

Directed evolution of enzymes and pathways for industrial biocatalysis

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Directed evolution has become a powerful tool for developing enzyme and whole cell based biocatalysts. Significant recent advances include the creation of novel enzyme functions and the development of several new efficient directed evolution methods. The combination of directed evolution and rational design promises to accelerate the development of biocatalysts for applications in the pharmaceutical, chemical and food industries.

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Abbreviations

ee	enantiomeric excess
epPCR	error-prone polymerase chain reaction
GGPP	geranylgeranyl diphosphate
IGPS	indole-3-glycerol-phosphate synthase
KDPG	D-2-keto-3-deoxy-6-phosphogluconate
PRAI	phosphoribosylanthranilate isomerase
RACHITT	random chimeragenesis on transient templates
SHIPREC	sequence homology-independent protein recombination

Introduction

The use of enzymes and whole cells as biocatalysts for industrial synthetic chemistry is rapidly growing [1•,2–5]. Biocatalysts exhibit exquisite catalytic power (high selectivity and environmental friendliness) unmatched by conventional catalysts. In the past few decades, biocatalysts have been successfully exploited for the synthesis of complex drug intermediates, speciality chemicals, and even commodity chemicals in the pharmaceutical, chemical and food industries. Recent advances in recombinant DNA technologies, high-throughput technologies, genomics and proteomics have fuelled the development of new biocatalysts and biocatalytic processes. In particular, directed evolution has emerged as a powerful tool for biocatalyst engineering [6,7•,8]. As shown in Figure 1, directed evolution is an essential component in the development of biocatalytic processes as in most cases the naturally occurring biocatalysts (enzymes and microorganisms) are not optimized for a given synthetic problem.

Directed evolution mimics Darwinian evolution in the test tube, and involves the generation and selection of a molecular library with sufficient diversity for the altered function to be represented [9,10]. Molecular diversity is typically created by various random mutagenesis and/or *in vitro* gene recombination methods [11•,12]. Functionally improved variants are first identified by a high-throughput

screening or selection method and then used as the parents for the next round of evolution. As a result, the success of directed evolution experiments often depends on the choice of diversity-generation methods and the availability of screening/selection methods.

In this review, we discuss the impact directed evolution has made in the past two years (2000 and 2001) and the potential impact it could have in the future on various applications in industrial biocatalysis. Specifically, we focus on the role of directed evolution in the fields of enzyme engineering and metabolic or biosynthetic pathway engineering. Applications of directed evolution to other areas such as gene therapy, therapeutic proteins and vaccine development will not be covered in this review and readers are referred to several recently published reviews [11•,13,14].

Directed evolution of enzymes

The successful implementation of enzymes as industrial biocatalysts requires the availability of suitable enzymes with high activity, specificity and stability under process conditions. However, naturally occurring enzymes are often not optimized to fulfill these requirements. Within this context, directed evolution is very effective in closing these functional discrepancies.

Specificity

The production of enantiomerically pure compounds is of increasing importance to the chemical and pharmaceutical industries. Using error-prone polymerase chain reaction (epPCR), saturation mutagenesis and screening, Reetz and coworkers [15,16] considerably increased the enantioselectivity of a *Pseudomonas aeruginosa* lipase towards 2-methyldecanoate — from $E = 1.04$ (2% enantiomeric excess [*ee*]) to $E = 25$ (90–93% *ee*) (where E is the enantioselective factor). None of the five amino acid substitutions in the best mutant was located near the substrate-binding pocket [17•]. Using a similar approach, Arnold and coworkers [18] successfully inverted the enantioselectivity of a hydantoinase from D selectivity (40% *ee*) to moderate L preference (20% *ee*). A single amino acid substitution was sufficient to invert the enantioselectivity. Highly D-selective mutants (90% *ee*) containing a single amino acid substitution were also found.

Altering substrate specificity by directed evolution has been very successful. The *E. coli* D-2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase catalyzes the highly specific reversible aldol reaction on D-configured KDPG substrate. Using epPCR, DNA shuffling and screening, Wong and coworkers [19] obtained a variant capable of

accepting both D- and L-glyceraldehyde as substrates in a non-phosphorylated form. Notably, the six functional mutations found in this variant were far from the active site. A double-mutant (Lys133Gln/Thr161Lys) of the same enzyme with considerably altered substrate profile was obtained using epPCR and site-directed mutagenesis [20]. Using DNA shuffling, saturation mutagenesis and screening, the substrate specificity of cytochrome *c* peroxidase from *Saccharomyces cerevisiae* was changed from the protein cytochrome *c* to small molecules including guaiacol and 2,2'-azino-bis(3-ethylbenzothiazole-6-sulfonic acid) [21,22]. Both the activity and substrate specificity of the mutants were significantly improved. Family shuffling of two homologous biphenyl dioxygenases created several mutants with altered substrate specificity [23]. Directed evolution of one of the two biphenyl dioxygenases using random-priming recombination yielded similar results [24]. Other studies dealing with the modification of substrate specificity include evolving *E. coli* β -galactosidase into a variant with β -glucosidase activity [25], converting *E. coli* β -glucuronidase into a β -galactosidase [26], introducing phospholipase activity into a *Staphylococcus aureus* lipase [27], and engineering a fatty-acid hydroxylating P450 monooxygenase with novel substrate specificity [28,29].

Stability and activity

Depending on the choice of screening/selection methods, enzyme stability and activity could be simultaneously improved or one property is improved at the cost of another. By screening for initial activity and residual activity at an elevated temperature, both the thermostability and activity of mesophilic subtilisin E [30], psychrophilic subtilisin S41 [31], and mesophilic *p*-nitrobenzyl esterase [32] were significantly increased using directed evolution strategies. By contrast, the evolved variants of a thermophilic indoleglycerol phosphate synthase using a genetic selection in *E. coli* exhibited increased k_{cat} values and decreased thermostability [33]. Using a similar selection method, Oshima and coworkers [34] were able to obtain four variants of a thermophilic 3-isopropylmalate dehydrogenase with enhanced specific activities at low temperatures. Two of these variants maintained the wild-type thermostability, whereas the other two variants exhibited decreased thermostability. A similar reverse correlation between activity and stability was observed in the thermostable *S. cerevisiae* 3-isopropylmalate dehydrogenase variants evolved using a genetic selection in an extreme thermophile [35]. Clearly, with no direct selective pressure on both properties, either activity or thermostability could drift randomly in the fitness landscape.

The potential application of biocatalysts in industrial biocatalysis would be greatly expanded if enzymes could function in non-natural environments such as organic solvents. Using random mutagenesis, recombination and screening, Song and Rhee [36] obtained three variants of phospholipase A₁ with enhanced stability and activity in organic solvents. Using a similar strategy, Arnold and coworkers [37] obtained a variant of horseradish peroxidase

with increased stability in the presence of H₂O₂, sodium dodecyl sulfate, and salts.

Novel specificity and activity

Engineering novel enzyme specificity and activity using directed evolution approaches remains a major challenge. Several recent reports describe significant advances made toward this goal. A particularly impressive example is the creation of novel enzyme substrate specificities and activities by the DNA shuffling of two highly homologous triazine hydrolases [38**]. The two enzymes, AtzA and TriA, differing by only nine amino acids, hydrolyze *s*-triazines by dechlorination and deamination, respectively, with little overlap in substrate preference. Permutation of the nine amino acid differences by DNA shuffling resulted in a set of variants that hydrolyzed five of eight triazines that were not substrates for either starting enzyme.

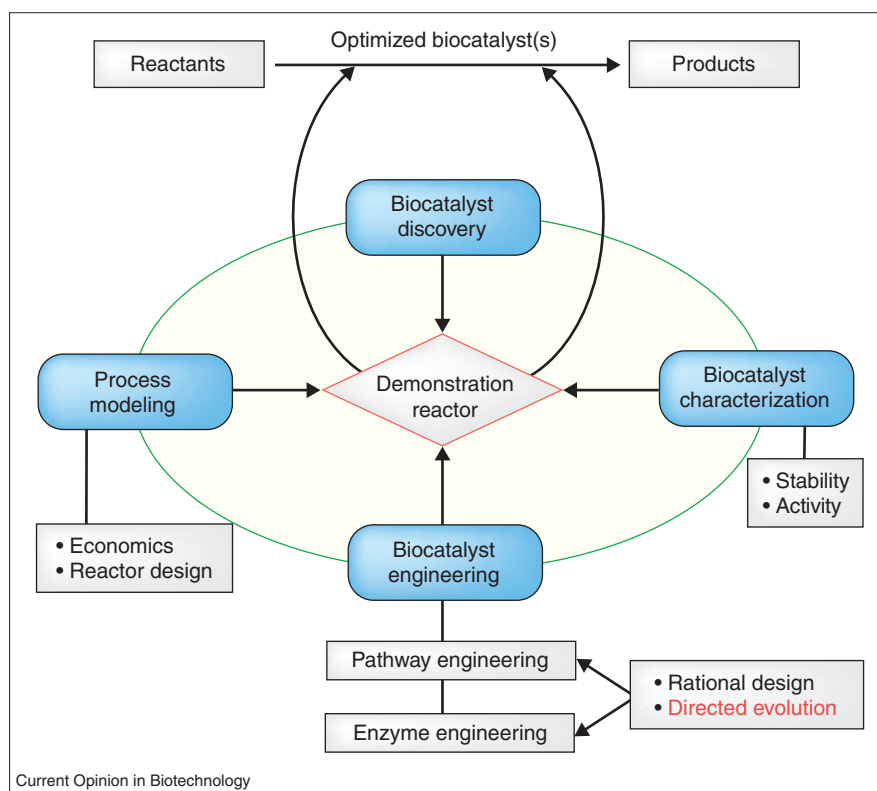
Another impressive example is the evolution of ampicillin-resistant activity from a functionally unrelated DNA fragment [39**]. The DNA fragment conferring very low ampicillin-resistant activity on *E. coli* was isolated from the genomic DNA library of a hyperthermophilic archaeon *Pyrococcus furiosus* in which no β -lactamase activity has ever been detected. After 50 rounds of DNA shuffling and screening at increasing ampicillin concentrations, the ampicillin-resistant activity of this clone on *E. coli* was significantly enhanced.

Directed evolution of pathways

Metabolic pathway engineering is a rapidly growing area with great potential to impact industrial biocatalysis [8,40*,41]. Successful applications of pathway engineering include the production of aromatics, carbohydrates, organic acids, alcohols, and secondary metabolites. Because of the central role played by enzymes in the pathways, directed evolution can be applied to optimize existing pathways as well as to create new pathways capable of synthesizing novel compounds.

In recent years, the engineering of carotenoid biosynthetic pathways in *E. coli* has come into the limelight, because the production of carotenoids in useful quantities using conventional synthetic means or from natural carotenogenic microorganisms has proved to be difficult and costly. Carotenoid pathways also constitute a well-suited target for directed evolution, as the bright color of many carotenoids makes them easy to screen from a library of variants. By performing directed evolution on geranylgeranyl diphosphate (GGPP) synthase, which catalyzes the rate-limiting step of production of the important precursor GGPP, Wang and colleagues [42] increased the production of carotenoids in *E. coli* twofold. Directed evolution has also been used to create novel carotenoid compounds. Schmidt-Dannert and colleagues [43*] shuffled two phytoene desaturases (encoded by *crtI*) and two lycopene cyclases (encoded by *crtY*), respectively, in the context of a carotenoid biosynthetic pathway assembled from different bacterial species, which

Figure 1



Flowchart illustrating the development of industrial biocatalytic processes. Four integral technical components – biocatalyst discovery, biocatalyst characterization, biocatalyst engineering and process modeling – need to be addressed to develop a pilot-plant scale bioprocess to demonstrate the favorable process economics. In terms of time and cost, directed evolution is the method of choice for biocatalyst engineering.

resulted in the production of novel carotenoids. Using epPCR and screening, Wang and Liao [44] evolved *Rhodobacter sphaeroides* phytoene desaturase (a neurosporene-producing enzyme) to produce lycopene.

Among other applications of directed evolution to metabolic pathways, the yield of *cis*-(1*S*,2*R*)-indandiol, a precursor in an engineered biosynthetic pathway for the production of the human immunodeficiency virus protease inhibitor CRIVAN[®], has been increased by random mutagenesis of the gene encoding toluene dioxygenase from *Pseudomonas putida* [45]. Kim and coworkers [46] used DNA shuffling to stabilize the structure of a linearly fused bifunctional *N*-carbamylase/D-hydantoinase enzyme, enabling a sixfold increase in the production of unnatural D-amino acids relative to the parent fusion enzyme [46]. As previously discussed, Arnold and coworkers [18] inverted the enantioselectivity of a D-selective hydantoinase from *Anthrobacter* sp. DSM 9771 to an L-selective enzyme. As a result of the evolution, the enzyme activity was also increased fivefold. The expression of this evolved variant with three additional enzymes in the methionine pathway has greatly increased the production rate of L-methionine. Similarly, random mutagenesis was used to broaden the substrate specificity of an enzyme in the biosynthetic pathway for histidine production, enabling the enzyme to also catalyze the corresponding step in the pathway for tryptophan production [47].

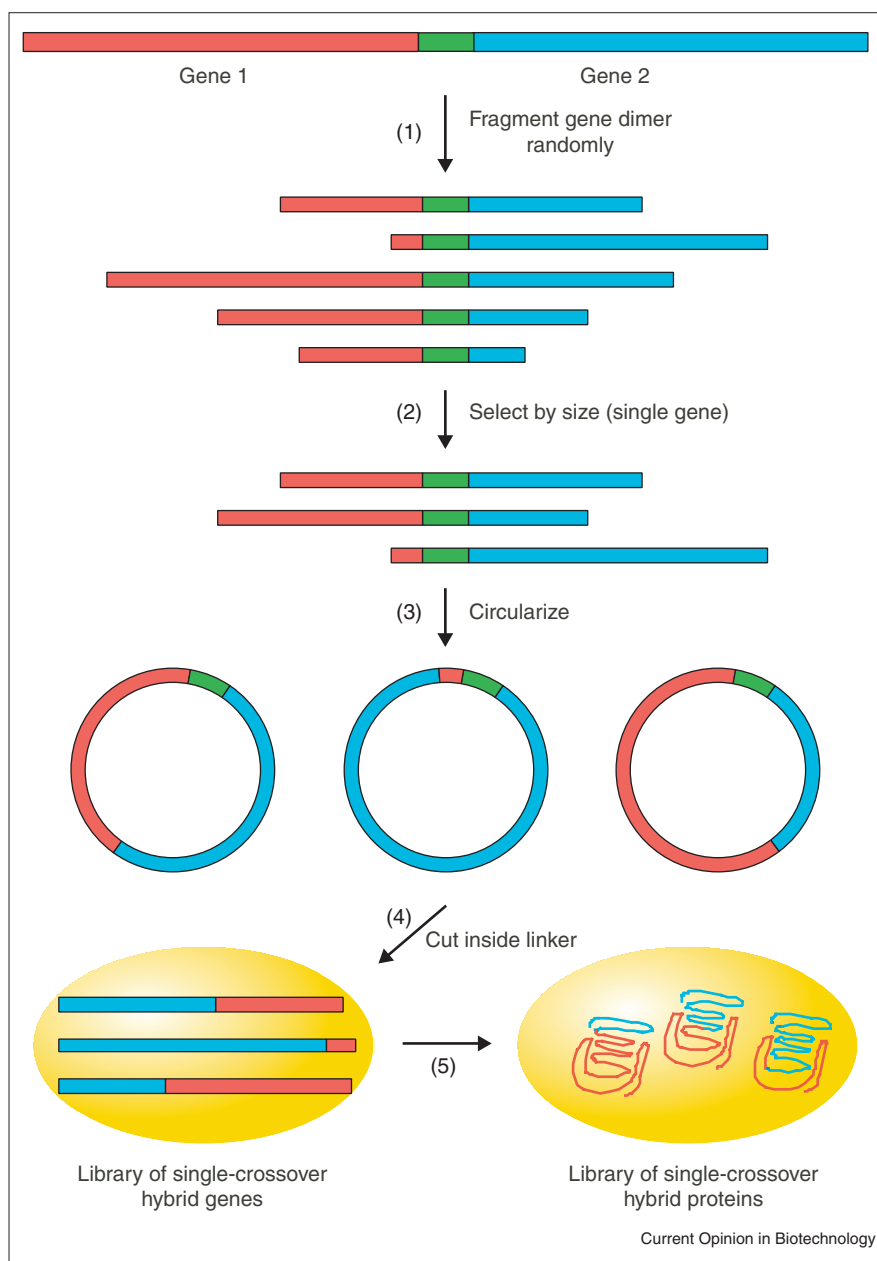
New methodological development

The past two years have witnessed the development of several new methods of diversity generation and advances in high-throughput screening or selection methods. An improved codon-level combinatorial mutagenesis method was developed to access all possible mutations at a given location in the gene [48]. Coco *et al.* [49[•]] developed an alternative DNA-shuffling method, random chimeragenesis on transient templates (RACHITT), which relies on the ordering, trimming and joining of randomly cleaved single-stranded parental gene fragments annealed onto a transient full-length single-stranded template. Compared with other recombination methods, this method has the advantages of higher recombination frequencies (crossover rates) and 100% chimerical gene products. As alternative ways to increase the recombination frequency and fraction of chimerical products of conventional DNA shuffling [50], combinatorial libraries enhanced by recombination in yeast (CLERY) [51] combining *in vitro* DNA shuffling with *in vivo* recombination in yeast, and family shuffling using single-stranded DNA [52] were developed.

In addition to random mutagenesis and homologous recombination methods, a few non-homologous recombination methods were recently developed to explore sequence space in bigger steps. Arnold and coworkers [53[•]] introduced a method for sequence homology-independent

Figure 2

Schematic overview of SHIPREC [53*]. Two genes of interest are connected to form a gene dimer through a linker sequence containing multiple restriction sites. (1) Blunt-ended fragments are generated from the dimer by random fragmentation and nuclease treatment. (2) Fragments of correct size are selected from the pool. (3) Selected fragments are circularized by intramolecular blunt-end ligation. (4) Circular DNA pieces are linearized by restriction digestion in the linker region to create a library of chimerical genes. (5) The chimerical genes are subcloned and expressed in a suitable host. (The figure was adapted from [53*] with permission.)



protein recombination (SHIPREC) that can create libraries of single-crossover hybrids of unrelated or distantly related proteins (Figure 2). Benkovic and coworkers [54] improved their previously developed non-homologous recombination method, incremental truncation from the creation of hybrid enzymes (ITCHY), by using nucleotide triphosphate analogs to create incremental truncation libraries. One of the main limitations with these two methods is the creation of a single crossover between the two parental genes. To address this limitation, Benkovic and coworkers [55] developed a new non-homologous recombination method named SCRATCHY, which combines ITCHY with DNA shuffling.

Thanks to the universal chemical nature of DNA, all the diversity-generation methods are generally applicable to any given gene or pathway. The wealth of these methods stands in sharp contrast with the relative dearth of high-throughput screening or selection methods. Fortunately, this situation has been improved dramatically in recent years. Interested readers are referred to an excellent recent review on this subject by Olsen and colleagues [56**].

Combination of directed evolution and rational design

The many unpredictable solutions to a given biocatalyst engineering problem uncovered by directed evolution

have underscored the power of this technology and highlight our limited ability to rationally design desired biocatalysts. Nonetheless, with the significant improvements in nuclear magnetic resonance spectroscopy and X-ray crystallography techniques as well as the availability of enhanced computing power in recent years, the capabilities of rational design are rapidly expanding, enabling a better understanding of protein folding, dynamics and structure–function relationships. An engineering approach that combines the best of rational design and directed evolution will probably represent the most powerful approach for biocatalyst development in the future.

As an impressive demonstration of this combined approach, Fersht and coworkers [57••] engineered a novel function in an α/β -barrel enzyme by completely converting the activity of indole-3-glycerol-phosphate synthase (IGPS) to that of phosphoribosylanthranilate isomerase (PRAI). Structure-based design was used to modify the IGPS α/β -barrel by the incorporation of the basic design of the loop system of PRAI, yielding a chimerical variant with very low PRAI activity. DNA shuffling, staggered extension process (StEP) recombination and genetic selection were then used to increase the PRAI activity. The final engineered variant exhibited sixfold higher activity than wild-type PRAI and no IGPS activity.

Other reports based on a similar combined approach include the redesign of the substrate specificity of isocitrate dehydrogenase using site-directed and random mutagenesis [58,59], the improvement of cytochrome P450 BM-3 activity [60] and KDPG aldolase substrate specificity [20], and the creation of new substrate specificity in castor Δ^9 -18:0-acyl carrier protein desaturase using structure-assisted design and combinatorial saturation mutagenesis [61].

The number of all possible protein variants in a directed evolution experiment is too large for any experimental screening or selection method to exhaustively explore. To address this limitation, structure-based computational methods have been used to reduce the search space for directed evolution. Voigt *et al.* [62] developed a powerful computational method to identify the likely sites for evolutionary improvement. Targeted mutagenesis at these selected sites would greatly reduce the experimental search.

Conclusions

Industrial biocatalysis is on the verge of significant growth. Directed evolution has rapidly become the method of choice for developing enzyme- and microorganism-based biocatalysts. The recent development of new efficient diversity-generation methods and high-throughput methods will further accelerate the development of biocatalysts. Furthermore, the ever-expanding capabilities of rational design will lead to more powerful biocatalyst design strategies that combine the best of both approaches. Advances in other related fields such as bioinformatics, functional genomics, and functional proteomics will also extend the

applications of directed evolution, rational design or a combination of both to many more industrial biocatalysts.

Update

A new diversity-generation method, random insertion/deletion (RID) mutagenesis, was developed by Murakami and colleagues [63•] to randomly introduce codon-based mutations in a target gene. This method enables deletion of up to 16 consecutive bases at random positions and, at the same time, insertion of a specific sequence or random sequences of an arbitrary number of bases at the same position.

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