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Recent Advances in Biocatalysis by Directed Enzyme Evolution

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Abstract: Naturally occurring enzymes are remarkable biocatalysts with numerous potential applications in industry and medicine. However, many of their catalyst properties often need to be further tailored to meet the specific requirements of a given application. Within this context, directed evolution has emerged over the past decade as a powerful tool for engineering enzymes with new or improved functions. This review summarizes recent advances in applying directed evolution approaches to alter various enzyme properties such as activity, selectivity (enantio- and regio-), substrate specificity, stability, and solubility. Special attention will be paid to the creation of novel enzyme activities and products by directed evolution.

Keywords: Enzyme engineering, biocatalysis, directed evolution, rational design, novel function.

INTRODUCTION

It is widely recognized that enzymes hold tremendous potential for industry. Currently, over 500 products across a wide spectrum of applications utilize enzymes in their manufacture, including the easily recognizable examples of ephedrine, aspartame, and amoxicillin [1,2]. The majority of industrial enzymes (65%) are used in technical applications by the detergent, starch, textile, leather, personal care and pulp and paper industries; the remainder are largely used in food processing (25%) or in animal feed supplements (10%) [2]. The exquisite specificity and selectivity (enantio- and regio-) of some enzymatic transformations makes them desirable for the production of fine chemicals and pharmaceutical intermediates. Their low environmental impact is also attractive: enzymes are drawn from renewable resources (generally from large-scale bacterial fermentation), are biodegradable, and typically operate in aqueous solvent under mild temperatures and pH conditions [2]. Unfortunately, in many cases naturally occurring enzymes are not optimal for the applications of interest, since after all they are evolved in nature for a purpose other than being the catalysts of industrial processes.

Three main different, yet complementary approaches have been used to develop enzymes with optimal catalyst performance in the past several decades (Fig. **1**). One approach is rational design, in which site-specific changes are made on the target enzyme with the aid of detailed knowledge about the protein structure, function, and catalytic mechanism. Another approach is directed evolution, which involves repeated cycles of random mutagenesis and/or gene recombination followed by high throughput screening or selection of the functionally improved mutants. This approach does not rely on *a priori* extensive structural and mechanistic information on the proteins. As a result, directed evolution has been very successful in engineering enzymes with optimal catalyst performance [3-5]. A third approach is biodiversity prospecting, or bioprospecting, in which new enzymes with better performance are isolated from living organisms. Given the extensive literature on similar topics, this review will only cover recent progress in enhancing biocatalysts by directed evolution, particularly focusing on examples of enzyme catalysts with current or potential industrial and therapeutic applications.

ENHANCING BIOCATALYST ACTIVITY

Enzymes are known to catalyze a broad range of transformations (and perhaps many more remain to be discovered), but often at levels of activity too low for commercial or therapeutic use. Castle *et al.* [6] isolated and improved the activity of a glyphosate tolerance gene by directed evolution. The herbicide glyphosate is used in conjunction with tolerant crops, commercially sold as Roundup Ready, and these genetically-engineered plants are the most prevalent genetically modified crops in use. Glyphosate targets the plant enzyme enolpyruvylshikimate-3-phosphate synthase, and current glyphosate tolerant crops possess a microbial version of the enzyme which is not inhibited by the compound. Glyphosate was shown to accumulate in the meristems of plants [7], which may disturb reproductive development or lower yields of tolerant crops [8]. Detoxification of glyphosate as a means of tolerance is advantageous, as it would allow application during reproductive development and provide a more robust tolerance. Therefore an enzyme capable of *N*-acetylation of glyphosate, which destroys its herbicidal activity, was desired. Screening bacilli resulted in the discovery of *N*-acetylation activity by *Bacillus licheniformis*, and the glyphosate *N*-acetyltransferase (GAT) enzyme was identified in three *B. licheniformis* strains. However, glyphosate is a poor substrate for this enzyme: heterologous expression of the *gat* genes in *Escherichia coli* did not confer resistance to glyphosate, nor did expression in tobacco and *Arabidopsis*. To improve the efficiency of the enzyme and its specificity for glyphosate, the three *gat* genes were recombined by family shuffling [9]. In further rounds of evolution more diversity was incorporated by including genetic information from related hypothetical proteins and employing the synthetic shuffling technique [10]. After a total of 11 rounds

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of directed evolution a GAT variant was isolated with catalytic efficiency improved 10,000-fold over the parent. Maize plants possessing the 11th round improved *gat* gene were unaffected by glyphosate application to 6-fold excess of the amount normally applied in the field.

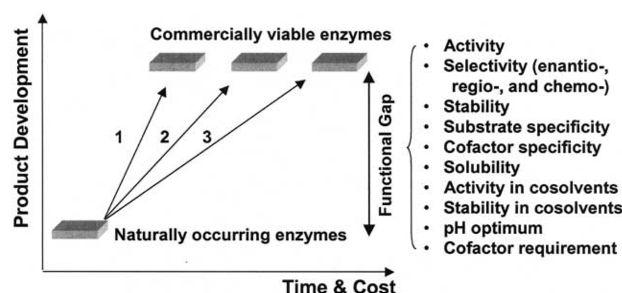


Fig. (1). Existing approaches for developing commercially viable enzymes that often require optimized features such as activity, selectivity and stability. Among the three most widely used approaches including directed evolution (1), rational design (2), and bioprospecting (3), directed evolution is considered as the most effective approach in filling the functional gap between naturally occurring enzymes and the commercially viable enzymes in terms of time and cost.

Low activity may also result from the desire to utilize an enzyme under circumstances for which it is not optimized. Extant proteins are evolved to work best in a certain environment, but application of enzymes as biocatalysts in industry, medicine, and agriculture may demand high activity at a different temperature, pH, or medium, or in the presence of unnaturally high levels of substrate and product. At the conditions required for use, an enzyme may retain only a fraction of its activity. In such cases, directed evolution can be used to improve the activity of an enzyme under specified conditions.

Overcoming Product or Substrate Inhibition

Enzyme activity may be inhibited by the presence of high substrate concentration or the accumulation of product to high concentration in a negative-feedback loop, and use of enzymes as biocatalysts is complicated by these forms of inhibition. Limited results are available regarding the use of directed evolution to specifically overcome product or substrate inhibition of enzymes. Kim *et al.* used directed evolution to increase the thermostability of an enantioselective esterase from *Pseudomonas fluorescens* KCTC 1767 and also found a decrease in substrate inhibition [11]. Commercial synthesis of the valuable compound (S)-ketoprofen, a common anti-inflammatory, seemed possible using this enzyme, but its low thermal and structural stability proved limiting. Random mutagenesis by error-prone polymerase chain reaction (epPCR) and staggered extension process (StEP) recombination were used in two rounds of evolution, and mutants were screened for residual activity after exposure to a temperature challenge of 45-60 °C. The best mutant isolated in this study ("6-52") showed increased thermal stability, as well as several other improvements: increased tolerance to alkaline pH and superior substrate tolerance. For low concentrations of substrate (<5 mM) conversion rates for the wild type and mutant enzymes were similar, but conversion yield for the mutant 6-52 was 1.5-1.7 fold higher over a range of higher substrate concentrations (10-100 mM). Although an additional round of evolution was attempted using mutant 6-52 as the parent, no further improvement was found.

Non-Aqueous Solvents

The application of enzymes in two-phase and organic solvent systems has lately gained interest, and is discussed in several reviews [12-16]. The requirement of aqueous media for biocatalysis represents a significant limitation, which is addressed by directed enzyme evolution. Some studies have found that improving the thermostability of an enzyme *via* directed evolution results in concomitant improvement of organic solvent tolerance [17,18], and it is also possible to directly screen for improved activity in organic media. The advantages of using non-aqueous solvents include solubilization of hydrophobic substrates, shifting of thermodynamic equilibrium to catalyze syntheses that are unfavorable in water, exclusion of unwanted water-induced hydrolysis or polymerization side reactions, easy removal of some products, enhanced enzyme thermostability, and avoidance of microbial contamination [19]. Adaptation of enzymes to organic media is particularly important for their use in the production of chiral synthons and intermediates in the pharmaceutical and fine chemical industries, as these compounds are often poorly soluble or insoluble in water [16].

Directed evolution was used by Wong *et al.* to increase the catalytic activity of cytochrome P450 BM-3 in organic cosolvents [20], which are required to solubilize the wide array of largely insoluble P450 BM-3 substrates (alkanes, alkenes, alcohols, fatty acids, amides, polyaromatic hydrocarbons, and heterocycles). Rather than the wild type enzyme, the parent sequence chosen for this study was a previously isolated mutant, P450 BM-3 F87A [21]. Random mutagenesis *via* epPCR was used in a first round of evolution, followed by saturation mutagenesis at a single residue of a double mutant ("F87AB5") isolated in that generation and also a second round of random mutagenesis utilizing mutagenic DNA polymerase. Catalytic activity of the libraries was screened in a 96-well plate format with and without the organic cosolvents dimethylsulfoxide (DMSO) and tetrahydrofuran (THF). Relative to the parent, the mutant F87A5F5, isolated in the second round of random mutagenesis, displayed increases in specific activity of 5.5-fold in 10% (v/v) DMSO and 10-fold in 2% (v/v) THF. Mutating the alanine at position 87 in that mutant back to a phenylalanine gave a new clone (W5F5) with nearly 6-fold higher specific activity in 25% (v/v) DMSO, but only a 3.4-fold increase in 2% (v/v) THF. Specific activities of the mutants W5F5 and F87A5F5 were also determined in four other water miscible cosolvents: acetone, acetonitrile, dimethylformamide (DMF), and ethanol. Both mutants retained higher catalytic activity than the parent in all four cosolvents. Further, enhancement of specific activity in acetone, acetonitrile, DMF and ethanol was comparable to the improvements observed for DMSO and THF, indicating that directed evolution for improved tolerance of a subset of cosolvents can concurrently enhance resistance to others that are not specifically used in screening.

Evolving pH Optimum

Application of enzymes in processes with a specified pH condition necessitates protein engineering to change pH performance. Rational design and site-directed mutagenesis were employed to improve the activity of phytases at lower pH [22] and to alter the pH optimum of xylanases [23,24] and β-amylases [25,26]. Directed evolution was applied to the α-amylase from *Bacillus amyloliquefaciens* (BAA) to improve its specific activity and activity at alkaline pH [27]. The wild type amylase from BAA has a pH optimum of 6, but application of the enzyme in detergent requires high activity and in particular activity at high pH. Wild type BAA amylase and two point mutants created by site-directed mutagenesis were used as the parent sequences for directed evolution, with epPCR employed in a first round of evolution. The library was screened by determining amylase activity at pH 7 and pH 10, resulting in the isolation of 16 mutants that were next subjected to DNA shuffling. From the shuffled library, two clones ("BAA 29" and "BAA 42") were isolated with improved overall activity and improved activity at pH 10. Characterization of these mutants revealed enhanced periplasmic expression as well as increased specific activity in both cases. Mutant 42 showed a broader pH activity profile and an increased pH optimum, now at pH 7, compared to the wild type amylase activity optimum at pH 6. However, the pH activity profile of mutant BAA 29 was largely unchanged.

Cold Adaptation

Psychrophilic microbes possess cold-adapted enzymes characterized by higher specific activity at lower temperature, enhanced conformational flexibility, and higher thermolability compared to related enzymes from mesophilic or thermophilic organisms [28]. In some commercial applications, an enzyme adapted for high activity at lower temperature may be desirable, but existing cold-adapted enzymes that perform well under low temperature process conditions often fare poorly at relatively higher temperatures and during storage [29]. By starting with a mesophilic or thermophilic enzyme and engineering for higher activity at lower temperature, a cold-adapted enzyme may be created which retains the parent's stability.

In addition to xylose and a variety of other sugars, xylose isomerase (XI) can isomerize glucose to fructose, a process used commercially to produce high fructose corn syrups. Currently high fructose corn syrups produced by XI are the dominant industrial sweetener, and XI is among the three most important industrial enzymes, amylase and protease being the other two [30-32]. Sriprapundh *et al*. [33] isolated a mutant of the hyperthermophile *Thermotoga neapolitana* xylose isomerase (TNXI), TNXI Val185Thr, which is more glucose-efficient than the parent, highly active at 97 °C and neutral pH, and extremely thermostable. However, the current industrial isomerization process proceeds at 60 °C, where the mutant TNXI Val185Thr reaches only 10% of maximum activity, and an acidic pH is desirable [32]. Thus directed evolution was used to create a new TNXI mutant adapted to the industrial XI glucose isomerization process [32]. The TNXI Val185Thr gene was subjected to random mutagenesis *via* epPCR and glucose isomerization activity was screened under varied pH and temperature. Two mutants were isolated which showed higher specific activity towards glucose compared to the wild type TNXI as well as the parent, TNXI Val185Thr, over the entire temperature and pH range tested. Of the two mutants, mutant 1F1 possessed a slightly lower temperature optimum of 90 °C, and nearly 5-fold higher activity at 60 °C, pH 5.5 versus the parent. Thermal inactivation experiments showed mutant 1F1 to be more stable than the wild type TNXI.

ALTERING ENANTIO- AND REGIO-SELECTIVITY

The inability of chemical catalysts to perform some regio- and stereoselective transformations [34] has propelled research in the evolution of biocatalysts for synthesis of chiral compounds. The potential of enzymes to provide enantiomerically pure compounds is of particular interest for the production of chiral synthons and intermediates for the pharmaceutical, agrochemical, and cosmetic industries [34,35]. A recent review by Jaeger and Eggert highlights progress in the improvement of enantioselective biocatalysts by directed evolution [35].

Refining and Reversing Enantioselectivity

The enantioselective epoxide hydrolase from *Agrobacterium radiobacter* (EchA) was improved by van Loo *et al. via* directed evolution [36]. Epoxide hydrolases add a water molecule to an epoxide forming exclusively a vicinal diol product, and both chiral epoxides and diols can be precursors for pharmaceutical or fine chemical syntheses. Site-directed mutagenesis was previously used to improve the relatively low enantioselectivity of *A. radiobacter* epoxide hydrolase with some success [37-39]. In a first round of evolution, epPCR was used to create a library of EchA variants. An agar plate assay was used as a pre-screen, exploiting the ability of *E. coli* to oxidize diols that are produced by epoxide hydrolase activity, resulting in an increased membrane potential and enhanced uptake of the colored compound safranin O from the media. Active clones isolated from this pre-screen were then subjected to further analysis by following the conversion of a racemic mixture of *para*-nitrophenyl glycidyl ether. From the library, fifteen clones were isolated which showed increased enantioselectivity, and these clones were subjected to DNA shuffling to recombine the discovered mutations. From the shuffled library, eight improved clones were identified, with enantioselectivity 5.5- to 13-fold greater than the wild type EchA. Of these eight clones, six also showed improved enantioselectivities with other 1,2-epoxides. Sequence analysis showed that for the most part the point mutations uncovered in this study were in residues near the active site of the epoxide hydrolase.

Rather than DNA shuffling or epPCR, which generate random point mutations across an entire enzyme, Park *et al.* recently used saturation mutagenesis of residues near the active site of a *Pseudomonas fluorescens* esterase (PFE) to improve its enantioselectivity [40]. In particular, the authors sought to improve the entantioselectivy of PFE hydrolysis for its substrate methyl 3-bromo-2-methylpropionate (MBMP), which is noted to be a valuable chiral synthon [41-45]. A homology model of PFE prepared with the first tetrahedral intermediate of hydrolysis for MBMP was analyzed to determine the amino acid residues nearest to the enzyme's stereocenter. Based on this model, four residues

were chosen for saturation mutagenesis: Trp28, Val121, Phe198, and Val225. Saturation mutagenesis was conducted separately for each of these positions, creating four libraries where each target residue was replaced by all 19 possible substitutions. The mutants were screened in a preliminary crude extract assay using hydrolysis of *p*-nitrophenyl acetate as an activity indicator. For active mutants the enantioselectivity of MBMP hydrolysis was first determined using the colorimetric Quick-E method [46], and confirmed using the endpoint method [47]. Screening and DNA sequencing identified five mutants with significantly enhanced enantioselectivity, and these five were all from the Val121 and Trp28 libraries; mutants in the Phe198 and Val225 libraries possessed enantioselectivities similar to or lower than the wild type enzyme. The two best mutants, Val121Ser and Trp28Leu, displayed approximately 5-fold higher enantioselectivity towards MBMP than wild type PFE. The enantioselectivity enhancement achieved in this study surpassed a 1.5-fold increase in PFE enantioselectivity that was found previously by random point mutagenesis across the entire gene [48].

Controlling Enzyme Regioselectivity

Site-directed and saturation mutagenesis were used by Fishman *et al.* to alter the regiospecific oxidation of toluene and naphthalene by *Ralstonia pickettii* PKO1 toluene *para*-monooxygenase (TpMO) [49]. Toluene monooxygenases are cited as particularly important due to their potential for use in bioremediation and chemical synthesis of important industrial compounds and pharmaceutical precursors. Wood and coworkers [50] previously characterized TpMO as producing 90% *p*-cresol, 10% *m*-cresol, and a negligible amount of *o*-cresol from toluene, and oxidizing these compounds to 4-methyl catechol. In this study the authors sought to (a) confer to TpMO *via* mutagenesis the ability to *meta*-hydroxylate substituted benzenes, (b) create from TpMO by site-directed or saturation mutagenesis a more exclusive *ortho*-hydroxylating enzyme, and (c) create from TpMO by site-directed or saturation mutagenesis a more absolute *para*-hydroxylating enzyme than the wild type, which already generates 90% *para*-hydroxylated product [50]. Amino acid positions 103 and 107 were chosen for saturation mutagenesis based on prior results obtained with other toluene monooxygenases [51-53]. The saturation mutagenesis libraries were first screened for mutants able to produce catecholic compounds, and the products of the TpMO variants were further analyzed by HPLC and gas chromatography. Mutants were identified as *meta*-, *para*- or *ortho*-hydroxylating based on the predominant isomer generated by toluene oxidation. Further characterization of mutants was done by examining the products of nitrobenzene (NB), methoxybenzene (MB), and naphthalene oxidation. Mutants isolated from the libraries and created by site-directed mutagenesis matched each of the three desired criteria listed above: (a) Based on previous results from the mutagenesis of toluene 4-monooxygenase from *Pseudomonas mendocina* KR1 [54], residue 100 of TpMO was mutated to a serine to enhance its *meta*-specificity. Mutant TpMO TbuA1 I100S produced 33% *m*-cresol, compared to only 10% for wild type TpMO, and also produced 65% *m*-nitrophenol (*m*-NP) from NB. A *meta*-hydroxylating enzyme was also found in the mutant libraries, and this variant, TpMO TbuA1 G103S, generated 29% *m*-cresol from toluene and 96% *m*-NP. The double mutant TbuA1 I100S/G103S was created by site-directed mutagenesis and displayed 75% *meta*-specificity for toluene and 100% *meta*-specificity for NB. The authors noted that this variant represents the first example of exclusive *meta*-hydroxylation by a toluene monooxygenase. (b) Mutant TpMO TbuA1 A107G produced 80% *o*-cresol from toluene, compared to 2% *o*-cresol production by the wild type TpMO, and 88% *o*-MB, which was not produced at all by the wild type. Additionally this mutant produced 97% 1-napthanol, while the wild type produced only 63%. The authors suggest mutant A107G exhibits similar regiospecificity to the toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4. (c) Mutant TpMO TbuA1 A107T displayed enhanced *para*-specificity, surpassing the wild type TpMO. Mutant A107T was found to hydroxylate toluene, NB and MB to their respective *para*-substituted phenols with greater than 98% specificity. In addition, 2-napthanol production was also enhanced in this mutant compared to the wild type TpMO.

ALTERING SUBSTRATE AND COFACTOR SPECIFICITY

Natural evolution has created a wealth of enzymes which are in some cases extremely substrate specific and in other cases highly promiscuous. As a result, adaptation of enzymes for desired applications may require the narrowing of specificity to a single molecule or a small set of molecules for a promiscuous biocatalyst, or alternatively substituting or broadening the selectivity of a more discriminating enzyme. Modifying substrate specificity is a common goal of directed evolution work and is the subject of many current research articles focusing on a wide variety of enzymes [55-71]. Cofactor usage presents an additional obstacle to the use of some enzymes as biocatalysts, and so it may also be desirable to change or expand cofactor preferences.

Substrate Specificity

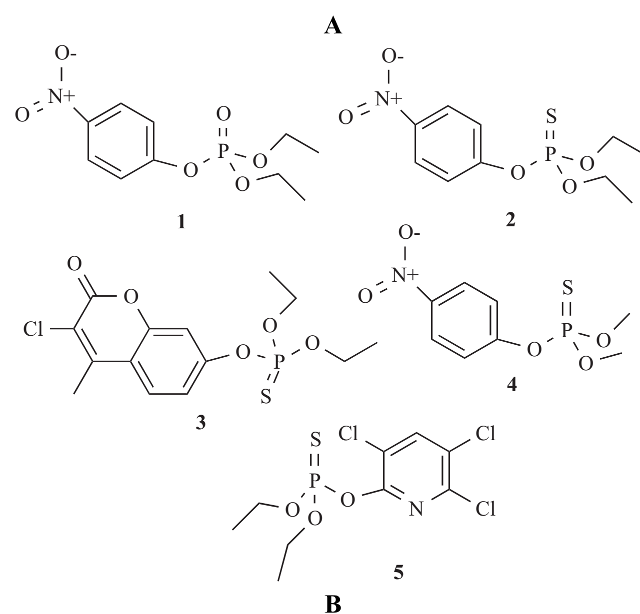
Cho *et al*. used directed evolution of the enzyme organophosphorus hydrolase (OPH) to increase its activity on chlorpyrifos (Dursban), a commercial pesticide [72]. OPH is able to hydrolyze toxic organophosphorus compounds including pesticides and chemical warfare agents, but with varied efficiency on different substrates: chlorpyrifos is poorly utilized, with hydrolysis 1000-fold less efficient than the organophosphorus compound paraoxon [73]. DNA shuffling using as templates the wild type gene and a previously isolated variant ("22A11") possessing improved methyl parathion activity [74] generated several improved variants in two rounds of evolution. Mutant B3561, the best variant, exhibited over 700-fold improvement in chlorpyrifos hydrolysis, giving activity similar to that of the wild type enzyme on its preferred substrate, paraoxon. Mutant B3561 also showed improvement ranging from 12- to 39-fold over the wild type enzyme in hydrolysis of paraoxon, parathion, coumaphos, and methyl parathion (Fig. **2**), making it a powerful tool for decontamination of toxic organophosphorus compounds.

While engineering improved substrate specificity by directed evolution often renders an enzyme more promiscuous, Varadarajan *et al*. sought to alter the specificity of a protease and simultaneously improve its

selectivity for the desired substrate [75]. A technique based on bacterial display and multiparameter flow cytometry allows for the concurrent positive screening of clones for a desired activity and negative selection of those clones which gain or retain activity against unwanted substrates. A library of mutants of an *E. coli* endoprotease (OmpT) was created *via* epPCR of the parent sequence, a previously isolated variant, "C5," which gives 60-fold higher cleavage at Arg-Val [76], and displayed on the surface of *E. coli* cells to facilitate fluorescence activated cell sorting (FACS). Two labeled synthetic peptides were used as substrates for the protease selection and counter-selection. For the undesirable substrate, a zwitterionic peptide containing an Arg-Arg bond, which is preferred by the wild type enzyme, was labeled with a single tetramethylrhodamine fluorophore. Cleavage of the peptide created a positively charged, labeled fragment of the peptide which adhered to the negatively charged cell by electrostatic interactions. The selection substrate consisted of a positively charged fluorescence resonance energy transfer (FRET) peptide with an Ala-Arg bond, the desired novel substrate for the protease, and was labeled with the BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid) fluorophore. FACS was used to select bacterial cells displaying protease clones with increased BODIPY fluorescence and decreased tetramethylrhodamine fluorescence, which represent increased activity against the novel substrate and decreased cleavage of the wild type substrate, respectively. The best mutant isolated by this study showed a reversal in specificity of over $3x10^6$ for the Ala-Arg peptide bond versus the natural substrate, the Arg-Arg peptide bond, as compared to wild type OmpT. The reversal in specificity for the mutant compared to the parent enzyme, OmpT variant C5, was approximately $1x10^6$.

Cofactor Specificity

The potential of oxidoreductases in biocatalysis is often hindered by their requirement of the soluble cofactors nicotinamide adenine dinucleotide (NAD(H)) and nicotinamide adenine dinucleotide phosphate (NADP(H)), which, through reversible reduction of the nicotinamide ring, in general drive enzymatic oxidations and reductions, respectively. The high cost of these cofactors renders infeasible their use in stoichiometric amounts during enzymatic transformations. Several *in situ* cofactor regeneration methods have been developed that permit usage of nicotinamide cofactors in catalytic rather than stoichiometric quantities, and these regeneration schemes also provide other advantages [77]. The lower stability and higher cost of NADP(H) versus NAD(H) makes reversal of cofactor specificity to NAD(H), as well as effective regeneration of NADP(H), particularly advantageous [78,79]. Several groups have reported relaxed or reversed cofactor specificity by targeted mutagenesis in the cofactor binding pocket. Rather than directed evolution, the complementary methods of rational design and site-directed mutagenesis have recently been used to alter the cofactor specificity of several dehydrogenases [78,80,81] (DHs) as well as *Corynebacterium* 2,5-diketo-D-gluconic acid reductase A [79].



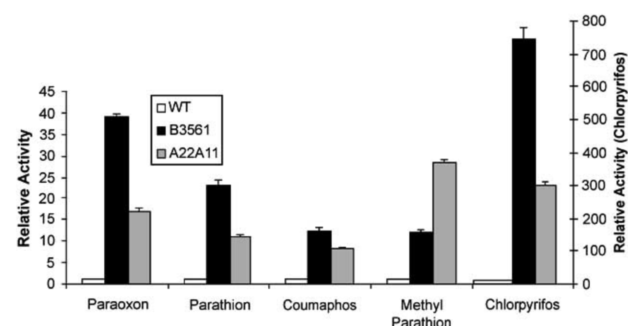


Fig. (2). (A) Paraoxon **1**, parathion **2**, coumaphos **3**, methyl parathion **4**, and chlorpyrifos **5**. (B) Hydrolysis of these compounds by variant B3561, the best mutant found in this study, far surpassed that of the wild type enzyme (WT) and also exceeds the activity of the parental mutant (A22A11) for all substrates except methyl parathion.

Woodyer *et al.* used homology modeling and site-directed mutagenesis to relax the cofactor specificity of *Pseudomonas stutzeri* phosphite dehydrogenase (PTDH) [78]. Two residues key to determination of cofactor specificity, Glu175 and Ala176, were identified by sequence alignment against other D-hydroxy acid DHs and creation of a homology model of PTDH. These residues were mutated separately and simultaneously to give three mutant PTDHs with broadened cofactor specificity and enhanced catalytic efficiency for both cofactors. The Ala175 and Arg176 double mutant performed best, with a 1000-fold higher catalytic efficiency with $NADP^+$ and 3-fold cofactor specificity for $NADP^+$, versus a 100-fold preference for NAD^+ in the wild type enzyme. The mutant PTDH isolated in this study represents a promising candidate for use in NADP(H) regeneration.

IMPROVING THERMOSTABILITY

The ability of an enzyme to retain function at, or at least withstand exposure to, elevated temperature is a prerequisite for many potential applications of biocatalysts [82-85], for example as time-release medicines, catalysts for industrial

transformations or food processing, or stable animal feed supplements [86]. If a thermophilic enzyme is not available for a certain application, the stability of a mesophilic enzyme can be enhanced to accommodate mandatory high-temperature conditions. Improving the thermostability of an enzyme may provide further advantages such as increased shelf life, extended life span during use at any temperature, both higher and lower, and a higher temperature optimum [87]. Strategies to improve thermostability include rational design [88], alignment of mesophilic enzymes with thermophilic equivalents to predict thermostabilizing residues, termed the consensus approach [86,89], and directed evolution. A review by Lehmann and Wyss provides an evaluation of these three approaches [90].

Xylan is the major component of hemicellulosic material, which makes up 20-30% of plant biomass, and both the compound itself and its products of xylanase hydrolysis (xylose and oligosaccharides) have current and potential uses in the pharmaceutical industry and as food additives [91]. Additionally xylanase enzymes find commercial use in the paper and pulp industries, serving as a "green" alternative to chlorine bleaching of pulp, and are used in food processing and other applications [91]. To improve the thermostability of a xylanase, Palackal *et al.* used two directed evolution technologies in combination [92]. Gene Site Saturation Mutagenesis™ (GSSM™) [93,94] whereby each position of a protein is randomized by substitution of all possible amino acids, was used to create a library of xylanase single mutants. The GSSM™ library was screened for improved thermostability by measurement of residual enzyme activity following exposure to 80 °C heat, which far exceeds the melting temperature of the wild type enzyme (61 °C). Nine unique thermostabilizing point mutations were isolated, and these nine mutants as well as the wild type enzyme were expressed heterologously in *E. coli* with a hexahistidine tag for purification purposes, and characterized. The discovered mutations all provided increased T_m values which ranged from 64 °C to 70 °C for individual mutants, and did not significantly affect specific activity, based on an assay of the initial rate of azo-xylan hydrolysis by the purified enzymes. A 9X mutant was created by site-directed mutagenesis, and the nine individual point mutations were also combined arbitrarily using GeneReassembly™ technology [95]. A T_m of 96 °C was measured for the 9X mutant, which is 35 °C higher than the wild type. The 9X mutant also retained more than 60% activity at 100 °C based on the initial reaction rate, whereas the wild type enzyme showed superior activity at lower temperatures but no detectable activity beyond 70 °C. The mutants derived from GeneReassembly™ all possessed T_m values at least 28 °C higher than the wild type xylanase and specific activity greater than the 9X mutant, with the best mutant ("6X-2") having a T_m of 90 °C and higher specific activity than the wild type enzyme. To the authors' knowledge, the increases in T_m obtained in this study are the highest T_m shifts reported for the use of directed enzyme evolution or rational design.

The *Pseudomonas stutzeri* PTDH enzyme, as mentioned earlier, possesses great potential for industrial NAD(P)H regeneration, but the low stability of the wild type enzyme is a major obstacle. Directed evolution was used by Johannes *et al*. [96] to improve its thermostability. Rather than the wild type PTDH, a previously isolated mutant possessing five point mutations that improve solubility and activity of the enzyme but do not significantly affect thermostability [97] was chosen as the parent enzyme. Random mutagenesis *via* epPCR was used to create libraries of PTDH variants in three rounds of evolution, and isolated mutations were combined by site-directed mutagenesis to create a most-improved parental enzyme for subsequent rounds. Mutants were screened in a 96-well plate assay in which initial activity was compared with residual activity following a temperature challenge of 42 °C, 57 °C, and 62 °C for the first, second, and third rounds of evolution, respectively. Dehydrogenase activity was monitored by a colorimetric assay based on the reaction of nitroblue tetrazolium (NBT) with reduced nicotinamide cofactors in the presence of phenazine methosulfate (PMS), which generates a blue-purple formazan that can be quantified by monitoring absorbance at 580 nm [98]. A total of twelve mutations were discovered after three rounds of evolution and screening using the NBT-PMS assay. These point mutations were incorporated into a final 12x mutant, for a total of 17 altered residues relative to the wild type sequence. Kinetic parameters, temperature optimum (T_{opt}), half-life of inactivation, and temperature required to reduce activity by 50% after a fixed incubation time of 20 minutes (T_{50}) were determined for the parent enzyme and select mutants. The T_{50} for the 12x mutant was increased to 59.3 °C, approximately 20 °C higher than the parent enzyme, and the T_{opt} was similarly increased by about 20 °C to a value of 59 °C. The half-life of inactivation at 45 °C was increased 7,000-fold for the 12x mutant relative to the wild type or parent enzyme. Improvements in thermostability were obtained without significant alteration of the enzyme's kinetic parameters. The 12x PTDH mutant was shown to be more stable and more active than the *Candida boidinii* formate dehydrogenase (FDH) enzyme that is the current standard in cofactor regeneration. In small-scale batch reactions at 40 °C, wild type PTDH, formate dehydrogenase, and the 12x PTDH mutant were coupled with *Bacillus cereus* leucine dehydrogenase (LeuDH) to produce L-*tert*-leucine from trimethylpyruvate and ammonia using regeneration of NADH (Fig. **3**). Under the conditions tested the 12x PTDH mutant reached 100% conversion and displayed an approximately 2-fold faster reaction rate than the commercially available FDH. The wild type PTDH precipitated rapidly and provided low conversion.

INCREASING EXPRESSION AND SOLUBILITY

Recombinant proteins may express poorly in microorganisms, or when overproduced, may be inactive due to aggregation and misfolding [99]. Strategies to improve functional expression include lowering expression temperature and expression under promoters of varying strength [100], fusion to solubility-enhancing proteins including maltose binding protein [101], NusA [102], and thioredoxin [103], or coexpression of molecular chaperones [104]. It may also be possible to improve functional expression through various amino acid substitutions that promote the innate folding ability and solubility of a protein. These advantageous mutations can be uncovered by traditional directed evolution techniques and an appropriate system for screening or selecting improved variants. Specialized screening methods for improvement of protein solubility were recently reviewed by Waldo [100], and an

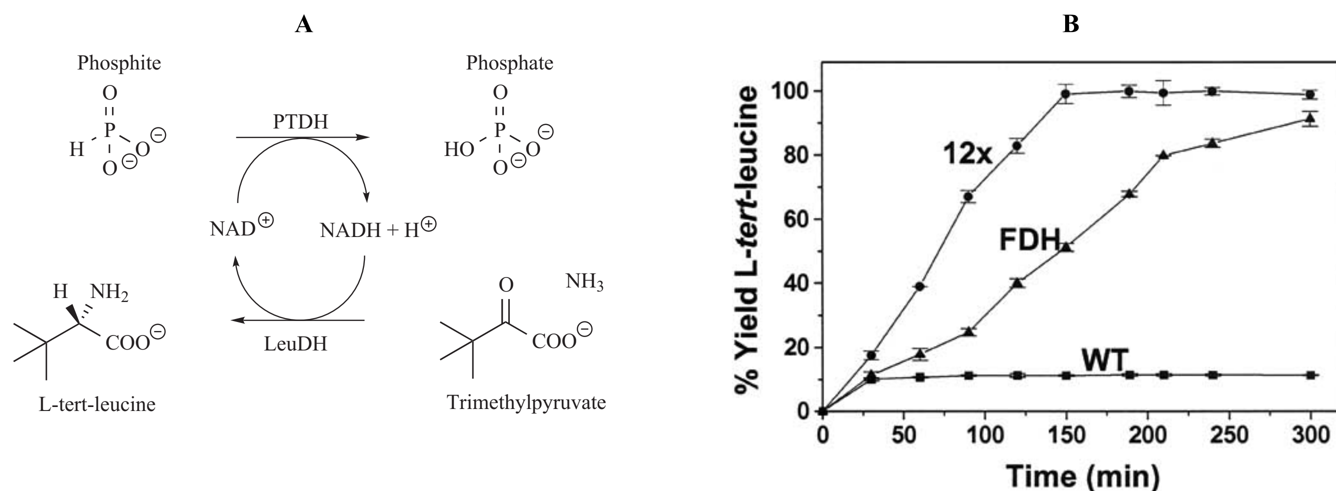


Fig. (3). (A) Production of L-*tert*-leucine from trimethylpyruvate and ammonia is facilitated by NADH regeneration. PTDH converts NAD^+ to NADH with concomitant conversion of phosphite to phosphate, and leucine dehydrogenase (LeuDH) then uses NADH to produce L-*tert*-leucine from trimethylpyruvate and ammonia. (B) In small-scale batch reactions, the 12x PTDH mutant (12x) outperformed a formate dehydrogenase (FDH) available from Jülich Fine Chemicals (Jülich, Germany). The wild type PTDH (WT) precipitated rapidly during the reaction and showed very poor conversion.

update on the latest progress in expression and solubility improvement is also available [105]. Fusion of a protein of interest to a green fluorescent protein (GFP) folding reporter was originally introduced by Waldo [99], and was recently used to improve the functional expression of *N*-carbamoylase [106]. An alternative strategy is use of chloramphenicol resistance as a solubility reporter by fusion of a protein of interest to chloramphenicol acyl transferase (CAT) [7].

When feasible, functional expression can also be improved by screening the total activity of an expressed protein. Aharoni *et al.* recently reported the functional expression of a mammalian paraoxonase (PON) enzyme in *Escherichia coli* [107]. PONs represent a poorly characterized group of enzymes which have gained interest due to their role in prevention of human disease. Through DNA family shuffling of four wild type PON1 genes derived from human, mouse, rat, and rabbit sources, a library of PON1 mutants was created and screened for esterase activity. Further analysis of several PON1 mutants showed that improvement was largely due to increased solubility rather than changes in kinetic parameters. While expression of wild type PON1 was nonexistant unless fused to a thioredoxin tag, mutants isolated in this study provided as much as ~12 mg/liter of pure, active PON1 expressed without the solublizing thioredoxin fusion.

NOVEL ACTIVITIES AND PRODUCTS

Perhaps the most ambitious goal of directed evolution is the creation of novel biocatalytic activities and bioproducts. While diversity generation methods can create libraries of mutants containing billions of members, widespread screening techniques based on microplates can efficiently screen only up to tens of thousands of clones, or agar plates up to several hundred thousand clones in a round of evolution. Limitation on the ability to screen for a specific activity represents a bottleneck to discovery of novel biocatalysts. As more advanced high-throughput and ultra-high-throughput techniques are established, generating novel activities and bioproducts by directed evolution becomes more feasible. Some high-throughput methods available to screen for novel biocatalyts were recently reviewed [108], and a general discussion of screening for novel industrial biocatalysts is also available [109]. Screening libraries of extant genetic diversity by high throughput culture of organisms [83,110], the metagenome approach for examining microbial communities and uncultivable organisms in particular [111-114], and querying databases of biocatalytic reactions [115] represent alternative methods for discovery of a desired bioactivity.

Pioneering work in novel bioproduct development has been begun in the field of natural product glycorandomization for the discovery of therapeutics. The efficacy of glycosylated natural products and their derivatives as antibiotics and anticancer agents is largely determined by attached sugar moieties, and this fact has piqued interest as to whether altering sugar conjugates could broaden or enhance the medically relevant activity of a molecule [116-120]. The *in vitro* glycorandomization (IVG) of a natural product scaffold, *via* promiscuous activity of sugar kinases or nucleotidylyltransferases which activate natural or unnatural sugars and a glycotransferase that attaches them, can create a library of modified glycoproducts theoretically limited only by the ability to synthesize unique sugar molecules. Directed evolution was used by Thorson and coworkers to broaden the substrate range of the *Escherichia coli* galactokinase GalK [118] as well as the nucleotidylyltransferase α-D-glucopyranosyl phosphate thymidylyltransferase from *Salmonella enterica* [120] in order to enzymatically activate a wide variety of natural and unnatural sugars. The potential of IVG was assessed using the compound vancomycin, a glycosylated natural antibiotic derived from *Amycolatopsis orientalis*, the vancomycin glucosyltransferase GtfE, which is known to accept a variety of substrates, and a library of 23 natural and unnatural nucleotide diphosphosugars [119]. Ultimately GtfE was able

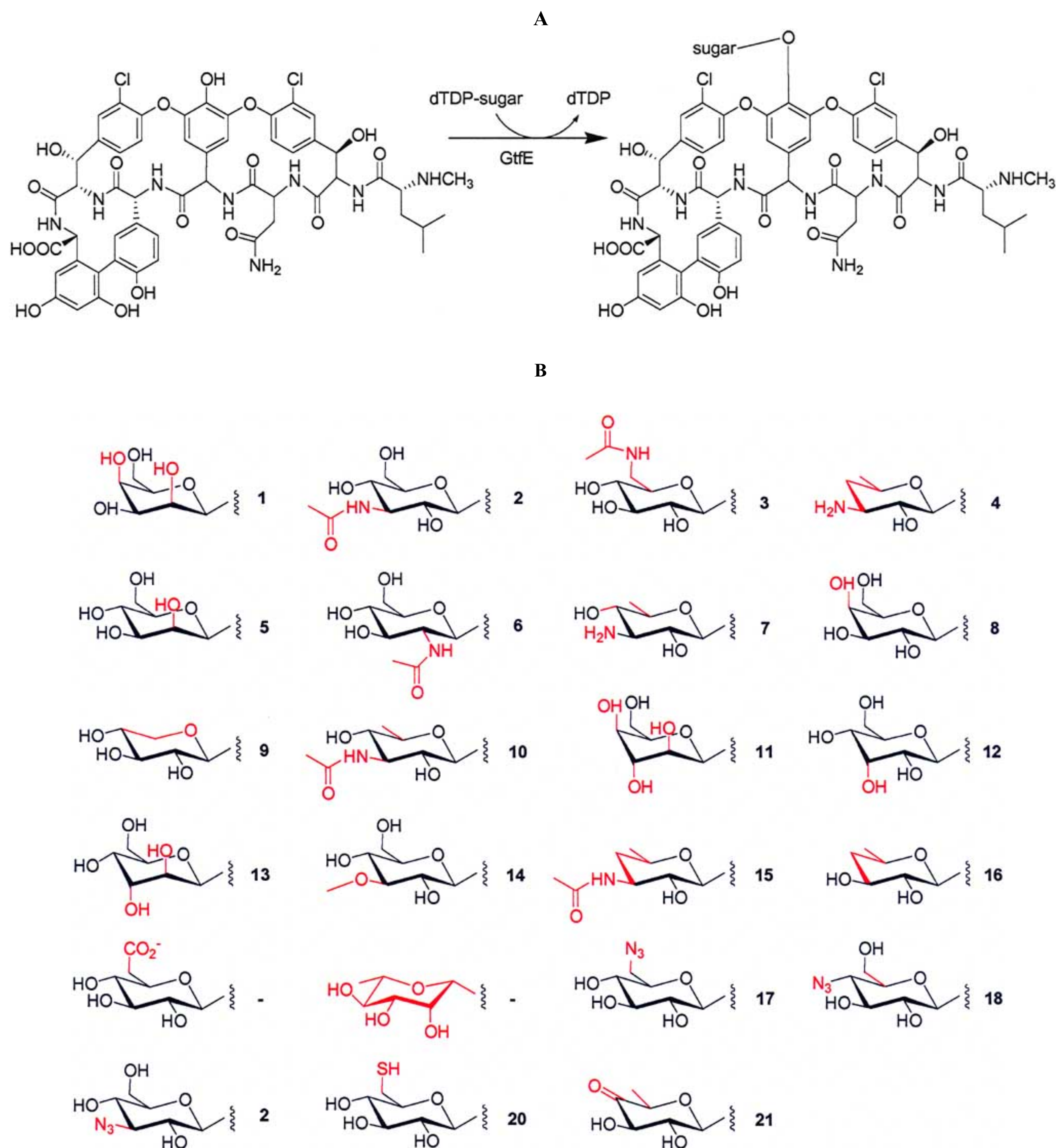


Fig. (4). (A) Vancomycin glycosyltransferase GtfE was able to use natural and unnatural NDP-sugars and produce the initial library of monoglycosylated vancomycin derivatives. (B) Numbers **1**-**21** represent products, and (-) indicates no conversion was observed. Changes from the wild type GtfE substrate, which is glucose, are indicated in red.

to utilize 21 out of 23 activated sugars as a substrate, creating a set of monoglycosylated vancomycin derivatives **1**-**21** each possessing a distinct conjugated sugar moiety (Fig. **4**). Chemoselective modification of the monoglycosylated vancomycin variant **17** led to the creation of derivative compounds with distinct antibacterial properties from the parent vancomycin, having a twofold increase in activity against *Staphylococcus aureus* and *Enterococcus faecium* but half the effectiveness versus *Bacillus subtilis*. Future directed evolution work to broaden the substrate repertoire of sugar kinases and sugar nucleotidylyltransferases as well as glycosyltransferases will further increase the capacity of IVG to enzymatically generate novel glycosylated compounds. Examination of substrate specificity and engineering of some glycosyltransferases has already been accomplished [117,121-124].

CONCLUSION AND FUTURE PROSPECTS

Directed evolution has emerged as a potent tool for adapting enzymes to current and potential use as biocatalysts in a variety of disciplines. Further advancement of enzymatic biocatalysis will require both the continued mining of new enzymes from nature's vast reserves, and use of directed evolution or rational design techniques to close the functional gap between existent and desired catalyst properties. Studies highlighted herein show the power of current evolutionary techniques to improve activity, stability, and soluble expression, and to change enzyme selectivity or substrate specificity. Creating novel enzymatic activity remains an elusive goal, and will require the development of innovative evolutionary strategies and ultra-high throughput screening systems to sort through immense mutant libraries. It should be noted that a method of stepwise *in vitro* co-evolution was recently reported for the creation of a novel protein function from an existing protein scaffold [125]. Ultimately new directed evolution techniques, in conjunction with continued discovery and traditional evolutionary or rational protein engineering approaches, will continue to fulfill the promise of biocatalysis in industrial, agricultural, and medical applications.

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ABBREVIATIONS

BODIPY = 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid
CAT = Chloramphenicol acyl transferase
DH = Dehydrogenase
DMF = Dimethylformamide
DMSO = Dimethylsulfoxide
EchA = *Agrobacterium radiobacter* enantioselective epoxide hydrolase
epPCR = Error-prone polymerase chain reaction
FACS = Fluorescence activated cell sorting
FDH = Formate dehydrogenase
FRET = Fluorescence resonance energy transfer
GAT = Glyphosate *N*-acetyltransferase
GFP = Green fluorescent protein
GSSM™ = Gene Site Saturation Mutagenesis™
GtfE = Vancomycin glucosyltransferase
LeuDH = Leucine dehydrogenase
MB = Methoxybenzene
MBMP = Methyl 3-bromo-2-methylpropionate
NAD(H) = Nicotinamide adenine dinucleotide
NADP(H) = Nicotinamide adenine dinucleotide phosphate
NB = Nitrobenzene
NBT = Nitroblue tetrazolium
OmpT = *Escherichia coli* endoprotease
OPH = Organophosphorus hydrolase
PFE = *Pseudomonas fluorescens* esterase
PMS = Phenazine methosulfate
PON = Paraoxonase
StEP = Staggered extension process
PTDH = Phosphite dehydrogenase
THF = Tetrahydrofuran
TNXI = *Thermotoga neapolitana* xylose isomerase
TpMO = *Ralstonia pickettii* PKO1 toluene *para*-monooxygenase
XI = Xylose isomerase

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