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Common Methods Employed in Directed Evolution and Their Application in Modification of Lipases

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ABSTRACT

Biocatalyst plays an important role in the catalysis of biochemical/chemical reactions. Unfortunately, the majority of natural occurring enzyme lacks characteristic features essentially required during industrial processes e.g. thermostability/solvent stability/enantio-selectivity. Protein engineering methods play an important role in altering the natural properties of the enzymes i.e. protein thermostability, catalytic efficiency, tolerance towards extreme pH, activity and stability in presence of organic solvents. Directed evolution has emerged as useful technique that can produce variants, prior the knowledge of three dimensional structures. This review article discusses most common methods that are employed in modification of industrially relevant enzymes. Since, past several years so our lab is working in lipases, therefore we are discussing the application of directed evolution in altering the function of lipases.

Keywords: Biocatalyst, directed evolution, error prone PCR, enantio-selectivity

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INTRODUCTION

Enzymes have evolved over billions of years as chiral catalysts in living cells, to improve the function of their perspective host. They perform enormous range of reactions during the bio-catalytic process. Therefore, identification of suitable enzyme is of most important [1]. A number of chemical industries are implementing biocatalysts for its promising applications in molecular, regio, enantio-selectivity and ultimately economy. The worldwide usage of enzymes being nearly $ 1.5 billion in 2002 [2] and increasing thereafter. With this rapid increase in use of enzymes in the biotechnology sector, there is pressing need to explore methods and technologies which will be cost effective and be beneficial for industrial processes [3-6]. Previously, these enzymes were produced using conventional fermentation technologies [7], which generally gave less yield of purified enzyme after recovery. However, at present most of these enzymes are now produced by recombinant DNA technology. Unfortunately, most of the naturally occurring biocatalysts are usually less stable under operational conditions [8], hence search for the enzyme with stability towards high temperature and pH is required for industrial processes. The use of biocatalysts in different industries will increase in the modern industrial processes, and therefore methods are needed to develop efficient technologies for fulfilling the industrial requirements. The properties of enzyme that it should possess for industrial application is (1) organic solvent stability (2) its high efficiency at high temperature (3) high substrate specificity (4) high pH stability. Despite, advances in the recombinant technology, majority of the natural occurring enzyme lack characteristics features which are importantly required during their industrial processes; hence, efficient methods are needed which can modify industrial relevant enzymes [9]. Protein engineering methods offers tool to alter enzymes function [10, 11]. Directed evolution further hold promises for producing large number of variants with better catalysis and thermostability or enzyme with the desired properties, it work on principle of Darwinian process, where desired and fittest mutants are selected from number of mutations accumulated during the random incorporations [12]. Below, we are discussing some common methods that are used in directed evolutions.

Error prone PCR:

This technique enables one to alter the properties of the enzymes without the prior knowledge of sequence and structure, and employed to generate variants with arbitrary mutations. The method comprises of following steps (1) construction of mutagenic library (2) screening of mutants with desired properties. The protocol is the modifications of standard PCR methods. The method enhances the error rate of the polymerase i.e. Taq DNA polymerase by altering one of the following factors e.g (1) lower concentrations of one or more dNTPs in the reaction mix, that result in low fidelity of the enzyme (2) addition of high concentrations of $MgCl_2$ in order to stabilize the non-complimentary pairs/ sometime $MnCl_2$ is also added to the reaction mix to increase the error rate (3) varying ratio of nucleotides in the reactions mix. After the amplification of the DNA under error prone PCR conditions, a library of variant clones is created in suitable cloning vector. The technique was first of all described by Leung et al. 1989 [13], who explored the intrinsic ability of thermostable DNA polymerases (lacking proof-reading activity), to introduce random mistakes during the extension of the new DNA strand. Taq DNA

polymerase introduce error rate in the range of 0.1×10^{-4} to 2×10^{-4}/nucleotide/pass of the polymerase. This enzyme introduces mutation in sequential fashion, where adverse mutations may also accumulate coincidently along with good one. Below, we are discussing some examples, where this approach has been utilized successfully for alteration of the existing properties of the enzymes i.e. p-nitrobenzyl esterase [14], subtilisins [15] Xylanase [16], β-glucosidase [17], 3-isopropylmalate dehydrogenase [18], amylases [19], xylanases [20, 21], and lipases [22-24], metagenomic lipase [25], Bacillus lipase [26].

DNA shuffling:

In this method a library of mutant's is generated by creating copies of the same gene with diverse mutations, which are randomly shuffled**.** This technique uses DNAase1for digesting libraries of the generated mutants, coupled with random rejoining of fragments, using self priming PCR. The effects of the various arrangement of mutation is then tested [27].

Rolling circle PCR:

This is an isothermal method that amplifies circular DNA, by a rolling circle mechanism and introduces mutations with the error rate of 3-4 mutation/ kb [28-30]. The process involve amplification of the whole construct (vector having wild type construct) under error prone conditions and eliminates the ligation step, which limits the library size in the conventional error prone PCR. This method does not require specific primers, since the random hexamers can be used as a universal primer for any template [31]. Also, this method does not require a thermal-cycler since the amplification reaction carried out at a constant temperature. The method has advantages of ease amplification of the circular DNA and direct transformation of E. coli. However, this method has disadvantage also, as whole plasmid can't be amplified as efficiently as single ORF sequence [32].

Mutators strain:

These are unique kind of strains that lacks three most important DNA repair pathways and are designated as mut S, mut D and mut T. These strains have mutation rate 5000-fold than the wild type. The method involves cloning of the wild type gene sequence into a plasmid followed by its transformation into the mutator strains e.g. one of such strain is supplied by stratagene's E. coli XL1-Red strain. The strain introduces mutations during the replication and may result in copy of the replicative plasmid having different sequence to that of wild type. It produces variety of mutations including substitutions, deletions and frame shifts. It has following disadvantages i.e. when the strain accumulate more mutations during the growth period, it become sick, therefore it is necessary to extract plasmid after the abundant growth and to retransform it in to new host followed by its re-growth so that a meaningful library could be generated.

Site saturation mutagenesis:

This method can produce library of mutants with all possible mutations at one or more determined target sites in gene sequence. Therefore, it becomes necessary to choose a site that contains one or more amino acids position crucial for success. The accurate choice of mutation depends upon the nature of catalytic properties under investigation e.g. if one is interested in enhancing the enantioselective properties of an enzyme, it's better to choose combinational active site saturation test, where every sites at the vicinity of complete binding pockets are considered. Next, if one is interested in enhancing the thermo stability, a different approach is followed (B-factor saturation test). Here, those sites will be considered important which exemplify higher plasticity (specified by respective B-factors data available from X-ray data). High inflexibility in these regions can be ascribing to increased thermostability. The method can be made more systemic by performing an iterative cycle of site saturation mutagenesis test e.g. a theoretical case in which four sites A, B, C and D are suggested as hot spot, the site saturation mutagenesis will be performed in each of these sites followed by screening of these libraries for catalytic properties of interest. Subsequently, the mutated genes act as template for next round. To perform site saturation mutagenesis several commercial kits are available e.g. Quick change ^{TM} [33]. Beside, researchers have also developed and described an efficient one step site directed and site saturation mutagenesis protocol based on oligonucleotide [34]. Several other molecular methods have been described for performing saturation mutagenesis [35-37]. Typically, the method is performed according to following ways.

1) Perform epPCR, identification of the hot spots followed by selection of important sites for saturation mutagenesis.
2) Perform site saturation mutagenesis systematically at all amino acids positions of a given enzyme.
3) The site for the saturation mutagenesis can be selected from previous information of the three dimensional data of the structural homolog's.

Mutazyme:

Mutazyme is mutant DNA polymerase produced by Stratagene. The enzyme introduces mutation more frequently than the Taq DNA polymerase. Interestingly, these high mutation frequencies are achieved under most favorable PCR conditions for the enzyme ($MgCl_2$, balanced nucleotides). Despite its inherent high error rate, it produces high PCR product yield over a broad range of DNA template concentrations. High mutational frequencies can be achieved by simply varying the initial target DNA concentration in the PCR reactions. In general, for the same PCR yield, the amplification achieved at low concentration of the target DNA undergo more duplications than targets amplified from high concentrations of DNA. Moreover, the number of times an amplicon is copied, it can yields ~0.5-10 mg of DNA. Interestingly, more time the target is replicated, the more errors accumulate. On the other side, lower mutation frequencies are achieved at higher DNA concentrations, the fewer PCR cycles can limit the number of target duplications. On an average 10 -100 ng of target DNA can produce mutation

frequencies up to three mutations per kb (0.3%), while PCR reaction carried out at10 pg to 1 ng of DNA template can have error rate of three to seven mutations/ kb (0.3%-0.7%). Furthermore, mutation frequency exceeding 0.7% is achieved by performing sequential amplifications on re-amplifying the portion of a PCR reaction. Mutation frequencies of 0.6% to 1.3% or 0.9% to1.8% can be created by performing two or three sequential PCR reactions, respectively.

Application of directed evolution in altering functional properties of the lipases:

Lipases are hydrolytic enzymes which are classically employed for the hydrolysis of triglycerides and produce free fatty acids in turn. The advances made in lipase discovery will help in expanding the synthesis and development of novel molecules (Bjorkling F et al. 1991). In past 12 years our lab at Panjab University Chandigarh is working on the lipase, we have reported many lipases from cultivable and uncultivable bacteria [26, 38-40]. These are versatile biocatalysts and catalyze novel reactions in both aqueous and non-aqueous media. They are highly stable in presence of organic solvents, show broad substrate specificity, stereo selectivity and positional selectivity [41]. They find immense application in modification of chiral drugs, synthesis of cocoa butter substituents, biofuels, personal care products and flavour enhancers [42,43]. These enzymes are produced in nature by diverse class of organisms [44]. Structurally, these enzymes posseses α/β hydrolase fold [45-48]. Due to increased stability and resistance to high temperature and chemical denaturation, lipases from thermopiles are expected to play a significant role in the industrial processes. In addition to higher thermostability, proteins from thermophiles often showed more stability toward organic solvent and exhibit higher activity at elevated temperatures. The extremophilic organisms, especially thermophilic bacteria can be isolated from the natural high temperature environments distributed throughout the world and found in association of tectonically active sites [49] (Brock TD. 1985). However, to satisfy the global requirement of the industrial processes, the enzymes showing optimal activities at extended pH, temperature and salt concentration are required. With the advancement made in molecular biological methods, many novel lipase genes with novel properties have been cloned from culturable and unculturable microorganisms. At the same time, directed evolution approach in combination with appropriate screening system was being used extensively as a novel approach to develop lipases with high thermal stability and enantioselectivity. Despite, availability of a number of lipases in nature their applications are limited. Directed evolution is being utilized successful for increasing activity, thermostability and enantio-selectivity of the lipases. Ahmad et al., [50] have evolved a lipase in vitro with six stabilizing mutations from mesophilic Bacillus subtilis in just two round of error prone PCR, when these mutations were engineered collectively, the variant so obtained showed remarkable 15°C shift in melting temperature and a several million fold decrease in the thermal inactivation. In another case, error prone PCR and DNA shuffling methods were used to improve thermostability and optimum temperature of the Rhizopus arrhizus lipase enzyme, where the mutant showed 10 ºC rise in the optimum temperature compared to wild type lipase. Moreover, the thermostability was also enhanced 12 fold at 50ºC [51]. Yet in another study, two mutant of p-nitrobenzyl esterase were produced, one of which showed 100-fold increased activity in aqueous organic solvents, while another resulted in 17°C increase in the thermostability. Next, error prone PCR

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and staggered extension PCR resulted in a mutant that displayed enhanced thermostability and activity over the wild type [52]. In our lab, we have produced a highly thermostable mutant lipase by directed evolution, having single mutation N355K at the vicinity of the active site [25]. The mutant showed 144 folds enhanced thermostability and 20 folds enhancement in the catalytic efficiency of the mutant compared to wild type. In another study from the lab error prone PCR was performed with the wild type lipase of a mesophillic Bacillus that increased the half life of mutant compared to the wild type lipase [26]. **Table1** will further represent the different lipases modified by directed evolution for various properties.

Table 1: Various lipases modified using directed evolution approach.

Method of mutagenesis	Target organism	Amino acid exchanged	Activity improved	References
Rational recombination of the two isozyme genes; site-directed mutagenesis	Geotrichum candidum lipase	I-348-II-406-I, I357A and L358F	enantioselectivity	[53]
Single-molecule PCR amplification	Burkholderia cepacia lipase	L17F, F119L, L167G and L266V	enantioselectivity	[54]
Error prone PCR and DNA shuffling	Pseudomona aeruginosa lipase	M16L, A34T	enantioselectivity	[55]
Combinatorial multiple-cassette mutagenesis	Pseudomona aeruginosa lipase	D20N, S53P, S155M, L162G, T180I and T234S	enantioselectivity	[56]
Error prone PCR and saturation mutagenesis	Pseudomona aeruginosa lipase	S149G, S155F, V47G, V55G and S164G	enantioselctivity	[57]
Error prone PCR and saturation mutagenesis	Bacillus subtilis lipase A	I22V, Q60L, Q60R, Y49C and N50S	enantioselectivity	[58]
Error prone PCR	Pseudomona aeruginosa lipase	S155F, L162G and S155F; L162G, S53P and S155F	enantioselctivity	[59]
Multiplex-PCR-based recombination	Bacillus subtilis lipase A	N18Q and Y49V	enentioselctivity	[60]
Site-specific saturation mutagenesis	Candida rugosa lipase lip4	A296I, V344Q and V344H	Change of the substrate specificity	[61]
Error prone PCR	Acinetobacter sp. lipase	S21F, A102G, S103F, D299E and N300H	Change of the substrate specificity	[62]
Cassette mutagenesis	Thermomyces lanuginosa lipase	S83T	Change of the substrate specificity	[63]
Error prone PCR	Candida antarctica lipase B	A281E and V221D	thermostability	[23]
Error prone PCR and Site-directed mutagenesis	Bacillus subtilis lipase	N66Y and A132D; N66Y, A132D and L114P	thermostabiltiy	[24]
Error prone PCR	Candida antarctica lipase B	N317Y	thermostability	[64]
Error prone PCR A15S and site saturation mutagenesis	Bacillus subtilis lipase	F17S, A20E, N89Y, G111D, L114P, A132D, I157M and N166Y	thermostability	[50]

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