Microbial Enzyme Engineering: Applications and Perspectives

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Abstract

Enzymes are fascinating the researchers because of their enormous power of catalysis and eco-friendly nature. In biotechnological processes, diversity of microbes is studied, and different metabolic reactions entitle a potential repository that direct valuable production of desirable products. Since community demands are getting more intensified, there is a continuous need to evolve the enzymes. There has been an immense development in techniques and computational tools that has developed the industries to meet the growing demands. The techniques such as protein engineering help in development of quality products by mutating the amino acids to make more stable and efficient product. Further, the techniques like enzyme immobilization give the opportunity to reuse the used enzyme with the same efficiency, thus a cost-effective measure for the industrial enzyme. Nanotechnology and CLEA formation are also incorporated in enzyme engineering to increase enzyme efficiency and their characteristics.

Keywords

Protein engineering • Immobilization • Nanotechnology • Microbial enzyme

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13.1 Introduction

The microbial enzymes are natural biocatalysts which are produced by different forms of microorganisms such as bacteria, fungi, algae, yeast, and actinomycetes to accelerate the rate biochemical reactions taking place in vivo system (Baweja et al. 2016; Kumar et al. 2014; Singh et al. 2016). These enzymes have been involved in all the processes such as DNA replication, transcription, translation, protein synthesis, metabolic reactions, and cycles which are essential for life, and their unique ability to carry out substrate-specific transformation has made them suitable for industrial processes. Enzymes are omnipresent ranging from living organisms to industries which catalyze the biochemical reactions to maintain the living system or synthesis of a product or degradation of a substance (Shrivastava et al. 2012, 2013; Kumar et al. 2014, 2016). The enzymes have replaced the chemicals from the industries, which are more eco-friendly and help to step up toward green environment. The industries are now totally enzyme dependent, and there is continuous demand for a number of microbial enzymes with novel characteristics and stability under industrial extreme conditions (Kumar and Shukla 2015). There is a continuous progress in microbiology, biotechnology, and bioinformatics to provide microbial enzymes with novel characteristics for the development of better industrial processes (Singh and Shukla 2012, 2015). The techniques of recombinant DNA technology, protein engineering, immobilization, nanotechnology, and metabolic engineering have progressed enough to support the industrial enzyme load. The industry demands operation stable enzymes with novel biochemical properties which should be economic and environment friendly. The continuous efforts are being done to meet all objectives of industrial demand of enzymes by utilizing a combination of various modern techniques of biotechnology, bioinformatics, microbiology, and nanotechnology. The enzyme engineering is proving as one of the finest techniques to improve the enzyme properties like operational stability, physical stability of enzyme, substrate specificity, and enhanced activity. There are several enzyme engineering techniques such as protein engineering, metabolic engineering, immobilization, and nanotechnology, and in silico methods; in this chapter, all these aspects will be described briefly. It will be helpful to understand the revolution in the microbial enzyme by changing at certain level or complete redesigning of the enzymes.

13.2 Improvement of Enzymes by Tailoring Their Protein Sequences

The industries continuously demand enzymes with high stabilities and substrate specificities. Since the native enzyme lacks the efficient system that can cope with hostile industrial conditions, there is a need to improve the enzyme that could withstand the harsh industrial conditions. With progress in recombinant technology and enzyme engineering, it has become possible to obtain customized enzymes. The improved novel enzymes that can fit into industry can be achieved by genetic manipulated microorganisms such as recombinant insulin production by using

Escherichia coli as host. The recombinant DNA technology makes possible to 100-fold increase in production of enzymes than the native expression, making them available at low cost and in large quantities. Thus, various food-processing enzymes and laundry enzymes can be tailored as per demand of the industrial process.

The protein engineering allows modification in protein itself to improve the properties for suitability to industrial process. In protein engineering, mutation is the key to improve the enzyme properties and to explore protein function. It is a method to alter a protein sequence to obtain a desired effect, such as change in the substrate specificity with increased stability toward extremes of pH and the temperature and in organic solvents. The protein engineering is divided into two types: (1) site-directed mutagenesis or rational design and (2) random mutagenesis.

13.2.1 Improvement of Enzyme Properties by Site-Directed Mutagenesis

The protein engineering is proving as stupendous technique to modify the enzyme to achieve customized biocatalyst. The major drawback with wild-type enzyme is that they cannot bear harsh experimental conditions. To overcome the snag of native enzyme, researchers are continuously adopting various methods to obtain refined industrial enzymes. Protein engineering is one such technique that works at the level of nucleotide to evolve the functional aspect of the protein. A comparison of native and engineered enzyme production has been described in Fig. 13.1. Site-directed mutagenesis, as the name suggests, is site-specific technique to improve enzyme. Thus, it is the technique for the proteins with full knowledge of structure and mechanism of action. The mutation type may vary as per requisite like point mutation, insertion, deletion, and substitution. Depending upon the number of mutation in a gene, it can be single site-directed mutagenesis and multiple site-directed mutageneses. The major application of site-directed mutagenesis is to introduce novel properties like enhanced specificity, stability, activity, solubility, expression, etc. to the biocatalyst. There are number of reports on improvement of industrial enzyme. A study was conducted to improve α-galactosidase features; the protein was mutated at specific site that improved the enzyme properties and also added the structural and functional information (Xu et al. 2014). In a similar study, the thermostability of the immobilized protease was improved by introducing Cys residues on surface of a cysteine-free mutant of a thermolysin-like protease from *B. stearothermophilus* and thus facilitated the site-directed immobilization of protease via single thiol group onto thiol Sepharose (Eijsink et al. 1995). It was reported by Rahimi et al. 2016 that mutation at nearby active site region is more promising in improving the protein function. A study was conducted to improve the keratinase enzyme to enhance its application at industrial level. Although the native enzyme itself had immense activity and pH stability, a truncation of PPC domain improved the tolerance to alkalinity, salt, chaotropic agents, and detergents (Fang et al. 2012). A study deduced that substitution of conserved residue Asn by arginine of γ-glutamyltranspeptidase (*Bl*GGT) by site-directed mutagenesis resulted in

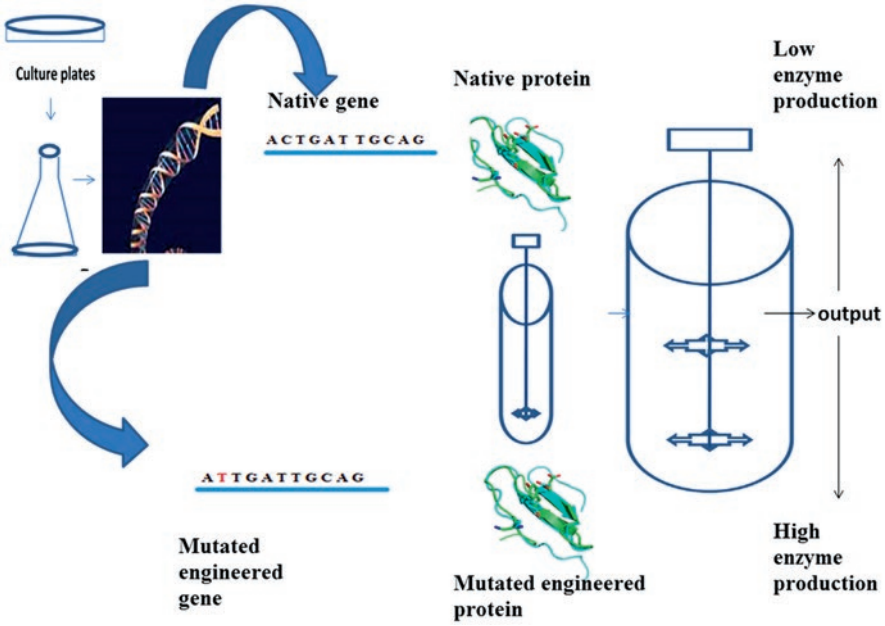

Fig. 13.1 A snapshot of industrial process describing high enzyme production using protein engineering

reduction in the catalytic activity (Lin et al. 2016). Wang and coworkers also elucidated the role of conserved amino acid residues by generating mutants by site-directed mutagenesis (Wang et al. 2015). The thermophilic archeal protein ST0452 was studied to comprehend the molecular machinery; after analyses, the researchers identified certain amino residues important for the glucosamine-1-phosphate and galactosamine-1-phosphate activities, viz., His308 is necessary for both GalN-1-P and GlcN-1-P AcTase activities, whereas Asn331 and Tyr311 are important only for the GalN-1-P AcTase activity (Zhang et al. 2015).

13.2.2 Improvement of Enzyme Properties by Random Mutagenesis

Random mutagenesis mimics the nature's process of variant generation following the unbiased approach. Since it involves mutation in randomized manner, thus it is a method of choice for those proteins whose structure has not been deduced (Baweja et al. 2016). Thus, it is quite an easy technique to employ but has cumbersome screening process since a number of variants produced are often large. There are various physical and chemical methods to create random mutation, such as chemical agents like ethyl methane sulfonate (EMS), methylnitronitrosoguanidine (MNNG), and ethylnitrosourea (ENU), using error-prone PCR by using less

FideliTaq polymerase instead of using pfu polymerase; using base analogs, altering the concentration of nucleotides; using heavy water during PCR, mutator strain, and many more; or using genetic recombination techniques like those based on gene recombination which are DNA shuffling, random chimeragenesis on transient templates (RACHITT), staggered extension process (StEP), recombined extension on truncated templates (RETT), and iterative truncation for the creation of hybrid enzymes (ITCHY) (Sen et al. 2007; Rasila et al. 2009; Baweja et al. 2015). The error-prone PCR is the most common technique in random mutagenesis with high success rates. The primary motto behind random mutagenesis is to characterize the open reading frame (ORF) and to modify the gene to obtain the desired product (Ramli et al. 2011).

There are various computational tools available that guide the library diversity and design, viz., ConSurf-HSSP GLUE, PEDEL, DRIVeR, and SCHEMA (Labrou 2010). There are various bioinformatics techniques available that reduce the cumbersome process of screening out libraries. Techniques like modeling and docking of enzymes prescreen the variants by giving the docking score that evaluates the enzyme-substrate relationship and effectiveness of their binding. The modeling and docking studies have been done in various enzymes like inulinase and xylanase (Karthik and Shukla 2012; Singh et al. 2016). The molecular dynamics simulation helps evaluate the stability of particular protein in particular milieu and thus filters out the variants during library screening (Singh et al. 2016).

13.3 Changing Pathways: Synthetic Metabolic Engineering

Industrial biotechnology promises to revolutionize conventional chemical manufacturing in the years ahead, largely owing to the excellent progress to reengineer cellular metabolism. It was evidenced that production of food stuffs and biofuels was enhanced after the era of metabolic engineering (Yadav et al. 2012). Metabolic engineering involves modification of metabolic pathways to screen the effect on the production of desired metabolite. To owe successful metabolic engineering, the first step is to crack metabolic pathway involved in the production of particular metabolite and to preclude the rate-limiting step in the reaction. The alteration in the rate-limiting step can be done by either overexpression of heterologous gene contributing the rate limitation or inhibiting the pathway in the network that halts the formation of desired product as shown in Fig. 13.2. To renew the production of metabolite, various experimental and computational tools are used to add beneficial traits to the system (Stafford and Gregory 2001). Metabolic flux plays an important role in valuation of the cellular phenotype; thus flux determination methods are inevitable components of metabolic engineering. Nowadays isotopic tracers are used to evaluate the balance of intracellular and extracellular metabolites. The 13C and 14C compounds were used to track the changes in and out of the cell (Klapa et al. 1999; Schmidt et al. 1997; Stafford et al. 2001). The introduction of multigene pathways into a host for heterologous production often faces flux imbalance because the host usually does not possess complex regulatory machinery to maintain such pathways.

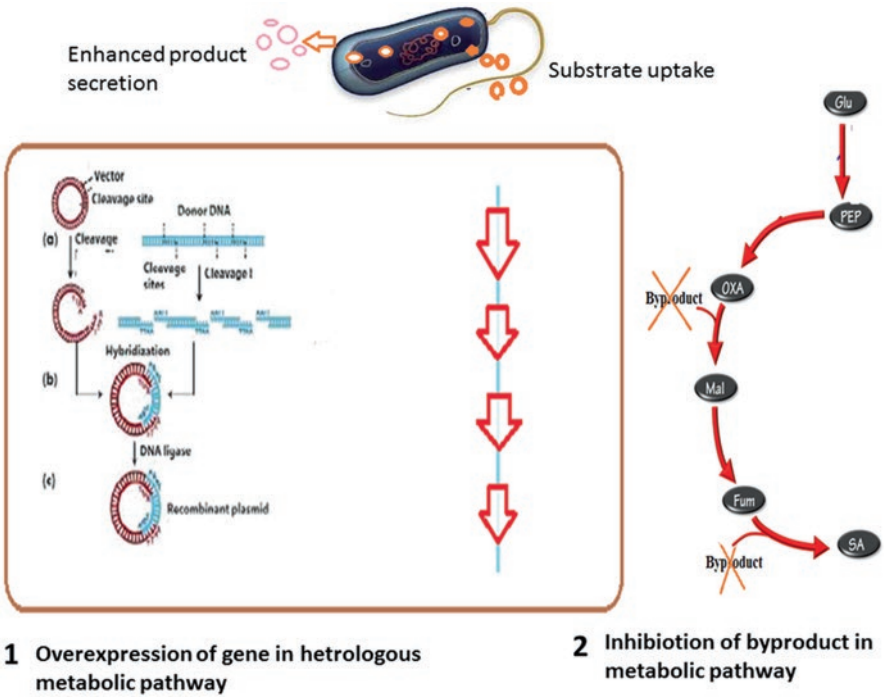

Fig. 13.2 Approaches of metabolic engineering for enhanced product secretion

However efforts have been done to resolve such problems by combining metabolic engineering tools with combinatorial genetics (Ajikumar et al. 2010; Lee et al. 2011). Although metabolic flux helps to deduce the pathway feature, it is not enough to decipher the system. Various high-throughput probes are used for complete revelation of metabolic networks. Among various molecular biological tools, efficient transformation system, viz., plasmids, hosts, and efficient promoters, is crucial for efficient product development and its modification using gene-editing approaches (Gupta and Shukla 2016; Keasling 1999). Gupta and Shukla (2015) described *E.coli* as suitable host during transformation.

13.4 Immobilization of Microbial Enzyme

Microbial enzymes catalyze a number of biochemical reactions efficiently and selectively; that's why they possess the ability to synthesize or to convert one compound to another. Immobilized forms of microbial enzymes have several industrial applications that are clear-cut as they provide a recycling method for the production of various compounds through biocatalyzed reactions. An immobilized microbial enzyme is the stable form of enzyme which is bound to an inert, insoluble material such as silica, chitosan, calcium alginate, copper alginate, agarose, polyacrylamide,

etc. which provides increased stability in changing conditions of pH or temperature during industrial processes. It allows enzymes to stay held on supported material throughout the reaction following which they are separated for recycling and reuse. Along with synthesis of desired compounds, immobilized microbial enzymes also have the ability to decompose harmful compounds making them suitable for additional field of industrial application in bioremediation and purification. Besides these applications, the enzyme immobilization techniques are basis to synthesize a number of biotechnological products that have various applications in biosensors and bioaffinity chromatography and diagnostics (Guibault et al. 1991). A therapeutic application of immobilized enzymes is in extracorporeal shunts (Chang 1991). In the history of three or four decades, immobilization techniques have been developed swiftly, but still there is a need for further development. Immobilization technique is systematically studied with the probability of modification and improvement of enzyme stability and characteristics for economic purposes. There are a number of microbial enzymes such as xylanase, phytase, laccase, inulinase, cellulase, and amylase which have been immobilized on various materials. Among this series, microbial xylanase, which catalyzes the hydrolysis of xylan, is considered one of the most significant hydrolases. It has numerous applications, but most extensively it is utilized in paper and pulp industry as a biobleaching and biodeinking agent. Kapoor and Kuhad in 2007 used a number of matrices to immobilize the xylanase enzyme using various methods from *Bacillus pumilus* such as entrapment using gelatin, physical adsorption on chitin, ionic binding with Q-sepharose, and covalent binding with HP-20 beads with maximum xylanase immobilization efficiency. Similarly, Nagar et al. (2012) used the immobilized xylanase enzymes to improve the digestibility of poultry feed. Aluminum oxide pellets charged with glutaraldehyde were used for the immobilization which results in increase of enzyme temperature optima from 50 to 60 °C and V_{max} from 3333.33 to 5000 IU/mL. Immobilized xylanase was biochemically active up to ten consecutive cycles with 60% of its initial activity. In the same series, xylanase enzyme has also been covalently immobilized on the beads of glutaraldehyde-alginate exteriorly which retains their efficiency more than 91% with an increase in kinetic parameters V_{max} (7092–8000 IU/ml) and K_m (0.9–1.49%) and an increase in pH optima 5–5.5 and temperature optima from 40 to 45 ° (Pal and Khanum 2011). The enzyme has been reused five times while retaining >85% of its starting activity. Recently, matrix entrapment method was carried out by Bibi et al. (2015) to immobilize microbial endo-β-1,4-xylanase produced by *Geobacillus stearothermophilus* KIBGE-IB29 within agar-agar gel beads.

Among the industrial enzymes, protease has taken a pivotal position in detergent industry and leather industry. The alkaline protease from *Bacillus mycoides* was immobilized on different carriers using various immobilization methods including physical adsorption, covalent binding, entrapment, and ionic binding. An alkaline protease preparation was physically adsorbed on chitosan, entrapped in 2% cross-linked polyacrylamide, covalently bonded on chitin and ionically bonded on Amberlite IR-120 that were observed with highest activities by Abdel-Naby et al. (1998). In previous year, chitosan-immobilized protease from *Bacillus*

licheniformis was applied in therapeutic use by Elchinger et al. (2015). They synthesized protease gains anti-biofilm activities after immobilization and was explored against biofilms formed by *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, etc. Similarly, immobilization techniques also employed for laccase enzyme to make them suitable for various bioremediation application and wastewater treatment. The laccase beads were synthesized by immobilizing laccase enzyme on copper-alginate beads, and additionally Fe_2O_3 was incorporated in the bead through magnetic force. These lac beads have been used for bioremediation of triclosan and Remazol Brilliant Blue R and subsequently for wastewater treatment (Thanh le et al. 2016). Laccase from *Trametes versicolor* was also covalently immobilized on the composite polymer particles of poly(2-chloroethyl acrylate), p(CEA), which were grafted on zeolite particles via surface-initiated atom transfer radical polymerization (SI-ATRP). The immobilized laccase on the zeolite-g-p (CEA) particles was applied in biodegradation of dye Reactive Red 120. Besides these enzymes, immobilization was carried out with several other microbial enzymes which have been summarized in Table 13.1 with their application and support material being used.

13.4.1 Microbial Enzyme Immobilization Using Nanotechnology

Nanoparticles exhibit some attractive properties like elevated surface reactivity, high catalytic efficiency, tough adsorption ability, and great surface-to-volume ratio which make them attractive agent for immobilization. Adsorption of microbial enzymes on nanoparticles leads to enhanced performance of microbial enzymes in terms of its catalytic activity (Lynch and Dawson 2008). The application of enzyme immobilized nanoparticles was started during the 1980s (Pereira et al. 2002; Soriano et al. 2005). A number of microbial enzymes such as xylanase, protease, amylases, and phytase have been immobilized on various nanoparticles such as Fe_3O_4-coated chitosan, 1,3,5-triazine-functionalized $Fe_3O_4@SiO_2$ nanoparticles, gold nanoparticles, carbon nanoparticle, etc. In order to characterize the structure, size, and magnetic properties of the immobilized xylanase, Fourier transform infrared spectra (FTIR), thermo-gravimetric analysis (TGA), transmission electron microscopy (TEM), vibrating sample magnetometer (VSM), and X-ray photoelectron spectroscopy (XPS) were used for analysis. The enzyme activity, thermostability, storage stability, pH stability, and reusability of the nanoparticles of microbial enzymes have exhibited significant superiority to the free microbial enzymes. The xylanase MNPs showed quite impressive stability after nine reaction cycles with about 65% of its initial activity (Soozanipour et al. 2015). Experimental results by Soozanipour et al. (2015) suggested that the 1,3,5-triazine-functionalized $Fe_3O_4@SiO_2$ nanoparticles could be the novel convenient magnetic carrier for xylanase immobilization. Similarly, recently, Shahrestani et al. (2016) synthesized 1,3,5-triazine-functionalized silica encapsulated magnetic nanoparticles to immobilize xylanase enzyme to apply in clarification of bear and juices with impressive stability even after ten reaction cycle. In the same sequence, xylanase from *Aspergillus niger* that

Table 13.1 Microbial enzyme immobilization on various supporting matrix and their application

Sr. No.	Enzyme	Supporting matrix	Type of immobilization	Application	References
1.	Xylanase	Aluminum oxide pellets charged with glutaraldehyde	Covalent	Digestibility of poultry feed	Nagar et al. (2012)
2.	Xylanase	Glutaraldehyde-alginate beads	Covalent	–	Pal and Khanum (2011)
3.	Xylanase	Agar-agar	Matrix entrapment method	Biodegradation of xylan	Bibi et al. (2015)
4.	Protease from *Bacillus licheniformis*	Chitosan	Surface adsorption	Anti-biofilm activities	Elchinger et al. (2015)
5.	Laccase	Copper-alginate beads	Entrapment	Bioremediation or waste water treatment	Thanh le et al. (2016)
6.	Laccase from *Trametes versicolor*	Poly(2-chloroethyl acrylate) zeolite-g-p(CEA) particles	Covalent adsorption	Biodegradation of Reactive Red	Celikbicak et al. (2014)
7.	Inulinase from *Aspergillus niger*	Chitosan beads	Covalent immobilization	Continuous inulin hydrolysis	Yewale et al. (2013)
8.	Inulinase from *Aspergillus niger*	Polyurethane foam	–	–	Silva et al. (2013)
9.	α-Amylase	Silica nanoparticles	Covalent adsorption	Formulation of detergent	Soleimani et al. (2012)
10.	α-Amylase from *Bacillus stearothermophilus*	Poly (urethane urea) (PUU) microparticles	Covalent attachment	–	Strakšys et al. (2016)

has been immobilized covalently on the surface Fe_3O_4-coated chitosan magnetic nanoparticles showed a high binding capacity (Liu et al. 2015). Xylanase MNPs can be used in a number of industrial applications under broader pH and temperature ranges, having long-term storage capability and permitting magnetically recycling of the enzyme for purification or reuse of the product. Similarly, cellulase enzyme has also been physically adsorbed through ionic bond on superparamagnetic nanoparticles with binding efficiency of 95% and used for long-term storage (Khoshnevisan et al. 2011). Amylase enzymes from *Streptomyces sp.* MBRC-82 also have been immobilized on gold nanoparticles which have various medicinal applications by Manivasagan et al. (2015).

13.4.2 Immobilization by Forming CLEAs

CLEAs are insoluble enzyme aggregates which are formed by cross-linking of protein precipitates using cross-linking reagents such as glutaraldehyde. CLEAs exhibited high stability and high activity in aqueous medium as well as in nonaqueous medium. These enzyme aggregates have also showed a high stability at high temperature (Sheldon 2007). CLEAs may have a combination of several enzyme activities; such CLEAs are called multipurpose CLEAs or combi-CLEAs. Extent of cross-linking often influences their activity morphology, stability, and enantioselectivity. Nadar et al. (2016) evaluated the effects of various cross-linkers and precipitating agent on amylase activity recovery of macromolecular cross-linked enzyme aggregates (M-CLEAs) of α-amylase. Precipitates of amylase enzyme cross-linked by dextran showed 91% activity, ammonium sulfate used as precipitating agent, but glutaraldehyde CLEAs (G-CLEAs) exhibited only 42% activity. Recently, Mahmod et al. (2015) manufactured multipurpose cross-linked enzyme aggregate (multi-CLEA) with lipase and protease activity.

13.5 Homologue Augmentation and Substitution

The rate-limiting step is the major issue in enhanced production of desired metabolite which can be altered by importing the homologous enzyme from different hosts, the process known as homologous augmentation. Furthermore, turnover of the heterologous pathway can also be increased by using homologues of nonnative enzymes, known as homologue substitution. Both of these techniques were employed to deduce the carotenoid production in *E. coli* (Yoon et al. 2009). Yadav et al. proposed chimeric pathways involving each enzyme from different host to construct MVA pathway for carotenoid production.

13.6 Conclusion and Future Perspectives

In this chapter a number of enzyme engineering techniques have been briefed to improve the enzymatic characteristics together with its recycling methods. Microbial enzymes with enhanced physiological properties have greater commercial application in the industries. Protein engineering technology along with other molecular techniques and nanotechnology approaches has occupied the major position in the proteomic studies. Here it is shown that microbial enzymes, isolated from a number of sources such as fungi, bacteria, actinobacteria, yeast, and metagenomic sample, were subjected to protein engineering to modify their characteristics such as activity, temperature, and pH stability to make them economic for industrial applications. A number of modern genomic techniques such as genome-walking PCR, TAIL-PCR, error-prone PCR, StEP recombination, and metagenomic approaches have been proved as successful tools to create structural modifications at protein translational level. A few reports on site-directed mutagenesis and directed evolution have been explained as successful techniques to enhance thermostability and

pH stability of microbial enzyme. The main focus of the chapter is laid upon modern enzyme engineering techniques to obtain a microbial protein with greater operational stability together with reusability. A combinatorial approach of metagenomic, proteomics, genomics, nanotechnology, and bioinformatics is required to obtain our goal of enzyme engineering.

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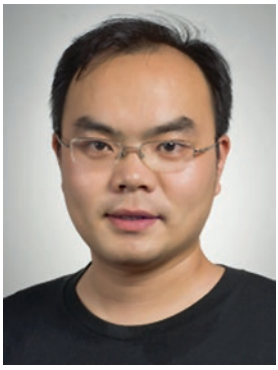

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