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Review

In vitro engineering of microbial enzymes with multifarious applications: Prospects and perspectives

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HIGHLIGHTS

- This review covers protein engineering of microbial enzymes with multiple uses.
- *In vitro* evolution tools for making alterations in the enzymes are discussed.
- Industrially relevant biophysico-chemical properties have been kept in focus.
- Protein engineered enzymes that entered into the market are also cited.

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ABSTRACT

The discovery of a novel enzyme from a microbial source takes anywhere between months to years, and therefore, there has been an immense interest in modifying the existing microbial enzymes to suit the present day needs of the industry. The redesigning of industrially useful enzymes for improving their performance has become a challenge because bioinformatics databases have been revealing new facts on a day-to-day basis. Modification of the existing enzymes has become a trend for fine tuning of biocatalysts in the biotech industry. Hydrolases are employed in pharmaceutical, biofuel, detergent, food and feed industries that significantly contribute to the global annual revenue, and therefore, the emphasis has been on engineering them. Although a large data is accumulating on making alterations in microbial enzymes, there is a lack of definite information on redesigning industrial enzymes. This review focuses on the recent developments in improving the characteristics of various biotechnologically important enzymes.

1. Introduction

The tailoring of the microbial enzymes has become a trend in the field of protein engineering to overcome the limitations of natural biocatalysts and to develop process-specific enzymes. Scientists have been attempting to generate enzymes which can withstand harsh and unfavourable conditions prevailing in industrial processes. The tolerance to high or low temperatures, exhibiting activity in the alkaline or acidic environments, high performance in non-aqueous media, increased protease resistance and others are a few of the requisite properties. The ultimate goal is to redesign the proteins in such a way that the industrial processes can be carried out in a more economic and greener way. In protein engineering, various methods are employed in modifying the target proteins. These are mainly rational design, directed evolution and semi-rational approaches for designing and constructing novel proteins. The rational methods require prior knowledge of the aa sequence, 3D-structure and the knowledge

Abbreviations: 2′-O-meNTP, 2′-O-methylribonucleoside triphosphates; aa, amino acid; CBM, cellulose binding module; CBM™, combinatorial beneficial mutagenesis; CGTase, cyclodextrin glycosyltransferase; CODH, carbon monoxide dehydrogenase; CSAT, combinatorial active site saturation test; CSSM, coevolving-site saturation mutagenesis; DB, disulphide bridge; EG III, endoglucanase III; ep-PCR, error prone polymerase chain reaction; GSSM, gene site saturation mutagenesis; GLNBP, galacto-N-biose/lacto-N-biose I phosphorylase; HMFS, human milk fat substitute; HRPL, high-redox potential laccases; IvAM, *in vivo* assembly of mutant libraries; LTM™, look-through mutagenesis; MD, molecular dynamics; Mod-PCR, mutagenic oligonucleotide-directed PCR; PDB, protein data bank; PGs, polygalacturonases; PLs, polygalacturonate lyases; RACHITT, random chimeragenesis on transient templates; REase, restriction endonuclease; SCP, single cell protein; SDM, site directed mutagenesis; SeSaM, sequence saturation mutagenesis; SLAC, small laccases; spCSR, short-patch compartmentalized self-replication; StEP, staggered extension process.

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of the structure–function aspects of the target proteins. So far, 95,052 protein structures have been solved and are available in the PDB. When mutations carried out by computational and random mutagenesis are compared, often the best mutants have been developed by either of the methods. Computationally developed muteins are obtained seldom by random mutagenesis, which explains the importance of rational design in terms of either analysing the effect of a single residue on protein stability or on folding and function of the mutein (Wunderlich et al., 2005). The directed evolution is a powerful tool to achieve more ambitious characteristics in proteins of interest. Unlike the rational design methodology, directed evolution does not require prior knowledge of the primary sequence of proteins and their function or structure. This method mimics natural selection *in vitro* and reduces the time required for evolution from millions of years to few weeks or months. The directed evolution can be employed by using a non-recombinant or recombinant approach. Non-recombinant approach includes techniques such as SeSaM (Gupta and Farinas, 2010) and ep-PCR (Song et al., 2012). Recombination based directed evolution involves techniques such as fragment shuffling (Bei et al., 2009) and random chimeragenesis on transient templates (RACHITT) (Coco et al., 2001). There are both advantages and disadvantages of using either rational or directed evolution methodology as a sole approach for protein engineering experiments. Thus, a combination of both the techniques would be more a promising approach to improve the properties and functions of the proteins. Depending on the number of sites chosen to mutate, mutagenesis can be divided into single site and multisite saturation mutagenesis. The popular single site saturation mutagenesis techniques include Mod-PCR, where degenerate primers are used to introduce mutations in DNA sequence elements (Chiang et al., 1993), codon cassette mutagenesis where a universal mutagenic cassette is used to introduce a single codon at the site of mutation (Kegler-Ebo et al., 1994). Multisite saturation mutagenesis is more complex process than the single site saturation mutagenesis. During the past few years, several attempts have been made to evolve and simplify multisite saturation mutagenesis. A Quik Change Multi Site-Directed Mutagenesis Kit sold by Stratagene Company (Stratagene, La Jolla, CA, USA) is employed for multisite saturation mutagenesis. Latest development in multisite saturation technique has been reported by a German group of researchers, which has come up with a cost effective Omni Change methodology (Dennig et al., 2011), wherein five independent sites can be saturated simultaneously in four simple steps. These techniques are becoming more and more useful to introduce changes in the proteins of interest. In this review, an attempt has been made to update the recent advances in ameliorating enzyme properties by protein engineering in order to achieve improved performance.

2. Redesigning microbial enzymes

Since the very beginning of evolution, nature has been constantly evolving proteins for survival and better adaptation of living organisms to their environments. The nature evolved enzymes are, however, not always best for industrial purposes. Some additional features are required to suit their applicability in industries. Sometimes two or three specific properties are needed in one protein at the same time. Moreover, natural evolution takes a very long time for the protein to acquire the desirable properties. This is where *in vitro* evolution takes the charge as it aids in tailoring and evolving microbial enzymes at a much faster rate (Fig. 1).

Different industrial processes require enzymes with unique properties to operate in the physicochemical environment of the process. For example, a protease must exhibit tolerance to detergents and bleaching agents in order to be used as a detergent additive (Joshi and Satyanarayana, 2013). On the other hand, an ideal xylanase suitable for the paper and pulp industry should be cellulase-free and thermo-alkali-stable (Verma and Satyanarayana, 2012). In order to generate biocatalysts that suit diverse industrial applications, various molecular approaches have been employed (Table 1, see Supplementary file, Table S1). In this review, we have discussed recent achievements in *in vitro* tailoring of various industrial enzymes.

2.1. In vitro changes in properties of laccases

The industrial importance of laccases arises from the fact that these versatile enzymes can oxidize both toxic and non-toxic substances. Laccases are utilized in a plethora of processes including pharmaceutical, textile, wood processing, food processing and chemical industries (Koschorreck et al., 2009; Sherif et al., 2013). It is known that subtle molecular changes in protein structure by mutations can significantly alter their biochemical properties (Ema et al., 2005; Mullaney et al., 2012; Verma and Satyanarayana, 2013). Mutagenesis has been the tool of choice to unleash secrets of biochemical properties of microbial laccases. Protein termini are known to have a significant role in determining enzyme properties (Wang et al., 2009).

Autore et al. (2009) found out the role of C-terminal extension of 16 aa residues in the stability and catalytic activity of *Pleurotus ostreatus* laccase (POXA1b). C-terminal truncated laccase was generated and properties of the full length and truncated enzymes were compared. The removal of C-terminal led to reduced stability at alkaline pH (pH 10), while increased stability at acidic pH (pH 5). As C-terminal is cleaved by proteolysis and is absent in mature laccases, removal of C-terminal eliminates the obstruction of T2/T3 channel, which is the route for oxygen entry and exit of water molecule, thereby aiding the catalysis by laccases (Autore et al., 2009). *Bacillus licheniformis* laccase CotA harbouring K316N mutation exhibited higher activity than the wild type enzyme, while the mutation D500G led to 11.4-fold higher expression (Koschorreck et al., 2009). The presence of glycine at position 500 creates a large space between Gly500 and Met502 residues and brings about structural changes in the protein that result in efficient protein folding and production of higher quantities of the enzyme. A combinatorial approach of random mutagenesis (IvAM) [Table S1] and SDM have resulted in 34,000-fold improvement in total activity of high-redox potential laccase (HRPL) (Mate et al., 2010) (Fig. 2). The mutations have been introduced in a fusion product of the prepro-leader sequence of the yeast α-factor mating pheromone and basidiomycete PM1 HRPL i.e. α-PM1. Initially mutations were introduced by mutagenic PCR followed by combined use of mutagenic PCR and *in vivo* DNA shuffling. The mutations that significantly improved α-PM1 properties but not selected for the final rounds of mutagenesis, were traced and introduced by SDM in the final stage of the experiment. Mutations in both the prepro-leader sequence and PM1 region contributed to the improvement in properties of α-PM1. The mutations in the prepro-leader sequence have been known to affect targeting of proteins to endoplasmic reticulum and secretion of the protein. Small laccases (SLAC, multicopper oxidases) from *Streptomyces coelicolor* have also been subjected to mutagenesis for understanding the effect of sequence variation (Sherif et al., 2013). Seventeen aa residues have been found to be important for the activity of SLACs. About ten histidine residues have been shown to coordinate copper during catalysis. Among the mutations studied, Y229A and Y230A increased SLAC activity by 10-fold. Higher activity of Y229A and Y230A was attributed to accumulation of higher copper amount than the wild type enzyme.