examples of enantioselectivity being enhanced and changed in enzymes. Liebeton et al. (23) were able to substantially increase the enantioselectivity of a lipase toward 2-methyldecanoate, 10 (Scheme IV). Using EP-PCR and an efficient screen they were able to increase the enantiomeric excess of the enzymatic-reaction product from 2% to 90%. Surprisingly none of the amino acid substitutions creating this change were found near the substrate-binding pocket. Enantioselectivity in lipases is important because several industrial companies utilize lipases for enantiomer resolution. Arnold and coworkers (24) were able to invert the enantioselectivity of hydantoinase from D (40% enantiomeric excess) to L (20% enantiomeric excess) in preference. Highly D-selective mutants (90% enantiomeric excess) were also found. These engineered mutants were licensed to Degussa Fine Chemicals, which is currently developing a biocatalytic process for the synthesis of various L-amino acids.

Hager et al. described an excellent example of how the reaction catalyzed by the enzyme can be altered by directed evolution (21). The enzyme chloroperoxidase (CPO) was subjected to directed evolution to improve the chiral epoxidation activity (Scheme V). The native enzyme has very low levels of epoxidation activity because of the high catalase activity, which robs the reaction of oxidant. Chiral epoxidations are important reactions in chemical synthesis because they generate optically active compounds when the epoxide is opened stereoselectively, either chemically or enzymatically. The importance of epoxides in synthesis has been widely recognized, but they are still currently generated by costly inorganic catalysts, which also result in waste disposal issues. A library of CPO genes was created by EP-PCR and transformed into the fungal host Caldarimyces fumago. The hosts were then screened for catalase activity and epoxidation activity. Three rounds of random mutagenesis coupled with screening led to several CPO mutants that had a 15-fold or greater enhancement of epoxidation activity on p-nitrostyrene, 11. The primary mutation responsible for the increased activity was the replacement of a heme ligand cysteine by either histidine or tyrosine. Interestingly, some of the mutants were also found to have increased activity in aqueous organic solvents.

The crystal structure of the evolved p-nitrobenzyl esterase mutant. The catalytic triad—S189, H399, and E310—is shown in light gray (red in color), the mutations are medium gray (green in color), and the loops that reorganize most significantly are in dark gray (yellow in color). (See p 3 for the color figure.) Most of the activating mutations are located far from the active site. For example, the distance between activating mutation H322R and the active site residue S189 is 21.8 Å. (Figure adapted from ref 22 with permission.)
Enzymes as true catalysts do not affect how much products are available at reaction equilibrium; they only speed up the process of achieving equilibrium between substrates and products. Therefore, it is common practice to increase the reaction temperature of endothermic reactions to shift the equilibrium towards products. Also, increasing reaction temperature can increase the overall reaction rates. Unfortunately, heat often denatures or severely reduces the catalytic power of enzymes.

Directed evolution has once again proven useful in creating biocatalysts that are stable at elevated temperatures. A recent example described by Zhao and Arnold was the conversion of a mesophilic enzyme, subtilisin E, into its thermophilic counterpart (25). In this work, alternating rounds of EP-PCR and a gene-recombination method, StEP, were used to create libraries of subtilisin E mutant genes (Figure 5). A 96-well plate based screening method was used to find the desired mutants, in which a chromagenic substrate was used to measure the enzyme activity spectroscopically. The mutants were grown into the 96-well plates and grown overnight. Two duplicate plates were prepared by taking an aliquot of the supernatant from each well of the growth plate containing the secreted subtilisin enzymes. The ratio between the enzyme activity before heat treatment for a certain time at a certain temperature and the enzyme activity after heat treatment was taken as the thermostability index. The criterion for selecting a positive mutant is that the mutant should have a higher thermostability index and a similar or higher initial activity compared to the parental enzyme. After five rounds of directed evolution, the resulting subtilisin E mutant was as stable as its naturally occurring thermostable counterpart, thermitase, isolated from a thermophilic microorganism. That mutant exhibited 200-fold increase in half-life of thermal inactivation at 65 °C compared to the wild-type enzyme. Also, that same mutant showed 5-fold higher activity than that of the wild type enzyme.

In addition to biosynthesis, directed evolution is gaining momentum in the area of environmental remediation. Over the past decades enormous quantities of industrial chemicals have been released into the environment. A large number of them, particularly those structurally related to natural compounds, are readily degraded by microorganisms found in soil and water. However, superimposed on the rich variety of natural organic compounds present in the environment is the increasing number of novel industrial organic chemicals heavily substituted with chemical groups (Cl, SO₃, etc.) not found or not extensively present in organics of biological origin. These xenobiotic compounds are usually catabolized slowly and tend to persist and accumulate in the environment.

Although natural microorganisms collectively exhibit remarkable evolutionary capabilities to adapt to a wide range of chemicals, the different evolutionary processes (mutation, gene transfer, gene rearrangement, and recombination) occur at an extremely slow rate (on the order of 10⁻⁴ to 10⁻⁸ per cell per generation), particularly when the acquisition of multiple catalytic activities is necessary. In such cases, acceleration of these events via directed evolution (such as DNA shuffling) may be helpful. In many cases, molecular techniques are the most useful way to enhance substrate specificity since microbial degradation usually occurs via cometabolism and does not exert a selective pressure on bacteria.
One example is the neurotoxic organophosphates that are used extensively as agricultural and domestic pesticides. Although an enzyme isolated from soil microorganisms, organophosphorus hydrolase (OPH), has been shown to degrade these pesticides, the effectiveness of hydrolysis varies dramatically. Some highly used organophosphorus insecticides such as methyl parathion, chlorpyrifos, and diazinon are hydrolyzed 30–1000 times slower than the preferred substrate, paraoxon (Figure 6). This reduction in catalytic rate is due to the unfavorable interaction of these substrates with the active sites involved in catalysis or structural functions. Site-directed mutagenesis has been applied to the various residues involved in the active sites, resulting in OPH mutants with improved catalytic characteristics against paraoxon, sarin, and soman. Although the 3D structure of OPH has been elucidated, identifying all the amino acids responsible for substrate specificity and those that might give rise to extended specificity remains an overwhelming challenge. To this end, in vitro directed evolution is perhaps the most useful way to sample this sequence flexibility in a simple and rapid fashion.

Sequential cycles of DNA shuffling and screening were used to “fine tune” and enhance the activity of OPH towards poorly degraded substrates. Owing to inaccessibility of these pesticides across the cell membrane, OPH variants were displayed on the surface of E. coli using the truncated ice-nucleation protein to isolate novel enzymes with truly improved substrate specificities. Two rounds of DNA shuffling and screening were carried out and several improved variants were isolated. One variant 22A11, in particular, hydrolyzes methyl parathion, 25-fold faster than the wild-type enzyme (Scheme VI).

**Beyond Enzyme Engineering**

The amazing diversity of compounds found in nature is not created by just one enzyme, but rather by multienzyme complexes or metabolic pathways. Some of the naturally
occurring pathways have been engineered to produce many industrially important compounds such as alcohols, aromatics, carbohydrates, and organic acids. As a logical extension of enzyme engineering, the methodology of directed evolution has been used to create and improve these metabolic pathways. In comparison with classical rational design-based metabolic engineering approaches, this new directed evolution-based metabolic engineering approach has an important advantage; that is, it can optimize not only single enzymes, but also interactions between enzymes and thus the overall metabolic flux of the system.

Schmidt-Dannert et al. (31) described a new strategy to engineer novel biosynthetic pathways by first assembling homologous genes from different microorganisms and then optimizing the synthesized pathway by directed evolution (Figure 7). The carotenoid biosynthetic pathway was chosen as a model system because carotenoids are important natural products and the bright color of carotenoids makes them easy to screen from a library of variants. The new pathways are those that produce more intense colors than the original or those that produce new colors. In a first step, two genes (crtE, geranylgeranyl diphosphate synthase, and crtB, phytoene synthase) responsible for the production of phytoene, a carotenoid precursor, were introduced into E. coli. Then, a library of desaturase mutants were created by DNA shuffling of two homologous phytoene desaturase genes (crtI) from Erwinia strains, which normally desaturate phytoene, 18, to lycopene, 19, and were introduced into the same E. coli strain. Several novel carotenoid compounds such as phytoene with few desaturations and tetrahydrolycopene, 20, were isolated. Further extension of the evolved tetrahydrolycopene pathway with a library of cyclase mutants created by DNA shuffling of two homologous lycopene cyclase genes (crtY) from Erwinia strains, which normally cyclize lycopene 19 to form β-carotene respectively, created many different colored variants. A bright red strain of E. coli was found to produce torulene, 21, a carotenoid common in red yeast, but never before found in bacteria.

Conclusions

Industrial biocatalysis is on the verge of significant growth. Recent development of novel highly effective gene discovery methods has greatly increased the availability of biocatalysts. In particular, the development of directed evolution methods enables the functional gap between naturally occurring enzymes and the desired biocatalysts used in a specific industrial setting to be rapidly closed. In contrast to earlier attempts at the rational design of improved enzymes, directed evolution is a much faster, more effective, and less expensive approach for biocatalyst development. With these new tools and other potential development in functional genomics, proteomics, and bioinformatics, the widespread use of biocatalysts in pharmaceutical, chemical, food, and agricultural industries will be ensured in the coming decade.

Literature Cited