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Directed Evolution: An Approach to Engineer Enzymes

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ABSTRACT Directed evolution is being used increasingly in industrial and academic laboratories to modify and improve commercially important enzymes. Laboratory evolution is thought to make its biggest contribution in explorations of non-natural functions, by allowing us to distinguish the properties nurtured by evolution. In this review we report the significant advances achieved with respect to the methods of biocatalyst improvement and some critical properties and applications of the modified enzymes. The application of directed evolution has been elaborately demonstrated for protein solubility, stability and catalytic efficiency. Modification of certain enzymes for their application in enantioselective catalysis has also been elucidated. By providing a simple and reliable route to enzyme improvement, directed evolution has emerged as a key technology for enzyme engineering and biocatalysis.

KEYWORDS biocatalyst, catalytic efficiency, engineering, evolution, enantioselective.

Enzymes are the product of biological evolution, which takes several million years. They are among the most remarkable biomolecules known because of their extraordinary specificity and catalytic power, which are far greater than those of man-made catalysts.[70] Moreover, as they are adjusted perfectly to their physiological role, the activity and stability of naturally occurring enzymes are often far away from what organic chemists, biochemists and biotransformists need.[59] This is true for the stability of enzymes in organic solvents and certain other reactions requiring high selectivity and, finally yielding industrially important compounds.[64] Nature has its own method of screening–natural evolution, i.e. it produces a large number of variants by mutation and subsequent selection of the "fittest" variant. This process can be mimicked in the test tube by using techniques such as mutation and recombination.[59] The collection of methods has been termed "directed" or "*in vitro*" evolution and provides a powerful tool for the development of biocatalysts with novel properties, without requiring knowledge of enzyme structures or catalytic mechanism.[11,59,132] Directed evolution has emerged as a key technology to generate enzymes with new or improved properties that are of major importance to the biotechnology industry.[54] It has been demonstrated that directed evolution can be used to produce enzymes with certain unique properties such as altered substrate specificity,[6,21,32,52,91] thermal stability,[9,46,82] organic solvent resistance,[119,145] and enantioselectivity.[58,99,107]

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It has been demonstrated that an enzyme is highly specific both in the nature of the substrate it utilizes and the type of reaction it catalyses. The lock and key hypothesis that was proposed long ago proved to be a fruitful way to depict the binding of enzyme to the substrate. With the passage of time, the need to modify the enzymes to show wide substrate specificity has arisen. The application of certain techniques for the same has produced some remarkable new insights. It has now become possible to alter catalytic activity and specificity of the enzyme in a rational manner.[48,98,133]

The use of enzymes in industry is often limited by lack of stability under extremophilic conditions. Most of the mesophilic enzymes are often not well suited for harsh reaction conditions required in industrial processes due to the lack of structural stability, which limits their use in industry. Additionally, increased reaction rates and reduced activity of other contaminating enzymes play a key role in promoting the industrial potential of enzymes active at higher temperatures. Most thermally adapted enzymes share some common structural features that enhance thermal stability. These include a decrease in loop length and concomitant increase in secondary structure, a decrease in labile residues (such as cysteines and glutamines), and also an increase in hydrophobic interactions, aromatic stacking, metal binding capacity and oligomerization. Thus for the commercial exploitation of proteins, directed evolution is an efficient method to evolve the above mentioned properties in normal mesophilic proteins. In addition it holds potential to be utilized as a tool to study the phenomena responsible for thermostability in wild type thermostable enzymes.[8,29,143]

The production of enantiomerically pure compounds is of steadily increasing importance to the world market for chiral fine chemicals, pharmaceuticals, agrochemicals and flavor compounds. The worldwide sales volume for chiral drugs is estimated to be US $200 billion in 2006.[110] The US Food and Drug Administration (FDA) today has very stringent guidelines for pharmaceutical companies and asks companies to rigidly evaluate whether or not a novel drug molecule can be produced as a single isomer. It is imperative to know how the growing requirements for the synthesis of optically active compounds can be met. There are two possible approaches to achieve this: asymmetric chemical catalysis[14,56,71,137] or biocatalysis using enzymes.[34,49,55,147] High throughput screening programs[152] have revealed a number of enzymes with the ability to catalyze enantioselective reactions.[12,106,129] It has been observed that, in most of the cases, enantioselectivity of a given enzyme is not as high as what is required for a desired reaction. Therefore, it is necessary to develop novel methods that allow creation of enantioselective enzymes.[107]

Evolution occurs through iterated replication with selection of large populations through many generations. However, the mechanisms that evolution discovers for achieving this ultimate goal are so convoluted and complex that the end product attained is limited within the constraints of the living organism. As a result, the enzymes are highly selective and optimized to perform the specific functions for which they have evolved and often perform poorly under industrially relevant conditions.[4,150]

Directed evolution is a process that directs molecular evolution in the test tube to produce the desired protein. This has resulted in the development of enzymes with improved properties for established technical applications and production of new enzymes tailor-made for entirely new areas of application. A directed evolution approach starts with the identification of a target enzyme to be optimized and the cloning of the corresponding gene. An efficient expression system is needed before the target gene is subjected to random mutagenesis and/or *in vitro* recombination, thereby creating molecular diversity. Subsequently improved enzyme variants are identified, preferably after being secreted into the culture medium, by screening or selection for the desired property. The genes encoding the improved enzymes are then used to parent the next round of directed evolution.

Various methods and strategies have been designed to achieve the above mentioned goal. The core algorithm of *in vitro* evolution involves certain basic steps, though the techniques used may vary.[57,102] The initial step involves the identification and isolation of the wild type gene responsible for encoding the desired enzyme. This DNA segment is then subjected to random mutagenesis such as error prone PCR (epPCR), DNA shuffling, incremental truncation or other modern methods. The library of mutant genes is further inserted into a suitable expression system. Improved variants are identified through screening such as high throughput screening, solid phase digital imaging, phage display and various other techniques. The inferior enzymes and genes are discarded and the improved enzymes are used as parents for the next

round of evolution, repeating the whole process as often as necessary.[105] The present review deals with the recent advances made in the use of directed evolution to improve or alter the enzymatic properties as well as new contributions to the technology and methods. The diverse applications of directed evolution, including improving the substrate range of enzymes, enantioselectivity, thermostability, protein solubility and expression, enzymatic properties of oxidases, lyases, recombinases, polymerases and various other enzymes of industrial applications, have been elucidated.

EXPLORING METHODS FOR THE EVOLUTION OF ENZYMES

A significant advantage of directed evolution of enzymes over rational designed methods is that new functional properties can be evolved in enzymes without the knowledge of details of the target protein.[18] The directed evolution approaches recognize the extreme complexity of the relationship between protein structure and function and promise to fill at least some of the gaps between our primitive state of molecular level understanding of proteins and the strong desire to engineer improved ones.[5] Therefore, a major step in directed evolution experiments is to efficiently explore sequence space through random mutagenesis, which can be done by achieving enzymatic molecular diversity in a library of mutagenized genes.

Error Prone PCR (epPCR)

Molecular diversity can be created in a number of ways, but the first method to achieve this was **e**rror **p**rone **PCR** (epPCR).[88,103,] This technique exploits the fact that the thermostable polymerase used lacks proofreading activity, for example *Taq* polymerase isolated from the thermophilic bacterium *Thermus aquaticus* incorporates wrong nucleotides at a frequency of 0.1×10^{-4} to 2×10^{-4} per nucleotide per pass of polymerase, during the extension of new DNA strand. Apart from this, several protocols have been developed with the aim of increasing the error rate of *Taq* polymerase, which can infinitely be varied by increasing the concentration of $MgCl_2$,[153] addition of $MnCl_2$,[15] using unbalanced concentrations of nucleotides,[16] using a mixture of triphosphate nucleoside analogs,[146] or a combination of all these to achieve higher rates of mutations. Similarly alcohol mediated epPCR is another method reported by Claveau *et al.*[24] to increase the error rates. In the presence of urea, isopropanol, propan-1-ol, butan-1-ol polymerases were active until only a critical concentration, beyond which they were progressively inhibited. A mutagenic effect occurred with vent (r) (exo-) DNA polymerase in the presence of 7.0 to 8.0% (v/v) propan-1-ol, affording mutation frequencies of up to 9.8×10^{-3} mutation/bp/PCR. Under these conditions the preferential replacement of Gs and Cs was observed, in opposition to standard epPCR that favors replacement of As and Ts. It was found that the increase in error rate due to propanol was due to partial destabilization of polymerase.

Cassette Mutagenesis

Another approach is used for mutagenesis of limited and defined gene segments, and it is called cassette mutagenesis. Short gene segments that are subjected to mutagenesis are either hot spots identified upon screening of epPCR libraries or are important domains of enzyme analyzed through structural data. Synthetic oligonucleotides can be created that carry random point mutations, when doped with nucleotide phosphoramidites. These synthetic oligonucleotides are used during the synthesis process for smaller gene segments. For larger size cassettes (>50 bp) epPCR methods are used. Cassette mutagenesis has an advantage in that it is relatively straightforward, but it is useful only when the targeted amino acids are in the same stretch of primary sequence. Also it has a disadvantage of being expensive.[30,131]

DNA Shuffling

A breakthrough in directed evolution was achieved by the development of DNA shuffling.[124,125] The major methods used earlier were epPCR and cassette mutagenesis. Generation of this technique overcame the various drawbacks of epPCR as elucidated. epPCR introduces random point mutations that may be too gradual to allow block changes that are required for continued sequence evolution. In addition, epPCR is useful when just a few cycles of mutation have to be applied, but rapidly becomes limiting when applied for multiple cycles.[124,125]

Staggered Extension Protocol (StEP)

However, DNA shuffling requires a large amount of template DNA, although **St**aggered **E**xtension **P**rotocol (StEP)[150] is yet another method that was developed

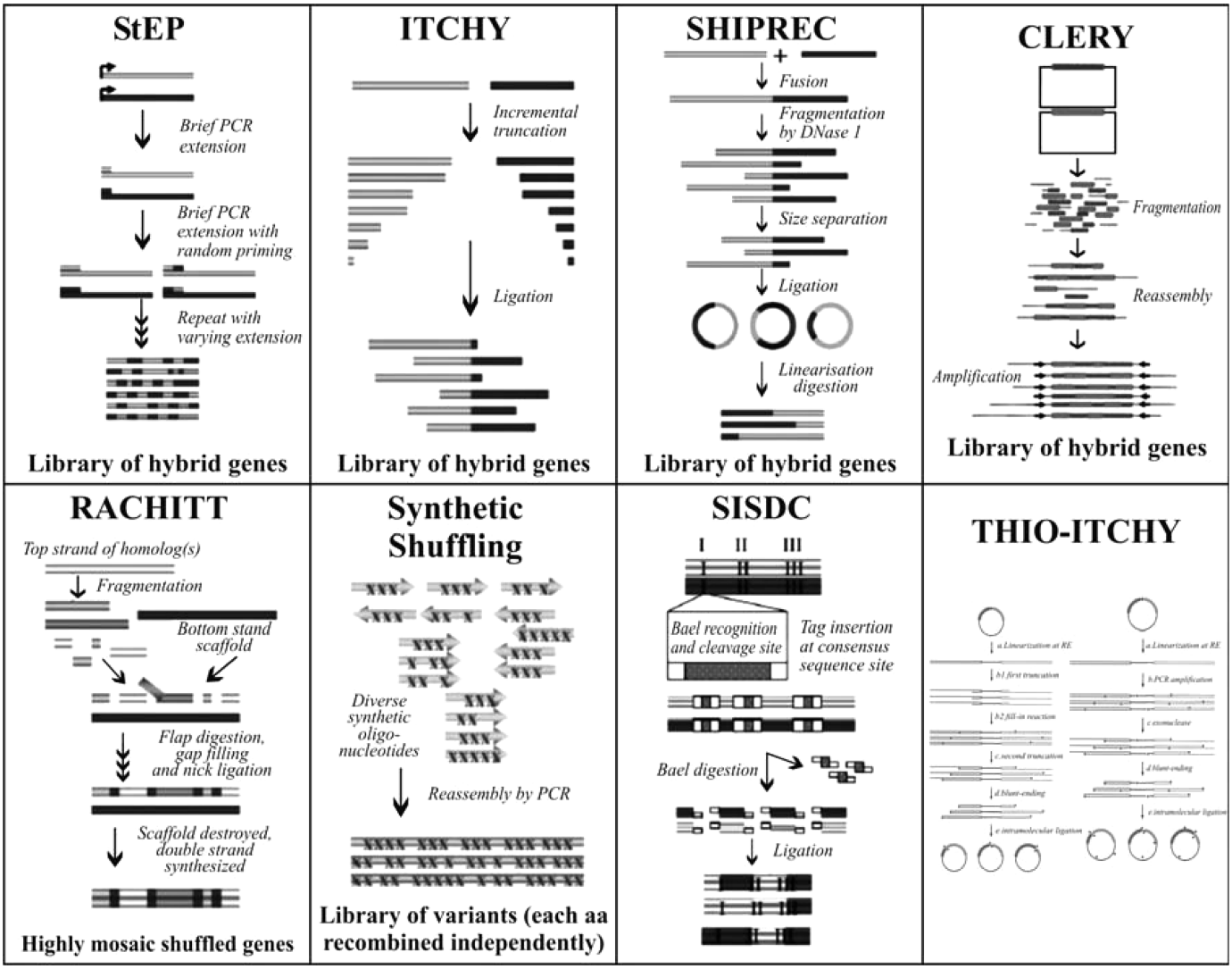

FIGURE 1 **Methods used for creating libraries using directed evolution [Staggered Extension Protocol, StEP (Valetti and Gilardi[131]); Iterative Truncation for the Creation of Hybrid enzymes, ITCHY (Valetti and Gilardi[131]); Sequence Homology Independent Protein RE-Combination, SHIPREC (Valetti and Gilardi[131]); Random CHImeragenesis on Transient Template, RACHITT (Valetti and Gilardi[131]); Synthetic Shuffling (Valetti and Gilardi[131]); Sequence-Independent Site-Directed Chimeragenesis, SISDC (Valetti and Gilardi[131]); Combination Libraries Enhanced by Recombination in Yeast, CLERY (Abecassis *et al.*[1]); THIO-ITCHY (Lutz *et al.*[75,76]).**

to overcome its limitations (Figure 1). This method does not require the DNaseI fragmentation step and yields chimeric genes through template switching. The template sequences go through repeated cycles of denaturation and extremely short duration annealing/polymerase catalyzed extension. In each cycle, the growing fragments anneal to different templates based on sequence complementarity and extend further. This is repeated until full-length sequences form. This technique has been used in the evolution of thermostable subtilisin. Five thermostabilized subtilisin E variants identified by a single round of epPCR and screening the StEP recombination library yielded subtilisin E whose half-life at 65°C was 50 times that of the wild type.[150]

Synthetic Shuffling

Further, it has been found that DNA shuffling allows recombination only in regions that share considerable sequence identity[60] and when fragments are sufficiently large to anneal to one another, blocks of parental sequences tend to be conserved in progeny sequences. To counter this, Ness *et al.*[87] devised synthetic shuffling, which allows genes to recombine independently ensuring variation of amino acids individually (Figure 1). A series of oligonucleotides, which encode all variations present in two parental genes, are designed. Differences in sequences between these genes are incorporated either as degeneracies in the oligonucleotides or

by using alternative non-degenerate oligonucleotides. This method is advantageous in various aspects and it is highly flexible as the genes are synthetic and codon usage can be altered to that of the desired expression host. It also allows generation of protein libraries directly from sequence database, thus eliminating the availability of parental genes. Variations, not present in a known protein, can also be incorporated and predicted to give the desired result. This method was used for obtaining chimeric subtilisin enzymes by recombination between 15 subtilisin genes that were screened for considerable activity at pH 7 and 10, and at pH 10 after heat treatment. Such a subtilisin, which is functionally active at alkaline pH and high temperatures, can be exploited for use in detergents. Four variants with improved activity compared to the commercially used Savinase® were isolated, two of them with a 2- to 4-fold greater activity after heat treatment, and more thermostable than any of the parents. The selected clones also displayed a higher ratio of activity at pH 10 over pH 7, meaning that a desirable higher stability to autolysis was achieved during enzyme production. Thus, synthetic shuffling explores the sequence space more extensively by accessing amino acid combinations and frequencies not found in parental proteins.[131]

Incremental Truncation for the Creation of Hybrid Enzymes (ITCHY)

To surpass the disadvantages of DNA shuffling, which can create crossovers only at homologous regions, Ostermeier *et al.*[94,95] created an approach to generate fusion libraries between two gene fragments called **I**ncremental **T**runcation for the **C**reation of **HY**brid enzymes (ITCHY) (Figure 1). Two parental genes are digested with exonuclease III in controlled conditions to generate truncated gene libraries with progressive 1bp deletions. The truncated 5′-fragments of one gene are fused to truncated 3′-fragments of the other gene, which yields a library of chimeric sequences, which are then expressed and screened or selected for improved enzyme activity. It allows creation of functional fusions of genes from overlapping amino or carboxy-terminal gene fragments independent of DNA sequence homology. However, this method has a lengthy protocol and requires extensive point sampling and to overcome these shortcomings an alternative procedure, termed THIO-ITCHY (Figure 1), was developed to create ITCHY libraries using nucleotide triphosphate analogs such as α-phosphothionate dNTPs. DNA is protected by the nucleotide analogs from exonuclease digestion and hence leads to the desired variation in truncation length upon nuclease treatment. The two targeted gene fragments can be combined into a single vector, as the generation of diversity is no longer a function of timed exonuclease digestion but instead based on the random distribution of the α-phosphothionate nucleotides.[75,93]

SCRATCHY

Even though ITCHY allows creation of comprehensive libraries, the main drawback is that the mutations are due to single crossover per gene. Therefore, a combination of ITCHY and DNA shuffling called SCRATCHY was devised by Lutz *et al.*[75,76] along with a computational model of eSCRATCHY that gives predictions on the expected results and biases for any given sequence. SCRATCHY was experimentally demonstrated by the generation of libraries with multiple crossovers, independent of sequence homology, between the glycinamide-ribonucleotide formyltransferase (GART) from *Escherichia coli* (*purN*) and human (*hGART*).

Sequence Homology-Independent Protein Recombination (SHIPREC)

Apart from DNA shuffling, another technique that utilizes recombination is **S**equence **H**omology-**I**ndependent **P**rotein **REC**ombination (SHIPREC) (Figure 1). SHIPREC has an advantage over DNA shuffling as SHIPREC can be used to create hybrid genes of distantly related sequences, since crossovers occur at positions that are structurally similar, and it depends on sequence length rather than sequence similarity. The first step is the fusion of two genes to form a heterodimer, which is then digested with DNaseI (in presence of Mn^{2+}) to yield fragments of random length, which are then isolated. These fragments are subjected to treatment with S_1 nuclease to produce blunt ends and the remaining ends of two genes are fused by circularization. The gene at 5′ position in the dimer will now be at 3′ and will donate the C terminus of the hybrid protein. This method was used to produce hybrids of membrane associated human cytochrome P450 (1A2) and a soluble heme domain of bacterial P450 BM3 from *Bacillus megaterium*. Therefore, SHIPREC was used to identify hybrid genes encoding soluble, folded human

P450 enzymes, which were more soluble in bacterial cytoplasm. The two parent proteins shared only 16% amino acid identity.[120]

In Vitro Compartmentalization (IVC)

***In Vitro* C**ompartmentalization (IVC) is yet another method developed by Griffiths and Tawfik.[42] In this system, artificial-cell-like compartments, which use water in oil emulsions, provide the linkage of genotype (DNA) and phenotype (protein) for directed protein evolution. It has been used for selection of enzymes such as methyltransferases,[27,68,128] a bacterial phosphotriesterase,[42] *Taq* DNA polymerase[40] and Fok I restriction endonuclease.[31] A modification of IVC that involves the use of nano-droplet delivery system has been reported by Bernath *et al.*[10] It allows the transport of various solutes, including metal ions, into the emulsion droplet. This method was used for identification of DNA nuclease inhibitors through the regulation of activity of colicin nucleases that were co-compartmentalized with genes, so that nucleases were activated by nickel or cobalt ions only after the potential inhibitor genes have been translated. Hence, it led to the isolation and amplification of the genes, which encoded the nuclease inhibitors. The stringency of selection could be easily modulated to give high enrichments (100–150 fold) and recoveries. Its utility was demonstrated by selecting libraries of the gene encoding the cognate inhibitor of colicin E9 (immunity protein 9, or Im9) for inhibition of another colicin (ColE7). As a consequence, the evolved inhibitors showed significant inhibition of ColE7 both *in vitro* and *in vivo*.

Random Chimeragenesis on Transient Templates (RACHITT)

Although several *in vitro* recombination methods have been reported, a continuous strive for the development of better recombination technique enabling fine resolution recombination between closely spaced parental alleles led to the development of **RA**ndom **CHI**meragenesis on **T**ransient **T**emplates (RACHITT) (Figure 1) by Coco *et al.*[25] RACHITT utilizes a single hybridization event, as opposed to the thermocycling steps used in PCR-based methods. This method involves using a single gene, of one of the two strains of a particular family of microorganisms, as a transient scaffold template and the other gene is used for generation of fragments. The former gene serves as a template for the filling of gaps. The gaps are filled between hybridized fragments, which allow inclusion of template sequences as mosaic insertions into the nascent chimeric strand. Unhybridized 5′ and 3′ termini or "flaps" can form on some annealed fragments because of overlap with adjacent fragments on it, or because of mismatches with respect to the scaffold, which are trimmed using nucleases. After flap trimming, gap filling and ligation, the heteroduplexed parental strand template is rendered non-amplifiable by uracil-DNA-glucosylase treatment and is replaced by a homoduplex chimeric strand during PCR. The technique was tested using *dszC* genes from *Rhodococcus erythropolis* IGTS8 and *Nocardia asteroides* A3H1 being 1.25 kb in length and having 89.9% identity. It results in the generation of chimeric genes having a rate of 1 mutation per 1,103 bases. *dszC* genes encode for dibenzothiophene monooxygenase (DBT-MO), which catalyzes the first and limiting step of *dszABCD* diesel biodesulfurization of fossil fuels, along with production of chiral sulfoxides and alkylaryl sulfonate surfactant intermediates. The library containing mutant clones was screened using a two-phase microplate format assay. It was aimed to increase enzymes substrate specificities resulting in a 20-fold improvement in conversion of indole into indigo. This method results in crossovers several fold higher than other shuffling methods, i.e. 14 crossovers per gene in addition to high recombinational frequency between closely linked sequence polymorphisms leading to an efficient exploitation of the allelic diversity inherent in parental proteins. Another advantage that has been reported is that the single hybridization step causes a greater binding specificity for DNA fragments of both low and high sequence identity. Also, trimming of flaps enables the incorporation of fragments much smaller than those generated by DNase I. Lastly, it prevents parental fragments from reannealing to their own complementary strands as a gene is there from just one parent as a template.

Orthogonal Combinatorial Mutagenesis (OCM)

A unique codon-level combinatorial mutagenesis method called Orthogonal Combinatorial Mutagenesis (OCM) has been devised by Gaytan *et al.*[39] This method makes use of two sets of deoxynucleoside-phosphoramidites protected on their 5′ hydroxy ends with two orthogonal protecting groups,

4,4′-dimethoxytrityl (DMT) and 9-fluorenylmethoxycarbonyl (Fmoc) for the synthesis of oligonucleotide libraries with controlled mutagenesis rates at codon level. Wild type and mutant codons are assembled with DMT phosphoramidites and Fmoc-phosphoramidities (or any R group instead of Fmoc), in an alternating fashion, but in the same synthesis column at a controlled, non-saturating rate (Figure 2). Condon level mutagenesis is achieved by selectively removing the DMT or R protecting group at a controlled rate. A binomial distribution of mutants is achieved through this method, which is given by the equation:

$$P = [n!/x!(n - x)!]\alpha^{x}(1 - \alpha)^{n-x}$$

- P-proportion of each set of mutants in the library.
- n-number of wild type codons to explore in the window.
- x-type of mutant in the library (e.g. for single mutant x = 1, for double mutants x = 2, etc)
- α-mutagenesis rate

The OCM method was tested by synthesizing oligonucleotides where four adjacent codons were subjected to various mutagenesis rates. Three oligonucleotide libraries were synthesized with the sequence 5′ TAG GAG GAT CCC CGG GTA CCG AGC TCG AAT TCA CTC GGA C-3′ where underlined triplets denote mutagenized codons. Primer was synthesized having a sequence 5′-GTCCGAGTGAATTCG-3′ complementary to the 3′ region of the oligonucleotide libraries, with DMT-phosphoramidites. Three libraries were generated with a mutagenesis rate per codon of 49.5, 78.9 and 10.61%. The mutated gene segments were electroporated into *E. coli* and analyzed by direct colony sequencing, which revealed a binomial distribution. This makes OCM method a viable alternative for constructing oligonucleotide libraries mutagenized at a codon level in non-saturating conditions, generating a binomial distribution roughly concordant with the mutagenesis rate.

Structure Based Combinatorial Protein Engineering (SCOPE)

Yet another technique that could be harnessed for creating mutants of a particular protein is **S**tructure based **CO**mbinatorial **P**rotein **E**ngineering (SCOPE). This method is particularly useful in creating multiple crossover libraries from non-homologous genes. Its application was demonstrated by taking two members of X-family of DNA polymerases i.e. rat DNA polymerase β (pol β) and African swine fever virus DNA polymerase X (Pol X). Libraries of chimeric genes with up to five crossovers were synthesized in a series of PCR reactions by employing hybrid oligonucleotides that code for variable connections between structural elements. Genetic complementation in *E. coli* allowed the screening of several novel DNA polymerases with enhanced phenotypes.[89]

Assembly of Designed Oligonucleotides (ADO)

Another method developed to overcome the shortcomings of the conventional method of DNA shuffling used as a tool for recombination of genes is **A**ssembly of **D**esigned **O**ligonucleotides (ADO). This method minimizes the self hybridization of parental genes and achieves a high recombination frequency with formation of full length products through controlled overlapping of the desired oligomers. It was used for the recombination of two lipase family genes from *Bacillus subtilis* (*LipA* and *LipB*). Several mutants from a library

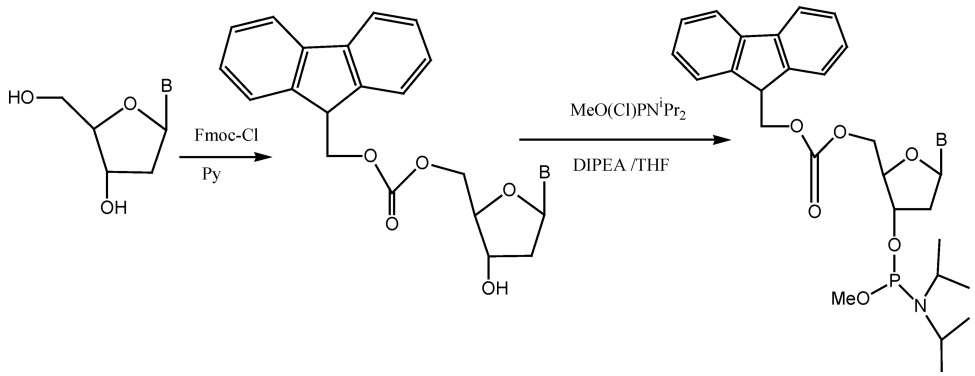

FIGURE 2 **Synthesis of Fmoc-phosphoramidites for orthogonal combinatorial mutagenesis. B = 6N-benzoyladenine, 4N-benzoylcitosine, 2N-isobutyrylguanine or thymine, Fmoc-Cl = 9-fluroenylmethoxycarbonyl chloride. Py = pyridine. DIPEA = N,N-diisopropylethylamine (Gaytan *et al.*).[39]**

of 3000 lipase variants showed increased enantioselectivity against the hydrolysis of meso-diacetate.[148]

Combination Libraries Enhanced by Recombination in Yeast (CLERY)

There are various ways to achieve recombination of genes and decrease the various limitations associated with the process. *In vivo* recombination is also one of the methods that yields low complexity chimeric enzymes. Abecassis *et al.*[1,2] developed a procedure, i.e. **C**ombination **L**ibraries **E**nhanced by **R**ecombination in **Y**east (CLERY) (Figure 1), which involved *in vitro* and *in vivo* recombination steps to construct a high complexity library containing low levels of parent genes. This technique was tested using human cytochrome P450 CYPIA1 and CYPIA2 as templates, which share 74% nucleotide sequence identity. The first step involved is the construction of an expression library of mosaic structures containing human *CYPIA1* and *CYPIA2* ORFs followed by DNase I catalyzed generation of fragments of the two vectors and thereafter subjected to progressive hybridization PCR program. It allowed the reconstruction of DNA fragments to the size of yeast vector (11 kb). Further PCR was done using primers located on the cDNA flanking regions followed by co-transformation in yeast of the PCR fragments with pYeDP60 yeast vector, leading to *in vivo* recombination events. The yeast library was then screened for statistical analysis using radiolabelled probes matching to the two parental sequences in regions of low sequence similarity. The statistical analysis of randomly selected clones showed a homogenous representation of the parental contribution among the sequences (55.8 $\pm$ 2.5% for a parental sequence IA2) and high proportion of chimeric genes (86%). Selection of the catalytically competent clones was done by colorimetric detection of activity in microtiter plates. The polycyclic aromatic hydrocarbon (PAH) hydroxylation was tested using naphthalene as a substrate. The sequence characterization was also reported for five randomly picked and five functionally selected clones, which allowed calculation of average length of sequence exchange and average number of mutations, which came out to be 8.3 $\pm$ 3.2 for the average number of functionally competent clones. The main advantage of this method is the direct construction of expression libraries in an eukaryotic microorganism, which allows direct expression and functional *in vivo* selection without the need for intermediate cloning steps in *E. coli*. This technique can be harnessed for improving the properties of multicomponent eukaryote complexes involving non-soluble enzymes.

Sequence-Independent Site-Directed Chimeragenesis (SISDC)

Another method that allows for the recombination of distantly related (or unrelated) proteins at multiple discrete sites is the **S**equence-**I**ndependent **S**ite-**D**irected **C**himeragenesis (SISDC) method (Figure 1). It is a general method that leads to recombination at multiple discrete sites with little sequence bias and in which all targeted fragments are recombined in the desired order.[51] To test the efficacy of this technique, β-lactamases TEM-1 and PSE-4 were recombined at seven sites, the library of 2^8 (256) chimeras were screened for functional enzymes. Sequencing the genes from clones having functional lactamases identified 14 unique chimeras.[51] The computational algorithm called SCHEMA was also developed to estimate the disruption caused in the three-dimensional structure of a protein by the inheritance of an amino acid from different parents upon recombination.[80]

Sequence Saturation Mutagenesis (SeSaM)

The epPCR method has certain limitations. The mutations rarely occur next to each other and the mutational bias of the employed DNA polymerases such as *Taq* polymerase favor A→T or T→A substitutions, resulting in limited mutation frequencies and low product yields. Sequence Saturation Mutagenesis (SeSaM) developed by Wong *et al.*[139] is devoid of such limitations. SeSaM can control the mutation incorporation through a universal base. The technique involves four steps that can be accomplished in 2–3 days. It includes DNA fragment generation with random lengths, enzymatic elongation of DNA fragments, using terminal transferase at 3′-termini, with the universal base deoxyinosine followed by full length gene synthesis using a single stranded template and replacement of universal bases with standard nucleotides. Mutations can be introduced in the target sequence at every single nucleotide position due to the promiscuous base pairing property of the universal base. Enhanced green fluorescence protein was used as a model system. Upon

sequencing of 100 genes, it was seen that a random distribution of mutations was achieved.

Codon Shuffling

Recently, a completely different technique has been devised by Rao *et al.*[104] called codon shuffling. This technique is based on the extension of a truncated parent gene with a new DNA fragment created *de novo*. The parent gene is extended through the ligation of 6bp DNA duplexes called dicodons, which are assembled from a set of 14 such dicodons, each of which are judiciously chosen to encode amino acids, such that all natural 20 amino acids are used in proportions that show their usage in *E. coli*, i.e. when they are expressed in *E. coli*.

In Vivo DNA Shuffling

A novel *in vivo* DNA shuffling, which allows successive homologous recombinations, has been devised by Xu *et al.*[142] This method involves chimera construction between two homologous genes based on the Rec A-dependent homologous recombination, which occurs naturally in *E. coli*. A three-way ligation of a vector and two homologous, *bphC* encoding 2,3-dihydroxybiphenyl 1,2-dioxygenases or the two-way ligation of the donor *bphC* gene and acceptor plasmid carrying the homologous *bphC* gene was performed to generate linearized DNA molecules. The homologous recombination between the genes on the linearized DNA molecules created the large chimeric *bphC* libraries in a recBC sbcA *E. coli* strain. After three rounds of recombinations and through high-throughput screening theromostable chimeric 2,3-dihydroxybiphenyl 1,2-dioxygenases were selected.[142]

SOME OF THE TARGET PROTEINS

Oxidoreductases

As the name suggests, the enzymes belonging to this class carry out both oxidation and reduction reactions. The substrate, which is oxidized, is regarded as hydrogen donor. Oxygenases are key enzymes that are capable of attacking the C–H bond in the presence of molecular oxygen in a highly selective and stereospecific manner. This makes them a potent biocatalyst for theoretical as well as practical interests. Protein engineering exercises can be successfully applied to these enzymes for achieving enhanced activity, stability and range of recognized substrates. It has been shown that these enzymes are organized in multiple domains with finely tuned and interdependent properties. This, combined with a low stability and complex mechanism of catalysis, makes them a challenging target of directed evolution.

Cytochrome P450 BM-3 catalyzes the hydroxylation of alkanes, alkenes, alcohols, fatty acids, amides, polyaromatic hydrocarbons and heterocycles in presence of polar organic cosolvents. However, to achieve high catalytic efficiency it needs to have consistent stability in organic cosolvents. Seng Wong *et al.*[119] improved the tolerance of P450 BM-3 in dimethylsulfoxide (DMSO) and tetrahydrofuran (THF), attaining an increase in specific activity up to 10-fold in 2% (v/v) THF and 6-fold in 25% (v/v) DMSO**.** The modified P450 BM-3 also showed resistance to acetone, acetonitrile, dimethylformamide and ethanol as cosolvents. Cytochrome P450 BM3 is also capable of epoxidation of various alkanes, including styrene. Cirino *et al.*[23] replaced 13 methionine residues in the cytochrome P450 BM-3 heme domain (463 amino acids) with the isosteric methionine analog norleucine, to improve the efficiency of the BM-3 peroxide "shunt" pathway through which peroxide can stimulate the catalysis, instead of NADPH and O_2. The directed evolution resulted in a two-fold increase in peroxygenase activity, though the thermostability was significantly reduced.

Biphenyl dioxygenase has an important application in the degradation of polychlorinated biphenyls (PCB), which are recalcitrant compounds and are recognized as serious environmental pollutants. Random recombination was done through DNA shuffling of the *bphA* gene of *Pseudomonas pseudoalcaligenes* KF707 and *bphA* gene of *Burkholderia* sp. strain LB400, followed by screening with biphenyl, 4-chlorobiphenyl (4-CB), 2,2′-dichlorobiphenyl (2,2′-CB), 4,4′-dicholorobiphenyl (4,4′-CB), 2,5,4′-trichlorobiphenyl (2,5,4′-CB), 4-methylbiphenyl (4-MB), diphenylmethane (DM) and dibenzofuran (DF). The evolved biphenyl dioxygenase was screened by selecting colonies showing a yellow pigment, indicative of the ring *meta*-cleavage product, and it was obtained by degradation of biphenyl, 4-CB, 4-MB, 4,4′-CB, DF, 2,2′-CB but not of 2,5,4′-CB and in addition to it, novel degradation activity for toluene, benzene and monocyclic aromatic compounds such as ethylbenzene, butylbenzene and isopropylbenzene was also gained (Table 1).[38,65]

TABLE 1 List of the enzymes engineered by directed evolution

Enzyme	Organism	Improved property	Method	Application	Reference
		Oxidoreductases			
1) Cytochrome P450 CYP102A2	*Bacillus subtilis*	6 to 9 fold increased activity with SDS, lauric acid and 1,4-naphthoquinone, ethacrynic acid and varepsilon-amino-n-caproic acid	epPCR + screening	Potential for biotransformations	7
2) Cytochrome P450 BM-3	*B. megaterium*	Regio and enantioselectivity towards octane (40% ee) and other alkanes greater than hexane (40–50% ee)	Directed evolution + site directed mutagenesis	Biotechnological applications and useful for asymmetric synthesis	101
3) Cytochrome c peroxidase (ccp)	*Saccharomyces cerevisiae*	20 fold increased activity and 70 fold increased specificity for 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).	DNA shuffling + saturation mutagenesis	Preparation of enzyme conjugated antibodies	53
4) Cytochrome P450 BM-3	*B. megaterium*	Thermostability	DNA shuffling + screening	Longer shelf life and biomimetic hydroxylase	115
5) Cytochrome P450 BM-3	*B. megaterium*	2 fold increase in peroxigenase activity in 10 mM H_2O_2	Site directed mutagenesis and screening	Decreased heme degradation in 10mM H_2O_2	23
7) Galactose oxidase	*Fusarium graminearum*	3.4-4.4 fold greater V_{max}/K_m with methyl galactoside and increased specificity	epPCR + screening	Derivatization of guar gum	135
8) Galactose oxidase	*F. graminearum*	Expression in *E. coli* and thermostability	epPCR + StEP recombination	Used for chemical synthesis	126
9) Galactose oxidase	*F. graminearum*	16 fold increase in activity and 3 fold decrease in K_m	epPCR + screening (digital imaging system)	Application in biosensors and diagnostics	28

10) Biphenyl dioxygenase	*Pseudomonas pseudoalcaligenes* and *Burkholderia* sp.	Degradation of biphenyl, 4-CB, 4-MB, 4,4′-CB,DF, 2,2′-CB, toluene, benzene and monocyclic aromatic compounds.	DNA shuffling	Degradation of PCBs	38
11) Toluene-4-monooxygenase	*P. mendocina*	Alteration of regiospecificity of nitrobenzene and toluene oxidation	Saturation mutagenesis (epPCR + screening)	Decontamination of toluene and nitrobenzene contaminated sites	35
12) Toluene dioxygenase	*P. putida*	Expression in *E. coli* and 5.6 times higher activity against 4-picoline and 20% more activity towards toluene	epPCR + Saturation mutagenesis + Screening	Dihydroxylates aromatic carbocycles	114
13) *p*-Hydroxyphenylpyruvate dioxygenase	*Streptomyces avermitilis*	HPD converted into HMS	Site directed mutagenesis	Bioremediation	44
		Transferases			
14) Aspartate aminotransferase	*Escherichia coli*	Increased specificity for phenylalanine over aspartate	DNA shuffling + site directed mutagenesis	Modulation of arginine switch mechanism	109
15) *Hae*III methyltransferase	*Haemophilus aegyptius*	670 fold improvement in catalytic efficiency and preference for AGCC	*In vitro* compartmentalization	Used in restriction modification system	27
16) Aspartate aminotransferase	*E. coli*	10^5-fold increase in the catalytic efficiency (k_{cat}/K_m) for β-branched amino and 2-oxo acids and a 30-fold decrease in that for the native substrates	DNA shuffling + selection	Diagnostic usage	144
17) Glutathione S-transferase	mammalian	15-fold increase in k_{cat} and up to 6-fold increase in k_{cat}/K_m	Random oligonucleotide-directed mutagenesis + selection	Modification of drug- and carcinogen-metabolizing enzymes to achieve desired resistance in both prokaryotic and eukaryotic plant and animal cells.	43

(Continued on next page)

Table 1 **List of the enzymes engineered by directed evolution *(Continued)***

Enzyme	Organism	Improved property	Method	Application	Reference
		Hydrolases			
18) Organophosphorus hydrolase	*P. diminuta*	25-fold improvement in hydrolysis of methyl parathion, 725-fold in k_{cat}/K_m for chlorpyrifos and 39-fold for paraoxon	DNA shuffling	Degradation of organophosphate pesticides	20
19) Phosphotriesterase	*Agrobacterium radiobacter* P230	Paraoxon used as sole phosphorus source	Cassette mutagenesis	Bioremediation (detoxification of phosphotriesterase pesticides and chemical warfare agents)	79
20) Phosphotriesterase	Bacterial	63 times higher k_{cat}	*In vitro* compartmentalization + selection	Bioremediation	42
21) Esterase	*P. fluorescens*	2-fold enhanced enantioselectivity towards 3-phenylbutyric acid resorufin esters	epPCR + screening	Enantioselective catalysis of commercially useful compounds	50
22) Lipase	*P. aeruginosa*	2-fold increase in amidase activity	Random mutagenesis + screening	Understanding lipase inability to hydrolyze amides	37
23) Lipase	*P. aeruginosa*	E = 25 improved from E = 1.1	epPCR + DNA shuffling	Pharmaceutical industry	105
24) Lipase B	*Candida antarctica*	20 fold increase in half life at 70°C	epPCR	Resolution and desymmetrization of compound	149
25) Hydantoinase	*Arthrobacter* sp.	Enantioselective hydantoinase expression in *E. coli* and 5 fold more productivity	Saturation mutagenesis + Screening	Production of L-Met (L-amino acids)	78
26) Subtilisin	*B. sphaericus*	Increased k_{cat}/K_m 9.6 times of the wild type and half life at 70°C 3.3 times less, with greater activity at low temperature	Random mutagenesis + recombination	Detergents	138
27) N-carabamyl-D-amino acid amidohydrolase	*A. tumefaciens*	18-fold higher oxidative stability and 8-fold higher thermostability	DNA shuffling	Higher shelf life and activity at high temperatures	90

28) Glutaryl (cephalosporin) acylase	*Pseudomonas SY-77*	10-fold improvement in catalytic efficiency on adipyl-7-ADCA	epPCR + selection	In manufacture of semisynthetic penicillin and cephalosporin	97
		Lyases			
29) Fructose bisphosphate aldolase	*E. coli*	Increased thermostablity and stability to treatment with organic solvent	DNA shuffling	Use in organic synthesis	46
30) Tagatose-1,6-bisphosphate aldolase	*E. coli*	80 fold improvement in k_{cat}/K_m and 100-fold change in stereospecificity	DNA shuffling + screening	Efficient syntheses of complex stereoisomeric products	136
31) Benzoylformate decarboxylase	*P. putida*	5-fold increase in carboligase activity	Random mutagenesis + selection + site directed mutagenesis	Application in bioorganic chemistry	73
		Isomerases			
32) Xylose isomerase	*Thermotoga neapolitana*	High activity on glucose at low temperature and low pH	Random mutagenesis + screening	Used in preparation of high fructose syrup	123
33) Triosephosphate isomerase	*Trypanosoma brucei*	11 fold increase in k_{cat} and 4 fold decrease in K_m	Random mutagenesis	Industrial exploitation for isomerization reaction	113
34) N′[5-(Phosphoribo-syl) formimino]-5-amino-imidazole-4-carboxamide ribonucleotide isomerase (HisA)	*Thermotoga maritima*	HisA able to catalyze phosphoribosyl anthranilate isomerase (TrpF)	Random mutagenesis + selection	Relation of $(\beta\alpha)_8$-barrel enzyme activities	62
		Ligase			
35) DNA polymerase I	*Thermus aquaticus*	Expansion of substrate specificity	Compartmentalized self replication (CSR)	Polymerizing DNA segments > 25 Kb	41
36) DNA polymerase I	*Thermus aquaticus*	Conversion into RNA polymerase	ep PCR + selection (phage display)	Biotechnological applications	141
37) Cre recombinase	Bacteriophage P1	Recognizes novel recombination site	epPCR + screening (FACS)	Cloning	116
38) Phage ΦC31 integrase	*Streptomyces* phage ΦC31	Increase in specificity and integration frequency	DNA shuffling + screening	Modification of genomes of higher eukaryotic cells	118

Similarly, *p*-hydroxyphenylpyruvate dioxygenase (HPD), a Fe^{2+}/oxygen dependent enzyme showing strong homology to *p*-hydroxy-mandelate synthase (HMS) was evolved. Gunsior *et al.*[44] reported conversion of *Streptomyces avermitilis* HPD into an engineered *S. avermitilis* HMS by site directed mutagenesis of four amino acid residues present within active sites and mutants were thereafter screened by HPLC assay analysis, which revealed the production of *p*-hydroxymandelate, along with homogentisate and an oxepinone derived from benzene-oxide intermediate hypothesized in HPD catalysis. The above enzyme evolution demonstrates the designability of this enzyme (Table 1).

Apart from improving enzymatic properties, one of the potential applications of directed evolution is also the identification of critical amino acid substitutions and combinations of substitutions outside the active site that are unlikely to be identified or predicted from X-ray crystal structures. In addition to the present mentioned application Kumar *et al.*[66] used *in vitro* evolution to improve the activity of cytochrome P450 2B1 towards a variety of substrates. The enzyme was subjected to epPCR and two rounds of mutagenesis and one subcloning step, which yielded a quadruple mutant V183L/F209A/L209A/S334P. The selection was done on the basis of high throughput screening, which used hydrogen peroxide (H_2O_2)-supported oxidation of the marker fluorogenic substrate 7-ethoxy-4-trifluoromethylcoumarin (7-EFC). However, due to decreased 7-EFC metabolism supported by NADPH, the V183L mutation was deleted. The resulting F202L/L209A/S334P triple mutant exhibited a 3- to 4-fold enhanced activity toward 7-BR (7-benzyloxyresorufin), benzphetamine and testosterone and 10-fold increase in stereoselectivity for testosterone 16α-hydroxylation. These mutants were also checked for metabolism of anti-cancer prodrugs such as cyclophosphamide and ifosfamide. L209A/S334P showed a 2.8-fold improvement of $k_{cat}/K_m$with cyclophosphamide (CPA) and V183L/L209A a 3.5-fold enhancement with ifosfamide (IFA) with the metabolism carried out via the 4-hydroxylation pathway. V183L/L209A did not show enhanced catalytic efficiency in the NADPH-dependent system with most of the substrates tested; however, it showed improved activity and regioselectivity for IFA. The solved structure of an inhibitor bound form of P450 2B4 was used for comparison of the locations of mutated residues of evolved P450 2B1 in different variants by observation of the 3-D protein structure. Hence, the modified P450 enzymes have potential synthetic, medical and environmental applications.[66]

Among the diverse applications of laboratory evolved enzymes, one of them is their use in the development of safe and effective pharmaceuticals. Before any drug enters the pharmaceutical market it is tested in many ways and one of the steps include the analysis of the toxicity and biological activity of the drug metabolites produced in humans. However, production of these metabolites using human cytochrome P450s is difficult due to high cost, poor stability, and slow activity of human enzymes *in vitro*. Hence, Otey *et al.*[96] used directed evolution to produce human metabolites of propranolol from *Bacillus megaterium* cytochrome P450 BM3. Variants of P450 BM3 were generated that could function via the "Peroxide shunt" pathway, using hydrogen peroxide in place of the reductase domain, oxygen and NADPH. Further evolution of the P450 BM3 heme domain peroxygenase was done to enhance production of the authentic human metabolites of propranolol by this biocatalytic route.[96]

Environmental pollution by xenobiotics and recalcitrants is a major concern today. The inability of microorganisms to degrade these harmful chemicals up to the desired permissible level necessitates the use of laboratory evolution to facilitate their mineralization. The hybrid pathway for 2-chlorotoluene degradation was engineered. Toluene dioxygenase of the TOD system of *Pseudomonas putida* F1 *(tod C1C2BA)*, which converts 2-chlorotoluene into 2-chlorobenzaldehyde (Figure 3), a second catabolic segment encoding upper TOL pathway from pWW0 plasmid of *P. putida* mt-2 (which catalyzes oxidation of benzyl alcohols to benzoates), and *ortho*-pathways, which catalyze aromatic ring cleavage, were assembled in separate mini-Tn5 transposon vectors. However, this attempt was unsuccessful as it failed to utilize 2-chlorotoluene as the sole carbon source although each individual part of the pathway was functional.[47]

Similarly, due to the increased environmental application of aryl-hydroxylating dioxygenases, as described above, it is necessary to design enzymes with broadened or altered substrate ranges that are capable of degrading technical mixtures of PCBs. Biphenyl dioxygenase (BphA) *Burkholderia xenovorans* LB400 was subjected to segmental random mutagenesis. A single mutagenic cycle yielded 10 beneficial variants, which

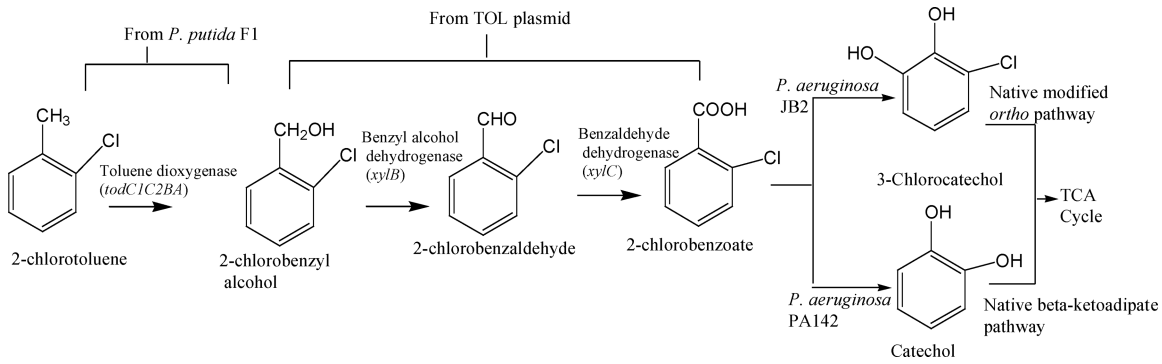

FIGURE 3 **Engineered hybrid pathway for 2-chlorotoluene degradation using toluene dioxygenase to produce 2-chlorobenzyl alcohol, the TOL plasmid upper pathway to convert 2-chlorobenzyl alcohol to 2-chlorobenzoate, and the native *ortho* or modified *ortho* pathways in the two host strains (Haro and de Lorenzo[47]).**

showed between a 5- and 200-fold increased turn over of chlorinated biphenyls, with substituent patterns that rendered them largely recalcitrant to attack by BphA-LB400. Pro334 and Phe384 were identified as amino acids, which prevent the acceptance of specific PCBs through sequence comparison of the mutagenized variants and wild-type enzyme.[154]

Oxidases, a sub-class of oxidoreductases, catalyze reactions that directly involve molecular oxygen. Certain enzymes utilize flavin as coenzyme, while others cause oxidation by removal of hydrogen. Horse radish peroxidase (HRP) is a widely used reporter enzyme though it presents considerable problems in being functionally expressed in commonly used expression hosts such as *Saccharomyces cerevisiae* and *E.coli*. A 40-fold increase in expression was brought about in *S. cerevisiae,* through random mutagenesis and screening, which identified mutants that accurately reflected the desired property along with consistent stability. This tailor-made HRP is now useful for diagnostic applications.[72,84,85]

Galactose oxidase (GO) has been reported to have direct applications in biosensors, chemical synthesis and diagnostics. GO was modified by random mutagenesis and screening using HRP, resulting in a 60-time increase in activity and considerable thermostability (Table 1).[126] To exploit the enzymes to their maximum potential, these enzymes are often modified in order to improve their enzymatic properties. However, a prerequisite for their alteration is expression in *E.coli*. Wilkinson *et al.*[135] reported the modification of GO and its expression in *Pichia pastoris*. GO has been evolved for yet another application, its use in industrial processes such as derivatization of guar gum, which is widely used as a food thickener and emulsifier. The variants showed 3.4–4.4 fold greater V_{max}/K_m with 1-methyl-α-D-galactoside as substrate and a reduction in K_m to 17 mM resulting in increased specificity (Table 1). Similarly, Delagrave *et al.*[28] also reported the optimization of GO through epPCR. A 16-fold increased activity and a considerable decrease (3-fold) in K_m was reported (Table 1).

Pyranose-2-oxidase (P2Ox) is a high-molecular-weight homotetrameric flavoenzyme, which carries out the oxidation of various mono- and disaccharides and specifically regioselective, C-2 oxidation of D-glucose. P2Ox has a potential application to yield dicarbonyl sugars, from the oxidation of the substrates mentioned above, which are used for the synthesis of rare sugars, fine chemicals and drugs. Bastian *et al.*[9] engineered P2Ox from *Peniophora gigantea* towards improved thermostability and catalytic efficiency for its efficient utilization in industrial applications. Initially site-directed mutagenesis was used to substitute a single residue (E542K) that resulted in enhanced thermo- and pH stability in addition to increased catalytic efficiencies (k_{cat}/K_m) towards D-glucose (2.0-fold) and 1,5-anhydro-D-glucitol (3.0-fold). The mutations were studied on the basis of structural information from *Trametes multicolor* and *Peniophora* sp. Decreasing or arresting the movement of the loop can be the cause of thermostability that was suggested by the fact that replacement of Glu542 with Lys created ionic interaction between the Lys amino residue and Asp124 carboxylic group, which resulted in fixation of the loop. The catalytic efficiency of this variant was further improved by application of epPCR, resulting in substitution of Lys312 with Glu. It led to a 59.9-fold increase in k_{cat}/K_m for D-xylose, a 69-fold for L-sorbose, a 5.3-fold for D-glucose, a 4.8-fold

for D-galactose, and a 2-fold for methyl-β-D-glucoside. Kinetic studies indicated that the increase in catalytic activity was due to increase in V_{max} and decrease in K_m except for D-galactose, for which only an increase in $V_{max}$was observed.[9]

Transferases

As the name suggests, transferases are group transferring enzymes, e.g., the glycosyl or methyl group from one compound (generally regarded as a donor) to another compound (generally regarded as an acceptor). In many cases the donor is a cofactor charged with the group to be transferred.

Rational redesigning of the substrate specificity of *E. coli* aspartate aminotransferase (eAATases) into that of *E. coli* tyrosine aminotransferase (eTATase) using directed evolution has been reported recently.[22] A number of eAATases mutants were characterized, including HEX (designed), HEX + A293D (design followed by directed evolution), and SRHEPT (directed evolution). The A293D mutation realized from directed evolution of HEX was modified into the SRHEPT by site directed mutagenesis, resulting in an enzyme (SRHEPT + A293D) with nearly the same ratio of k_{cat}/K_m (Phe) to k_{cat}/K_m (Asp) as that of wild type eTATase. The A293D substitution played an important role in specificity determinant by selectively disfavoring interactions with dicarboxylic substrates and inhibitors compared to aromatic ones. It was found that in both SRHEPT + A293D and HEX + A293D, the additional mutation held the Arg 292 side chain away from the active site to allow increased specificity for phenylalanine over aspartate.

Cyclodextrin glycosyltransferase (CGTase) preferably catalyzes transglycosylation reactions, whereas many other α-amylase family enzymes are hydrolases. Hence, Leemhuis *et al.*[69] converted CGTase into a starch hydrolase by subjecting it to epPCR and thereafter screening *Bacillus circulans* strain 251 CGTase mutants with increased hydrolytic activity. Three rounds of mutagenesis resulted in a 90-fold increase in the hydrolytic activity. It also helped in understanding the role of induced fit mechanism, in which sugar acceptor binding at subsite +1 activates CGTase in a transglycosylation reaction. The structure of the mutation with the largest effect, A230V, suggested that the larger valine side chain hindered the substrate binding at the acceptor site +1, although not to the extent that catalysis is impossible. The much higher hydrolytic than transglycosylation activity of this mutant indicated that the use of sugar-acceptor was hindered.

Restriction-modification (R-M) systems are widespread in bacteria with over 240 DNA specificities known.[108] The R-M system is a defense mechanism against phage infection, containing a DNA methyltransferase which methylates a specific sequence and a restriction enzyme that digests unmethylated (foreign) DNA. However, even if a large diversity of natural R-M systems exist, there is a continuing effort to engineer systems with novel specificities, as both methyltransferases and restriction endonucleases are extremely valuable tools in molecular biology. It has been observed that many enzymes have promiscuous activity against molecules that are not their principal substrate and that new enzymes may evolve by improvements in the enzyme's ability to catalyze the conversion of one of these poor substrates. This approach has been exploited by Cohen *et al.*[27] *Hae*III methyltransferase (M.*Hae*III) methylates the internal cytosine of the canonical sequence GGCC, but there is promiscuous methylation of a variety of non-commercial sites, the most frequently methylated star site being AGCC (Table 1).[26] *In vitro* compartmentalization (IVC) has been used to evolve M.*Hae*III with novel sequence specificity. The selection of variant M.*Hae*III was done by a restriction enzyme, which digested the genes encoding inactive enzymes, leaving the methylated genes encoding active enzymes intact. Some restriction sites may be protected from digestion if they overlap methylation sites. One such site is *Nhe*I. A two step mutagenesis strategy, involving initial randomization of DNA contacting residues followed by randomization of the loop that lies behind these residues yielded the best mutant T29 which showed a dramatic change in both catalytic efficiency and sequence specificity. T29 had five amino acid changes and was found to methylate *Nhe*Isub over 600 times faster than M.*Hae*III. Using 10 nM substrate DNA, the T29 mutant was found to methylate *Nhe*Isub (AGCC) very efficiently, with an initial rate of 4.1 M/min/M enzyme, 24 times faster than methylation of GGCC by the wild type enzyme. This variant was also found to methylate *Hae*IIIsub (GGCC), but the initial rate of 0.36 M/min/M enzyme was 11 times slower than methylation of *Nhe*Isub (AGCC). The k_{cat} for methylation of AGCC was increased to 4.6 fold compared with the wild type and K_m^{DNA} was 140 fold lower than

the M.*Hae* III. It was found that this mutant efficiently methylated CGCC, but other star sites including TGCC and GGCT were methylated more slowly[26]. Consequently T29 catalytic efficiency (k_{cat}/K_m^{DNA}) was improved by 670-fold on AGCC and it showed a 3-fold preference for methylation of AGCC over GGCC in terms of k_{cat}/K_m^{DNA}.

The human O^6 alkylguanine-DNA alkyltransferase (hAGT) has potential to be used for *in vivo* labelling of fusion proteins with synthetic reporter molecules but it has to be first modified for efficient activity in order to achieve the above-mentioned goal. The hAGT had been reportedly[61] altered by directed evolution and the mutants were selected by the phage display system depending on their capability of efficiently reacting with the inhibitor O^6 benzylguanine by the irreversible transfer of the benzyl group to a reactive cysteine residue. It had been modified in such way that it allowed a highly efficient covalent labeling of hAGT fusion proteins *in vivo* and *in vitro* with small molecules and therefore can be used as an efficient tool for studying protein function in living cells. In addition to various applications in proteomics, the selected mutants also had the ability to give information regarding interaction of the DNA repair protein hAGT with its inhibitor O^6-benzylguanine.

Hydrolases

These enzymes catalyze hydrolytic cleavage of C–O, C–N, C–C and some other bonds including phosphoric anhydride bonds. A number of hydrolases acting on ester, glycosyl, peptide, amide or other bonds are known to catalyze not only hydrolytic removal of a particular group from their substrates, but also transfer this group to suitable acceptor molecules. In principle all hydrolytic enzymes might be classified as transferases, since hydrolysis itself can be regarded as transfer of a specific group to water as the acceptor. Yet in most cases the reaction with water as the acceptor is considered as the main physiological function of the enzyme. Since this class is capable of showing diverse types of reactions, directed evolution can be applied to obtain enzymes that are capable of showing more than one possible activity at a time.

Use of site directed mutagenesis as an annexure to directed evolution, as described in earlier applications, improves the targeted enzymatic property by many fold as compared to the application of any of these techniques individually. Hence, this practice is becoming more and more popular. Yet again, Morillas *et al.*[86] used this technique to extend the substrate specificity of penicillin acylase. The enzyme was first subjected to directed evolution, which led to its modification so that the *E. coli* strains containing penicillin acylase acquired the ability to use glutaryl-L-leucine for growth. Two of these mutants were selected and further mutation was induced by replacing the B-chain residue Phe (B71) with either Cys or Leu. The 100-fold increase in the values of k_{cat}/K_m for glutaryl-L-leucine was observed. The mutant proteins were also found to be resistant to urea denaturation. The analysis of the protein structure revealed that the substrate specificity was enhanced by the reduction of steric constraints for substrate binding.

β-glucuronidase (GUS) is used as a reporter in the gene expression studies, however it has certain limitations associated with it. It suffers from loss of activity during tissue fixation by glutaraldehyde or formaldehyde. To overcome this hurdle Matsumura *et al.*[77] incorporated resistance in GUS through directed evolution against both glutaraldehyde and formaldehyde. A variant with eight amino acid changes was isolated after three rounds of mutation, i.e. DNA shuffling and screening. An alteration in the surface chemistry of the enzyme was observed with amino acid changes generally occurring near conserved lysines. It was also reported that cell lineages in *Xenopus* embryos could be traced using the GUS variant, allowing double staining when used in conjugation with other reporters.

Directed evolution has also been used to predict antibiotic resistance, which involves the accumulation of mutations beneficial to the pathogen but keeping the function preserved by maintaining residue interactions and core packing. TEM-1 β-lactamase has been reportedly modified through the directed evolution technique, which uses a hypermutator *E.coli* with cefotaxime selection. Consequently, a 500-fold increased resistance was reported by the combination of mutations E104K/M182T/G238S and upon comparison to the database of clinical isolates, it was found to be equivalent to clinical isolate TEM-52. Upon the determination of structure of TEM-52 to 2.4Å, it was observed that G238S widened access to the active site by 2.8Å and E104K stabilized the reorganized topology. The stabilization of the new enzyme structure appeared to be carried out by a global suppressor mutation, which turned out to be M182T mutation located at 17Å from the active site.[92]

Lipases do not hydrolyze amides despite the similarities to serine proteases in the active site structure and the reaction mechanism. However, lipase can be altered through directed evolution to a general acyl transfer catalyst for the biotransformation of amides. Fujii *et al.*[37] identified five mutants with 1.7–2-fold increased relative amidase activities by subjecting lipase from *Pseudomonas aeruginosa* to a single round of random mutagenesis, followed by screening for hydrolytic activity for oleoyl 2-naphthylamide as compared with that for oleoyl 2-naphthyl ester. A 2-fold increase was reported in a double mutant F207S/A213D, which gave the highest molecular activity of 1.1 $min^{-1}$for the amide substrate (Table 1). Further structural analysis of lipase showed that the mutations occurred at the sites near the surface and away from the catalytic triad, but close to the calcium binding site.

The enantioselective enzymes are widely used in pharmaceutical industry to obtain enantiomerically pure or enriched organic compounds. Reetz[105] demonstrated the application of directed evolution in the development of enantioselective enzymes (Figure 4). Through epPCR and saturation mutagenesis, enantioselectivity of lipase increased from (enantiomeric excess) ee = 20% (E = 1.1) for wild type enzyme to ee = 90–93% (E = 25) for the best mutants (Table 1). It is worth noting that a single amino acid substitution at any one of the hot spots (e.g. at position 155 it was discovered that phenylalanine (F) is the best amino acid) was sufficient to lead to the highest degree of enantioselectivity.

Bacillus subtilis lipase A is an essential enzyme for the preparation of the pharmaceutically relevant chiral compound 1,2-O-isopropylidene-sn-glycerol (IPG). PCR mutagenesis with spiked oligonucleotides was employed by Droge *et al.*[33] for saturation mutagenesis of a stretch of amino acids near the active site. Mutants with inverted enantioselectivity were selected through a phage display system. Dual selection with (S)-(+)- and (R)-(−)-IPG stereoisomers covalently coupled to enantiomeric phosphate suicide inhibitors (SIRAN Sc and Rc inhibitors, respectively) was used for variant isolation. A structural insight was obtained into the mechanism of enantioselectivity of enzyme through the study of 3D structures of the Sc and Rc inhibitor-lipase complexes.[33]

The goal is to achieve an efficient and minimalist design to obtain a desired property in proteins, which can be acquired by using the structural basis of selection. This technique has been utilized by Acharya *et al.*[3] for the evolution of a thermostable *Bacillus subtilis* lipase. Random mutations were generated by epPCR, then screened for enhanced thermostability and further selection of the mutants by harnessing their structural information. A 300-fold increase in half life of denaturation was observed through three point mutations generated by two cycles of epPCR. The structural basis of selection involved the analysis of high-resolution crystal structures of mutants, which showed subtle changes such as stacking of tyrosine residues, peptide plane flipping and better anchoring of the terminus. These changes were responsible for increased thermostability.

Directed evolution has been used to alter enzymes that have major applications such as bioremediation and in national security. One of these enzymes is organophosphorus hydrolase. Organophosphorus (OP) compounds are highly toxic because they inhibit acetylcholine esterase in the central nervous system synapses, leading to a subsequent loss of nerve function. These compounds are a threat as nerve agents for chemical warfare and are used as agricultural insecticides. Organophosphorus hydrolase has potential applications since it may be used for the hydrolysis of different insecticides as well more toxic chemical warfare agents such as soman and VX. This enzyme has been isolated from *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC 27551, and the enzyme from these organisms can hydrolyze OP. OP hydrolase can degrade a wide spectrum of OP compounds but with varying degrees. Hydrolysis of paraoxon is limited to diffusion-controlled limit; also it is several orders of magnitude slower for malathion, chlorpyrifos and VX. Directed evolution has been reportedly used to bring

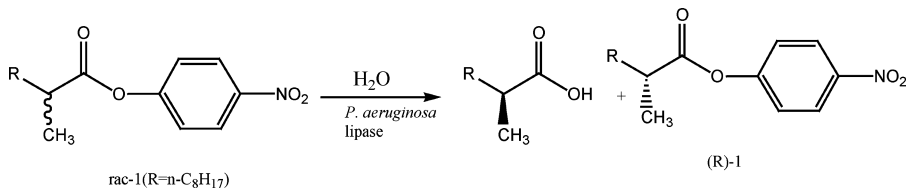

FIGURE 4 **Enantioselective catalysis by the *P. aeruginosa* lipase [rac-1 is a racemate which was enzymatically resolved to give the pure enantiomer (R)-1] (Reetz[105]).**

about 25-fold improvement in hydrolysis of methyl parathion, a substrate that is hydrolyzed 30-fold less than paraoxon.[19] To further improve the enzyme, the best variant 22A11 obtained from improved degradation of methyl parathion was used along with the wild-type *opd* gene and subjected to two rounds of DNA shuffling. One of the variants B3561 exhibited a 715-fold increase in specific activity against chlorpyrifos at a rate similar to the wild-type OPH rate of hydrolysis of paraoxon and had the measured k_{cat}/K_m value of $2.2 \times 10^8\ M^{-1}s^{-1}$. The hydrolytic rates for all other substrates were improved by 12- to 39-fold. The variant B3561 also holds the ability to hydrolyze paraoxon 39-fold better than the wild type enzyme.[20]

Semisynthetic cephalosporins and penicillins are the most widely used antibiotics. All clinically important semisynthetic cephalosporins are made from 7-aminocephalosporanic acid (7-ACA) or 7-amino-desacetoxycephalosporanic acid (7-ADCA). The 7-ACA is derived from cephalosporin C (aminoadipyl-7-ACA), which is obtained by fermentation using the fungus *Cephalosporium acremonium*. Deacylation is performed either chemically or by a two-step enzymatic process using a D-amino acid oxidase and a glutaryl acylase. The latter enzyme can be found in several *Pseudomonas* and *Acinetobacter* species as well as in some gram-positive bacteria. The 7-ADCA is produced from pencillin G made by *Penicillium chrysogenum,* involving several polluting chemical steps, followed by enzymatic deacylation by penicillin acylase. For the deacylation of this novel β-lactam, an adipyl acylase is needed. Since presently identified acylases show little or no activity towards adipyl-7-ADCA (AD-7-ADCA), Otten *et al.*[97] converted glutaryl acylase into an adipyl acylase. The glutaryl acylase used in the experiment was isolated from *Pseudomonas* SY-77 as it was proven to be suitable for the development of an industrial process for deacylation. Five libraries were constructed of overlapping gene fragments of β-subunit of SY-77 and they were mutagenized by epPCR. Since deacylation of β-lactam compounds cannot be used for growth selection, the five libraries were selected for diamino hydrolyzing capability of the acylase. Sequencing of 20 randomly picked clones showed that the mutation frequencies lay between 1 and 3 bp substitutions per mutant. In total, 24 out of 41 plate selected mutants were found to have a significantly improved ratio of adipyl-7-ADCA versus glutaryl-7-ACA hydrolysis. Frequent mutations were identified at positions Asn-266 and Phe-375. Crystallographic models have pointed that the role of Phe-375 is in the determination of substrate specificity. The mutant SY-77^{N266H} showed a 10-fold improvement in catalytic efficiency (k_{cat}/K_m) on AD-7-ADCA, resulting from a 50% increase in k_{cat} and a 6-fold decrease in K_m, without decrease in the catalytic efficiency on glutaryl-7ACA. On the contrary, the improved adipyl/glutaryl activity ratio of the mutant SY-77 ^{F375L} was mainly due to the reduced catalytic efficiency towards glutaryl-7-ACA (Table 1).[97]

Hydantoinase is used for the commercial production of D- and L-amino acids and is usually used as whole cell catalyst (Figure 5). Hydantoinase from *Arthrobacter* sp. DSM9771 is used for the industrial production of L-amino acids from D,L-5-monosubstituted hydantoins.[78] However, this enzyme is not commercially exploited for the production of certain L-amino acids such as L-methionine (L-Met) as the enzyme shows preference for D-5-(2-methylthioethyl) hydantoin (D-MTEH), hence showing D-selectivity. The product from this reaction, i.e. D-N-carbamoyl-methionine (D-C-Met), leads to low productivity of L-Met as the conversion is very slow. Therefore, the inversion of enantioselectivity of D-hydantoinase to L-hydantoinase could lead to the development of a suitable process for production of L-Met. May *et al.*[78] successfully achieved this goal using directed evolution. epPCR was used to generate the first generation of mutants having an average of one amino acid substitution per gene. Screening of L-enantioselective biocatalyst was done by a pH indicator test and confirmed by HPLC analysis of the products of enzymatic reaction with racemic hydantoin. Significantly inverted enantioselective enzymes were not obtained and the variants were subjected to a second round of mutation, followed by saturation mutagenesis. Sequencing of positive clones of the first generation library revealed that substitution at position 95 relaxed enantioselectivity of the enzyme. On substitution with phenylalanine, the Q2H4 mutant was identified with an ee of 20% at ~30% conversion. Recombinant whole cell catalysts were prepared by coexpressing the evolved hydantoinase with a hydantoin racemase and an L-N-carbamoylase in *E. coli*. It was found that more than 90% conversion was achieved in less than two hours as compared to ten hours for the wild type enzyme. In addition there was a 4-fold decrease in the accumulated D-C-Met intermediate concentration. Despite its low L-selectivity, the evolved hydantoinase represented

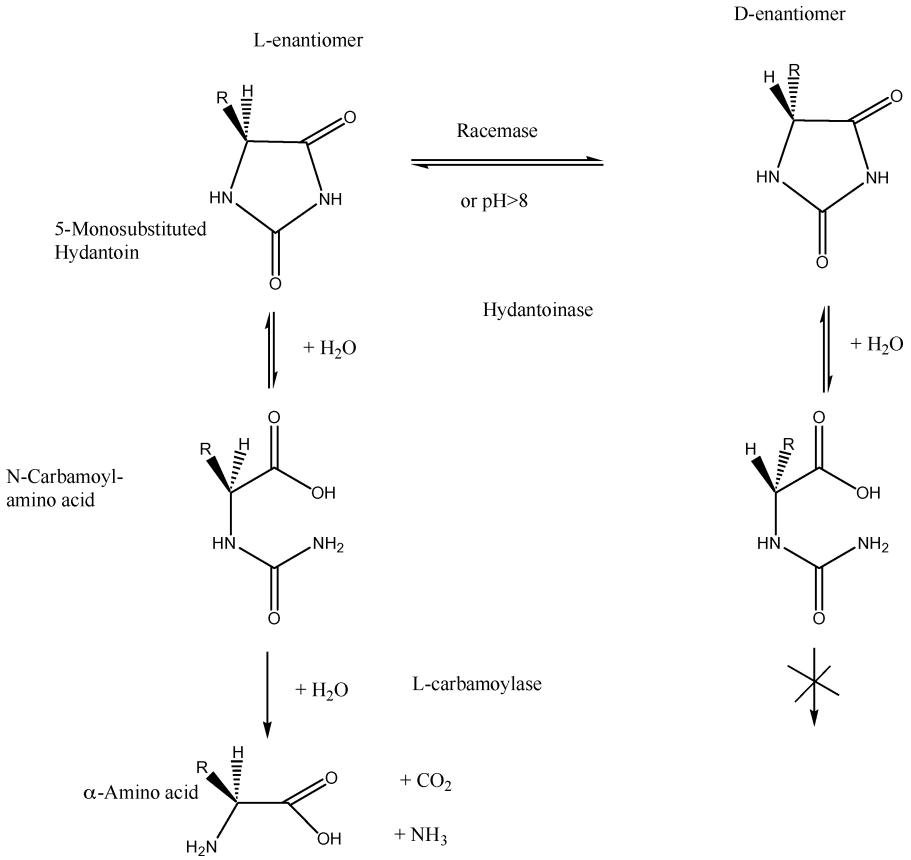

FIGURE 5 **Reactions and enzymes involved in the production L-amino acids from racemic hydantoins by the three-enzyme hydantoinase process (May *et al.*[78]).**

a significant improvement in the multienzyme pathway for the production of L-Met (Table 1).

A successful attempt to improve the thermal stability of prolyl endopeptidase, which specifically cleaves at proline, was made by Uchiyama *et al.*[130] An efficient expression system for the enzyme in *E. coli* was established using the prolyl endopeptidase gene from *Flavobacterium meningosepticum*. A method for screening thermostable variants was developed by combining heat treatment with active staining on membrane filters. An increase in thermostability was observed with each step of random mutagenesis by epPCR and screening, which was repeated three times. It was found that the mutant PEP-407 had a half-life of 42 minutes at 60°C, which was 60 times longer than that of the wild type enzyme. The thermostable mutant was also more stable with a high concentration of glycerol, which is a necessary condition for *in vitro* amidation.

Kim *et al.*[63] reported an appreciable increase in the thermostability of a maltogenic amylase produced from *Thermus* sp. strain IM6501 (ThMA). The half life of the mutagenic enzyme (ThMA-DM) was recorded as 172 minutes at 80°C, a temperature at which the wild type ThMA was completely inactivated in less than 1 minute. This was achieved by random mutagenesis using DNA shuffling. Four rounds of DNA shuffling and subsequent recombination of the mutations produced the highly thermostable mutant enzyme ThMA-DM, which had a total of seven individual mutations.

IVC has been used by Griffiths and Tawfik[42] for directed evolution of phosphotriesterase (PTE). This enzyme has been the focus of directed evolution due to its potential application for the degradation of organophosphate pesticides and nerve agents such as soman, sarin and VX (Table 1). Organophosphate degradation (OPD) genes encoding PTE were selected by their incubation, after biotinylation, with streptavidin-coated beads of diameter 1 μm, then coated with biotinylated anti-HA antibodies. Incubation of these microbeads in the cell-free translation mixture allowed the translation of genes and capture of the HA-tagged

protein by the anti-HA antibody. The microbead-displayed gene-protein complexes created in the first emulsion were re-compartmentalized in a second emulsion. Selection of enzymes was done by the hydrolysis of a paraoxon derived substrate, ethylated nitrophenol-caged biotin (EtNP-cgB) in which an ethyl group is replaced by a linker connected to caged-biotin (Figure 6). The emulsions were incubated for 16 hours to allow the hydrolysis of the substrate and then irradiated to yield the biotinylated product or substrate, which binds to the streptavidin-coated bead in the compartment. Thereafter the product-coated microbeads were sorted by flow cytometry. Kinetic characterization of selected mutants revealed k_{cat} 1.4×10^5 s^{-1} being 63 times higher than wild type. A study of 3D structure of isolated mutants showed that at position 132, the wild type residue isoleucine was present in two clones with very low activity. At the same position, i.e. 132, the wild type residue phenylalanine was the second most common residue after leucine. Although the substrate binding site of PTE is reported to be hydrophobic, a potential hydrogen bond between $N^{\varepsilon 1}$ of Trp 131 and the phosphoryl oxygen of the substrate was identified, which prevailed in the more active clones.[42]

Another enzyme that has been modified by laboratory evolution and is used for cephalosporin synthesis is *p*-nitrobenzyl (*p*NB) esterase. Cephalosporins are one of the most important antibiotics used, but during the synthesis *p*-nitrobenzyl alcohol (*p*NB-OH) is used to protect carboxylic acid functionalities. Deprotection usually involves catalytic zinc in organic solvents. However, this generates large amounts of solvent and zinc in waste material, causing serious environmental pollution. The *p*NB esterase from *Bacillus subtilis* was reported as an alternative to this problem as it can be used for hydrolysis of *p*NB-protected cephalosporins. First, second, third, and fourth generation antibiotics were synthesized using *p*NB-ester. The most stable fourth generation antibiotic loracarbef was shown to be synthesized using *p*NB esterase enzyme, which enables its catalytic deprotection. However, the substrates used are only sparingly soluble in water and the presence of even small

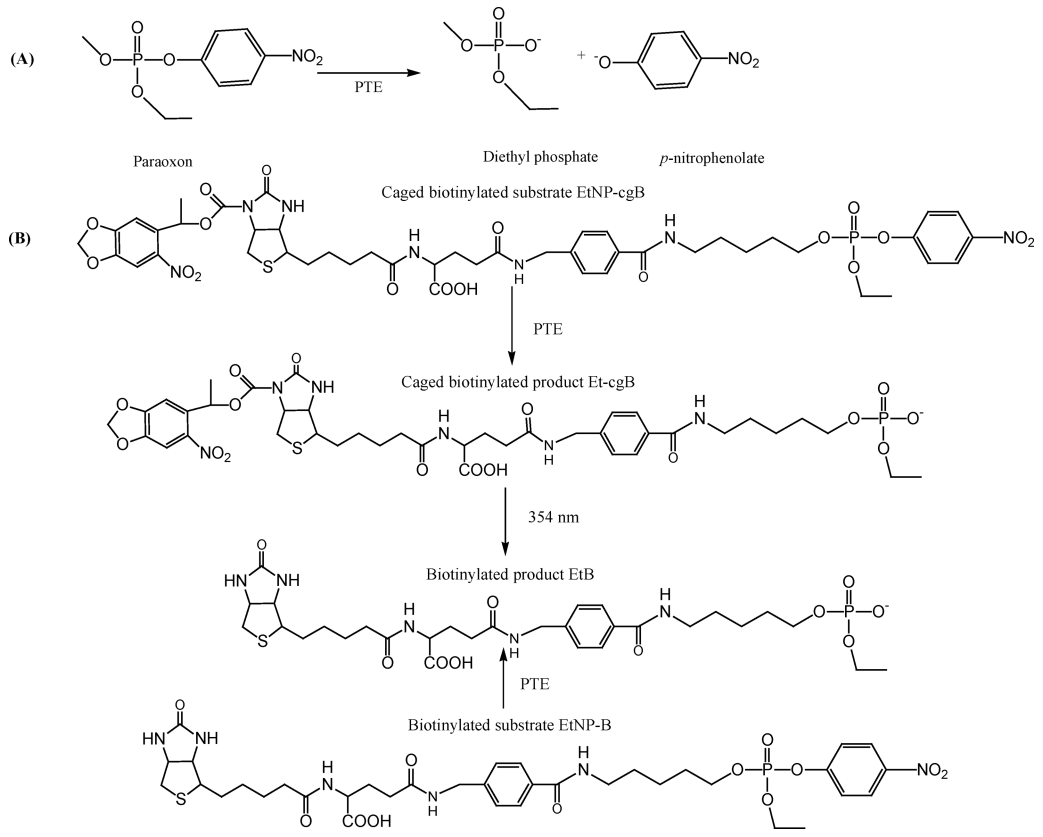

FIGURE 6 **PTE substrates. (A) PTE catalysed hydrolysis of paraoxon. (B) PTE catalysed hydrolysis of the modified substrate EtNP-cgB (paraoxon modified by substituting an ethyl group with a linker connected to caged-biotin). Hydrolysis resulted in *p*-nitrophenol and corresponding phosphodiester Et-cgB. Irradiation at 354 nm released the caging group and carbon dioxide to yield the (uncaged) biotinylated substrate (EtNP-B) or product (Et-B) (Griffiths and Tawfik[42]).**

quantities of water-miscible organic solvents greatly decrease the enzyme's catalytic efficiency. Hence, *p*NB esterase was subjected to directed evolution to enhance its activity in organic solvents.[83] The screening was not easy, using LCN-*p*NB as substrate since the reactant and products do not absorb in the visible region and their UV spectra are very similar. *p*-Nitrophenyl acetate (*p*NPA) enabled the screening of the *p*NB esterase variants as the resonance structures of the nitrophenol product gave a characteristic yellow color, but this substrate was sterically and chemically different from LCN-*p*NB (Figure 7). Therefore, a new substrate was designed to include *p*NP chromophore and loracarbef nucleus. The *p*NB esterase gene was subjected to four rounds of epPCR resulting in a variant that showed activity in 30% dimethylformamide (DMF), yielding a 16-fold increase in the esterase activity. To further enhance the activity, the variant was subjected to one recombination step, causing a 50–60 fold increase in total activity.[83]

Due to the extensive release of toxic and non-degradable compounds such as various recalcitrants and xenobiotics, research is being directed to the isolation of microbes capable of mineralizing these compounds. One such compound is 1,2,3-trichloropropane (TCP), a toxic synthetic chlorinated hydrocarbon used as a chemical intermediate in organic synthesis, as a solvent and as an extractive agent. Major release of this compound in the environment is as a by-product from the manufacture of epichlorohydrin and from the soil fumigant D-D, a mixture of 1,3-dichloropropene and 1,2-dichloropropane, which contains TCP as a contaminant. A possible pathway leading to the bacterial degradation of TCP involves haloalkane dehalogenases. These catalyze the hydrolytic removal of chlorine from TCP to yield 2,3-dichloro-1-propanol which is a good

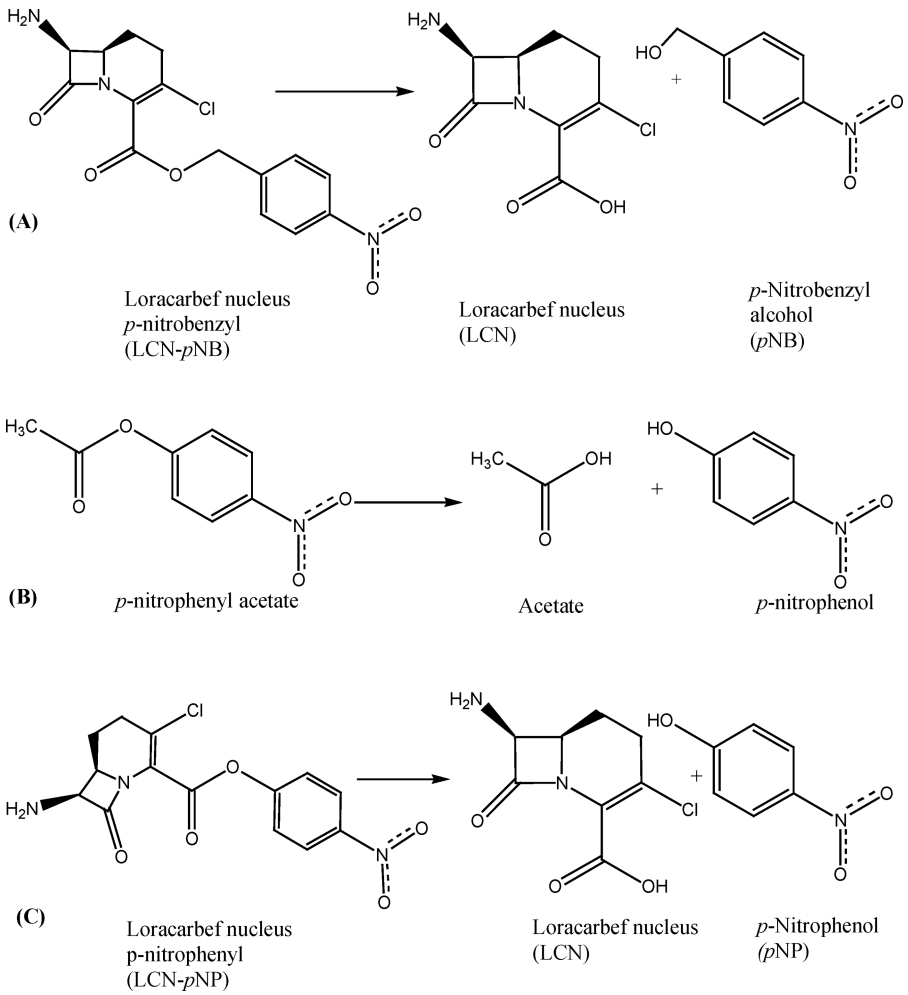

FIGURE 7 **Substrates and products of reactions catalyzed by *p*NB esterases. (A) Hydrolysis of *para*-nitrobenzyl loracarbef nucleus. Screening reaction: (B) esterase catalyzed hydrolysis of *p*-nitrophenyl acetate, (C) hydrolysis of *p*-nitrophenyl loracarbef nucleus (Moore and Arnold[83]).**

growth substrate for various gram negative bacteria. The haloalkane dehalogenase (DhaA) from *Rhodococcus* sp. m15-3 acts on TCP to produce 2,3-dichloro-1-propanol (DCP) releasing halide ion. The heterologous expression of *dhaA* gene in DCP utilizing bacterium *Agrobacterium radiobacter* strain AD1 would yield a productive degradation pathway (Figure 8). Hence, the *dhaA* gene was targeted for directed evolution to overcome its various shortcomings. The activity of DhaA on TCP is too low to provide sufficient energy for biosynthetic processes and bacterial growth. In addition, due to slow catabolism, cells are exposed longer to the toxic effects of TCP, which arrests growth. Randomly mutated *dhaA* libraries were generated by random mutagenesis (DNA shuffling/epPCR) and screened by a plate screening assay based on the indicator eosin methylene blue (EMB plates). After two rounds of mutation, best variant M2 was selected due to its highly colored red colony as compared to the wild type and blue colored colonies of *dhaA* negative clones. M2 exhibited more than 30% improved activity on 10 mM TCP. It was observed that M2 was 8 fold more efficient on TCP due to the double mutation, Cys176Tyr and Tyr273Phe. $DhaA_f$, a homologue of DhaA, showed mutations at similar positions. This enzyme was found in 1,2-dibromoethane (DBE) utilizing bacteria *Mycobacterium* sp. in strain GP1. Position 273 showed an identical substitution, whereas the cysteine residue at position 176 was replaced by a phenylalanine side chain. The structural models gave an insight into the molecular basis for the enhanced dehalogenase activity. The cap domain of this protein partially defines the active site cavity and contributes to the structural basis for differences in substrate specificity between haloalkane dehalogenases. Replacement of Cys176, being located in this domain, by a larger tyrosine side chain resulted in a 2-fold reduction in the volume of the active-site cavity, which may push small substrate molecules closer to catalytic residues and cause a better average positioning of the Cα of the substrate for nucleophilic attack, explaining improved activity of the evolved enzyme on both DBE and TCP. In addition, the Tyr 176 side chain shields the bulk water from the active site generating a favourable hydrophobic microenvironment necessary for the enzyme's reaction. The second substituted residue, Tyr 273, was found to be located in the main domain adjacent to His 272, the base catalyst of the catalytic triad. The variants were further analyzed on the basis of their interactions with the substrate through computer docking. It was found that the DBE binds in the *gauche* conformation with a distance between the nucleophilic Asp106-$O_{\delta 2}$ and the Cα atom of DBE of 3.5Å, which is similar in both the wild type and the variant. This conformation is suitable for S_N2 displacement of bromide by nucleophilic attack. Studies with TCP showed that in M2 Cα atom of TCP was shifted to a position that resembles that of the Cα atom of DBE in the wild type DhaA, i.e. with shorter distance between the Cα atom of TCP and the nucleophilic aspartate, which makes it more susceptible to a reaction. The direct interaction of the aromatic ring of Tyr 176 and chlorine atom on Cγ of TCP resulted in this type of binding mode.[13]

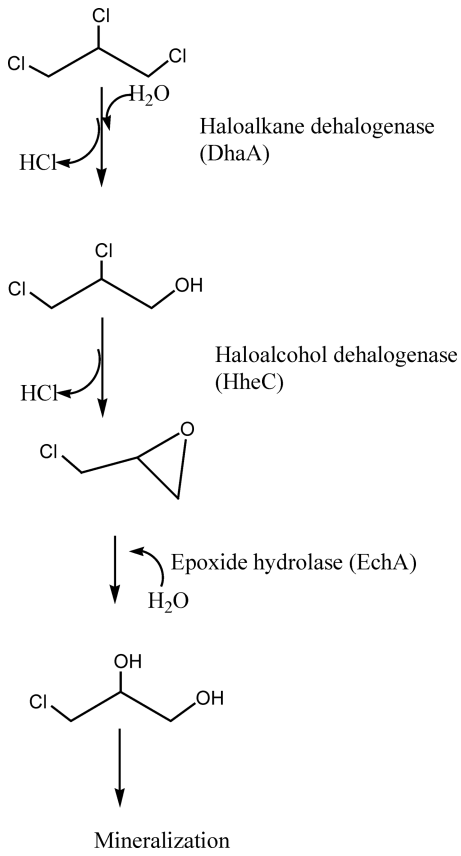

FIGURE 8 **Reactions and enzymes involved in the proposed degradation pathway of TCP for *A. radiobacter* AD1(pTB3) (Bosma *et al.*[13]).**

Epoxide hydrolases belong to the hydrolase class of enzymes as they hydrolyze an epoxide corresponding to the vicinal diol by addition of water. Epoxide hydrolases play an important role in the degradation of certain xenobiotics. The oxirane moiety of epoxides is electrophilically reactive and forms adducts with various cellular components including

DNA, RNA and proteins. Such epoxides are also produced during the aerobic degradation of chlorinated ethenes, a major pollutant. In mammalian systems glutathione (GSH) S-transferases in conjugation with epoxide hydrolase carry out conversion of harmful epoxides into less active diols. For biodegradation of chlorinated ethenes such as trichloroethylenes (TCE) and *cis*-1,2-dichloroethylene (*cis*-DCE), epoxide hydrolase is required in the degradation of primary intermediate, chlorinated epoxyethanes, which have several detrimental effects ranging from covalent modification of cellular components, inactivation of enzymes, even to cell death. Toluene *ortho*-monooxygenase (TOM) has been reported to be evolved *in vitro*. It can carry out the detoxification of TCE, *cis*-DCE, trans-1,2-dichloroethylene and it has been named as TOM-green. TOM-green was evolved using DNA shuffling of nonhememonooxygenase.[17] Hence, Rui *et al.*[111] exploited the activity of these two enzymes to enhance the aerobic mineralization of *cis*-1,2-dichloroethylene. Saturation mutagenesis of *Agrobacterium radiobacter* AD1 (EchA) was used to achieve the desired mutation at particular sites, which were chosen because of their vicinity to the catalytic triad residues. The variant of EchA was screened for its activity by co-expressing it with TOM-Green for enhanced *cis*-DCE degradation. A 10-fold enhancement in *cis*-DCE mineralization was observed on whole cell screening in high concentrations (540 μM) of *cis*-DCE. In addition this variant also showed enhanced activity for 1,2-epoxyhexane and natural substrate epichlorohydrin. A novel glutathione S-transferase from *Rhodococcus* sp. strain AD45 having activity toward *cis*-1,2-dichloroepoxyethane was also coexpressed with TOM-Green, in *E. coli*. Significant detoxification of reactive epoxide intermediates from *cis*-DCE, *trans*-1,2-dichloroethylene and TCE was also observed (Figure 9).[112]

Lyases

Lyases are enzymes cleaving C–C, C–O, C–N and other bonds by elimination, leaving double bonds or conversely adding groups to double bonds. Various subclasses of lyases include peridoxal phosphate enzymes that catalyze the elimination of β- or γ-substituent from a α-amino acid followed by a replacement of this substituent by some other group. In the overall replacement reaction, no unsaturated end product is formed; therefore, these enzymes might formally be classified as alkyl transferases.

Aldolases are carbon bond forming enzymes that have proved to be useful tools for an organic chemist, though their utility is limited due to their narrow substrate utilization. Hence, Wymer *et al.*[140] have reportedly modified the substrate specificity of *E. coli* 2 keto-3-deoxy-6-phosphogluconate (KDPG) aldolase through

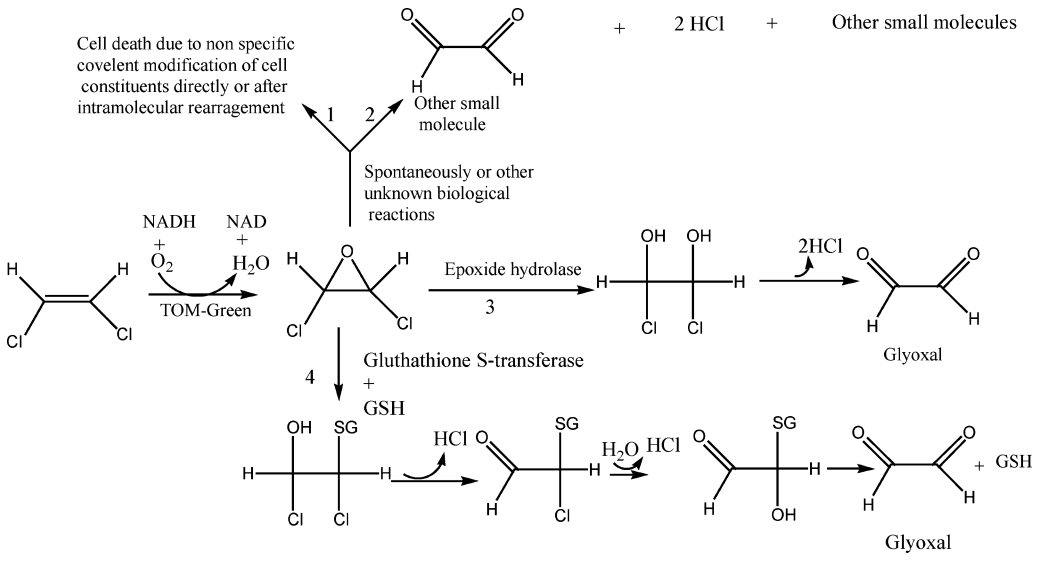

FIGURE 9 **Metabolic engineering done to enhance *cis*-DCE mineralization by cloning an evolved epoxide hydrolase along with an evolved toluene *ortho*-monooxygenase (TOM-Green). Steps 1 and 2 are the two possible spontaneous transformation pathways for *cis*-DCE epoxide. Steps 3 and 4 represent two major detoxification strategies through which *cis*-DCE epoxide may be biologically converted by either an epoxide hydrolase or glutathione S-transferase (IsolLR1) (Rui *et al.*[111]).**

directed evolution. The double mutant created was identified to have an alteration at the position of the active site lysine from one β-strand to a neighboring strand. The evolved enzyme was found to be different from existing aldolases with respect to the location of its active site to secondary structure. Another work done on the same enzyme was by Fong *et al.*[36] As the wild type enzyme is highly phosphate- and D-sugar-dependent, the KDPG aldolase was evolved to improve its catalytic efficiency, altered substrate specificity and stereoselectivity. This experiment enabled the evolved KDPG aldolase to accept both D- and L-glyceraldehyde in non-phosphorylated form as substrates and pyruvate for reversible aldol reaction.

Hao and Berry[46] improved fructose 1,6-bisphosphate (FBP) aldolase with respect to tolerance at high temperatures and organic solvents through directed evolution (Table 1). Genes of *E. coli* class II FBP-aldolase and *Edwardsiella ictaluri* class II FBP-aldolase were randomly mutagenized by family shuffling. After four rounds of mutagenesis, the T_{50} (temperature at which 50% of initial enzyme activity is lost on incubation for 10 minutes) was increased more than 11°C as compared to the wild type *E. coli* FBP-aldolase and over 12°C than *Ed. ictaluri* FBP aldolase. In addition the half-lives of the fourth generation mutant were increased by 190-fold (1490 min) compared with *E. coli* FBP aldolase (7.7 min) and 360-fold compared with *Ed. ictaluri* FBP aldolase (4.1 min). It has been found that thermostability and tolerance to organic solvents are often related. Hence, the aldolase variants were tested for resistance to inactivation by organic solvents using hydrazine-based assays involving 20% (v/v) of three polar organic solvents [methanol, acetonitrile and N,N-dimethylformamide (DMF)] and three non-polar organic solvents (toluene, chloroform and hexane). The mutants showed considerable activity in all solvents as compared to the wild type enzyme, although the level of activity in DMF was very low for all variants. Seven mutations V45A, K71I, A111T, A124V, A210V, M321K and K346R were observed to be responsible for the 9.5°C increase in T_{50} value between the first and fourth generation variants. Mapping these mutations on the 3-D structure of wild type *E. coli* enzyme revealed that four of them were in α-helices and the remaining three in the loops. No mutation was found in any of the β-strands in catalysis in $(\beta/\alpha)_8$ barrel enzymes. All mutations were at least 11Å from the nearest active site zinc atom and five of the seven residues were significantly exposed on the surface of the enzyme dimer. Only one position, 71, was directly involved in the dimer interface. The surface mutations can be easily accommodated in the protein structure than buried mutations, which are unlikely to increase stability without compensating changes elsewhere. Study of thermostability mechanisms showed that removal of methionine residues could be a possible reason for thermostability, as these residues can undergo thermally induced modifications, which can lead to aggregation, denaturation and/or loss of enzyme activity. In addition, the lysine residue 346, which lies in α-11 helix, was replaced with arginine. This mutation has been reported to generate thermostable proteins as the longer side chain of arginine can interact with more distant amino acids, without the requirement for a bridging solvent molecule and without affecting overall charge and the resonance capacity of the guanidinium moiety. This favors formation of salt bridges and hydrogen bonding with more than one residue.[46]

Aldolases are a key target for modification of reactivity since they have considerable potential as tools for the synthesis of stereochemically complex carbohydrates. An interesting approach was tested on tagatose-1,6-bisphosphate aldolase (TBPA) with the aim of altering the stereochemical course of the bond forming step, instead of evolving the enzyme to accept an unnatural stereoisomer as substrate. Fructose 1,6-bisphosphate aldolase (FBPA) catalyses the condensation of the same two reactants recognized by TBPA, i.e. dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P) with a similar reaction mechanism but a completely inverted stereochemical attack (the DHAP enediolate intermediate attacks G3P on the *Si*-face in FBPA and on the *Re*-face in TBPA generating products that only differ in C4 stereochemistry (Figure 10). Three rounds of DNA shuffling were applied and thereafter screening yielded an evolved aldolase that showed an 80-fold improvement in k_{cat}/K_m toward the non-natural substrate fructose 1,6-bisphosphate, resulting in a 100-fold change in stereospecificity (Table 1). ^{31}P-NMR spectroscopy was used to show that, in the synthetic direction, the evolved aldolase catalyzes the formation of carbon-carbon bonds with unnatural diastereoselectivity, where the $>99{:}<1$ preference for the formation of tagatose 1,6-bisphosphate was switched to a 4:1 preference for the diastereoisomer, fructose 1,6-bisphosphate. The implications are relevant for complex synthetic chemistry, since this demonstrated the possibility of developing new biocatalysts

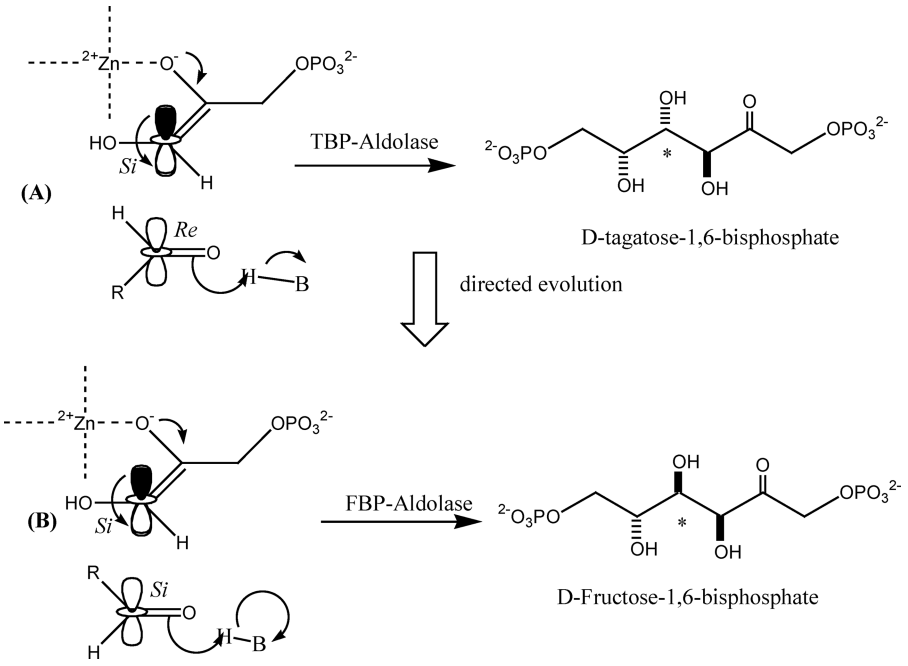

FIGURE 10 **Stereochemistry of the reaction catalyzed by aldolases. (*A*) The mechanism of TBP aldolase. The DHAP ene-diolate is formed after abstraction of the 1-*proS* proton from DHAP and polarization by the catalytic zinc cation. Attack of the activated DHAP C1 from its *Si* face onto the G3P C1 *Re* face generates the 3*S*, 4*S* product tagatose 1,6-bisphosphate, and proton donation by H-B (Asp-82) converts the C4 carbonyl to a hydroxyl group, completing TBP synthesis. (*B*) The mechanism of FBP aldolase. The DHAP ene-diolate is formed after abstraction of the 1-*proS* proton from DHAP by Glu-182 and polarization by the catalytic zinc. Attack of the activated DHAP C1 from its *Si* face onto the G3P C1 *Si* face and proton donation by H-B (Asp-109) convert the C4 carbonyl to a hydroxyl group, completing the synthesis of the 3*S*, 4*R* product fructose 1,6-bisphosphate. FBP and TBP are epimeric at C4, and this position is marked with an asterisk. R = $CH(OH)CH_2OPO_3^{2-}$ (Williams *et al.*[136]).**

that are able to generate rare stereoisomers of certain natural products obtained, in turn, by other wild type enzymes.[136]

Pectin degrading enzymes have potential applications in the textile industry. It has been found that these enzymes are valuable candidates for industrial usage because they can reduce the number of toxic alkaline chemicals being currently used in the textile industry. Keeping this potential application in mind Solbak *et al.*[121] used high throughput screening to screen complex environmental DNA libraries and, consequently, obtained more than 40 novel microbial pectate lyases. Several enzymes were found that possessed pH optima and specific activities on pectic material in cotton fibers, compatible with their use in the scouring process. However, none exhibited the desired temperature characteristics. Hence, to further improve properties, a candidate enzyme was selected for molecular evolution and 36 single site mutants exhibiting improved thermotolerance were produced using gene site saturation mutagenesis, a trademark technology. After another round of mutation, nineteen variants with improved thermotolerance were identified and tested for both thermotolerance and performance in the bioscouring application. The optimal temperature of the evolved enzymes was 70°C, which was 20°C higher than the wild type. It was found that the best performing variant contained eight mutations, and a melting temperature 16°C higher than the wild type enzyme, while retaining specific activity at 50°C. The evaluation of these mutant enzymes in bioscouring revealed that they are significantly better than the chemical scouring process and hold potential to replace it industrially.

Lingen *et al.*[74] altered the substrate specificity of a thiamine diphosphate (ThDP)-dependent benzoylformate decarboxylase (BFD) by epPCR. Screening identified two mutant enzymes, L476Q and M365L-L461S, which were able to accept *ortho*-substituted benzaldehyde derivatives as donor substrates leading to formation of 2-hydroxy ketones. Both of these mutants were able to selectively catalyze the formation of enantiopure (S)-2-hydroxy-1-(2-methylphenyl) propan-1-one, which cannot be efficiently carried out by the wild type enzyme. Different *ortho*-substituted benzaldehyde derivatives, such as 2-chloro-, 2-methoxy- or 2-bromobenzaldehyde, were accepted as donor substrates

by both the variants and conversion with acetaldehyde resulted in the corresponding (S)-2-hydroxy-1-phenyl-propan-1-one derivative. A 3D structure analysis revealed that the reduction of the side chain at position L461 resulted in an enlarged substrate binding site and facilitated the initial binding of *ortho*-substituted benzaldehyde derivatives to the cofactor ThDP.

Similarly, directed evolution applied to l-aspartase through four rounds of epPCR and three rounds of DNA shuffling resulted in a 28-fold increased k_{cat}/K_m and 4.6-fold decreased K_m, while the thermostability and pH range were also enhanced. Seven base changes resulted in three amino acid changes, namely N217K, T233R and V367G, which caused the enhancement of properties.[134]

Xylanase is yet another enzyme that falls under the category of enzymes-lyase. Recently, a combination of directed evolution techniques, i.e. random point mutagenesis, saturation mutagenesis and DNA shuffling, were used to generate a thermostable variant, Xylst of glycosyl hydrolase family-11 xylanase from *Bacillus subtilis*. The half-inactivation temperature for the Xylst variant compared to the wild type enzyme after incubation for 10 minutes elevated from 58°C to 68°C. Study of the three-dimensional structure revealed that increased hydrophobicity around Cys 179 was responsible for the enzyme's stability.[82]

Isomerases

These enzymes catalyze geometric or structural changes within one molecule. According to the type of isomerases, they may be called racemases, epimerases, *cis-trans*-isomerases, tautomerases, mutases, etc. At times the interconversion in the substrate is brought about by an intramolecular oxidoreductase. Since hydrogen donor and acceptor are the same molecule and no oxidized product appears, they are not classified as oxidoreductases, even if they may contain firmly bound $NAD(P)^+$. Directed evolution has been applied keeping all types of isomerases in mind.

Almost all known $(\beta\alpha)_8$-barrel proteins are enzymes and are capable of catalyzing diverse reactions. Hence, this scaffold is commonly used for engineering proteins through directed evolution. The most important step in the whole algorithm of *in vitro* evolution is the screening or selection step, which allows the identification of the variants possessing the desired property. However, the compatibility of $(\beta\alpha)_8$-barrels with *in vitro* selection methods has not been greatly explored. Recently, an efficient plasmid display technique has been formulated by Patrick *et al.*[100] Phosphoribosylanthranilate isomerase (PRAI) was engineered which carried a FLAG epitope in the active site forming loop 6. This yielded a highly sensitive method of selection which enabled the selection of FLAG-PRAI variants from 10^6 fold excess of FLAG-negative competitor proteins in three rounds of *in vitro* selection simply by binding of monoclonal antibody (mAb) M2 (a mAb to FLAG) to the epitope. Further, these epitope residues were randomized and libraries were created containing approximately 10^7 clones. Four rounds of selection for Ab binding identified and enriched a variant in which a single nucleotide insertion produced a truncated $(\beta\alpha)_8$ barrel consisting of $(\beta\alpha)_{1-5}$ $(\beta)_6$. It was observed in the clone (trPRAI) that there was a 21-fold increase in the mAb M2 affinity as compared to full length FLAG-PRAI. This truncated barrel was also found to be soluble, structured, thermostable and monomeric, indicating that it represented a genuine subdomain of PRAI. Another $(\beta\alpha)_8$ barrel enzyme is N-(5′-phosphoribosyl)-anthranilate isomerase Trp1p from *S. cerevisiae*. It has recently been reported that directed evolution has been used to transform the protein into a split-Trp protein sensor, which can be used for the analysis of protein interactions in the cytosol as well as the membrane. The screening was carried out by the growth of trp1 cells on medium lacking tryptophan and the generated split-trp protein sensor has a potential as a new tool for high-throughput interaction screening.

The patchwork model of enzyme evolution postulates that ancestral enzymes were relatively unspecific and therefore were capable of catalyzing chemically similar reactions in different metabolic pathways. Genes encoding these enzymes would have duplicated in the course of evolution and would have subsequently specialized by diversification. A way to support the common ancestry of two enzymes from an ancestral enzyme of broader substrate specificity is through directed evolution, if the substrate specificity of the two enzymes from different metabolic pathways can be interconverted. This has been observed in two enzymes N′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) isomerase (HisA) and phosphoribosylanthranilate (PRA) isomerase (TrpF), which catalyze similar reactions in the biosynthesis of the amino acids histidine and

tryptophan. Both of them catalyze an Amadori rearrangement, which is the irreversible isomerization of an amino aldolase to an amino ketose (Figure 11). Also, both the enzymes belong to the same enzyme family of $(\beta\alpha)_8$-barrels consisting of eight repeating units of $\beta\alpha$ modules; even though they lack any amino acid similarity, they belong to this most frequent fold among single domain proteins. As already described above, to support the hypothesis that HisA and TrpF may have evolved from one common ancestral enzyme with low substrate specificity, Jurgens *et al.*[62] used random mutagenesis and selection *in vivo* to generate HisA variants that can catalyze the TrpF reaction as well, both *in vivo* and *in vitro*. The stable HisA protein from *Thermotoga maritima* (tHisA) was subjected to DNA shuffling and variants selected by genetic complementation of an *E. coli* strain that lacked trpF gene on its chromosome, thereafter plating the transformants on selective medium lacking tryptophan. Three mutants were isolated, though the sequencing revealed that two of them were identical. Steady state kinetic studies revealed K_m^{PRA} values of the mutant enzymes were several orders of magnitude larger than that of tTrpF, whereas their k_{cat} values were at least 10% that of tTrpF and one of the variants retained significant HisA activity (Table 1).

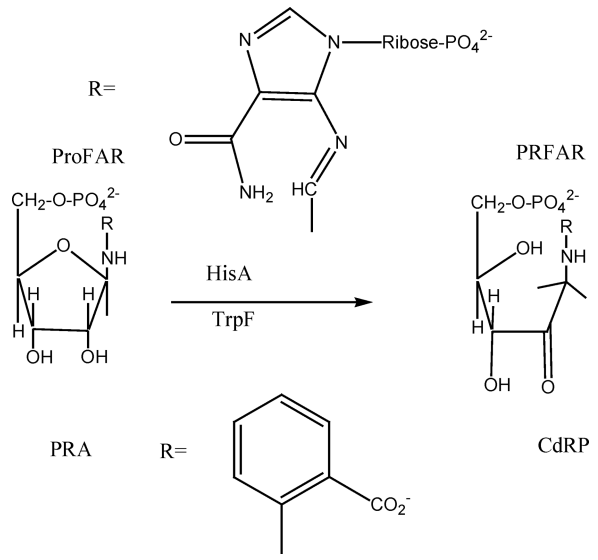

FIGURE 11 **HisA and TrpF catalyze similar reactions in histidine and tryptophan biosynthesis. HisA and TrpF catalyze the isomerizations of the aminoaldoses *N*′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) and phosphoribosylanthranilate (PRA) to the aminoketoses *N*′-[(5′-phosphoribulosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (PRFAR) and 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate (CdRP) (Jurgens *et al.*[62]).**

Xylose isomerase (XI) is an intracellular enzyme found in bacteria that can utilize xylose as a carbon source for growth. The xylose isomerases used industrially are obtained from mesophilic organisms (e.g., *Streptomyces* sp. and *Actinoplanes* sp.), though they suffer from a limitation that the reaction temperature has to be limited to 60°C because of by-product and color formation that occurs at high temperature and alkaline pH and also the isomerases themselves are not highly thermostable. The industry has a great demand for thermostable XIs, as they would allow faster reaction rates, higher fructose concentration at equilibrium, higher process stability, decreased viscosity of substrate and product streams, as well as reduced by-product formation. In an effort to improve the wild type XI the valine residue at 185 position was substituted by threonine and the mutant obtained TNXI V185T was more active, more glucose efficient and highly thermostable at 97°C, but it suffered from a serious drawback as it was poorly active (10% of maximal activity) at 60°C, which is the current industrial isomerization temperature and requires a neutral pH for optimal activity.[122] Therefore, the mutant TNXI V185T was subjected to directed evolution for improvement in its activity at low temperature and low pH (Table 1) and epPCR was carried out using the TNXI V185T gene as a template and yielding an average of one or two mutations per gene. Variant 3A2 was identified in screening to be more active at 60°C and at pH5.2 and thereafter mutagenized by the second round of epPCR. Screening revealed a second mutant 1F1 having 80% and 40% increases in activity on glucose at 80°C (pH5.2) and 60°C (pH7.0), respectively. In addition, 3-fold higher activities were obtained at 90°C for 1F1 and 95°C for 3A2. Activation energy, calculated using the equation $A = A_o\, e^{-Ea/RT}$, observed for TNXI was 87 kJ/mol though 3A2 and 1F1 showed reduced E_a (activation energy) as 57 and 44 kJ/Mol, respectively. These lower E_a values explain why 3A2 and IF1 are 7.3 and 12.3 times more active, respectively, than TNXI at 60°C, but only 4.2 and 4.8 times more active than TNXI at 90°C. These variants (3A2 and 1F1) also showed more activity at pH 5.5 than TNXI and TNXI V185T activity at pH 7.0. In addition the kinetic analysis revealed that a 5-fold increase in V_{max} for 1F1 resulted in 1.7-fold greater activity at 60°C (pH5.5) than TNXI V185T at 80°C (pH7). Thermal stability of the two mutants was determined by EDTA treatment of enzymes in 10mM MOPS (pH7) containing 0.5 mM $CoCl_2$. All inactivation data were best fit by a two exponential terms

equation of the form

$$y = C_1 e_1^{-kt} + C_2 e_2^{-kt} + C_3$$

where y is residual activity, t is time in minutes and C_1, C_2, C_3, k_1 and k_2 are constant parameters. At pH7, both 3A2 and 1F1 were kinetically more stable than TNXI and TNXI V185T. 1F1 was found to be most stable enzyme at pH 7.0 due to its lower inactivation rate constants k_1 and k_2, and higher residual activity than TNXI and TNXI V185T. The amino acid substitutions responsible for the change in property were identified through sequencing. Two mutations were identified in TNXI 1F1 in addition to V185T, namely C282P and F186S. Leu 282 is close to the inter-subunit interface of the enzyme tight dimer. The L282P caused a 35% decrease in activation energy for the activity of 3A2 on glucose; however the structure analysis revealed unfavorable van der Waals contacts between Pro 282's pyrrolidine ring and the local backbone structure. The L282P mutation occurred in regions whose stability is not limiting for the stability of the whole enzyme, as it was observed that the mutation resulted in a kink in helix α_7 of 3A2's $(\alpha/\beta)_8$ barrel and did not destabilize the enzyme at pH7.0. F186S is located in the back of the active site adjacent to Thr 185. This mutation created a cavity that increased mobility in the active site, resulting in dramatic improvement of 1F1's low temperature activity. Mutation F186S disturbed a potentially stabilizing four-residue network of aromatic interactions, but the mechanism underlying the increase in kinetic stability of 1F1 remained unknown.[123]

Ligase

Ligases are enzymes catalyzing the joining together of two molecules coupled with the hydrolysis of a pyrophosphate bond in ATP or a similar triphosphate. The bonds most often found are high energy ones. Directed evolution has been used to create enzymes capable of catalyzing such type of reactions.

Hager and Szostak[45] reported the isolation of novel ribozymes, which ligate adenosine 5-monophosphate (AMP) activated RNA substrates using directed evolution. RNA molecules (10^{15}) were subjected to ten rounds of *in-vitro* selection, including three rounds involving epPCR. The RNA molecules contained mutagenized adenosine triphosphate (ATP) aptamer flanked by long random sequences. The evolved ribozymes were selected on the basis of ligation of an oligonuleotide to the 5′-capped active pool RNA species. The selected ribozymes showed 0.4 ligations per hour, corresponding to rate acceleration of $\sim 5 \times 10^5$ over the templated. Three such isolated ribozymes catalyzed the formation of 3′-5′-phosphodiester bonds and were highly specific for activation by AMP at the ligation site. These ligases provide a promising starting point for the evolution of more efficient template-directed ligases or polymerases capable of sequence-independent recognition of substrate RNAs or monomers equipped with an AMP "handle". Similar attempts were made by Miyamoto *et al.*[81] to isolate evolved ligase ribozymes that accelerate the ligation reaction of an oligonucleotide under acidic condition. The ribozymes were selected after four rounds of randomized mutations. The best mutant isolated had a reaction rate of 8000 times more than the original (b1) ribozyme at pH 4. However, the reaction rate of evolved enzyme was reduced in the presence of 3′ primer, which indicates that the ribozyme 3′ region plays a role in the ligation reaction of the ribozyme. The 3′ primer sequence was complementary to the 3′ primer-binding site of the ligase ribozyme.

DNA polymerase I from *Thermus aquaticus* (*Taq*) has large importance in biotechnological applications and hence it has been a focus of modification by directed evolution. Ghadessy *et al.*[41] used a compartmentalized self-replication (CSR) method for the laboratory evolution of *Taq* polymerase for generic expansion of their substrate specificity (Table 1). Two best variants M1 and M4 were identified that could extend A.G (primer.template) and C.C mismatches, also including other strongly disfavored mismatches such as G.A, A.A and G.G. They showed 427-fold and 82-fold improved efficiency to extend C.C 3′ mispair as compared to wild type *Taq*. In addition it was found that the processivity of M1 and M4 was equal to (or higher than) wild type *Taq* even in presence of blocking agents such as an abasic site, *cis-syn* cyclobutane pyrimidine dimer (CPD) and 5-nitroindol (5NI), that are responsible for termination of DNA replication by wild type *Taq*. One of the advantages of using a mutated version of *Taq*, i.e. M1, is that it does not require the addition of a proofreading polymerase as with wild type *Taq*. It has the ability to extend mismatches itself, as well as amplify targets greater than (>) 25 kb. It was also shown that M1 was capable of extending DNA fragments containing non-cognate nucleotide substrates such as 7-deaza-dGTP and phosphorothioates (αS dNTPs), which arrest

the wild type *Taq* activity. An interesting application of these polymerases could be the generation of highly fluorescent microarray or *in situ* hybridization (FISH) probes, expansion of the chemical repertoire of deoxyribozymes. Based on their ability to cause replication of DNA bearing dye labeled nucleotides, M1 was able to amplify DNA targets of 0.4 kb labeled with fluorescein (FITC)-12-dATP or with much reduced efficiency using Rho-5-dUTP and DNA fragments up to 2.5 kb in length with Bio-16-dUTP. The reason for the promiscuous activity of M1 and M4 was reported to be a more open active site compared to the wild type *Taq* polymerase. Due to an open active site, it leads to a more relaxed geometric selection, which enables it to extend 3′ mismatches and incorporate as well as replicate a diverse collection of unnatural substrates. A relaxation of steric constraints extending to the DNA duplex binding region may also be responsible for processive replication of DNA that is fully or partially substituated with αS and biotin- or dye-labeled bases as the local diameter of the DNA duplex at the place of substitution increases. The improved ability of M1 to use the unnatural nucleobase 5NI, thus may also have indicated decreased dependence on minor-groove interactions. 5NI is comparable in size to a purine base and favors the anti position as observed by NMR. A 5NI.A base-pair would therefore resemble purine-purine mismatches, which are efficiently extended by M1. However, 5NI is also devoid of any hydrogen-bonding potential. Elegant experiments using isosteric non-hydrogen bonding base analogs have shown that lack of Watson-Crick hydrogen bonding *per se* does not preclude efficient insertion or extension. Apart from the other applications mentioned above these polymerases can also be used as a tool in mutagenesis and DNA shuffling as the strong bias of *Taq* towards transition mutations restricts the sequence space that can be accessed effectively using PCR mutagenesis.[41]

Topoisomerases are enzymes that act on the topology of DNA by cleaving and thereafter resealing the phosphodiester backbone of DNA in two successive transesterification reactions. These enzymes have been classified into type I and type II DNA topoisomerases. Topoisomerase I (Top I) is an essential enzyme in multicellular organisms, though it's not necessarily found in yeast. Top I bind, cleave and open transient gates in supercoiled DNA, in order to allow the passage of another single or double-stranded DNA segment. Chemical agents that are able to interfere with DNA Top I are widespread in nature and some of them have outstanding efficacy in human cancer. One such anti-cancer drug is compotothecin (CPT), which hinders the rejoining reaction, thus leading to the stabilization of the DNA-Top I complex in which the cleaved DNA strand is covalently linked to a tyrosine residue. The DNA replication is arrested by the generation of irreversible double-stranded DNA cuts and causes cell death. The laboratory evolution of human Top I expressed in yeast was reported by Scaldaferro *et al.*[117] to increase its CPT sensitivity. As yeast cells lacking the *top I* gene are viable and insensitive to CPT, a *S. cerevisiae* strain was used for expression of mutagenized human *top I*. After three rounds of DNA shuffling, 10- to 25-fold increased sensitivity to CPT was observed in the variants, as compared to the wild type *top I*. To observe the amino acid mutations of the evolved *top I*s, the cDNAs of twelve selected *top I* were fully sequenced. They found 28 base mutations (25 transitions and 3 transversions) and no base deletions or insertions. The linker showed more mutations than the C-terminal domain due to its high conservation. D677G, V703I and I728T were found to be responsible for the enhanced CPT sensitivity in the *top I* variants.

Lee *et al.*[67] reported the directed evolution of farnesyl diphosphate (FPP, C15) synthase (IspA) of *E. coli*. The enzyme was modified by epPCR and selected through a color complementation screen. The variants showing enhanced C40 carotenoid precursor geranyl geranyl diphosphate (GGPP, C20) were selected using C40 carotenoid pathway enzymes. Structural analysis of 12 mutants showed mutations in the conserved regions of prenyl diphosphate synthases. Certain mutations were found near or before the conserved first aspartate rich motif (FARM), which is believed to be involved in the mechanism for chain elongation reaction of all prenyl synthases. Therefore, study of the mutations revealed the structural determinants of chain length specificity, which also included substitutions of the 1st and 9th amino acids upstream of the FARM. In addition, a mutation on a helix adjacent to the FARM within the substrate-binding pocket (D115G) displayed a novel mechanism for chain length determination. One mutant carried a substitution of C155G at the second amino acid upstream of another conserved region, which was found necessary in controlling the chain elongation of a Type III GGPP synthase.

Directed evolution has been so far used mostly for bacterial enzymes; however, Zhou *et al.*[151] used a

laboratory evolution strategy to identify glyphosate-resistant mutants of rice 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS). EPSPS is the key enzyme in the shikimate pathway, which is targeted by the wide-spectrum herbicide glyphosate. The rice (*Oryza sativa*) EPSPS gene was subjected to epPCR and selection was done on minimal medium by functional complementation of the *E. coli* strain, AB2829, in which the mutagenized EPSPS was introduced. A 3-fold increase in glyphosate resistance of *E. coli*, expressing one of the enzyme variants, was observed. This variant also showed a 70-fold and 4.6-fold decrease in the affinity for glyphosate and phosphoenolpyruvate, respectively, compared to wild-type EPSPS.[151]

Directed evolution can also be used to create a novel enzymatic function, which is a challenge from a theoretical perspective. Xia *et al.*[141] devised an activity-based selection method to evolve DNA polymerase activity. Three mutants were isolated to be efficient catalysts towards polymerization using triphosphate ribonucleotides (rNTP) at rates compared to those measured on the wild type using dNTPs. The variants were screened by displaying the Stoffel fragment (SF) of the *Thermus aquaticus* DNA polymerase on a filamentous phage. The mutants were selected on the basis of their ability to incorporate rNTP and biotinylated UTP (assayed on a DNA template also anchored to the same phage surface) via recovery with streptavidin coated beads. The mutants isolated after four rounds of screening contained mutations at nine different sites (Table 1).[141]

CONCLUSIONS

The use of directed evolution as a strategy for engineering novel enzymes and catabolic pathways has yet to reach its full potential. There are certain challenges for future directed evolution that include developing workable strategies for evolution of new catalytic functions evolving biosynthetic or degradative pathways, evolving single and new enzyme pathways for large scale chemical production and evolving enzymes that are difficult to handle in terms of functional expression, stability and assay development. There is a need to clearly understand what one means by a "good" library for evolution and we certainly need high throughput screening tools and new assay formats to address the challenging problems to which these tools are now being applied. *In vitro* evolution has been reported to be a powerful and efficient tool for tuning enzyme performance in a wide range of applications. Directed evolution needs to be exploited to the maximum in certain areas such as designing novel allosteric biocatalysts, enzymes capable of chiral resolution, i.e. to direct the enzyme to produce the desired enantiomer. This technology needs to be used in active site protection of the chiral building blocks. Directed evolution can also be used in designing enzymes that are able to recognize certain recalcitrant entities and are able to modify them to decrease or eliminate their toxicity and hence can have a major role in decontaminating the environment. One of the most critical areas is the ability of the enzymes to show activity under non-aqueous or anhydrous conditions. Laboratory evolution can help us in obtaining these types of "super enzymes" that can catalyze certain industrially important reactions. As a reliable catalyst improvement technology, directed evolution is expected to play a major role in removing bottlenecks of biocatalysis process technology.

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