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# Molecular biology interventions for activity improvement and production of industrial enzymes

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**Abstract**

Metagenomics and directed evolution technology have brought a revolution in search of novel enzymes from extreme environment and improvement of existing enzymes and tuning them towards certain desired properties. Using advanced tools of molecular biology i.e. next generation sequencing, site directed mutagenesis, fusion protein, surface display etc. now researchers can engineer enzymes for improved activity, stability, and substrate specificity to meet the industrial demand. Although many enzymatic processes have been developed up to industrial scale, still there is need to overcome limitations of maintaining activity during catalytic process. In this article recent developments in enzymes industrial applications and advancements in metabolic engineering approaches to improve enzymes efficacy and production are reviewed.

**Keywords:** Enzyme; cosmetic; pharmaceutical; metagenomics; directed evolution; fusion protein

## 1. Introduction

With the revolution in the chemical industry, there is a new challenge to extend microbes and enzymes based chemical synthesis processes (Hazeena et al., 2019; Rajesh et al., 2019). For a transition from a petrochemical-based industry to a green chemistry-based economy, we must ensure that our ability to explore novel enzymes, engineer biocatalysts, and design artificial enzymes does not become a bottleneck. There is an enzyme for each natural compound and these enzymes also have capability to catalyze nonnatural chemical reactions (Sheldon & Pereira, 2017). Further, these enzymes are evolving continuously in vivo to metabolize new unnatural chemicals. Various applications of enzymes have been reported in many industries such as the food and beverage industry (amylase, lipase, pectinase), textile and leather industry (laccase, cutinase, protease), cosmetic industry (superoxide dismutase (SOD), oxidase), pharmaceutical (chitinase, streptokinase), chemical industry (epimerase, lipase), etc. (Moreno et al., 2020; Patel et al., 2019; Raveendran et al., 2018). Enzymes carry out the reactions under ambient temperatures, pressures, physiological pH, in aqueous solutions as well as in organic solvents (Bollinger et al., 2020). Further, enzyme-mediated reactions do not require any additional steps for either activation or reactivation of surface functional groups in the active sites (Wang et al., 2016). But with every process comes the limitations to be addressed; these enzymes are often selective in nature, enantioselective to specific substrates, and are not in favourable condition with non-natural substrates (Arnold, 2018). Few of the industrial processes do require catalysts that could withstand extreme temperatures and pressures, but these enzymes lose their specific activity at those conditions resulting in lower end-product yield and productivity (Hammer et al., 2017). Sometimes enzymes isolated from the natural resources or in their native form do not have desired properties which enforce researchers to search for novel enzymes. Nowadays metagenomics is routinely practiced for microbial taxonomy and finding novel enzymes (Kumar Awasthi et

al., 2020). Recently many enzymes have been discovered from diverse habitats such as endoglucanase from termite gut (Guerrero et al., 2020), α-amylase from sheep rumen (Motahar et al., 2020), esterase from lotus pond sludge (Qiu et al., 2020), etc. using a metagenomic approach. With the advancements of genetic engineering technology genes from uncultured, pathogenic, and slow growing microbes can be cloned into genetically recognised as safe (GRAS) microbes for higher production (Sharma et al., 2019). Despite of exploration of different habitats for novel enzymes still, their industrial applications are hampered by low activity, stability, and specificity. To overcome these limitations a variety of engineering approaches such as directed evolution, fusion protein, surface display, the introduction of unnatural amino acids have been applied to improve enzymes (Emond et al., 2020; Guirimand et al., 2019a). Information generated on the basis of gene sequence, structure-function relationship, enabling researchers to design new enzymes having novel function (Mayer et al., 2019).

In the present scenario, enzymes are an important part of our routine life and many industrial applications. With the advancements in biotechnology and molecular biology tools, researchers have capability to engineer and design new enzymes having improved and novel functions. The main objectives of this article are to provide an updated view on industrial applications of various enzymes and different molecular biology approaches to search for new enzymes and improve the existing ones for increased activity, and stability.

## 2. Industrial applications of enzymes

From ancient times microbes and enzymes are used in food processing and preparation of beverages (Far et al., 2020). During the last few decades, applications of various enzymes extended to various sectors such as food and feed, textile & leather processing, cosmetic, pharmaceutical, and bioenergy sectors (Table 1) (Raveendran et al., 2018). All this is possible

due to advances in technologies involved in microbial cultivation, throughput screening, and microbial fermentation technologies. A greater share of the enzyme is produced utilizing microorganisms (bacteria and fungi) due to their rapid growth, minimum nutrient requirements and easy to culture at a large scale. Almost 75% of the enzyme market is covered by hydrolases (protease, lipase, amylase, cellulases) and their demand is increasing continuously (Jemli et al., 2016). In the food industry enzymes are mostly used to perform hydrolytic reactions to improve solubility and clarification (Mahmoodi et al., 2017). Recent trends in the food industry include the development of functional food (prebiotics and probiotics) and artificial sweetener etc. (Choi et al., 2015). In the textile industry the fibres require different processing steps such as seizing, bleaching, dying, and functionalization. These all steps are energy intensive and require various chemicals. Enzymes are getting interest in the textile field and can be used to perform a function such as desizing, wrinkle proof, and improve other properties (Duarte et al., 2020; Wang et al., 2018). In the cosmetic industry a variety of chemicals are used and produced from petrochemical based material. Nowadays consumers are looking for cosmetic products based on natural compounds, so companies are now looking for various enzymes have cosmetic related applications. The use of various enzymes such as superoxide dismutase (Li et al.), protease, and lipase, etc. have been explored for cosmetic related applications (Gomes et al., 2020).

With the introduction of advanced technologies such as sequencing, metagenomics, site directed mutagenesis, fusion protein, bioinformatics, and computational tools it is become possible to improve the existing enzymes and design new enzymes that have novel properties. These advancements in enzyme research shifting the enzyme market towards other sectors such as the synthesis of chiral molecules (amino acids, amino alcohols, and amines) required for pharmaceutical purposes. The utilization of enzymes in the synthesis of

various pharmaceutical important compounds are already reviewed and discussed (Choi et al., 2015). Energy demand is increasing continuously, and overexploitation of fossil-based resources will lead to the depletion of these energy resources in the next few decades. There is a need to find alternate sources of energy that are sustainable, renewable, and eco-friendly. Lignocellulose is the abundantly used raw material and can be utilized as raw material for microbial fermentation to produce biofuel. Various enzymes such as cellulases, hemicellulases, laccases, lignin peroxidases, are used to pre-treat and hydrolyse lignocellulosic biomass into free sugars (Østby et al., 2020). Other enzymes like carbonic anhydrase, fatty acids decarboxylase are also used in the energy sector to produced advanced biofuels (Bhatia et al., 2019; Jiang et al., 2019).

**3. Molecular biology approaches for improved enzyme production**

Enzymes as biocatalysts are used in almost all industrial sectors to fasten the rate of biological and chemical reactions. Microorganisms are considered the best source of enzymes as these can be easily cultivated at much faster rates in fermenters and thus are the preferred choice for mass production of industrial enzymes. Also, microorganisms have been isolated from the widest ecosystems and extreme environments – hot springs, cold glaciers, and ice bergs, high pressure, acid mine drainages, hypersaline environments (Mirete et al., 2016). These microbes have evolved and adapted themselves to extreme conditions and are known to produce extremozymes – which may be well suited for industrial processes (Bhatia et al., 2020a). Enzymes having unique properties can be isolated from uncultured microbes using a metagenomics approach. Alternatively, we can modify or engineer the existing enzymes to enhance their utility for commercial applications using various molecular biology techniques such as directed evolution promoter screening, engineering of translation and transcription factors, codon optimization and protein fusion, etc.

### 3.1 Metagenomics and genome mining for novel enzymes

Metagenomics, also known as population genomics, community genomics, and environmental genomics, is the study of genome structures of microbial communities present in diverse environmental conditions. This technique eliminates the need of culturing the microbe using conventional culture techniques and makes use of direct sequencing techniques, to identify the gene of interest. Environmental samples are directly used to isolate gDNA and create metagenomic libraries, in suitable vectors. Depending on the insert size, several options have been explored. These include plasmids, cosmids, fosmids, Bacterial Artificial Chromosomes (Thimoteo et al.), λ phage (Madhavan et al., 2017). The vectors are further transformed into suitable expression systems – *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas,* and other eukaryotes (Lorenz & Eck, 2005). This process allows the mining of new enzymes avoiding the problems associated with non-culturable microbes through sequence based and functional based screening. Sequence based metagenomics involves sequencing of DNA from environmental samples, genome assembly, gene identification, a search of the complete metabolic pathway, and compare organisms of different communities. With the advent of next generation sequencing (NGS) it is possible to generate a large amount of data in a fraction of the cost as compared to traditional sequencing technologies (i.e., Sanger) (Koutsandreas et al., 2019). NGS and advanced computational tools allow to analyse a large set of sequencing data, but annotation of genes totally relies on sequence similarity of the already characterized genes in the public database. Due to this limitation, almost 40-50% of genes in the genome are routinely reported as hypothetical. In functional metagenomics, metagenome libraries are screened for the desired protein function. Metagenomics is not able to distinguish the active member from the inactive member of the microbiome community. To improve enzyme screening and selection process it is suggested that samples should be analysed by integrating metagenomics with other omics techniques

such as meta-transcriptomics and meta-proteomics etc. Using metatranscriptomics i.e. RNA sequencing (RNASeq) of expressed transcripts within a microbiome at a time point under a set of environmental conditions, actively expressed proteins can be easily screened (Shakya et al., 2019). In the metaproteomics approach, the entire protein content of environmental microbiota at a given point in time is analysed. The use of metaproteomics for the analysis of extracellular proteins from natural environmental samples is a complicated task as these proteins cannot be easily and reproducibly separated and concentrated from the extracellular matrix (Speda et al., 2017). Because of this issue majority of metaproteomics studies are performed for the study of the intracellular fraction of metaproteome. Bioinformatics analysis is further used for sequence analysis, Pfam analysis, structure prediction, and understand its phylogeny (Prayogo et al., 2020).

Several cellulases, proteases, lipases, α-amylase, chitinase, β-glucosidase, and endoglucanses have been isolated from extreme environmental conditions, using a metagenomic approach and patented (Prayogo et al., 2020). Such enzymes have shown much better thermostability and thus are better suited for industrial applications. Identifying and choosing the unique environments and niches, is the most important step for the successful identification of novel enzymes, with improved properties. Innumerable examples of such intelligent screens are available in the literature. Metagenomic libraries obtained from mangrove sediments have successfully been used to isolate proteases, enzymes commercially used in the food industry, and bioremediation processes. Candidate PR4A3, an alkaline metalloprotease enzyme, has demonstrated high thermotolerance and thermostability. It has shown promising results for biotechnological applications (Pessoa et al., 2017).

Cellulases are widely used in biofuel production, detergent, paper, and cotton industry. Thermostable cellulases have been isolated from the cellulose rich environment of the bamboo paper pulp industry (Prayogo et al., 2020). Metagenomic cellulases have also

been isolated from diverse niches like soil, animal and insect microbiomes, faeces, and compost samples (Tiwari et al., 2018). Lipases are other important enzymes, widely used in food, detergents, paper, and pharmaceutical industries. Metagenomics has proved an effective tool for mining of lipases from uncultured microflora (Almeida et al., 2019; Tang et al., 2017). These metagenomic lipases – LipS, LipG9 and LipR1 have shown promising enantioselectivity for resolution of alcohols, carboxylic acids, esters from racemic mixtures, biodiesel ester, and flavour ester synthesis (Almeida et al., 2019). Amine transaminases (ATAs) have particularly emerged as valuable biocatalyst for the production of drug intermediates and stereoselective chemical compounds like sitagliptin, dilevalol, formoterolo, and mexiletine (Koszelewski et al., 2009; Savile et al., 2010). A limited number of ATAs has conventionally been isolated from bacterial and fungal strains, after repeated rounds of directed evolution. These enzymes require tremendous improvement in properties like thermostability, stability in organic solvents, and storage, for industrial applications. Metagenomes from hot springs of China, Italy, and Iceland, have yielded three thermostable ATAs (Ferrandi et al., 2017). Thermotolerant Ω-TA has also been isolated from metagenomes isolated from hypersaline environments of Triassic salt mines. These novel enzymes have adapted to halophilic conditions with better stability in organic solvents (Kelly et al., 2019). Xylanases, isolated from mesophilic fungi *Trichoderma ressei*, have found usage in wide industries ranging from the food and feed industry, paper and pulp industry, bioethanol, and biofuel production. But these enzymes have lower stability at high industrial temperatures. Thermostable xylanases, with higher activity compared to *T. ressei* xylanase have been isolated from metagenomes derived from lignocelluloses enriched composts (EllilÃ et al., 2019). Furthermore, 25 families of thioesterases have been identified to play active role biocatalysis of protein, lipids, and aromatic compounds metabolism. Metagenomic approach has been used by the researchers to isolate novel thioesterases belonging to family

13, from activated sludge samples, which play important role in biocatalysis of phenylacetic acid and 4-chlorobenzene, both compounds associated with environmental pollution (Sanchez-Reyez et al., 2017).

The efficiency of the strategies can be improved by using specific enrichment techniques or using high through-put screening systems (Lorenz & Eck, 2005). The simpler screening techniques make use of chromogenic and fluorogenic substrates like methylumbelliferyl (MU)-glycoside substrates, for identifying the activity of enzymes. Another approach makes use of substrates with self immolative linkers, specifically for screening of unsaturated glucuronidases (Nasseri et al., 2018). Substrate induced gene expression screening (SIGEX), which contains GFP as a reporter gene is another interesting approach to identify novel enzymes from metagenomic libraries. It is based on the fact that the expression of GFP is induced by the production of certain metabolites (Madhavan et al., 2017). Recent advances in the use of ultra-high thoroughput screens (uHTS) can be further explored to increase the speed of novel enzyme discovery from metagenome libraries. Traditional agar-based screenings can be replaced by 96-well microtitre plate (MTP) assays to quickly screen numerous chromogenic and fluorogenic substrates, thus decreasing the screening time of metagenomes. Genetic selection systems based on either gain of function or loss of function strategies, are one such complex examples of uHTS. These methods have successfully been employed for the isolation of novel β-galactosidases and vitamin biosynthesis genes (Cai et al., 2018). Droplet and flow cytometry based sorting of metagenomes has also been used to screen large biosynthetic clusters, which can further be sequenced (Markel et al., 2019). Despite all these studies, bioprospecting for novel enzymes still remains a challenge. Choosing the unique environments, appropriate sampling of the environmental samples followed by smart screening techniques is a prerequisite for the success of the metagenomics approach.

### 3.2 Engineering for enhanced enzyme production

A quest for novel enzymes, with optimum properties for industrial usage, can be further accentuated by using metabolic engineering approaches. Genetic material contains all the information which decides its structure, function, secretion, and regulation. Thus, strategies involving modifications of genetic structures have been proved as an effective tool to enhance their production.

#### *3.2.1 Promoter optimization*

A very first approach for engineering for enhanced enzyme production can be at the level of a promoter. The promoter region provides the site for RNA polymerase to bind and initiate the transcription. A strong constitutively expressing promoter with high affinity must be ideal for large scale production of biotechnological products (Qin et al., 2011). Breakthrough in sequencing methods and bioinformatics tools has provided data for the screening of new promoters, in non-model organisms. This approach has been used to identify new promoters in *Clostridium thermocellum*, a promising candidate for the production of ethanol. Promoters 2638 and 815 were found to have higher activity for reporter genes (Olson et al., 2015). Promoter engineering is the need of the hour. This can be achieved either by mutagenesis, engineering of native promoters or by screening for the strong synthetic promoters. Random mutation inside the inulase promoter and signal peptide of *Kluyveromyces marxianus* has shown to improve the secretion of lignocellulolytic enzymes (Zhou et al., 2018). Alternatively, a strong promoter can be cloned along with the gene of interest, to increase its production. Cbh1 is considered as a strong promoter and employed to overexpress the gene of cellobiohydrolase II (*cbhII*) to increase its production in *T. ressei* (Fang & Xia, 2013). In another study, the production of cellulases has been improved in *S. cerevisiae*, by placing the native SOD1 and PSE1 under *pgk1* promoter (Kroukamp et al., 2013). Rational engineering approaches have also provided good results. *kasOp** is one such example of engineered

promoters from *Streptomyces coelicolor.* This promoter has been modified in two steps, by removing the binding site of its two regulators ScbR2 and ScbR (Wang et al., 2013).

$P_{GAP}$ is one most commonly used promoter used for heterologous protein expression in *P. pastoris* (Zhang et al., 2009). It has been used for the constitutive expression of lipases in high cell density conditions (Wang et al., 2012). Efforts have been made to further improve it by generating its promoter library using Error prone PCR. These mutants provided many folds increased expression, which can further be explored for engineering interventions (Qin et al., 2011).

### *3.2.2 Increased expression by transcription regulation*

The fine interplay between activators, inducers, and repressors molecule in any organism, is responsible for the regulation of biosynthetic pathways, at the transcription level. Transcription factors are required for the expression and production of enzymes. CLR4 is for example one such conserved regulator for the production of cellulases in *Neurosopra crassa* and *Myceliophthora thermophila* (Liu et al., 2019). Overexpressing the transcription factors has been used as an effective strategy to increase the expression of target genes in *S. cerevisiae* (Alazi et al., 2018; Chua et al., 2006). The same phenomenon has also been observed in *T. Ressei* and *A. oryzae*. Overexpression of XlnR, a transcriptional activator of cellulolytic genes in *A. oryzae* has been shown the levels of hydrolytic enzymes (Noguchi et al., 2009). Genetic modification of Xyr1, has been utilised as a promising strategy to increase the production of cellulase, cellobiosidase and β-D-glucosidase in *T. Ressei* (Jiang et al., 2016). The production of several important enzymes, in presence of substrates like glucose has been shown to be regulated by creA mediated carbon catabolite repressor, thus requiring presence of inducers in culture conditions to produce them (Niu et al., 2015).

Few interesting studies have successfully shown inducer independent hyper production of enzymes like cellulases. This has been achieved by overexpression of

transcription regulator XlnR, under the control of strong copper responsive promoter $P_{tcu1}$ (Lv et al., 2015). Such copper responsive promoters have also been shown to overexpress the genes in *Cryptococcus neoformans* (Lamb et al., 2013). Thus, this strategy opens horizons for hyper production of specific cellulases, under non inducing conditions, with higher purity and reduced downstream processing costs. In another study hyper production of pectinases from *A. Niger*, has been achieved by overexpression of transcription factor GaaR and deletion of creA (Alazi et al., 2017).

Furthermore, the role of sigma factors can also be explored to increase the rate of transcription in different fungi. Such engineering studies have been done in cyanobacteria to increase the bioproduction of valuable products like ethanol, fatty acids, hydrogen etc. (Srivastava et al., 2020).

***3.2.3 Increased expression by translation regulation***

Bioproduction of industrially relevant enzymes requires engineering at multiple gene expression levels. Translation processes can be explored in this direction. Translation processes are not only responsible for the synthesis of protein from mRNA, but also affect their folding, structure, and secretion of proteins. To achieve higher production of enzymes, all mRNAs must be translated into proteins and folded into correct structures. Quantitative proteomics studies have indicated the role of ~17 different translation related factors in increasing the production of xylanases in *P. pastoris* (Lin et al., 2013). Heterologous gene expression of enzyme phytase, in engineered microbes, like *P. pastoris* can be improved by overexpression of Bcy1, a regulatory subunit of protein kinase A (Liao et al., 2020). Certain transcription factors like Fhl1p have shown a role in rRNA processing and transcription of ribosomal proteins, thus regulating the rate and efficiency of translation processes. Higher expression of Fhl1p, has shown to increase the level of phytases and pectinases (Zheng et al., 2019). Ribosome Binding Sites (RBS) are other effective targets that must be considered to

improve the translational efficiency. Several RBS libraries are available and can be screened to increase the flux of translation reactions (Jervis et al., 2019). Such studies have been reported in *E. coli* and cyanobacterium *Synechocystis* sp. (Thiel et al., 2018).

The effectiveness of enzyme production is also limited by their accumulation in inappropriate compartments or improper translocation. This can be overcome by engineering the signal peptides. The native signal peptides can be replaced with more efficient and proven signal peptides. Higher production of penicillin G acylases (PGAs), cloned into *E. coli* have successfully been done using this approach (Pan et al., 2018). Original signal peptides were replaced with more efficient pelB and MalE signal peptides, resulting in overexpression of PGAs. Gene expression levels can also be regulated by using different induction systems. For large scale production of enzymes, high density growth of microorganisms is desired. Then, this can be followed by induction of gene expression. The most common systems used for this purpose are *lac* and *tet* systems (Llanes & McFall, 1969; Orth et al., 2000). The main disadvantage of these systems is their low-level expression, in non-inducing conditions. These systems can be replaced by more promising theophylline inducible riboswitches, for enzyme expressions (Kamiura et al., 2019).

### *3.2.4 Increased expression by codon optimization*

Codon optimization is a technology used to redesign a gene to improve the protein expression level without altering the amino acids. However, a protein is comprised of only 20 different types of amino acids and there are 64 codons. Out of these, 61 codons code for 20 amino acids (2 amino acids coded by 1 codon, 9 with 2 codons, 1 with 3 codons, 5 with 4 codons, and 3 with 6 codons) and three codons do not specify any amino acid and function as a termination codon. Codon degeneracy provides several options to encode a protein e.g. a 375 amino acid protein can be encoded by $10^{207}$ various sequences (Şen et al., 2020). All the possible codon sequences are not equally observed in nature; however, organisms prefer a

synonymous codon over the other (codon bias) which directly corelated with the expression level of a gene. For example, in *Homo sapiens* amino acid leucine mostly (39.5%) coded by CUG codon, while the same codon is used only 11.1% times to code this amino acids in *S. cerevisiae* (Şen et al., 2020). Codon optimization is considered an important factor to affect the gene expression level in different hosts. Using frequent and more host specific codons instead of the rarely observed ones level of protein expression can be improved by $10^5$ folds (Gustafsson et al., 2004). Various sequence design software such as Codon optimizer, Gene Designer, and OPTIMIZER are in trends to optimize the frequency of individual codon occurrence. The main factors which affects the expression of recombinant proteins include a selection of the proper host, the sequence of open reading frame and upstream and downstream regulatory elements (Ghahremanifard et al., 2018).

*Codon optimization in E. coli*: *E. coli* is a widely used host for heterologous protein production due to its fast growth, easy to genetic modification, and requirement of inexpensive culture medium. Usage of codon biased methods improves the expression of heterologous proteins expression in *E. coli*. The competition for rare tRNA also affects enzyme expression level e.g. AGA and AGG codon which code for arginine are rare in *E. coli* and may cause translation errors or low expression. The codon bias can be mitigated by expressing the genes code for rare tRNA or using mutagenesis to change the rare codons with more commonly used codons (Liu et al., 2012a). Various codon optimizations examples are discussed in table 2.

*Codon optimization in Pichia pastoris*: *P. pastoris* is a widely utilized host for heterologous protein expression for research and industrial scale. However, the expression level of different proteins is affected by factors linked to mRNA translation, post translation modification, and secretion efficiency (Tian et al., 2012). Tian et al. cloned an acetyl xylan esterase (AXE1) cDNA from edible mushroom (*Volvariella volvacea*) into *P. pastoris* and

reported lower expression level 1.88 IU/ml after 8 days, after codon optimization by reducing GC content from 55.49% to 48.62% they able to increase expression of AXE1 up to 136.45 U/ml (Tian et al., 2012). *T. reesei* cellulase has many industrial applications including paper, and textile industry, Akcapiner et al. applied codon optimization for endoglucanase 1 (EGI) to express it in *P. pastoris* and able to increase its activity by 1.24 fold as compared to native EGI (Akcapinar et al., 2011) (more examples are discussed in Table 2).

## 4. Engineering for improved and new catalytic activity

Through the decade's biologists, and process engineers opted physical, structural, and genetic modification strategies to re-design the existing enzymes to provide high yields, selectivity, tolerance to high substrate concentrations with low enzyme loading. The advent of new recombinant tools and the availability of genome databases led to the development of efficient and economical processes resulting in enzymes performing natural and non-natural reactions. The strategies employed to improve the specific activity, stability, and selectivity of the enzymes are site directed mutagenesis, directed evolution, fusion of multiple enzymes using linkers, cell surface display technology, and introduction of uncanonical amino acids.

### 4.1 Directed evolution

The scenario of synthetic catalysts for reactions of commercial interest can be replaced with biocatalysts. These biological catalysts can perform highly enantio and regioselective reaction mechanisms on the substrates of interest with an enhanced specific reaction rate. But the commercial reaction processes and their physiological conditions do not favors the naturally occurring enzymes. Directed evolution is one such technique that complements novel or improved functionalities on the enzymes. Evolution is the Darwinian principle of natural selection, directed or laboratory evolution mimics the Darwinian theory, and forward engineer the enzymes to adapt and recombine to yield a recombinant enzyme with enhanced properties at a higher pace (Arnold, 2018). How does directed evolution work? The

mechanism couple random mutagenesis, DNA recombination, selection, and high throughput screening strategies (fig. 1a). In the concept of directed evolution, a mutant library is created, then selected or screened based on the desirable trait, the process is repeated 'n' times until the desirable trait is observed. Higher mutants in the library can be observed if there is a probability of an increased number of mutations, the mutations can be induced in the peptide in the genomic level either by error prone PCR, site saturation, chemical mutagenesis, and DNA shuffling. Approximately 5.6 mutations can be observed per amino acid, then if the peptide is 20 amino acid long, the mutant library obtained have a very high chance of having the mutant with the desired phenotype. The mutant gene obtained will be cloned into a compatible plasmid and transformed into a chassis strain for functional expression of the recombinant protein. Then the important approach is the selection and screening of the large number of mutants generated by directed evolution. In a study whole cell biocatalyst *Bacillus amyloliquefaciens* SS35 was irradiated with UV light to generate the mutants that could produce endogluconase and carboxy methylcellulase (CMCase) enzyme with improved activity and stability. The CMCase from the mutants displayed pH stability in the alkaline range 7.0 – 11.0, with 1.6 - 4.1-fold increase in activity. The enzymatic hydrolysis of lignocellulosic biomass using the mutant enzyme resulted in 1.8-fold increase in sugar release (Singh et al., 2020b). Similarly, the rate limiting enzyme of cellulose hydrolysis, and the component of cellulase, the β-glucosidase, is sensitive to the end product glucose, although various glucose tolerant enzymes were discovered, a novel Bgl6 with inhibitory concentration $IC_{50}$ ~ 3.5 M glucose, with poor thermal stability at 50 $^{o}$C, has undergone random mutagenesis, the resultant M3 mutant showed 3 fold increase in catalytic activity ($K_{cat}/K_m$). The cellobiose hydrolysis using the mutant M3 enzyme has 20% increased conversion efficiency than the wild type (Cao et al., 2015). Directed evolution generates new functional variants but the major challenge involved is a theoretically small fraction of the whole

sequence is accessible for screening and selection. Hence generation of wide quality and quantity of mutant libraries is desired. Some of the enzymes that are evolved to perform new functions are P411 engineered from Cytochrome P450. The mutant P411 is able to perform intermolecular benzylic C-H amination with >99% enantioselectivity. The *Rhodothermus marinus* cytochrome C oxidase evolved enzyme with chemoselective for Si-H insertion, could catalyse chiral organosilicon product formation from ethyl-2-diazoproponate and phenyl dimethyl silane, and the same enzyme was observed to catalyse chiral organoborane product formation from carbene precursors and borylating agents (Arnold, 2018). An unspecific peroxygenases was observed as the missing link between heme and cytochrome P450 peroxidases, these new peroxidases are soluble extracellular and highly stable observed as in *Cyclocybe aegerita.* The gene responsible was expressed in *S. cerevisiae* and undergone directed evolution by inducing 4 mutations in the native signal peptide and 5 mutations in the mature protein, resulting in 27-fold increase in secretion and 18-fold increase in $K_{cat}/K_m$ (Alcalde, 2015). Hence directed evolution can fine tune the enzymes to perform new reactivities. Few other examples of enzymes improved by directed evolution are tabulated (Table 3).

**4.2 Site directed mutagenesis**

Site directed mutagenesis (SDM) is a specific tool to perform rational or semi rational mutagenesis, which involves editing of an amino acid at a particular site that do alter the functionality of the protein (fig. 1b). SDM is possible in enzymes with known sequence, structure, active site conformation, evolutionary relations, and reaction mechanism (Baweja et al., 2016). Either by insertion or deletion at a specific site, in less time, the recombinant protein with increased specificity, stability, activity, and solubility can be obtained. The commercially available enzymes like proteases and lipases have improved effectively. For example, natokinase, a bacterial serine protease, having applications in developing

cardiovascular drugs, catalytic efficiency, and stability was increased by 2 rounds of SDM. Similarly combined random mutagenesis and SDM of Harobin, a serine protease improved its fibrinolytic and antithrombosis effect (Baweja et al., 2016). A series of site directed mutagenesis in *Bacillus pumilus* W3 resulted in a mutant S208G/F227A, in which *CotA* laccase, the enzyme responsible for decolorization of dyes and degradation of toxic substances was observed to have mutations at Gly208 and Ala227 sites, that decreased the thermal stability but increased the catalytic efficiency by 5.1-fold (Xu et al., 2016). Hence the knowledge on the structure and the configuration is very much important before inducing SDM, the amino acid change in one or more sites may have a positive or negative influence on the resulting protein. In a L-arginine biosynthesis strain *Corynebacterium crenatum*, in a rate limiting enzyme N-acteyl-L-glutamate kinase, D311 and D312 amino acids are replaced by arginine, resulting in 3.7-fold and 14.6% increase in inhibitory concentration and enzyme activity respectively (Zhang et al., 2015). In a study carried out by Fang and associates ornithine carbamylase that catalyse the formation of ornithine and carbomoyl phosphate from citrulline and arginine was engineered through SDM, at 9 different sites, out of which H140A, Q143W mutants displayed 2-3 times increase in catalytic activity and increased tolerance to ethanol. Another mutant with a point mutation D236R has shown a 1.4-fold increase in thermal stability (Fang et al., 2020). Either single or multiple mutations in the mature protein with a sophisticated screening technique would be an ideal approach for generating desirable enzymes with improved functionalities.

**4.3 Fusion protein linkers**

Either in natural or non-natural biosynthesis reactions, the end-product may not be produced in a single step, so the complex coupled reactions have various limitations in the form of stability, productivity, insufficient functional expression, and tolerance to the intermediates. Even the traditional site directed and random mutagenesis with high throughput screening

methods makes the selection of protein with desirable phenotype tedious. But the answer is? In nature, the complex biosynthesis pathways and other cascade reactions are finely regulated either by extracellular or intracellular open reading frames, subcellular reaction compartments, membrane associated complexes, protein clusters, and modular fusion proteins. If we can mimic the natures ability to bring the different enzymes in proximity, we can overcome the limitations in the commercial scale. A new technology of fusing the two or more catalytically complementary enzymes resulted in chimeric or recombinant protein with enhanced catalytic efficiency. For example, a 230 KDa cellulases from *Anaerocellum thermophilum* are the natural enzyme clusters with active endo and exogluconase activity. Later various dehydrogenases and transaminases are artificially fused using the linkers like fusion of alcohol dehydrogenase and cyclohexanone monooxygenase. The fused product could mediate two step reaction for the conversion of cyclohexanol (alcohol) into ε-caprolactone (ketone) (Aalbers & Fraaije, 2017). With the fused enzyme cascade or multistep experiment, complex molecules can be produced in a single step without the need of separation and purification of the intermediates. It was also observed that covalently linked enzymes outperformed the mixture of enzymes in expression levels, conversion rates, stability, and activity (Aalbers & Fraaije, 2017). So how these enzymes are fused? Multiple genes coding for these proteins are arranged in an open reading frame without stop codons in between each gene, wherein a sequence encoding peptide linker is introduced, further transcription, translation and posttranslational modifications yields a fused protein (fig. 1c). The activity and the stability of the fused protein depends on order of the genes and the linker of choice, for example Pazmiño and co-workers fused phosphite dehydrogenase (PTDH), NADPH cofactor recycling enzyme with Baeyer-Villiger monooxygenase (BVMO) enzyme, the fused enzyme has displayed complete catalytic conversion efficiency, in either of the orientations PTDH-BVMO or BVMO-PTDH. However in a redox neutral cascade reaction

by coupled alcohol dehydrogenase (Tb ADH) from *Thermoanaerobacter brockii* and cyclohexanone monooxygenase (Tm CHMO) from *Thermocrispum municipale*, Tb ADH – Tm CHMO has almost retained wild type activity, and Tm CHMO – Tb ADH has four fold reduced ADH activity (Aalbers & Fraaije, 2019). Similarly, the composition of the linker, physical characterisitics (flexibility, rigidity, hydrophilicity, hydrophobicity), length has an impact on the stability, activity, and turnover of the fused enzyme. In a study of effect of linker length, repetitive units of three amino acids proline, alanine, and serine of different length say PAS 60, PAS 40, and PAS 20 sequences are used to fuse alcohol dehydrogenase and transaminase, which converts alcohols to amines. Final observations were interesting to find, how important is the length of the linker; PAS 60 has the highest soluble expression levels, PAS 40 highest retaining of specific activity, and PAS 20 has 2-fold increased conversion efficiency than the individual enzymes. Various research groups have worked on the characterization of linkers, its efficiency, based on the simulations and structure mediated studies resulted in the construction of linker libraries. It was observed that glycine rich linkers are flexible, and alanine and lysine rich linkers are rigid in nature (Chen et al., 2017). These linkers are not just the connections between two proteins, it has a major role in post-translational modifications, enzyme orientation, display of an active site for substrate binding and further reaction rate (Chen et al., 2017). These fusion proteins can display their activities in various fields like pharmaceuticals, food and beverages, bulk, and specialty chemicals. For example, astaxanthin, a well-known food grade additive, red-colourant, having anti-oxidant activity is produced by various plants and microorganisms. In a study two bacterial enzymes, 3,3'-β-carotene hydroxylase (CRTZ) and 4,4'- β-carotene oxygenase (CRTW) are fused with different linker combinations, the fused protein can convert β-carotene to astaxanthin. After the expression in *E. coli* the fused protein with glycine rich small (10 a.a) and medium (20 a.a) size linkers has higher activity than the longer (29 a.a) linker, as shorter linkers might

have enhanced the substrate and intermediate channelling between the proteins (Nogueira et al., 2019). These hybrid or fused proteins gaining new functionality, derived from individual enzymes or domains, can either misfold, or lead to impaired activity after the post-translational modifications without the presence of linkers. Linker with composition (GGGGS)n (n<6); enhance flexibility and solubility, similarly (EAAAK)n (n<6) imparts rigidity and helical linker between the proteins (Chen et al., 2017). Usually flexible linkers are more preferred, but in a study related to monooxygenase activity of cytochrome P450s, *B. subtilis* flavodoxin was fused with *E. coli* flavodoxin reductase using flexible (GGGGS)n linker and rigid (E/LPPPP)n linker, due to greater separation the fused protein with rigid linker outperformed (Bakkes et al., 2017). Similarly, in a leucine biotransformation, where cofactor regeneration is necessary, formate dehydrogenase and leucine dehydrogenase enzymes are fused using a rigid linker, that tends to form a tunnel like structure favouring the intramolecular movement between C-terminal FDH and N-terminal LeuDH. The combination FDH-3 rigid linkers (R3)-LDH increased relative specific activities up to 145 and 103% respectively (Zhang et al., 2017). We observed that linker could enhance the activity, but linkers have a functional role in solubility and stability of the protein, the work carried out by Degregorio and associates explains the increased solubility of human liver cytochrome P450s by fusing with reductase from *Bacillus megaterium* BM3 using 3 and 5 glycine residues as linkers. Both the fused proteins displayed stability, increased solubility, fused protein with 3 glycine linker has highest redox potential and NADPH oxidation state, whereas in 5 glycine linker fusion protein Vmax increased 2-fold (Degregorio et al., 2017). Basic pre-requisites to be considered in designing fusion proteins are; (i) efficient translation machinery, (ii) orderly fashion of proteins to be fused based on structure, size and confirmation, and (iii) designing of linkers for proper flexibility to retain functionality and catalytic efficiency (Elleuche, 2015). Usually longer the linker, the peptide will be prone to proteolytic cleavage thereby

separating the fused proteins, hence optimum length of linker to be accessed either through literature search or by computational simulations.

**4.4 Unnatural amino acids**

The proteins and peptides are synthesized using canonical amino acids, that do regulate biological complex reactions, and recruit co-factors or undergo post-translational modifications for additional bioconversions (Drienovská & Roelfes, 2020). For example, covalent binding of cyclins to kinases, chaperons to HSP90, NAD to sirtuins, and AMP to AMP activated kinase results in an increase in catalytic efficiency ($K_{cat}/K_m$) (Darby et al., 2017). In the usual mRNA translational process L-amino acids (aa) are incorporated for the peptide chain biosynthesis, but few D-amino acids are found which are the derivatives obtained through enzymatic conversion of L-aa. The non-ribosomal, ribosomal, and post translational modified peptides have a racemic mixture of D and L aa's, and further, undergo conformational resolution by a few downstream enzymes. For example, in dehydratases and hydrogenases L-serine / L-threonine are converted to D- alanine / D-ethyl glycine (Vagstad et al., 2019). The concept of a designer or recombinant enzymes has brought great attention due to their capability to catalyse abiological transformations or the reactions which are not existed in nature, improve the catalytic nature, substrate binding capacity, or the specific activity in the existing biocatalysts. How do these unnatural or non-canonical amino acids have a role in the engineering the enzymes? Incorporation of these non-canonical amino acids during translation yields a peptide with unique reactive functional groups as the side chains, because of which the catalytic repertoire of the individual enzyme could be expanded (Hu & Wang, 2016). These changes could also impart novel functionalities and improve the specific activity of the enzymes. So how to alter the genetic code for the incorporation of unnatural amino acids. Sung Lee and co-workers have explained in detail the two ways of incorporation, (i) site specific incorporation, wherein a stop codon or 4 base codon is

assigned for unnatural amino acids following orthogonal translation system, or Re-assigning or replacement of one natural amino acid residue with unnatural amino acids. For example, genetic code UUU re-assigned to L-3-(2-napthyl) alanine (LNA), but in translation AU base pairing dominates GU pairing hence instead of LNA higher concentrations of natural phenylalanine got incorporated into the peptide chain. As the genetic code is degenerate, if a rare codon is re-assigned like AGG, decoded by less abundant tRNA's and 1 out of 6 codons for arginine, the resulting peptide chain is incorporated with abundant unnatural amino acids (Lee et al., 2015). The active site confirmation and the surface residues are very much important in maintaining the substrate specificity, and reactivity of the enzyme. Incorporation of these unnatural amino acids could assist in fine tuning of the active site, but the approach is not just confined to the active site, in 2001, 4 phenylalanine residues in Pvu II restriction enzyme were replaced with ortho (o), meta (m), and para (p) – fluorophenylalanine, the enzyme incorporated with m-fluorophenylalanine has 2-fold improvement in its specific activity than the wild type (Dominguez Jr. et al., 2001). Similarly, replacement of tyrosine with m-flurotyrosine in transaminase, increased the enzyme stability and final titers of phenyl ethylamine, replacement of methionine with norleucine in cytochrome P450 peroxidase increased its activity by 2-fold (Drienovská et al., 2018).

The major requisites for cell free protein or peptide synthesis are co-factors, salts, amino acid residues (standard and unnatural), tRNA's, mRNA template, and energy source. The translation can be carried out either via frameshift suppression, global suppression, amber suppression, or sense codon re-assignment as discussed above to incorporate unnatural amino acids (Gao et al., 2019). Introduction of these unnatural amino acids in the cellular protein machinery would improve the chemical and thermal stability, enable access to metal ligands, co-factors, resulting in hybrid enzymes catalysing physiologically irrelevant but commercially important reactions.

**4.5 Surface display technology**

The concept is comparable to the immobilization technique wherein enzymes are either adsorbed on to an inert matrix, covalently linked with a support, entrapped in a medium through ionic interactions or hydrogen bonding. The immobilization technique has offered the biocatalysts the means to increase its stability, resistance to harsh process conditions, and more importantly the ability to reuse the catalyst. In a whole cell or enzyme mediated biosynthesis, the major drawbacks usually observed are substrate transport and enzyme purification. The surface display technology (SDT) addresses these limitations by presenting the proteins or peptides on the extracellular surface of the cell, where the reaction could occur in the external medium preventing the substrate transport inside the cell (Madhavan et al., 2017). So how does the protein or peptide gets displayed? The protein to be displayed (Traveller protein) is fused with the native or heterologous anchor (Carrier) protein. Later the signal peptide in the carrier assists the traveler in transfer from inner membrane, periplasm and finally anchored on the outer membrane. The orientation of the traveler protein, and size is dependent on the carrier protein. The SDT can be applied in a different range of host cells from viral capsids, bacterial spores, other prokaryotes, and eukaryotes (Smith et al., 2015). The presenting of proteins or peptides on the cell surface assist in protein library screening, recombinant viral vaccines, biosensors, whole cell biocatalysts in bioremediation, bioconversion, biotransformation, and biodegradation processes (Gallus et al., 2020). The successful display of the passenger protein involves a series of networks in the host cells, beginning with an efficient signal peptide to direct the traveler towards the secretory pathway, recombining with the anchor or surface motifs, and further tagging of epitope for expression and detection. The most widely used prokaryotic surface motifs are a fused protein outer membrane protein A (OmpA)-lipoprotein (LPP), an autotransporter and Ice nucleation protein (INP), these transporters differ in their efficiency like OmpA-LPP is

common carrier protein, but INP is a new carrier protein with higher display efficiency and have a linker to display at a farther distance. The display of proteins or peptides on phages or prokaryotes have few limitations like simple post translational modifications and size of the traveler protein, and as bacteria have low tolerance to organic solvents, enzymes that regulate complex reactions in the presence of organic solvents cannot be expressed. Although yeast has a lower growth rate and transformational efficiencies, the ability to tolerate harsh conditions, available genetic tools, and efficient post translational modifications make the yeast as the suitable host for the homologous or heterologous cell surface protein display (fig. 1d). In a study wherein cell surface, heterologous expression of xylose reductase and β-D-glucosidase on the *S. cerevisiae* cells resulted in simultaneous co-fermentation of cellobiose and xylose to xylitol with 2.5-fold increase in the yields (Guirimand et al., 2019b). Similarly, expression of glucoamylase and glucose oxidase using a – agglutinin as the anchoring motif resulted in 4-fold increase in the reaction rate in comparison to the individual rates (Fan et al., 2020). Even in the case of biodegradation experiments wherein the whole cells tolerance to those unfriendly substrates makes the degradation process uneconomical, here a novel enzyme PETase that could degrade polyethylene terephthalate (PET) was displayed on *Pichia pastoris* (*Komagataella phaffii*) resulted in increased thermal and pH stability, and 36-fold increase in enzyme turnover rate and stable reactive specific activity after 7 repetitions (Chen & Arnold, 2020). In a prokaryotic system INP from *Pseudomonas syringae* as the anchor protein, carboxylesterase encoding gene (Car EW) from *Bacillus* sp. K91, and a gfp protein was expressed in *E. coli* BL21 (DE3) cells using pET-28a (+) vector. At optimal temperature of 45 °C and pH-9.0, 1.5 mg/ml of diisobutyl phthalate was degraded by 10 U of carboxylesterase displayed on the cell surface (Ding et al., 2020). In comparison to the conventional whole cell biocatalysis, where intracellular enzymes are used in biosynthesis or

biodegradation processes, the cell surface display could reduce the mass transfer limitations by preventing the transport of substrates or products to or from the cell.

**5. Challenges and future perspectives**

Microbes are ubiquitous in nature and considered as the most prominent source for biochemicals and functional enzymes (Thapa et al., 2019). The enzyme industry is the dominant industry in the world and there is always a demand for new enzymes having improved activity, stability, and substrate specificity. Despite advances in biotechnology still, more than 99% of microbial flora is unexplored. The interaction between physical and chemical factors of the environment modulate the microbiological universe thus it's important to study these factors to predicts the micro inhabitants and vice versa. The recent advances in metagenomics, genome mining, and high throughput sequencing technologies are generating a huge amount of data that is not proportional to its biological significance (Alves et al., 2018). With the advanced bioinformatic tools, metagenomics data analysis could be able to transform raw data into useful biological information. Other challenge is to integrate the outcomes of the data analysis with already existing knowledge. Along with these challenges the success of novel enzyme research depends on factors such as gene size and its abundance in metagenomic sample, availability of host vector system, efficiency of screening systems. There is a need to find new hosts, broad host range vectors and develop biosensors to speed up screening methods for identification of positive clones in thousands of metagenomic library.

Further research is required to explore the novel techniques of enzyme production to full fill the increasing consumers demand at an industrial and commercial scale. With the advancements of molecular biology tools (promoter engineering, translation and transcriptional factors regulation, codon optimization, etc.) researchers able to increase enzyme production many folds. Use of synthetic gene may help to overcome the barriers of

codon biasing, allow the introduction of site-specific mutation and expression under choicest promoters, enhancer, and terminators for high level production. To make the enzyme-based processes more efficient enzymes should have higher activity, stability, and selectivity. Regardless of recent advances in enzyme engineering techniques (site directed mutagenesis, surface display technology, fusion proteins, use of unusual amino acids, etc.) it is a complicated task to mutate the enzymes for desired properties. Understanding structure modeling, computational enzyme engineering, and associated fields will be helpful in engineering designer enzymes have high activity, stability, and specificity properties required for industrial applications.

## 6. Conclusions

Green chemistry is getting attention around the world as enzymes-based reactions are more advantageous due to the formation of fewer side products. There are many successful examples of novel enzyme discovery and improvement using advanced molecular bio-technology approaches like metagenomics, site directed mutagenesis. However, applications are limited with lab scale research and further strategies are needed to integrate in industrial scale to strengthen the enzyme-based industry. In the future, the association of experimental and advanced computational tools of enzyme engineering will lead to the discovery of novel designer enzymes having multifunctional activities and desired industrial applications.

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**Figures caption**

**Figure 1a:** Illustration of enzyme engineering through directed evolution (A) and site directed mutagenesis (B).

**Figure 1b:** Illustration of fusion of multiple proteins using linker proteins.

**Figure 1c:** Illustration of enzyme engineering through incorporation of unnatural amino acids; (A) native peptide synthesis, (B) Re-assigning of rare t-RNA molecule of natural aminoacid to unnatural aminoacid, and (C) Re-assigning of stop codon to unnatural aminoacid.

**Figure 1d:** Illustration of enzyme engineering through surface display technology. E1-E3: three enzymes catalysing a process to convert substrate to end product.

**Table. 1 Industrial application of various enzymes**

| Industry | Enzyme | Function | Major applications | Reference |
|---|---|---|---|---|
| **Food and Feed** | Cellulase | Decompose cellulolytic material | Used to improve extraction and clarification of fruit juice. | (Sharma et al., 2017) |
| | Xylanase | Catalyze hydrolysis of xylan | Forage crops pre-treatment to improve digestibility of ruminant feeds and facilitate composting. | (Ran et al., 2019) |
| | Lactase | Hydrolyze lactose | To prepare lactose free milk for lactose intolerant people. | (Raveendran et al., 2018) |
| | Glutaminase | Aminohydrolase enzyme produce glutamate from glutamine | Increase the protein solubility and improve flavour of fermented food. | (Amobonye et al., 2019) |
| | Glucose oxidase | Catalyze oxidation of β-D-glucose | $H_2O_2$ improves the viscoelasticity of dough.<br>Removal of glucose and oxygen to increase self-life of food.<br>Biosensing applications and food analysis. | (Dubey et al., 2017) |
| | Levansucrase | Catalyze synthesis of fructose polymer | Levan used as fiber and sweetener. | (Bersaneti et al., 2018) |
| | Protease | Hydrolyze peptide bonds | Used to reduce mixing time, reduce dough consistency, and improve texture and flavour. | (Raveendran et al., 2018) |
| | Lipase | Hylrolyze triglycerides | Flavour development in cheese by hydrolyzing milk fat.<br>Used as emulsifier in baking industry. | (Guerrand, 2017) |
| | Pectinase | Breakdown pectin | Degrade pectin and decrease its water holding capacity and results in increased juice yield. | (Mahmoodi et al., 2017) |
| | Amylase | Involved in hydrolysis of starch | Used in baking and brewing industry for starch liquefication. Also used for clarification of beer and fruit juice or for the treatment of animal feed to improve the digestibility. | (Far et al., 2020) |
| **Textile and leather industry** | Laccase | Oxidize phenolic substrates | Denim washing and biopolyshing, shape memory polymers (SMP).<br>Removal of hazardous dyes from industrial effluent. | (Kanelli et al., 2015) |
| | Cellulase | - | Biostonewashing of the fibre to remove exposed fibril. | (Araújo et al., 2008) |

| | | | | |
|---|---|---|---|---|
| | Amylase | - | For efficient desizing without affecting fabric. | (Saha et al., 2018) |
| | Lipase | - | Degreasing of fat from animal skin during processing. | (Chandra et al., 2020) |
| | Savinase | Type of protease hydrolyze peptide bonds | Treatment of wool fibers for shrinkproof finishing. | (Wang et al., 2018) |
| | Cutinase | Belongs to serine esterase and break down ester bond | Used in the modification of PET fiber to improve hydrophilicity. | (Sooksai et al., 2019) |
| | Transglutaminase | - | Cross linking leather proteins with filler (glucose, flour and gum) to provide smooth surface. | (Duarte et al., 2020) |
| | Keratinase | Type of serine protease and hydrolyze peptide bond | Removal of hairs during the leather processing. | (Vidmar & Vodovnik, 2018) |
| **Cosmetics** | Superoxide dismutase | Dismutation of superoxide radicals | To mitigate the effect of reactive oxygen species (ROS) in antiaging cream. | (Gomes et al., 2020) |
| | Protease | - | Exfoliation of dead skin. | (Gomes et al., 2020) |
| | Photolyases | Eliminate thymine dimer | Reversal of sun damage to skin. | (Gomes et al., 2020) |
| | Oxidase, peroxidase | Catalyze oxidation-reduction reaction | Used in hair dying products. | (Hirose, 2017) |
| **Bioenergy** | Cellulase | - | Hydrolyze cellulose into fermentable sugars. | (Huang et al., 2018) |
| | Xylanase | - | Xylanase degrade xylan and increase accessibility of cellulase to cellulose. | |
| | Laccase | - | Have role in lignin degradation and detoxification of various aromatic compounds which results in increased fermentation. | (Bhatia et al., 2020b) |
| | Lipase | Catalyze esterification and transesterification | Transesterification of oil with alcohols to produce biodiesel. | (Bhatia et al., 2017) |
| | Fatty acid decarboxylase | Catalyze decarboxylation | Used for the decarboxylation of fatty acids into alkene. | (Jiang et al., 2019) |
| | Carbonic anhydrase | Interconversion of $CO_2$ and $H_2O$ into carbonic acid | Capturing carbon dioxide for bioenergy production. | (Bhatia et al., 2019) |

| | | | | |
|---|---|---|---|---|
| **Biochemical Production** | Lysine decarboxylase | Catalyze decarboxylation reaction | Conversion of lysin into cadaverine a precursor for nylon 66. | (Moon et al., 2019) |
| | Lysine decyclodeaminase | Catalyze deamination reaction | Used in biotransformation of lysine into pipecolic acid. | (Han et al., 2020) |
| | Lipase | - | Used in synthesis of commercially important esters. | (Chandra et al., 2020) |
| | Arylacetonitrilase | Catalyze deamination reaction | Reported for production of acids from their corresponding arylacetonitriles. | (Bhatia et al., 2014) |
| | Epimerase | Epimerization | D-allulose 3-epimerase was used for D-fructose conversion into D-allulose | (Patel et al., 2020) |
| **Pharmaceuticals** | Chitinase | - | Used in the treatment of inflammatory or fibrotic diseases. | (Singh et al., 2020a) |
| | Nattokinase | - | It has fibrinolytic activity, lipid-lowering, and neuroprotective effects. | (Chen et al., 2018) |
| | Lipase | - | Catalyze the resolution of enantiomeric pharmaceuticals or drug precursors (ibuprofen, ketoprofen and atenolol etc.). | (Contesini et al., 2012) |
| | L-asparaginase | Hydrolyze L-asparagine | Used in the treatment of acute lymphoblastic leukaemia. | (Brumano et al., 2019) |
| | Streptokinase | Promote lysis of Arg/Val bond in plasminogen | Used in the brake down of clot in case of myocardial infarction. | (Ucar, 2019) |
| **Detergent industry** | Lipase | - | Decompose fatty materials that are major components of oil related stain. | (Kumari et al., 2019) |
| | Cellulase | - | Used to improve colour brightness and fabric softening | (Patel et al., 2019) |
| | Amylase | - | Act on stains contains starch by degrading starch into short sugars. | |
| | Laccase | - | Used in cloth washing to remove the odor from fabric. | (Moreno et al., 2020) |
| | Mannase | Degrade mannan | Used for mannan stain removal. | (Al-Ghanayem & Joseph, 2020) |

**Table. 2 Improved production of enzymes by codon optimization**

| Host | Gene origin | Enzyme | Comment | Activity/Improvement | Reference |
|---|---|---|---|---|---|
| *Bacillus megaterium* | *Paenibacillus macerans* | α-Cyclodextrin glycosyltransferase | To ensure protein expression and secretion of enzyme a strong inducible promoter Pxyl and the signal peptide SPLipA was used. | 48.9 U/ml | (Zhou et al., 2012) |
| *E. coli* | *Bacillus* sp. NR5 UPM | β-Cyclodextrin glycosyltransferase | Codon optimization and supplementation of glycine resulted in increased secretion and improved activity. | 2.2 fold | (Nik-Pa et al., 2020) |
| | *Paenibacillus macerans* JFB05-01 | α-Cyclodextrin glycosyltransferase | Total protein yield and extracellular enzyme activity improved. | 326% | (Liu et al., 2012a) |
| | *Candida antarctica* | Lipase B | Molecular dynamic simulation, codon optimization and statistical analysis approach was used. | 2.1 fold | (Ghahremanifard et al., 2018) |
| | Human | Superoxide dismutase | Codon optimization followed by fed batch fermentation. | 46541 U/mg | (Yang et al., 2020) |
| | *Rhodococcus rhodochrous* J1 | Nitrile hydratase (NHase) | High activity and shorter culture time were achieved. | L-Nhase = 400 U/mg<br>H-Nhase = 234 U/mg | (Lan et al., 2017) |
| | *Thermococcus litoralis* | Steryl glucosidase | Promoter optimization, co expression of chaperones with development of high cell density fermentation. | 200 fold | (Eberhardt et al., 2017) |
| *Saccharomyces cerevisiae* | *Aspergillus awamori* | Glucoamylase | - | 31-40% | (Favaro et al., 2012) |
| *Streptomyces lividans* | *Streptomyces hygroscopicus* | Transglutaminase | Codon optimization coupled with negative element removal from promoter results in improved enzyme yield. | 5.73 U/ml | (Liu et al., 2016) |

| | | | | | |
|---|---|---|---|---|---|
| *Pichia pastoris* | *Aspergillus niger* | Endopolygalacturonase | A combination of codon and promoter optimization strategy was used. | 1.19 fold | (Karaoğlan & Erden-Karaoğlan, 2020) |
| | *Aspergillus niger* | α-glucosidase | Codon optimization leads to enhanced translation efficiency and stable mRNA structure. | | (Liu et al., 2012b) |
| | *Bacillus licheniformis* LS04 | Laccase | Combination of strategies; site directed mutagenesis and optimization of culture conditions were used. | 9.3 fold | (Wang et al., 2017) |
| | *Phanerochaete chrysosporium* | Lignin peroxidase | Codon optimization followed by fed batch fermentation optimization. | 4480 U/ml | (Majeke et al., 2020) |
| | *Starmerella bombicola* | Lactone esterase | Codon optimization followed by temperature optimization and coexpression of transcription factor *HAC1*. | 32 fold | (De Waele et al., 2018) |

**Table. 3 Improved activity and performance of the enzymes by directed evolution.**

| Enzyme | Microorganism | Technique | Property modified | Improvement | Reference |
|---|---|---|---|---|---|
| Endo-β-1,4-glucanase (Cel5A) | *Bacillus subtilis* | Error-prone polymerase chain reaction (PCR) and DNA shuffling | Mutations at V74A and D272G | Improved pH tolerance and thermostability | (Lin et al., 2009) |
| Xylanase | *Bacillus amyloliquefaciens* | Error-prone touchdown PCR | Point mutation S138T | 3.5 times improved specific activity | (Xu et al., 2016) |
| Protease | *Bacillus alcalophilus* | Error-prone polymerase chain reaction (PCR) | Mutants E110A and E134A | 3.6 times increased in activity | (Liu et al., 2014) |
| Lipase | *Bacillus pumilus* | DNA shuffling | Three-point mutations G14S, A15G, and V109S | 8 times improved activity and 9 times longer half life | (Akbulut et al., 2013) |
| Amylase | *Bacillus* sp. *TS-25* | Error-prone polymerase chain reaction (PCR) and DNA shuffling | A point mutation T142A | 20% increase in activity, and 50% increase in thermal stability | (Jones et al., 2008) |
| Laccase | *Coprinopsis cinereal* | Error-prone polymerase chain reaction (PCR) | Three amino acid changes E116K, N229D, I393T | Improved pH stability (in alkaline conditions) | (Yin et al., 2019) |
| Carboxy methyl cellulase | *Bacillus amyloliquefaciens* SS35 | UV Irradiation | Aspartate 233 was substituted by glycine in CMCase-UV2 | 1.6 – 4.1-fold increased activity and enhanced pH stability | (Singh et al., 2020a) |
| Cellobiohydrolase Cel7A | *Hypocrea jecorina* | Directed evolution and genome shuffling | - | Improved thermal stability and 44-fold greater half life | (Goedegebuur et al., 2017) |
| L-asparaginase | *Bacillus megaterium* H-1 | Error-prone polymerase chain reaction (PCR) and DNA shuffling | Reduction of hydrogen bonds, and incorporation of flexible residues at an active site Thr15 | ~21 fold improvement in catalytic activity, tolerance wider pH range and temperatures | (Lu et al., 2019) |

**Fig. 1a**

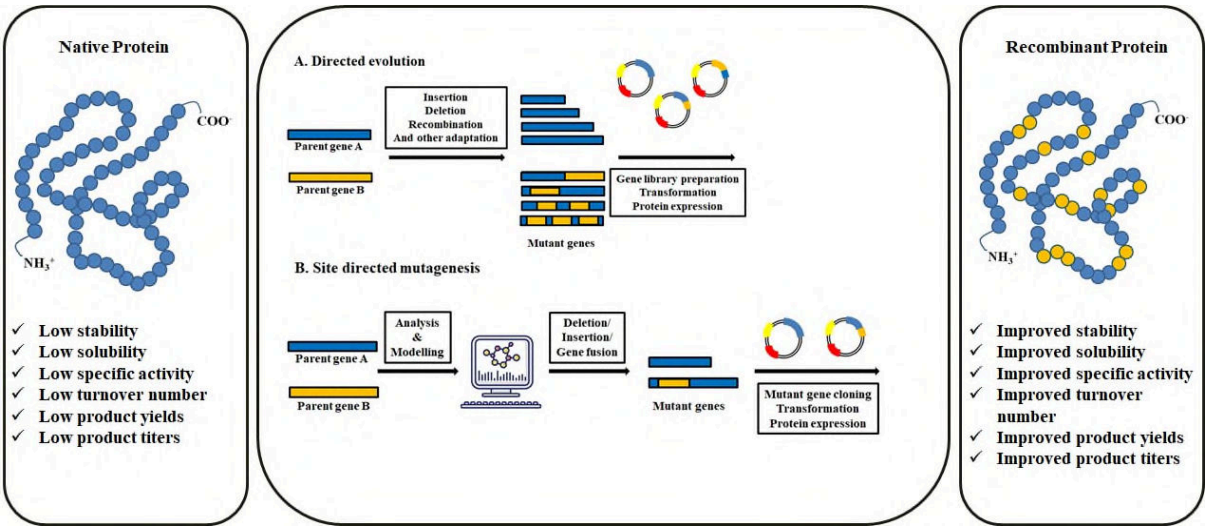

Native Protein
COO⁻
NH3⁺
✓ Low stability
✓ Low solubility
✓ Low specific activity
✓ Low turnover number
✓ Low product yields
✓ Low product titers
A. Directed evolution
Parent gene A
Parent gene B
Insertion
Deletion
Recombination
And other adaptation
Mutant genes
Gene library preparation
Transformation
Protein expression
B. Site directed mutagenesis
Parent gene A
Parent gene B
Analysis
&
Modelling
Deletion/
Insertion/
Gene fusion
Mutant genes
Mutant gene cloning
Transformation
Protein expression
Recombinant Protein
COO⁻
NH3⁺
✓ Improved stability
✓ Improved solubility
✓ Improved specific activity
✓ Improved turnover number
✓ Improved product yields
✓ Improved product titers

**Fig. 1b**

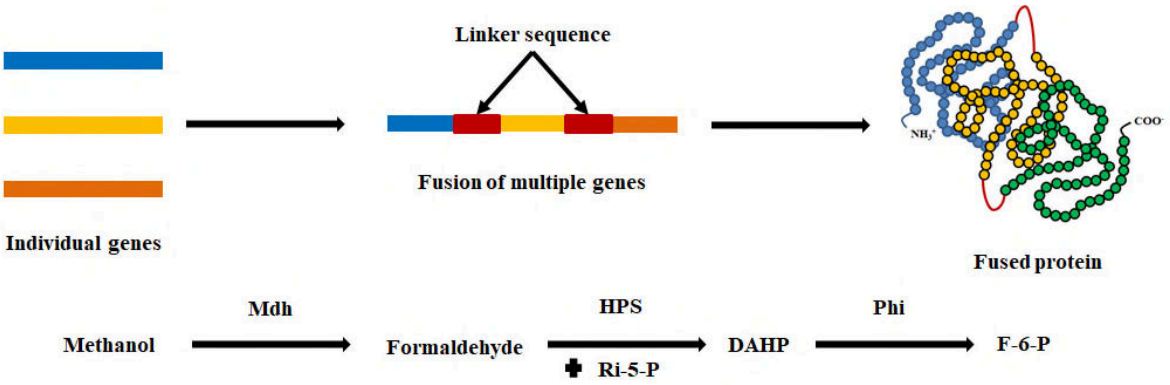

Linker sequence
Fusion of multiple genes
Individual genes
Fused protein
NH3⁺
COO⁻
Methanol
Mdh
Formaldehyde
HPS
Ri-5-P
DAHP
Phi
F-6-P

**Fig. 1c**

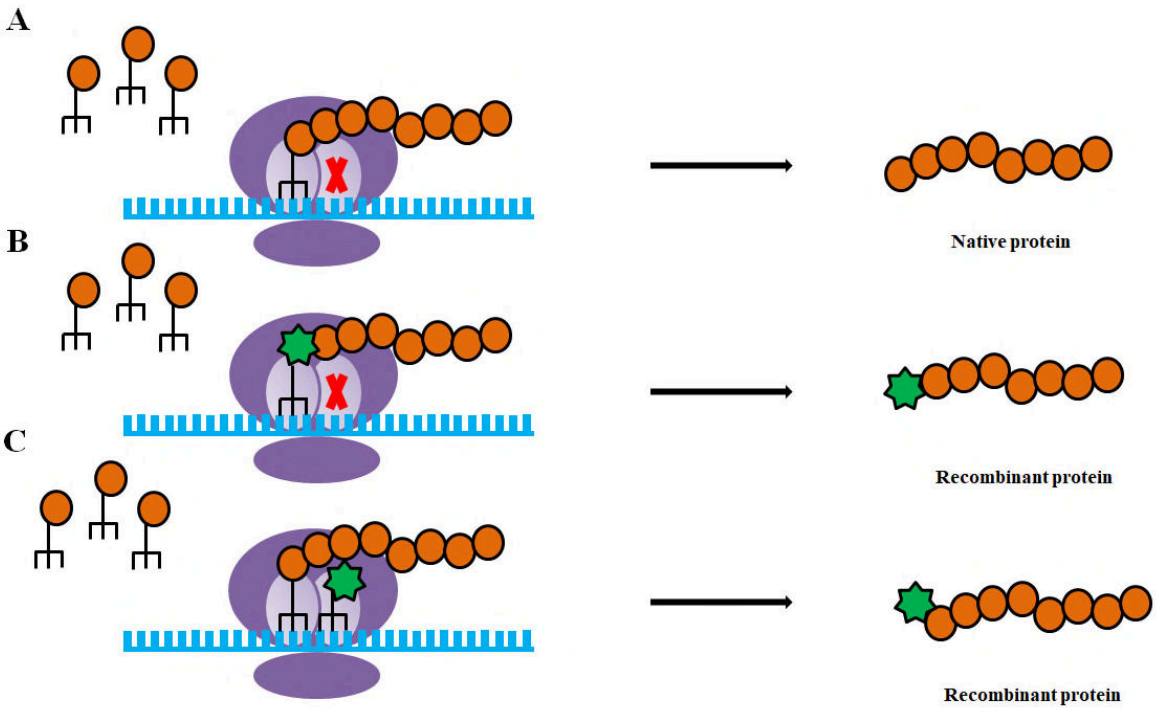
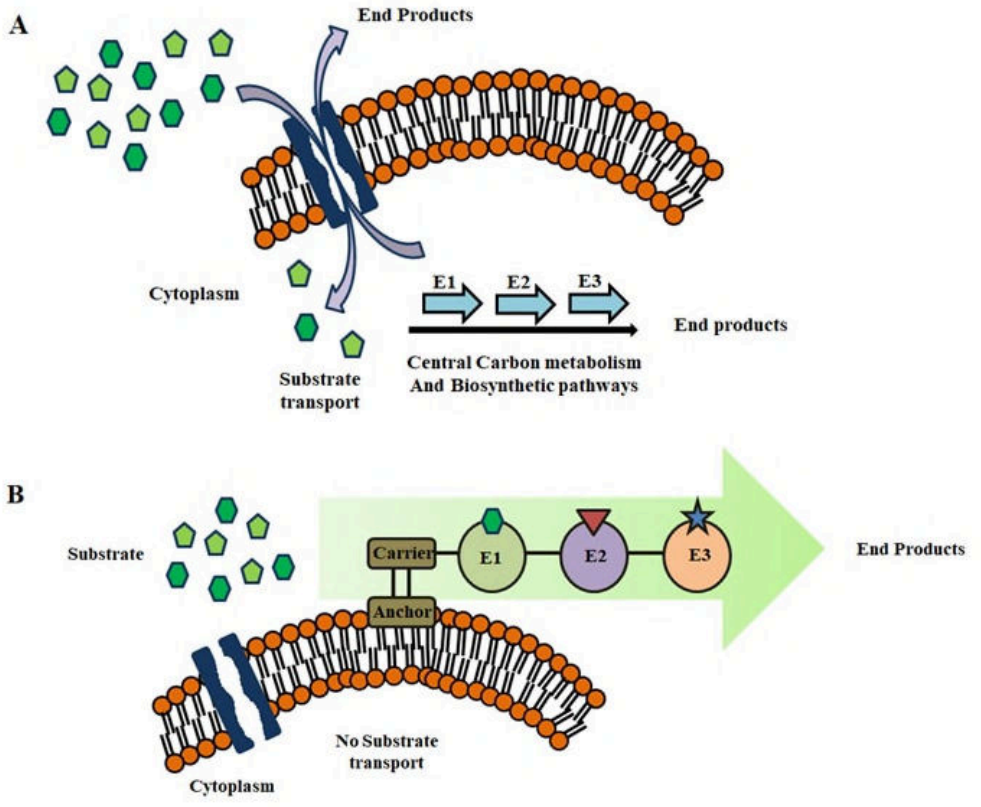

A
B
C
Native protein
Recombinant protein
Recombinant protein

**Fig. 1d**


A
End Products
Cytoplasm
E1
E2
E3
End products
Central Carbon metabolism
And Biosynthetic pathways
Substrate
transport
B
Substrate
Carrier
E1
E2
E3
End Products
Anchor
No Substrate
transport
Cytoplasm

# Molecular biology interventions for activity improvement and production of industrial enzymes

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