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Review article

Current status and emerging frontiers in enzyme engineering: An industrial perspective

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ABSTRACT

Protein engineering mechanisms can be an efficient approach to enhance the biochemical properties of various biocatalysts. Immobilization of biocatalysts and the introduction of new-to-nature chemical reactivities are also possible through the same mechanism. Discovering new protocols that enhance the catalytic active protein that possesses novelty in terms of being stable, active, and, stereoselectivity with functions could be identified as essential areas in terms of concurrent bioorganic chemistry (synergistic relationship between organic chemistry and biochemistry in the context of enzyme engineering). However, with our current level of knowledge about protein folding and its correlation with protein conformation and activities, it is almost impossible to design proteins with specific biological and physical properties. Hence, contemporary protein engineering typically involves reprogramming existing enzymes by mutagenesis to generate new phenotypes with desired properties. These processes ensure that limitations of naturally occurring enzymes are not encountered. For example, researchers have engineered cellulases and hemicellulases to withstand harsh conditions encountered during biomass pretreatment, such as high temperatures and acidic environments. By enhancing the activity and robustness of these enzymes, biofuel production becomes more economically viable and environmentally sustainable. Recent trends in enzyme engineering have enabled the development of tailored biocatalysts for pharmaceutical applications. For instance, researchers have engineered enzymes such as cytochrome P450s and amine oxidases to catalyze challenging reactions involved in drug synthesis. In addition to conventional methods, there has been an increasing application of machine learning techniques to identify patterns in data. These patterns are then used to predict protein structures, enhance enzyme solubility, stability, and function, forecast substrate specificity, and assist in rational protein design. In this review, we discussed recent trends in enzyme engineering to optimize the biochemical properties of various

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biocatalysts. Using examples relevant to biotechnology in engineering enzymes, we try to expatiate the significance of enzyme engineering with how these methods could be applied to optimize the biochemical properties of a naturally occurring enzyme.

1. Introduction

Enzymes are proteinous biocatalyzers that regulate intricate metabolic pathways and other cellular functions in addition to determining the rate of biological reactions [1–4]. Enzymes as biocatalysts are known to exhibit high efficiency and versatility. Reactions proceed far more quickly when enzymes are present than when they are not [5]. These biological molecules can identify the chemicals that require transformation with extreme specificity. Over the course of development, enzymes have evolved on their own, giving rise to innovative biocatalysts with superior qualities and a wide range of biological applications [5–8]. All forms of life are now capable of undergoing a vast range of alterations because of their regulated evolution over time. Throughout the course of evolutionary history, all forms of life have undergone continuous adaptation and modification, leading to the development of diverse biological traits and capabilities. This evolutionary process, driven by natural selection and genetic variation, has enabled organisms to acquire a wide array of characteristics that enhance their survival and reproductive success in changing environments. As a result of this regulated evolution over time, organisms possess inherent flexibility and adaptability, allowing them to undergo a vast range of alterations in response to environmental pressures, genetic mutations, and other factors. This capacity for adaptation is a fundamental feature of life on Earth, shaping the diversity and resilience of living organisms across different ecosystems and evolutionary time scales. This emphasizes the dynamic nature of evolution and the role it plays in shaping the biological diversity and adaptability observed in all forms of life. As green biocatalysts, they are widely used in a wide range of industries, including baking, beverages, food, textiles, leather, feed, pharmaceuticals, and detergents [9,10]. This is because they can reduce the use of natural resources, minimize adverse effects on the environment, increase efficiency, be economically viable, and produce high-quality products [5–8, 11–13]. Furthermore, microbial enzymes have the ability to either convert or degrade harmful chemical substances found in household and industrial waste, including nitriles, amines, phenolics, and xenobiotics [2,7,14]. Microbial and plant-based enzymes like hydrolases and pectinases have been utilized extensively in manufacturing food items like juice, milk and its derivatives, beer, and bread [15]. Highly selective, effective, and stable enzymes have been developed for widespread application in various industries, including detergent, textiles, pharmaceutical synthesis, waste treatment in bioremediation, pulp and paper. Enzymes isolated from natural sources or in their native forms may fall short of what customers in the market need.

The main challenge with enzyme-based catalysis is the lack of effective biocatalysts for numerous essential chemical processes with commercial relevance [16,17]. Natural biocatalysts are limited in industrial processes by the possibility that it will be extremely inefficient when catalyzing reactions with non-cognate substrates and vulnerable to the severe conditions of chemical reactions in laboratories. Therefore, enhancing the biocatalytic efficacy of native enzymes under chemical reaction settings by higher orders of strength represents the ultimate challenge in biocatalysis-assisted chemical reactions. This problem is solved via enzymatic engineering [17–19]. Enzyme engineering is now frequently used in some situations to increase the activity of enzymes and satisfy the demands of intricate industrial applications. As a result, great efforts are being made to modify enzymes in order to meet industry demands. Biocatalysts' resistance to high temperatures, severe pH values, and high levels of organic solvents has been increased by the application of enzyme engineering [16,18,20]. Enzyme engineering techniques are also frequently used to engineer other features of enzymes, such as specific activity, substrate specificity and binding, and enantio, regio, and stereo selectivities. Additionally, enzyme engineering, can be used to simultaneously improve the kinetics and stability of the enzyme (catalytic enzyme characteristics) and the yield of the enzyme and ease of downstream processing (enzyme productivity). Furthermore, the most recent advancement in enzyme technology is enzyme engineering, which uses different genetic engineering techniques to target several biological molecules' properties at once [21–23]. Unfortunately, determining which mutations are essential when developing a novel enzyme is difficult [24], which extends the time needed to engineer proteins [25–28]. By developing a variety of new and enhanced biocatalysts suitable for a wide range of biocatalysis-based processes in industries, recent developments in genetic engineering techniques, in addition to the introduction of numerous highly efficient computational tools, have revolutionized the field of protein and enzyme engineering. Most biocatalysis-based sectors, including the pharmaceutical industry, will likely rely on highly efficient and specifically designed enzymes in the near future to produce bulk and fine chemicals [22,28,29]. Lactobacillus kefir ketoreductases (KRED) are employed to catalyze the conversion of a ketone intermediate into a chiral alcohol by reduction. Ketoreductase-mediated processes are employed in the production of intermediates for diverse pharmaceuticals, including montelukast, atorvastatin, duloxetine, ezetimibe, crizotinib, and phenylephrine [30,31]. A highly promising instance of protein engineering involves the synthesis of antidiabetic sitagliptin by Codexis and Merck [11,27,28]. The process involved utilizing a specifically engineered enzyme called R-selective transaminase, which was produced through directed evolution. This enzyme was employed to carry out the asymmetric amination of prositagliplitin ketone, resulting in the production of sitagliptin with an enantiopurity of 99.95 % [28]. Diagnostic enzyme development is receiving attention in pharmaceutical and medical research. Shahbazmohammadi et al. employed an engineered fructosyl peptide oxidase (FPOX) as a diagnostic enzyme for diabetes mellitus [32]. In a particular study, ginger peroxidase (GP) was immobilized through adsorption using a nanocomposite material consisting of polypyrrole-cellulose-graphene oxide [33]. The outcome yielded an enhanced decolorization efficacy of Reactive Blue 4 Dye (RB4) by 11 % compared to the free GP. Jacquet et al. created a modified version of a phosphotriesterase-like lactonase derived from the hyperthermophilic archaea *Sulfolobus solfataricus* [2]. The tremendous advancement in this field has been made possible by the rapid growth of several biological fields, including system and structural biology, as

well as other omics techniques. This review focuses on current status and emerging frontiers in enzyme engineering as well as the application of machine learning (MA)and artificial intelligence (AI) for enzyme screening.

2. Challenges hindering the widespread application of enzymes in industries

An enzymatic process must be at least as economically efficient as the conventional one it aims to replace [34]. Ideally, it should not require significant and costly changes to the existing production line. This is true despite the encouraging potential and success stories surrounding the application of enzymes in industrial processes. If not, it won't inspire change or be appealing [35,36]. Enzymes employed in the enzymatic synthesis of pharmaceutical intermediates may be cost-prohibitive at large scales. For instance, lipases used in the resolution of chiral compounds for pharmaceutical synthesis often require purification from microbial sources, which can be expensive and labor-intensive [37]. Developing microbial fermentation processes for the cost-effective production of recombinant lipases or exploring alternative enzyme sources, such as enzyme immobilization on inexpensive supports, can mitigate production costs and facilitate scale-up for pharmaceutical manufacturing [38,39]. Most enzymes from wild-type organisms tend to lose their activity during various industrial applications that last for hours at extreme pH, pressure, and temperature [40]. This particular challenge makes wild-type organism -produced enzyme very vulnerable in their numerous industrial applications thereby making the entire process to be less economically feasible [21]. In order for that to be feasible, the enzyme or enzymes must be able to withstand the frequently severe conditions of the processes (such as pressure, temperature, nonaqueous solvents, and pH) and have a fast enough catalytic rate to avoid impeding the productivity of the entire production line [40]. Enzymes used in the production of bio-based plastics, such as polyhydroxyalkanoates (PHAs), face stability challenges during processing and storage [41]. PHA-producing enzymes, such as polyhydroxyalkanoate synthases (PHA synthases), can lose activity or denature at elevated temperatures or in the presence of solvents [41]. Improving the stability of PHA synthases through protein engineering or formulation strategies is crucial for maintaining enzyme activity and prolonging shelf-life, thus enabling the cost-effective production of biodegradable plastics.

High concentrations of substrate and/or product are another requirement for industrial enzymes [24]. Since these enzymes evolved to perform their physiological function in aqueous fluids, they rarely exhibit product-feedback inhibition as an auto-regulation mechanism, they do not need to be very fast or stable in hostile environments, and obtaining enzymes with such features is extremely difficult [42]. Enzymes used in the production of specialty chemicals may exhibit limited substrate specificity, restricting their application to specific feedstocks. For instance, nitrile hydratases employed in the synthesis of carboxylic acids from nitrile substrates may exhibit narrow substrate specificity, limiting their utility to a subset of nitrile compounds [43]. Engineering nitrile hydratases through directed evolution or rational design to broaden their substrate scope and improve catalytic efficiency can enhance their versatility and enable the synthesis of diverse carboxylic acids from renewable feedstocks [43]. The majority of known enzymes, in fact, have catalytic rates that fall into a very narrow range of values [6]. While this makes them ideal for biological systems' metabolism and unmatched at physiologic temperature, it may not be appealing to industry, which can use much higher temperatures to overcome the room temperature rate of enzymes and make up for the lower efficiency of chemical catalysis [44]. On the other hand, the sequestration of a particular enzyme in the membrane which is the result of multiple *trans*-membrane domains leads to an intracellular secretion of wild-type organism enzymes [45]. These transmembrane domains anchor the enzyme within the lipid bilayer of the membrane. As a consequence, the enzyme may undergo intracellular secretion, meaning it remains within the confines of the cell rather than being released outside. This phenomenon can occur naturally in wild-type organisms, where certain enzymes are localized to specific cellular compartments or organelles for their respective functions. This particular challenge leads to low synthesis of that particular enzyme thereby making the entire process to be less effective. Furthermore, it may take years and significant financial resources to research and develop enzymatic processes to their maximum productivity and scale, which discourages enterprises from adopting them. It can be difficult to get some enzymes on an industrial scale, especially those derived from prokaryotes. Substrates and cofactors are costly and difficult to access in industrial quantities. This is crucial for redox biocatalysis in particular, as less expensive substitutes for redox equivalents are being sought. The advent of enzyme engineering, which has received a lot of funding and attention recently, aims to optimize the many features of enzymes in order to develop the best biocatalysts possible and avert these challenges.

3. Strategies for improving the catalytic properties of enzymes through enzyme engineering

Enzymes are used in industry because of advances in biotechnology that have made it possible to produce a sufficient amount of these molecules. Enzymes specifically tailored to the operating conditions of industrial processes can now be developed due to enzyme engineering. The development of enzymes with desired properties at relatively inexpensive production costs has become possible through the use of genetic engineering [46]. In order to assist with substrate solubility and product extraction, the enzymes can be designed to function on a variety of substrates and concentrations, extremes pH, temperatures, and pressure, and stability in non-aqueous solvents [7,14]. Since discovered enzymes are adapted to their natural surroundings, they naturally lack such characteristics [10]. Because of this, natural enzymes would work best in the physiochemical conditions seen in nature. Consequently, in order to achieve the essential stability and activity, the enzyme must be modified [7]. Protein engineering presents remarkable opportunities for the design of commercial enzymes at reduced production costs [14]. There are three primary approaches to enzyme engineering: directed evolution, rational design, or semi-rational design, which combines the first two techniques. The practicality of high-throughput screening (HTS) technology, the availability of a protein's structure and function, and an understanding of the desired characteristic all influence the method of choice (Fig. 1). To select the most suitable strategy and process for the engineering of enzymes, a number of scientific and technical issues pertaining to engineered enzymes must be considered. This would overcome the

difficulties that enzyme engineering imposes.

3.1. Directed evolution

By including random mutations in the gene encoding the target enzyme, directed evolution incorporates the theory of natural selection [47,48]. By using the gene encoding the target enzyme to generate DNA libraries and then selecting enzyme variants with improved desired characteristics, directed evolution mimics the process of natural evolution [47]. The two primary stages of directed evolution for enzyme engineering involve generating a library of variants in genes by random mutagenesis or recombination, and then selecting the variants that have the desired function [49]. High-throughput screening techniques are used in selecting the most desirable enzyme variant based on its improved characteristics [4]. One of the most effective methods for developing enzymes with substrate specificity, enhanced thermostability, catalytic activity, and stereoselectivity in enzyme engineering is directed evolution. When there is little understanding of the structure-function interactions within the enzyme, this enzyme engineering technique is frequently used [47]. It offers an effective solution for the shortcomings of natural enzymes, primarily due to the lack of a need for in-depth structural and biochemical understanding.

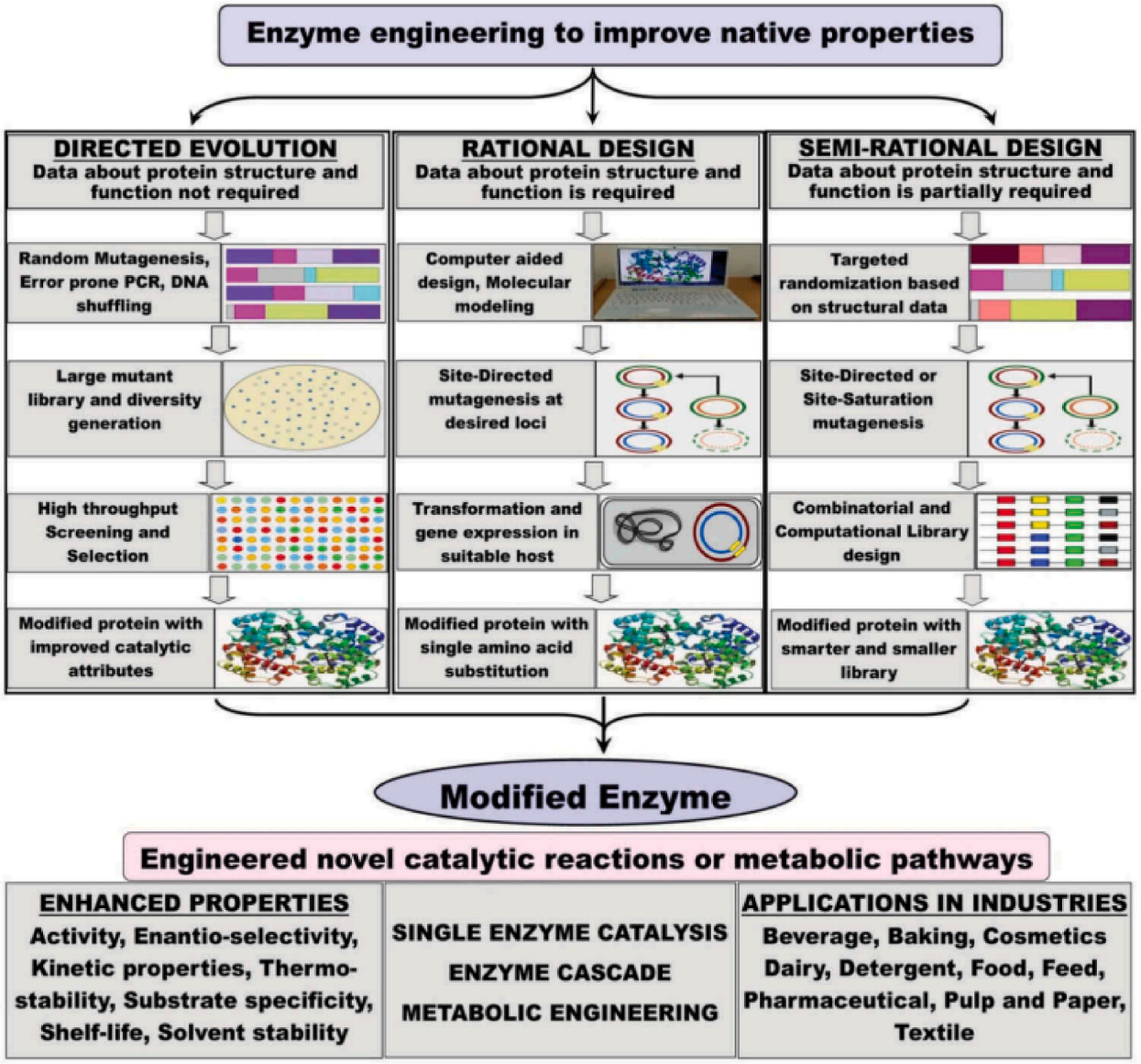

Fig. 1. Major enzyme engineering strategies [15]. The enzyme engineering strategies are depicted in dark blue charts, while the methods employed to implement each strategy are shown in light blue charts. As can be seen from the connecting lines, the semi-rational strategy incorporates methods from directed evolution and rational design.

As illustrated in Fig. 1, directed evolution integrates two distinct methods, random mutagenesis and HTS, to determine the appropriate enzyme variations. This method generates enormous libraries of DNA by introducing random mutations in the genetic assembly encoding the desired protein using methods like DNA shuffling, staggered extension procedure, and error-prone polymerase chain reaction [47]. Ye et al. report that the error-prone PCR technology, which is commonly used in laboratories to produce random genetic variants [50], replicates DNA under less-than-ideal conditions for mutant populations whose potential is decreased to the same or more than that of the original enzyme [51]. DNA shuffling is a distinct method of creating random mutations. It was first published by Stemmer in 1994 and includes using DNase to fragment various homologous genes, then amplifying the results by PCR without primers to create new random combinations [52]. Nevertheless, these methods of random mutagenesis produce large genomic libraries, which complicates the task of locating the target variation [53,54]. The most complicated aspect of this approach is screening these libraries to look for the enzyme variant with the desired properties [55]. The process of screening and selection has been aided by the use of a variety of analytical techniques, from fluorescence, NMR spectroscopy, chromatography, or to HTS methods like in-vitro compartmentalization, microtiter-plate screening, and fluorescence-activated cell sorting (FACS). Because HTS can analyze at a pace of 104 or 105 variations per day, it can expedite the identification process [4]. Consequently, in order to detect mutants utilizing directed evolution, it is essential to select an appropriate HTS approach based on the yield and properties of the target enzyme [4]. It is well known that in-vitro selection systems such as phage-assisted continuous evolution (PACE) and ribosome display or mRNA display are effective tools for speeding up the evaluation of enzyme variations [56]. These systems are commonly used in enzyme engineering and directed evolution studies to rapidly screen large libraries of enzyme variants for desired properties such as enhanced activity, stability, or substrate specificity. PACE is a selection system that allows for the continuous evolution of enzymes or other biomolecules within the context of a phage display system. It involves the iterative enrichment of enzyme variants with improved properties through cycles of mutagenesis, selection, and amplification, thus accelerating the evolution process compared to traditional methods. Ribosome display is a technique that enables the in-vitro selection of peptides or proteins displayed on ribosomes. It involves the immobilization of ribosome-mRNA-protein complexes on solid supports, followed by selection and amplification of desired variants. Ribosome display is particularly useful for selecting enzyme variants with high affinity or specificity for a target substrate. mRNA display is a method for selecting peptides or proteins based on their encoding mRNA molecules. It involves the linkage of mRNA molecules to their encoded proteins through a covalent bond, allowing for the physical association of genotype and phenotype. mRNA display enables the selection of enzyme variants with desired properties directly from large libraries of mRNA sequences. The directed evolution of enzymes was first conceptualized and experimentally carried out by Arnold and colleagues [57]. In this groundbreaking study, a variant of the subtilisin enzyme—which is employed in the food processing, detergent, and cosmetic industries—was developed by multiple rounds of consecutive mutagenesis and screening, and it had around 256 times more enzyme activity than the wild-type enzyme [58]. It worthy to note that numerous screening techniques have been developed to assess mutant libraries. These include plaque screening, computer screening, adding indicators to construct a multienzyme system, and choosing between in vitro and in vivo processes. Furthermore, metabolites are frequently used as markers for exceptional performance; as a result, directed evolution has shifted from molecular to process engineering [50]. One example of directed evolution in action is the dehalogenation of halohydrin dehalogenase, which uses less reagent and doesn't require pricey equipment. This technique shows the promise of digital image calorimetry in conjunction with paper-based whole-cell screening as a viable strategy for the identification of industrially significant enzymes [59].

3.2. *Rational design*

A thorough understanding of the structure and associated functions of enzymes is the basis of rational design. A more accurate evaluation of the most promising residues to employ as mutation targets is made possible by the examination of the enzyme's structure, the alignment of many sequences, and the application of computational techniques to perform molecular dynamics simulation [56]. This technique requires three steps: selecting the suitable enzyme, identifying the residues to be mutated, and characterizing the mutants [60]. It is based on the mutation of individual residues or a group of residues at an identified location in the structure of a protein (Fig. 1). The rational design technique typically produces smaller libraries than those produced via directed evolution; nevertheless, the selection of the residues to be changed requires prior knowledge of protein function, structure, and catalytic site [61].

Sequence-based and structure-based methods are typically used to obtain information regarding the structure and function of proteins [62]. The sequence-based method involves aligning the target protein's sequence with a well-characterized homologous protein sequence [63]. In order to find potential amino acids that could be altered to enhance the characteristics of an enzyme, these two sequences are compared [64]. The active site can be redesigned using an enzyme's three-dimensional crystal structure with the structure-based method [51]. It is possible for the residues to change in either scenario through substitution, deletion, insertion, or substitution with amino acids that differ in physicochemical characteristics and size [65]. In a recent study, site-directed mutagenesis technique was employed for the modification of two amino acid residues (L167W or P172L) at the active site of a β-glucosidase from *Trichoderma reesei* [66]. Result from this study shows that the obtained enzyme exhibited improved thermal pH, stabilities, and glucose sensitivity despite having only two mutations.

Recently, methods like structure-guided consensus, which integrates structure- and sequence-based methods, have been employed to supply protein information on structures for rational design [3,5]. Rational design has various drawbacks, notwithstanding the fact that it has some apparent advantages over directed evolution, such as the ability to generate smaller libraries and simpler screening, particularly when simultaneous substitutions are needed [67]. Directed evolution, on the other hand, relies on building libraries and testing mutations to find proteins with better qualities [4,68]. Three key components of enzyme information—access, quality, and organization—are necessary for the development of tools and models utilized in rational design [67]. Public databases with curated data published by academics, such as the Universal Protein Resource [69], and Protein Data Bank [70] can provide some insight about

these characteristics.

Conclusively, enzyme structure analysis, multiple sequence alignment, computational methods, and are the three basic strategies to determine the specific residues for mutations. Residues that line the enzyme's binding pocket and demonstrate direct interaction with the substrate can be found by examining the crystal structure of the enzyme [3]. Results from a recent study show that these residues have been identified as the targets for mutation since it would be advantageous to change these locations in order to obtain the desired function. For the *in-silico* identification of the hotspots, a number of web tools are also accessible, including IntFOLD, Protein Homology/AnalogY Recognition Engine (PHYRE2) Swiss-Model, Rosetta, Threading ASSEmbly Refinement (I-TASSER) [71]. Multiple sequence alignment of homologous proteins identifies a protein's evolutionary conserved particular amino acids. High conservation scores for particular amino acids indicate that they are crucial to the structure and functioning of the enzyme and cannot be altered. Conversely, residues with low conservation scores can be targeted for engineering because they are not crucial to the structure and function of the enzyme [4]. As computational biology has progressed, techniques for identifying enzyme engineering hotspots have been developed. In this sense, molecular dynamics (MD) simulation has made significant progress since it provides atomistic details on the dynamic molecular interactions that dictate the stability and functionality of proteins [72]. A computational methodology is used to determine the target residues for laccase engineering by fusing quantum mechanics and molecular mechanics with simulation was recently developed [13].

3.3. Semi-rational design

In order to address the drawbacks that arise from using rational design and direct evolution alone—such as the need for a lengthy screening process and the lack of comprehensive structural information about enzymes—enzyme engineering employs a combination of approaches in semi-rational design [56]. Semi-rational design considers the use of sequence and structure-based information about the target enzyme to identify potential target sites that could be modified using a combination of site-directed mutagenesis and random mutagenesis (Fig. 1). Furthermore, computational techniques aid in anticipating and removing the potential for deleterious mutations. This method produces tiny libraries containing functional content, aiding in the intended modification of protein conformation. It has been discovered that this method of designing enzymes, which centers on the impact of amino acid substitution within the catalytic domain of the enzyme, is incredibly effective for developing novel enzymes and improving the stability, stereoselectivity, and catalytic activity of already-existing enzymes. The engineering of novel enzymes with features favorable to industry biocatalytic processes relies on the development of in-vitro experimental assays. These assays are dependent on information pipelines for the modification of current proteins. Strategies based on sequencing, structure, and random mutagenesis are typically employed in semi-rational design. Computational approaches are a major component of these strategies.

A mutability map, generated by software like the HotSpot Wizard [73] server, aids protein engineers in locating hot spots within a protein sequence. Moreover, molecular dynamics simulations and modeling tools can be combined with sequence-based data. The progress in computational biology has broadened the scope of protein engineering and enabled the development of de novo engineering, which modifies synthetic proteins by utilizing the three-dimensional structures found in natural proteins and their folding topologies [74]. The catalytic characteristics of the active site are intimately linked to the amino acid sequences present inside it. Numerous techniques, such as multiple sequence analysis and phylogenetic tree construction, provide insightful information about the conservation of homologous protein sequences and evolutionary history. This makes choosing amino acid residues that can be substituted during the enzyme engineering process simpler. The development of compact and intelligent libraries is made easier by the identification of amino acid residues in enzyme active sites.

Enzyme structure is considered in structure-based semi-rational design in order to maximize enzymatic activity [75]. Enzyme models can be constructed utilizing sequence information in the lack of crystal structure through homology modeling and threading techniques. Using the existing rotamer libraries and backbone recognition, computational evaluation of these models is performed based on the energetics of amino acid changes in the enzyme [76]. By computing the difference in their free energies, forcefield-based algorithms like as TANGO, SNPEffect, and FoldX [8,77,78] can score the mutants of the enzyme. It is also possible to develop de novo enzymes with certain catalytic characteristics by employing computational techniques. Understanding the relationships between enzymes and substrates is especially helpful with this method [79]. Only a few numbers of designs for the in-vitro engineering of enzymes are filtered out by virtual modifications in amino acid substitutions to provide desired properties in the unique enzyme. The precise positions of amino acid residues within the enzyme's active site are predicted using quantum mechanical calculations and molecular dynamics simulation. Programs like YASARA and RosettaMatch [80,81] can be used to accomplish this.

The semi-rational approach could be utilized in the following enzyme engineering processes.

3.3.1. De-novo design

De-novo design is one of the semi-rational approaches of enzyme engineering that is based on computer-aided programs to design a new pathway or reconfigure an existing one. This technique involves mainly mutagenesis by insertion with substitution at specific points. This procedure involves utilizing computer-stimulating programs to design the protein structures and predict sequences that could optimize the enzyme biochemical properties. It takes account of the previous skeleton structures of the enzymes to discover large results of the new de-novo design that could perform the same function as that particular protein structures. The initial process of de-novo design begins with the design of a new active site. This involves a sophisticated computer-aided procedure to construct an active site capable of participating in a specific transition state of a reaction. This is followed by the incorporation of crucial residues for transitional state stability, selectivity with activity probably. Being able to redesign a simple relationship that is non-covalent based on the polarity and bonding activities gives room for the novel substrate to bind to those binding pockets of the enzymes located at the

required site. With the help of software tools like iterative computational protein library redesign and optimization (IPRO), an algorithm developed by Pantazes et al. [82], enzymes that react to the novel substrates with an increase in activity have been developed. An example is the engineering of *Escherichia coli* thioesterase TesA that [83]. Conversion of medium chain acyl- ACP thioesterase (C_6–C_{12}) to enhanced C_{12}–C_8-specified variant with high activity esterase catalyzed reaction was observed after the enzyme engineering procedure. This brought about 27 mutants with enhanced substrate specificity of thioesterase which is utilized in the production of medium-chain oleochemicals like amines, fatty alcohol, and ester [83]. Also, Throckmorton et al. used the IPRO program to explore a ser-specific domain of non-ribosomal peptide synthetases from *Escherichia coli* [84]. Mutation at these site-directed spots ensured increased specificity of this domain. The Quantum mechanical calculation was employed for a spatial conformation of esterase catalytic triad residues and oxyanion hole that fits the substrate binding site [85]. The researchers used the Rosetta software program to embed the conformed catalytic triad residues and oxygen anion into a protein scaffold. The newly designed mutant was capable of catalyzing the hydrolysis of p-Nitrophenyl acetate.

3.3.2. Enzyme fusion for strain development

Tight regulation of transcription and translation processes, an addition of desired gene copies using suitable high copies of various vector-plasmid with the inclusion of strong promoters are strategies employed in a semi-rational approach of enzyme engineering that involves enzyme fusion for strain development [86]. On the other hand, vector optimization, RNAi-induced gene cut-off of an enzyme inhibitors, translational fusion partners with secrete system engineering involving strain selection has being proven to ensure enzyme overproduction in microbial enzymes too. Pessentheiner et al. established an endoxylanase–laccase by an incorporation of specific domain to the endoxylanase which gave rise to a more thermostable and proteolysis-resistible enzyme [87]. Furtado et al. established a novel enzyme that is consists of glucanase-laccase fusion by incorporation of a specific domain to the glucanase which gave rise to a more functional enzyme that hydrolyzed sugarcane bagasse more than the enzyme cocktail [88]. Diogo et al. established the xylanase-xylosidse from chimeric fusion that gave rise to a more thermostable and functional enzyme which is responsible for the release of xylose [89]. Han et al. established a more improved functional enzyme by fusing the catalytic domain contained at the C terminus of cyclodextrin glycosyltransferase to a substrate domain of α-amylase that consist of the carbohydrate-binding module (CBM). This engineered enzyme showed increased production by more than 5-fold of 2-O-D-glucopyranosyl-Lascorbic acid compared to the wide-type [90].

3.3.3. Enzyme immobilization

The concept of immobilizing enzymes is to fix or trap an enzyme with the solid support material. These support materials ensure stabilization and preservation of the enzyme structure which in turns renders it more resistible to its surrounding environment. The act of immobilizing enzymes also ensures the recovery of both enzyme and the support system. Enzyme immobilization has become a key technology for the transition of biocatalytic processes from batch reactors to continuous feed-back reactors. The continuous feedback reactions require that the enzymes do not leach during the reaction processes. Finally, immobilization ensures the alteration of biocatalysts from homogeneous form to heterogeneous form after the linking, thereby ensuring a separate enzyme that synthesizes products with high purities. The mechanism of enzyme immobilization could be divided into two categories; the physical and chemical mechanisms. The physical mechanism involves the encapsulation and entrapment process. The chemical mechanism relies on covalent or non-covalent interactions between the target enzymes and the support. Protein engineering is one of the means used to introduce covalent and non-covalent affinity tags that mediate specific interactions between the enzyme and the surface [91]. The formation of a covalent bond between the enzymes and a carrier will ensure that the enzyme remains bound to the carrier for the duration of its operations.

Incorporating protein engineering in enzyme immobilization procedures is a new frontier in modern biology [92]. This aspect is very important because most immobilized enzymes tend to lose their functions once they are linked to their carriers. The loss of activity is due to reactions that occur between the enzymes and the carriers. Cross–linking of enzymes to their carriers covalently led to chemical reactions at the surface of the immobilized enzymes. This limitation could be overcome by introducing the directed evolution process to optimize the function of immobilized enzymes. One of the early studies carried out that proofed the incorporation of protein engineering in enzyme immobilization procedure [93]. The researchers utilized two-cycle of error-prone PCR to optimize the activity of formate-dehydrogenase (FDH) for the immobilization process in a polyacrylamide gel. The process produced a variant that had a 4.4 fold higher reaction activity compared to the wild type [93]. The variant was observed to possess some substituted amino acids in an active site that previously contained lysine, glutamic acid with cysteine residues. The immobilized enzyme that didn't undergo error-prone PCR was believed to have undergone a chemical reaction with the carrier through the lysine residue present at the surface of the carrier which led to the decrease in activity. Substitution of residues located at the surface of the enzyme could also avert this limitation by providing multiple attachment points and allowing optimum activity to take place at the surface. On the other hand, Li et al. made use of specific site mutagenesis to integrate a non-standard amino acid that possessed a functional group which could facilitate cross-linking activity in a supervised procedure to avoid reaction that could occur with glutaraldehyde which will give rise to loss of enzyme activity [94]. Finally, protein engineering could be utilized in the fusion of polyester synthase to a target protein (polyhydroxyalkanoates) using an in-vivo mechanism of immobilization. This procedure gives rise to an insoluble polyhydroxyalkanoates bead that showcases the target enzyme which is linked covalently in its full optimum activity [95].

3.3.4. Metagenomics

The application of enzyme engineering in metagenomics is widely gaining interest in modern biology. These processes involve the screening of sequence with its function to ensure that over-activities occur during the entire techniques. These techniques consist of

utilizing isolated DNA-fragment from the prescribed environment (soil or water), to construct libraries of genes that will be cloned to a suitable expression vector which ensures that sheared genomic DNA from the environment and introduced vector is established in the appropriate vector to express the target gene. Screening and identification of novel enzymes are performed by function-based or sequence-based. Hu et al. proved that suspended particulate-matter that are present in nitrate-contaminated estuarine river contained genes of dissimilatory nitrate reductase (*nirBD*) using metagenomics libraries [96]. They were able to prove that diverse alpha-and gamma-proteobacteria that are present in the suspended particulate matters played a crucial role in the conversion of nitrate to ammonia and therefore regarded as key active heterotrophic bacteria. This conversion helps to balance the ecosystem in the nitrate-contaminated estuarine river [96]. Finally, metagenomics libraries could also assist to screen newly synthesized *Escherichia coli* that are assembled from different DNA gene circuits which are comprised of transporters, promoters with terminators. These newly synthesized *Escherichia coli* cells are designed as host cells or vectors to carry out a particular function so the screening helps to eliminate genes that are not necessary for this particular function which gives room for non-excess metabolic activities and also makes the entire process to be cost-effective [97].

3.3.5. Development of enzymes with conjugates or co-factor specificity

The semi-rational design approach could be utilized in developing of enzymes with conjugate or co-factor specificity intending to explore its application in several industrial processes. The application of the L-asparaginase enzyme for therapy of lymphoblastic leukemia with acute myeloid and non-Hodgkin's lymphoma has been greatly limited by several factors. These limitations include rapid serum clearance of drugs, enzyme inactivation by proteolytic enzyme degradation, and active allergic reactions triggered by short spans and pancreatic issues. Furthermore, the enzyme itself has a short lifespan, ranging from 2.5 h in mice to about 18 h in humans. These challenges have hindered the widespread effectiveness and application of L-asparaginase in cancer therapy. These limitations could be overcome by developing of co-factor L-asparaginase with mono-methoxyl polyethylene glycol which expands the enzyme therapeutic potential. Kurtzberg et al. proved that developing the L-asparaginase enzyme with conjugate led to 52 % retainment of its activity compared to the native enzyme [98]. This enzyme with its cofactor has been applied in the treatment of malignant, canine tooth ache with human brain-tumor. On the other hand, Campbell e*t al.* constructed mutants of alcohol dehydrogenase from the aldo-ketoreductase protein family due to its highly efficient and alcohol-tolerant properties [99]. The mutation was targeted at binding pockets of the co-factor to broaden its selectivity. The generated mutant was able to establish a bio-fuel enzyme co-factor pair with a non-native nicotinamide mono-nucleotide. This engineering approach transformed the substrate selectivity of temperature-tolerant alcohol dehydrogenase D. It achieved this by incorporating an active loop region derived from aldose reductase obtained from human origin. This procedure converted both enzyme substrate selectivity and enzyme co-factor selectivity [99].

3.3.6. Development of new chiral chemicals with metalloenzymes for non-native reactions

Asymmetric catalysis [100], and biocatalysis [101] using enzymes are the only two possible approaches to synthesis pure enantiomeric compounds. This process is followed by high-throughput screening or selection [102], which proves that quite a number of these synthesized biocatalysts are capable of catalyzing the enantioselective reactions [103]. In the context of enzyme engineering, high throughput screening or selection involves the simultaneous testing of numerous enzyme variants for specific traits such as enhanced activity, stability, or substrate specificity. This is typically achieved using automated systems or robotic platforms capable of performing thousands to millions of individual assays or measurements in parallel. High throughput screening methods can vary depending on the desired outcome and the nature of the enzyme or target being studied. Common techniques include colorimetric assays, fluorescence-based assays, chromatographic methods, and mass spectrometry, among others. These assays are often designed to rapidly detect changes in enzyme activity, binding affinity, or other relevant properties. Most enantiomeric pure compounds with new chiral chemicals that are selected from high throughput screening tend to perform lower than their desired requirement; therefore, it is necessary to design novel procedures that will induce over activities; one such procedure is the directed evolution technique [104]. This technique tends to alter some naturally occurring enzyme-restricted bond types of relevance to biology (e.g., C–C, C–O, C–N, C–S, etc.), notwithstanding the fact that the evolution process has programmed its profile in developing the mechanism that produces the same bond type over and over again (e.g., chiral amines) [105]. Furthermore, most reactions that are known for organic synthesis are currently unknown in our evolved biology, and there is a need to expatiate this unknown with directed evolution which has led to promising and active areas that are required for enzyme engineering. This implies that many chemical reactions commonly utilized in organic synthesis, particularly in laboratory settings, are not naturally occurring or prevalent in biological systems shaped by evolutionary processes. In other words, while organic chemistry has developed numerous methods for synthesizing complex molecules, these methods often involve reactions that do not occur spontaneously or efficiently within living organisms. Therefore, there is a disparity between the repertoire of chemical reactions available to synthetic chemists and those utilized by biological systems for essential processes such as metabolism and biosynthesis. This has also ceded highly optimistic outcomes [106, 107]. Reactions like carbon-carbon and carbon-halide bond formation that are already known to be existing in nature but are too restrictive to alter for broad application in biocatalysts (e.g., narrow substrate-range aldolases or low-specificity haloperoxidases Windle et al., when challenges of these nature arrive, novel enzymes that are of de novo design with bonds which consist of C–C bonds forming are utilized [108]. Also, Friedel−Crafts acylase derived recombinant acyltransferase of *Pseudomonas proteges* [109] and redesigning the synthesis of flavin-dependent halogenate RebH from *Lechevalieria aerocolonigenes* (Payne et al., 2015).

The activities of nitrogenase during the nitrogen fixation processes are proof of metalloenzyme activities that consist of natural enzymes utilizing metal ions or metal-based cofactors to enhance or improve the catalysis of the enzyme. Nitrogenases are made up of heterometal-complex that are situated at the active site which consist of iron and sulfur that contained cofactors responsible for enhancing these biocatalysts activities. On the other hand, being able to focus on the chemical reaction space accessible to protein iron-

dependent metal groups (cytochromes *c* and P450), researchers have made available some new natural chemicals that can undergo successful reactions. This mechanism is been utilized for the synthesis of enzymes that can catalyze the transfer of carbene [1] and the formation of carbon-silicon [110] with carbon-boron [111]. The initial de novo designed enzyme is usually characterized by low turnover numbers, but the introduction of a directed evolution approach gives rise to more improved enzyme properties. Cyclopropane products are synthesized by subsequent transfer of carbene to styrene substrates however, this reaction is instigated when cytochrome p450 reacted in Fe II state with ethyldiazoacetate to synthesize the iron-carbenoid, an intermediate product that took part in the synthesis of the cycloproapane products [112]. P411 (a new variant that was generated by error-prone PCR when cysteine was changed to serine at the haem-ligating residue. This variant was observed to increase the rate at which reduction took place at the ferric state situated at the center of iron. This reaction made room for the endocellular reduction of Nicotinamide adenine dinucleotide phosphate (NADP) to reduce the ferric state and increase the activity that involves carbene transfer [112]. Researches that are based on the use of biocatalyst for wholly novel activities is still an emerging field, and most of the recently engineered enzyme has not been utilized for large-scale production. Moreover, these recent advances have laid the foundation that could expand the list of chemicals that are available for biocatalysis in future prospective.

4. Enhancing enzyme stability and product yield through immobilization

These biocatalysts must be able to be recovered and reused in order for enzyme-based catalysis to be commercialized and for the catalytic process to be cost-effective. Enzyme immobilization is a process crucial for commercializing enzyme-based catalysis, involving the attachment of enzymes onto a support matrix [113]. This attachment confines the enzymes to a specific location or structure, offering several advantages for industrial applications. The process typically begins with selecting a suitable support matrix, such as porous materials like silica, cellulose, agarose, or various polymers. This matrix choice impacts the immobilized enzyme's stability, activity, and reusability. Following support matrix selection, the matrix might need activation to facilitate enzyme binding. This activation can involve chemical modification or functionalization of the support surface to introduce reactive groups. Once activated, the enzyme is attached to the support matrix. Depending on the chosen method, this attachment can occur through physical adsorption or covalent binding. Physical adsorption involves non-covalent interactions, while covalent binding forms chemical bonds between the enzyme and support matrix.

Enzyme immobilization is a highly profitable technology in businesses where the economic viability of biocatalysts is highly dependent on their use. This method ensures that the procedure is tuned to raise the overall turnover number and regulate the enzyme's useable operating duration. When the enzyme is needed to function in unusual circumstances, it can also be utilized to change the equilibrium of the reaction through enzyme immobilization. This is entirely dependent upon the most important aspect of enzyme immobilization—the optimization of the enzyme-support system. Immobilization enhances enzyme stability, making enzymes more resistant to harsh conditions encountered during catalysis, such as changes in temperature, pH, or the presence of inhibitors. This stabilization extends the operational lifetime of the enzyme and improves its resistance to denaturation or degradation. After immobilization, the properties of the enzyme are characterized to ensure activity, stability, and other parameters meet application requirements. Immobilized enzymes exhibit improved stability and reusability compared to their soluble counterparts. This improved stability allows for long-term use in industrial processes where stability is crucial. Additionally, enzyme immobilization enables easier separation and recovery of enzymes from reaction mixtures, facilitating their reuse in multiple cycles. This reusability leads to cost savings and increased efficiency in industrial processes. Moreover, immobilization simplifies downstream processing by facilitating the separation of products from reaction mixtures, resulting in higher product yields and purity. Furthermore, immobilized enzymes can be used in various reactors, including packed-bed reactors, fluidized-bed reactors, and membrane reactors. This versatility allows for greater flexibility and optimization of process conditions, contributing to process intensification and efficiency.

The development of an efficient immobilization technique involves several essential steps. These steps include identifying and characterizing the enzyme to be immobilized, selecting an appropriate matrix that can bind to the enzyme and provide stability without altering its catalytic properties, and optimizing the operational efficiency of the resulting enzyme-support system. The aim is to enhance the enzyme's yield and activity while considering the reaction requirement [51]. Enzyme immobilization can be accomplished by covalently attaching the enzyme to a support material that contains chemicals or groups, such as glyoxyl, glutaraldehyde, and vinyl sulfone. These compounds have the ability to form covalent bonds with the amine and thiol groups present in the enzyme [114]. This approach ensures the stability of the enzymes when exposed to different situations that can render them inactive. Osuna et al. employed chitosan-coated magnetic nanoparticles for the immobilization of Aspergillus niger lipase [115]. Glutaraldehyde and glycidol were employed to create covalent bonds between the enzyme and the polymer. The immobilized enzyme exhibited improved stability against variations in pH and temperature and maintained 80 % of its original catalytic activity after undergoing 15 hydrolytic cycles. Another immobilization technique involves inducing weak forces between the enzyme and the support material by adsorption. Recently, carriers have been modified by adding hydrophobic groups such as octyl and phenyl moieties to their surface. This modification allows the carriers to be adsorbed onto the hydrophobic patches of the enzyme. Rueda et al. conducted a study where they examined the effectiveness of octyl-agarose and octyl-glyoxyl agarose in immobilizing lipases from Candida antarctica (form B), *Thermomyces lanuginosus* (TLL), or *Rhizomucor miehei*. They used both hydrophobic adsorption and covalent bonding approaches for immobilization [26]. The investigation revealed that the enzymes exhibited improved stability and activity when exposed to organic solvents. Additionally, the enzymes were found to be capable of being reused for five hydrolytic cycles without experiencing significant loss in activity when immobilized on a heterofunctional substrate, specifically octyl-glyoxyl agarose. Both covalent attachment and adsorption can be combined to enhance enzyme-support interactions and provide high enzyme stability. The basic steps involved in enzyme immobilization are shown below (Fig. 2).

When choosing a matrix for enzyme immobilization, consider factors like compatibility, stability, porosity, and surface area [116]. Ensure the matrix is chemically and mechanically stable, compatible with the enzyme, and has sufficient surface area for efficient binding and catalysis [114]. Biocompatibility is crucial for applications in medicine or food processing [116]. Seek ease of modification, cost-effectiveness, and the ability to regenerate the enzyme for sustainable use. Environmental considerations, including biodegradability and recyclability, are important [117]. By addressing these factors, you can select a suitable matrix that optimizes enzyme performance and enables successful enzyme-based catalysis in various applications [118].

5. Extremozymes as industrially significant biocatalyst

Enzymes have been widely employed across the beverage and food sector to augment both the flavor and nutritional composition of various products. Extremozymes, which are enzymes derived from extremophiles capable of functioning under extreme conditions, have gained significant importance as industrially significant biocatalysts [119]. These extremozymes offer unique properties that make them valuable for various industrial applications [120]. Recent research and advancements in biocatalysis have highlighted the potential of extremozymes in industrial processes due to their stability under harsh conditions and efficiency in catalyzing reactions. Immobilization techniques on novel matrices, metabolic and genome mining for enzyme improvement, enzyme cascade systems, and computational biology are some of the emerging strategies being explored to enhance the performance of extremozymes for industrial use [121]. Xylanases and other starch processing enzymes, including pullulanases, transglutaminase, and amylases, are extensively employed in the manufacturing of food additives. Enzymes are frequently employed at elevated temperatures in industrial applications. Consequently, the optimization of enzyme engineering techniques to boost the temperature stability of enzymes has been shown to improve their overall efficiency and increase the yield of the desired product. Pang et al. conducted a study aimed at enhancing the thermostability and activity of a type II pullulanase derived from the thermophilic microorganism, *Anoxybacillus* sp. WB42 [122]. This was achieved by the introduction of surficial residue replacements and the incorporation of disulphide bonds. Xylanases have been recognized for being beneficial in the biofuel sector due to their capacity to enzymatically break down hemicellulose, a crucial constituent of plant cell walls. Hegazy et al. employed xylanase derived from *Geobacillus stearothermophilus* that exhibits thermostability in their study [25]. The researchers utilized random mutagenesis techniques to generate xylose-tolerant mutants, which shown notable enhancements in catalytic efficiency. In this particular scenario, due to the unavailability of the 3D structure of the xylanase-xylose complex, a comprehensive library was generated through the application of error-prone polymerase chain reaction, involving random mutation of the entire xylanase gene. The library screening process yields mutants that exhibit tolerance to xylose, namely with the mutations of L133V, M116I, and L131P. These mutations are located within the N-terminus of α-helix 3. When compared to the wild-type, the most optimal mutant with xylose-tolerance exhibited a 3.5-fold increase in ki value and a 3-fold increase in catalytic ability. In addition to xylanases, the biofuel sector also utilizes endoglucanases and lipases in the manufacturing of biodiesel.

Noey et al. employed molecular dynamic simulations and quantum mechanical calculations to develop enantioselective mutants of Lactobacillus kefir ketoreductase [12]. These mutants were found to exhibit enhanced efficiency with 3-thiacyclopentanone compared to 3-oxacyclopentanone. The method employed in this study utilizes molecular dynamics to determine the association between the relative fraction of catalytically viable poses for enantiomeric reductions and the empirically observed enantiomeric ratio. The findings demonstrate that specific mutations induce changes in the geometry of the binding site, resulting in an expansion of the binding pocket

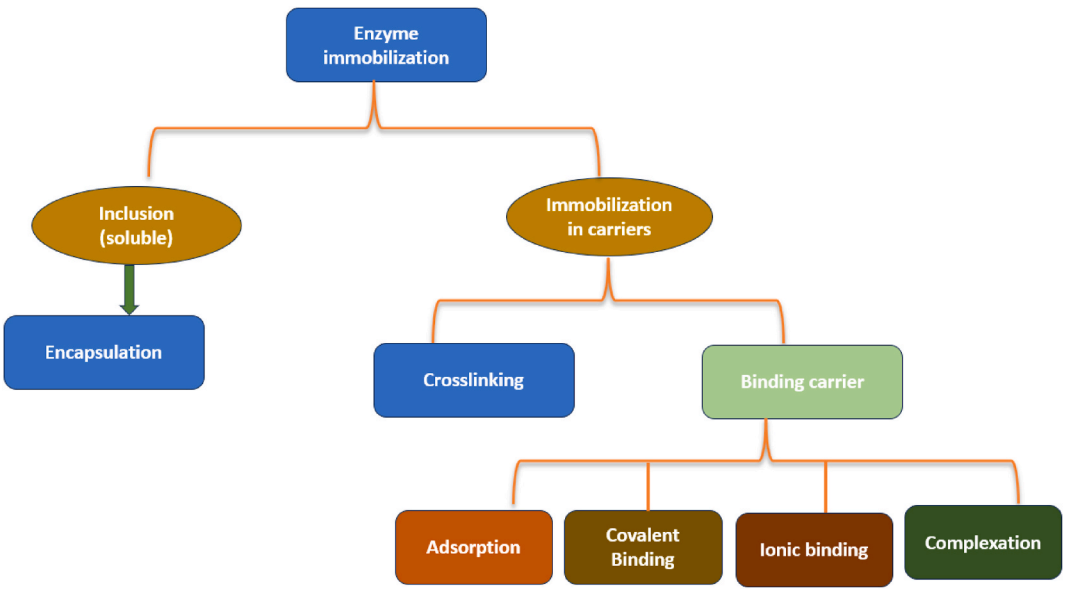

Fig. 2. Basic steps involved in enzyme immobilization.

to accommodate the bigger sulfur atom. Consequently, this modification enhances the selectivity towards S-isomers when interacting with 3-thiacyclopentanone. The surfactant business extensively employs several lipases and proteases as biologically derived detergents. Enzyme engineering techniques are employed to enhance the stability and preserve the activity of these enzymes within the formulations. Lactonase, an enzyme derived from *S. solfataricus*, plays a crucial role in the process of detoxifying organophosphorus chemicals [2]. The utilization of enzymes presents a highly promising approach for the selective degradation of potentially hazardous substances, resulting in the transformation of these compounds into safer molecules. Some industrially important extremozymes are presented in Table 1 below.

6. Current procedures involved in screening mechanism

6.1. Display mechanism of screening

Screenings are utilized to develop proteins or enzymes that possess a particular biochemical or biophysical property. Examples of these properties include enhanced functions and stabilities with enhanced binding affinities. It is also important to note that you are likely to get what you screen for; therefore, it is important to select suitable measures for any particular screening type. Display mechanism of screening for bacteria [132], ribosome display mechanism [19], yeast surface display mechanism [133], had a positive effect. These mechanisms target the displaying protein of interest at the surface of various organisms followed by screening for target antigen binding affinity. Additional properties like resistance to acid or basic induced environment, increase solubility and enhanced thermostability could also be achieved by modifying the chosen environment (pH, temperature, solubility of solvent) to sort for mutants that possess stable properties under various harsh conditions. Combing the next generation sequencing with these display procedures gives rise to high-throughput specific variants or mutants. Finally, mechanisms like the magnetic or fluorescence-activated sorting are employed to distinguish and enrich cells with the desired protein of interest and improved features [134].

6.2. Protein reporter biosensor

This mechanism involves the integration or fusion of the reporter biosensor to the protein of interest. This biosensor helps to distinguish the improved protein when they undergo the prescribed sensor screening. This mechanism is based on the fact that these biosensors could split into two halves and also generate fluorescence that acts as signals that could correlates with stability, increased activity with solubility when they are non-covalently bounded to the protein of interest. Example of cases where this mechanism is utilized includes (a) Insertion of protein of interest into the loop of *Escherichia coli* uroporphurinogen-III methyl transferase ($CysG^{A}$) a reporter biosensor which are responsible for the catalysis and formation of fluorescence compounds [135] (b) β-lactamase was used as a reporter biosensor to correlate antibody resistance of *Escherichia coli* with aggregation resistance, thermostability and folding of protein as biochemical properties to look out at the protein of interest [136].

7. Application of machine learning and artificial intelligence in engineering enzymes

Machine learning (ML) is an emerging method for designing novel biocatalysts that has garnered significant interest in recent decades [137]. These technologies enable the exploration of the vast combinatorial space of enzyme sequences, allowing for improved selectivity, solubility, and activity of enzymes through approaches like restricted mutagenesis and combinatorial techniques [138, 139]. Contrary to the model-driven rational design, this approach is data-driven as it utilizes known data patterns to forecast qualities of similar input that have not been observed before. Machine learning-based design, unlike the iterative selection of existing mutants

Table 1
Industrial important extremozymes.

Enzymes	Organism sources	Enzyme engineering approach	Uses	References
Lactonase	*Sulfolobus solfataricus*	The rational method employing a structure-based approach.	Organophosphorus compouds bioremediation	[2]
Endoglucanase	*Alicyclobacillus acidocaldarius*	Amplification of DNA by PCR with enzyme template	Biofuel production from plant biomass degradation	[123]
Cyclodextrin glycosyltransferase	*Bacillus circulans* STB01	Site directed mutagenesis	Used in pharmaceutical and food industry	[124]
Lipase	*Bacillus* sp.	Site directed mutagenesis	Applied in production industries	[125]
Chitinase	*Paenibacillus pasadenesis* CS0611	Site directed mutagenesis	Chito-oligosaccharides production	[126]
Type II pullulunase	*Anoxybacillus* sp. WB 42	Site-directed mutagenesis utilizing overlap extension PCR	Used in chemical and food industries for debranching of starch	[127]
Laccase	*Thermus thermophilus* SG0.5JP17-16	Site directed mutagenesis	The manufacturing of paper and its usage in the food companies	[128]
α-amylase	*Klebsiella pneumoniae* (CCICC no. 10018)	Site-saturation mutagenesis	Application in food processing and biosynthesis	[129]
Pullulanase	*Bacillus acidopullulyticus*	Site directed mutagenesis	The enzymatic hydrolysis of starch for the purpose of producing high-glucose syrup	[130]
β-1,4-glucosidase chimeric enzyme and Endo-glucanase	*Clostridium thermocellum*	Site directed mutagenesis	Biofuel and ioethanol production	[131]

with directed evolution, has the ability to develop novel and promising variants that have not been observed before. This is achieved by analyzing patterns in the obtained data. In a similar manner as the interplay between rational design and directed evolution, machine learning is being employed in conjunction with both approaches [140,141]. The surge in its popularity can be attributed to its remarkable proficiency in tasks that were previously considered impractical or exceedingly challenging from an algorithmic standpoint [142,143]. These tasks include facial and handwriting recognition, spam and fraud detection, natural language processing, and web search, among others [137]. The latest developments in the examination of human genetic variation data in the fields of biology and healthcare enhance the attractiveness of this method for creating advantageous mutations [144]. The synergy between big data generated by platforms like Ginkgo Bioworks and AI models has led to remarkable breakthroughs in enzyme optimization. For instance, Ginkgo's AI tool, Owl, has been instrumental in fine-tuning enzymes for specialized roles by leveraging large-scale data generation to refine predictive models iteratively [142,143].

Several machine learning algorithms have been employed in enzyme engineering. Notable examples of machine learning techniques used in bioinformatics include random forests for predicting protein solubility [145], support vector machines [146]and decision trees for predicting changes in enzyme stability due to mutations [147], K-nearest-neighbor classifiers for predicting enzyme function [148] and mechanisms [149], and various scoring and clustering algorithms for rapid functional sequence annotation [150]. The primary appeal of machine learning in enzyme engineering arises from its generalization capacity. Once trained on a known input, referred to as a training set, a machine learning algorithm has the potential to swiftly generate predictions for new variants. On the other hand, the rational design technique typically necessitates the creation of a novel model, which can entail many months of rigorous calculations and processing. Similarly, the directed evolution approach will likely entail several months of extensive experimentation. Nevertheless, the effectiveness of a machine learning predictor for data that has not been encountered before is significantly influenced by both the caliber of the data used for training and the efficacy of the underlying algorithm. The extensive variety of enzyme mechanisms, reactions, and experimental conditions poses a significant obstacle in utilizing machine learning for biocatalyst design. This is primarily due to the need for stringent quality control in data collection and reporting, the challenge of standardizing data formats, the scarcity of large and uniform data sets for model training, and the slow process of collecting new data for model testing.

The work-flow for utilizing the machine learning process involves the following steps.

(1) Coalition of data is the initial step involved in machine-learning-assisted enzyme engineering process. These data that are being collated are usually generated by screening, sequencing or, data from past literature so as to generate sequences that are functional in a library form.
(2) The next step involves engineering of feature vectors from previous extracted data in step one to generate feature libraries that are utilized for model training. These feature vectors engineering could be sequenced based on structure or energy minimization calculations. It could also involve data from different generated databanks or collections from descriptors. Carrying out the normalization and standardization process on these engineered feature vectors are usually of great importance in an effort to minimize the possibility of the engineered feature vector to be propensive in n-dimensional feature space.
(3) The next step involves computing of the various model performances from the generated datasets. This process takes account of the learning and verification of these datasets by splitting it into subsets. The cross-verification process is utilized at the subsets data that had been initially split repetitively.
(4) Finally, these last generated data are utilized for model training. This process involves trained machine which possesses the ability to predict variants that could perform better from a narrow unknown sequence. The predicted sequence is finally verified in the laboratory and this verification will be utilized for elucidation of model effectuation in making instances for new cases. These new cases could be registered in the machine for model advancement.

8. Current challenges associated with machine learning-aided techniques

The most significant challenge in utilizing machine learning for enzyme engineering arises from the inherent multidisciplinary nature of the technique. In order to provide clear objectives, rigorous training and analysis, and to prevent errors in methodology and misinterpretations, it is imperative for molecular biologists, biochemists, mathematicians, computer scientists, and bioinformaticians to establish a shared language. Although ready-to-use software packages are undoubtedly beneficial for standardizing the training of machine learning algorithms for individuals without specialized knowledge, it may not be the most effective approach to simply gather all available data and apply a variety of machine learning algorithms to determine the best predictor. The No Free Lunch theorem [151] asserts that there is no ML approach that is inherently superior to others; thus, it is crucial to have a comprehensive understanding of the data types and challenges at hand in order to design efficient predictors [152]. The "No Free Lunch" theorem in the context of search and optimization is a fundamental concept in computational complexity and optimization. It states that for certain types of mathematical problems, the computational cost of finding a solution, averaged over all problems in the class, remains the same for any solution method. This theorem implies that there is no universal optimization strategy that outperforms all others across all possible problems. The name "No Free Lunch" alludes to the idea that no method offers a shortcut or advantage over others when considering all possible problem instances within a class [153]. In essence, the theorem suggests that different search algorithms may achieve varying results on specific problems, but when averaged across all problems, their performance is indistinguishable [153]. Therefore, if an algorithm excels on certain problems, it will likely perform less effectively on others. This concept underscores the importance of tailoring optimization strategies to the specific structure and characteristics of individual problems to achieve superior performance. The theorem highlights the need for specialized approaches that leverage problem-specific information to optimize

solutions effectively [151]. The ongoing trend towards adopting advanced machine learning techniques, such as combining multiple algorithms into hybrid meta-predictors, optimizing hyperparameters with multiple training cycles, learning features, and integrating machine learning -based and classical bioinformatics tools into a single predictor, will pose additional challenges in fostering interdisciplinary collaboration for the development of effective and reliable predictors in enzyme engineering [154]. Hybrid meta-predictors are advanced models that combine multiple prediction mechanisms to enhance accuracy and performance [155]. These predictors integrate different prediction strategies, such as local and global predictors, and use a meta-predictor to determine the final prediction based on the most effective approach [155]. By leveraging a combination of prediction methods, hybrid meta-predictors can achieve superior results compared to individual predictors alone. These models are designed to optimize prediction outcomes by utilizing the strengths of diverse prediction mechanisms and selecting the most reliable prediction based on their collective performance [153].

Unfortunately, the enthusiasm surrounding innovative uses of machine learning in enzyme engineering appears to be overshadowing another crucial aspect of the method. The primary objective of science is not alone to enhance prediction capabilities, but also to possess the ability to elucidate the obtained outcomes. Only a small number of studies go beyond basic ROC analysis. For instance, they may employ resample cross-validation to determine the statistical significance, investigate the causes of poor predictions, and examine learning curves. What factors contribute to the superior performance of a specific predictor? Which features are essential for the efficacy of a predictor on a worldwide level? What are the ranges of feature values and which areas of the feature space are most crucial for accurately classifying a specific data point? Several articles on the subject lack this type of analysis, which restricts our comprehension of the fundamental molecular concepts.

The rising popularity of machine learning applications in enzyme engineering highlights the growing significance of robustly comparing different predictions. The comparison of methodologies is hindered by the absence of standardized techniques and data sets for testing, posing significant challenges [156]. Inconsistencies in performance measurement methods, insufficient or unclear information in research papers, and the difficulty in finding reviewers with deep expertise further exacerbate these obstacles [156]. Addressing these urgent challenges is critical to advancing the field and facilitating meaningful comparisons among methodologies [156]. Scientists engaged in bioinformatics research, particularly in areas like protein structure and function predictions, have developed multiple platforms for comparing machine learning predictors. Notable examples include Critical Assessment of Function Annotations (CAFA), Enzyme Function Initiative (EFI), COMputational BRidge to Experiments (COMBREX), and Critical Assessment of protein Structure Prediction (CASP). Other applications have not yet witnessed comparable endeavors due to the absence of three crucial elements: (a) a substantial group of researchers dedicated to the advancement of such applications, (b) a consistent influx of new, top-notch data, and (c) a proactive leader willing to assume responsibility and devote time and effort to organize this undertaking. Furthermore, it is important to acknowledge that competitions of this nature are not without their flaws. Their introduction has resulted in an unintended consequence: a heightened level of secrecy and a prolonged delay in the dissemination of newly developed methods, as a result of the competition deadlines. This, in turn, has had a detrimental impact on the pace at which scientific knowledge is circulated. In addition, although industry players are encouraged to join, they often possess a competitive edge, such as access to confidential data, and are typically not obligated to disclose their codes publicly.

9. Potential ethical implications or biases associated with machine learning algorithms in interdisciplinary fields like enzyme engineering

Machine learning algorithms in interdisciplinary fields like enzyme engineering can introduce ethical implications and biases that need careful consideration [1]. These technologies have the potential to revolutionize enzyme design and optimization but may also raise concerns related to fairness, transparency, privacy, and accountability [2]. Biases in data can lead to suboptimal enzyme designs or favor certain characteristics over others, impacting the diversity and inclusivity of enzyme applications. The complexity of machine learning algorithms can make it challenging to understand how decisions are made, hindering researchers' ability to validate results or comprehend the reasoning behind specific enzyme designs [1]. Additionally, enzyme engineering projects involving sensitive biological data may raise privacy issues if not handled appropriately, necessitating strict adherence to data protection regulations.

Ensuring accountability for the outcomes of machine learning models in enzyme engineering is crucial [2]. Researchers must implement measures to detect and mitigate biases, address errors, and take responsibility for the ethical implications of their work. Integrating ethical considerations into machine learning-driven enzyme engineering projects is essential. Establishing clear ethical guidelines, promoting diversity in dataset collection, ensuring informed consent for data usage, and prioritizing fairness and equity in algorithm design are key steps to mitigate ethical risks [2]. By addressing these ethical implications and biases associated with machine learning algorithms in enzyme engineering, researchers can foster responsible innovation, uphold ethical standards, and promote the equitable and ethical use of advanced technologies in biocatalysis and biotechnology.

10. Industrial applications of engineered enzymes

Engineered enzymes have found their application in various industries ranging from pharmaceutical and medicine to bioremediation to biofuel to detergent formulation and finally to the food industry and animal feed production. These enzymes were altered to elevate their ability to remain stable during harsh conditions, high selectivity of various substrates with high catalytic reaction rates. Applications of most engineered enzymes in various industrial aspects are listed in the next section.

Table 2
Engineered enzymes with medical and pharmaceutical uses.

Enzyme	sources	Enzyme engineering approach	Uses	Objective	Modification made	References
Fructosyl peptide oxidase	*Eupenicillium terrenum*	Site-directed mutagenesis	Diagnosis of diabetes mellitus	Enhanced specificity	Tyr261Trp	[32]
Keto-reductases (KRED)	*Lactobacillus kefir*	Quantum mechanical calculations and molecular dynamics simulations	Enantioselective reduction of 3-triacy-clopentanone and 3-oxacyclopentone	Enhanced enantioselectivity	Glu145 and Ala94Phe	[163]
Carbonyl reductases (KRED)	*Sporobolomyces salmonicolor* AKU4429	Directed evolution	β-Amino ketones enantioselective reduction	Enhanced activity and improve enantioselectivity	Pro170His–Leu174Tyr and Pro170Arg–Leu174Tyr	[158]
TICKLE switch (Thymidine kinase)	Herpes simplex type 1	Directed evolution	Cancer treatment	Enhanced specificity	HSV-TK/CH1 fusion	[164]

10.1. Medical and pharmaceutical applications

A highly promising instance of protein engineering involves the synthesis of antidiabetic sitagliptin by Codexis and Merck [11,27,28]. The process involved utilizing a specifically engineered enzyme called R-selective transaminase, which was produced through directed evolution. This enzyme was employed to carry out the asymmetric amination of prositagliplitin ketone, resulting in the production of sitagliptin with an enantiopurity of 99.95 % [28]. Boceprevir, a chiral amine, is synthesized by the use of altered monoamine oxidase. It is employed for the treatment of chronic hepatitis C. The researchers discovered that the process yield significantly increased by 150 %, while water usage reduced by 40 % [157]. Lactobacillus kefir ketoreductases (KRED) are employed to catalyze the conversion of a ketone intermediate into a chiral alcohol by reduction. Ketoreductase-mediated processes are employed in the production of intermediates for diverse pharmaceuticals, including montelukast, atorvastatin, duloxetine, ezetimibe, crizotinib, and phenylephrine [30,31]. Possible KRED enhancements encompass techniques such as directed evolution and process optimization. KRED demonstrates exceptional enantioselectivity, with a value above 99.55 % [30]. Zhang et al. developed two mutants (P170H/L174Y and P170R/L174Y) by saturating the active site of a KRED enzyme, which facilitated the reduction of 3-(dimethylamino)-1-phenylpropan-1-one and 3-(dimethylamino) -1-(2-thienyl)-propan-1-one to its (R)- γ-amino alcohols with a maximum enantiomeric excess of 95 % [158]. Chiral γ-amino alcohols play a crucial role in the production of certain inhibitors of serotonin reuptake, including tomoxetine and fluoxetine [158]. The demand for chiral chemicals is growing due to the potential variations in biological activities and toxicities exhibited by enantiomers of the same substance [22].

Oxidative biocatalysis has found applications in the pharmaceutical sector, where some compounds like esomeprazole have been synthesized utilizing oxidases [29]. The monoamine oxidase derived from *A. niger* was modified using a mix of approaches, including rational design, and directed evolution, in order to demonstrate a wide range of substrates [29,159]. A chiral variation was employed in a process of separating enantiomers in pharmaceutical intermediates, specifically for the synthesis of solifenacin and levocetirizine. The researchers stated that the R enantiomer yielded a production rate above 97 % [159]. Transaminases are a significant category of modified enzymes that have been thoroughly investigated for their ability to produce chiral amino compounds [23,29]. Midelfort et al. identified an aminotransferase (Vfat) in Vibrio fluvialis that may be used to produce (5)-ethyl 3-amino-5-methyl octanoate [23]. This compound is an intermediary in the manufacture of imagabalin, which is being considered as a potential treatment for generalized anxiety disorder. By employing a combination of varied protein engineering methods and high-throughput screening approaches, they successfully developed a mutant that exhibited a remarkable 60-fold enhancement in the starting velocity, surpassing that of the wild type [23].

Physcomitrium patens, a species of bryophyte, has demonstrated encouraging outcomes in enhancing the glycan structure to produce beta-glucocerebrosidase and alpha-glucocerebrosidase enzymes, which are utilized in the treatment of Gaucher's and Fabry diseases [160,161]. Physcomitrium patens has exhibited enhanced stability, absence of cytotoxicity, and improved pharmacokinetics in comparison to other cell culture systems. These qualities position it as a highly attractive candidate for the development of safe and efficacious medicines. Furthermore, P. patens may have applications beyond lysosomal storage diseases and might be investigated for the treatment of various other medical illnesses as well [162].

Diagnostic enzyme development is receiving attention in pharmaceutical and medical research. Shahbazmohammadi et al. employed an engineered fructosyl peptide oxidase (FPOX) as a diagnostic enzyme for diabetes mellitus [32]. Fructosylvalylhistidine, derived from haemoglobin A1c, serves as a reliable biomarker for the detection of diabetes mellitus and the assessment of glycemic levels. Nevertheless, FPOX facilitates the synthesis of unglycated amino acids, glucosone, and hydrogen peroxide from various fructosyl amino acids, resulting in a less precise diagnostic outcome. Multiple iterations of FPOX were manipulated to enhance the selectivity towards fructosylvalylhistidine as the substrate. The researchers employed computational and experimental methods to introduce the Tyr261Trp mutation in FPOX, thereby enhancing its selectivity by minimising the influence of other amino acids [32]. Other engineered enzymes applied in medical and pharmaceutical industries are listed in the table below (Table 2).

10.2. Enzymatic bioremediation

The pursuit of sustainable solutions is a prominent objective of the current era, and numerous technologies are being devised to accomplish this aim. The bioremediation process offers a compelling ecological option in response to the escalating release of harmful substances. Plants, microbes, and their enzymes have the ability to break down xenobiotics, which are substances that are foreign to biological systems. When comparing the use of genetically modified microorganisms to bioremediation using enzymes, the latter offers various benefits. These include simpler regulation and safer and more convenient disposal methods. Additionally, enzyme-based bioremediation is ecologically friendly and does not have any negative ecological effects due to competitive relationships. Furthermore, it does not require living cells to have specific nutritional requirements and is subject to fewer regulatory restrictions [165,166]. The aforementioned characteristics are consolidated with the capacity to produce these enzymes at a reduced expense, augmenting durability, effectiveness, and selectivity on a larger magnitude through the utilization of molecular engineering methodologies. It plays a significant role in ensuring economic sustainability by enabling an environmentally friendly procedure that promotes long-term economic growth.

The presence of cyanide in industrial effluent is a significant issue due to the elevated levels of cyanide-associated toxicities. Nitrilases have been recognized as a cost-efficient approach for the detoxification of cyanides. Cyanide hydratases and cyanide dihydratases (CynDs) are promising alternatives due to their independence from secondary substrates and cofactors. Cyanides are present in environments with alkaline conditions, when the pH is greater than 11, impeding their enzymatic treatment. Several engineering strategies have been employed to enhance the catalytic activity and ability to withstand varying pH levels of these enzymes.

Wang et al. utilized error-prone PCR and HTS techniques to produce alkali-tolerant mutants, aiming to address the issue of pH tolerance [167]. Two mutants, H7 (E35K, E327G, and 322R) and C5 (D254E, E96G, and Q86R), were shown to possess the ability to degrade cyanide at high temperature (42 °C) and pH [167]. Crum et al. developed 3 CynD mutants, namely 7G8 (A202T, D172 N), CD12 (E327K), and DD3 (K93R), utilizing the identical method of in vivo activity screening [168]. The mutations exhibited enhanced thermal stability at a temperature of 42 °C and demonstrated catalytic activity. The combination of all these mutations in a triple mutant resulted in a synergistic outcome, demonstrating higher improvement compared to the individual mutations [168].

The residues generated by textile industry pose a significant environmental threat due to their high toxicity and ability to cause damage even at very low concentrations of contaminants. The dyes produced by these corporations have the ability to alter both the cloudiness and purity of water, while also posing a risk of causing cancer and toxicity to plants and animals. Multiple experiments conducted by Ali et al. demonstrate significant enhancements in enzyme decontamination through the use of various methods involving immobilized enzymes [169]. In a particular study, ginger peroxidase (GP) was immobilized through adsorption using a nanocomposite material consisting of polypyrrole-cellulose-graphene oxide [33]. The outcome yielded an enhanced decolorization efficacy of Reactive Blue 4 Dye (RB4) by 11 % compared to the free GP. Additionally, there was a significant increase in the maximal velocity, which was 3.3 times greater, as well as an augmented affinity towards RB4. GP was immobilized through electrostatic interaction with a nanocomposite of polypyrrole-zirconium (IV) selenoiodate cation exchanger. This immobilization resulted in enhanced stability (both pH and temperature), improved decolorization efficiency of Methyl Violet 6B Dye (96 % compared to 88 % for free GP), and a tenfold increase in maximal velocity [169]. Both systems employ nanoparticles, which offer numerous benefits due to their extensive surface area, efficient enzyme loading, and strong mechanical properties. Utilizing nano substances for enzyme immobilization has significant potential for industrial waste purification.

Polycyclic aromatic hydrocarbons (PAHs) are highly poisonous and cancer-causing chemical molecules. They are emitted into the environment through the burning of oil, tobacco, petrol, coal, and wood, as well as through natural events such volcanic eruptions and forest fires [170,171]. Multiple research teams have manipulated cytochrome P450 enzymes due to their capacity for polycyclic aromatic hydrocarbon (PAH) cleanup. These enzymes play a crucial role in the breakdown and processing of xenobiotics, fatty acids, and steroids, in mammals. Due to their instability, poor activity, and ecotoxicological concerns with human P450 [172], these enzymes are unsuitable for practical usage in the field. Cytochrome P450 enzymes found in various organisms such as plants, bacteria, fungi, archaea, viruses, and protists have been utilized for their potential in bioremediation [172]. Harford-Cross et al. developed a modified version of cytochrome P450 with a high NADH turnover rate [173]. This modification greatly increased its effectiveness in breaking down the PAHs fluoranthene, phenanthrene, benzo(a)pyrene and pyrene. The authors performed site-directed mutagenesis to introduce substitutions at the active residuesY96 (Y96A mutants and F87A-Y96F) and F87 in CYP101. The NADH oxidation rate exhibited a 31 % increase compared to the camphor oxidation rate catalyzed by the wild-type P450 enzyme [173]. Syed et al. selected CYP5136A3 from the fungus *Phanerochaete chrysosporium* to enhance the oxidation of the PAH molecules phenanthrene and pyrene, based on prior research [172]. Through the process of rational design, which was subsequently validated by activity testing, two mutants (namely, one single mutant, L324F, and one double mutant, W129F/L324F) were discovered to exhibit superior performance compared to the wild-type enzyme. The single mutant exhibited a 23 % increase in the rate of NADH oxidation for phenanthrene and a 144 % increase for pyrene. Similarly, the double mutant showed a 29 % increase for phenanthrene and an 187 % increase for pyrene [172].

Organophosphorus (OP) chemicals are a significant focus of bioremediation due to their widespread application as agricultural insecticides. Enzymes that break down OP compounds exhibit an optimum temperature range of 25–37 °C, which restricts their application in field circumstances [174]. Jacquet et al. created a modified version of a phosphotriesterase-like lactonase derived from

Table 3
Engineered enzymes employed in the process of bioremediation.

Enzyme	sources	Enzyme engineering approach	Uses	Objective	Modification made	References
Triple mutant Cyanide dehydratase (CD12,7G8, DD3)	*Bacillus pumilus*	Error-prone PCR Directed evolution	Bioremediation of cyanide	Enhanced activity	CD12 (Glu327Lys), 7G8 (Asp172Asn). DD3 (Lys93Arg), and Ala202Thr), Triple mutant (Lys93Arg-Asp172Asn-Glu327Lys)	[168]
Ginger Peroxidase	*Zingiber officinale*	Immobilization using polypyrrole-cellulose-graphene oxide nanocomposite	Anthraquinone dye bioremediation	Increase affinity, V_m and activity	–	[169]
phosphotriesterase-like lactonases (SsoPox-αsD6)	*Sulfolobus solfataricus*	Site-directed mutagenesis	Organophosphate bioremediation	Enhanced activity	Val27Ala–Tyr97Trp–Leu228Met–Trp263 Met	[2,175]
Ginger Peroxidase	*Zingiber officinale*	Immobilization using polypyrrole-zirconium (IV) selenoiodate cation exchanger nanocomposite	Cationic dye bioremediation	Increase stability, V_m and activity	–	[169]

the hyperthermophilic archaea *Sulfolobus solfataricus* [2]. The observed form exhibited exceptional thermal stability, with a melting temperature (Tm) of 82.5 °C. Additionally, it demonstrated a substantial enhancement in activity, indicating superior effectiveness in decontamination assays. Various research has concentrated on these characteristics in order to develop enzymes for enhanced bioremediation [2]. Poirier et al. assessed the ability of the phosphotriesterase-like lactonase from *S. solfataricus* to remediate OP chemicals by biodegradation [2]. The findings demonstrated a reduction in the death rate and an enhancement in the ability to move of freshwater planarians, specifically *Schmidtea mediterranea* [175]. Table 3 displays a compilation of modified enzymes employed in the process of bioremediation.

10.3. Biofuels

Global energy demand has been steadily increasing in recent years. Given that fossil fuels are not renewable, it is imperative to explore alternative energy sources that are more environmentally friendly and can be renewable. There is a growing interest in the manufacturing of biofuels. Enzymes are capable of breaking down many substrates to generate biodiesel and ethanol, making them suitable for biofuel production. Nevertheless, a significant number of enzymes lack the inherent resilience required for commercial applications [176,177]. Xylanases are extensively employed enzymes in the field of biofuel production. These enzymes have the ability to break down xylan, which is the primary constituent of plant biomass that is responsible for hemicellulose degradation. Hence, the agroindustrial wastes that are typically disposed of by the industry can serve as substrates for xylanases [178]. Teng et al. employed site-directed mutagenesis to introduce two disulfide links into recombined xylanases derived from *Penicillium janthinellum* MA21601, as part of their xylanase engineering study [179]. The mutant DB-s1s3 exhibited a significant increase in the ideal temperature range, increasing from 50 °C to 70 °C. Additionally, it demonstrated a 4.76-fold improvement in specific activity when compared to the wild-type xylanase [179]. The production of xylanase on a large scale is restricted due to the inhibitory effects of xylose, which is the primary result of xylan breakdown. In order to address this constraint, Hegazy et al. developed genetically modified xylanases that are tolerant to xylose through the process of directed evolution utilizing error-prone PCR [25]. The variants were found to exhibit three mutations: Leu131Pro, Leu133Val, and Met116Ile. The most efficient mutant exhibited a threefold augmentation in catalytic activity and a 3.5-fold enhancement in the inhibition constant (ki) [25].

Additionally, enzymes can also be employed as fuel cells to transform chemical energy into electrical energy. Enzymatic fuel cells provide a notable energy density and serve as eco-friendly biocatalysts that can produce energy without causing harm to the environment [180]. Ma et al. employed a directed evolution technique to enhance the tolerance of the 6-phosphogluconate dehydrogenase enzyme, which functions as an enzymatic fuel cell, to highly acidic pH levels [181]. The researchers noted that the mutant enzyme exhibited a 42-fold enhancement in catalytic ability at a pH of 5.4, in comparison to the natural enzyme [181].

Furthermore, lipases facilitate the breakdown of triacylglycerides into free fatty acids and glycerol through hydrolysis. Lipases are a group of enzymes that are extensively utilized in the production of biofuels. Fatty acids are highly useful molecules in the oleochemistry sector, particularly for applications such as the production of biodiesel [182,183]. Dror et al. employed two methods, specifically structure-guided consensus and error-prone PCR random mutagenesis, to induce mutations in a lipase derived from *Geobacillus stearothermophilus* T6 [182]. Their goal was to obtain a lipase that possessed enhanced transesterification activity, higher methanol tolerance and stability, all of which are crucial features in biodiesel production. Both engineering methodologies could generate enzymes with a prolonged half-life in a solution containing 70 % methanol. In addition, the mutant Gln185Leu, which was the most favorable variant obtained from the random mutagenesis library, exhibited a significant 23-fold enhancement in stability but a reduction in methanolysis activity. Nevertheless, the consensus approach yielded a particularly encouraging variant, His86Tyr/-Ala269Thr, which exhibited a remarkable 66-fold enhancement in stability, a significant increase in thermostability (+4.3 °C), and a 2-fold increase in soybean oil methanolysis [182]. Table 4 comprises a compilation of designed enzymes that are suitable for use in the biofuel production.

10.4. Detergent formulation

Engineered enzymes from protease and lipase are the most sustainable alternatives that can substitute the use of harmful chemical compounds for the removal most difficult stains. The use of enzyme-engineered formulated detergents also limits environmental pollution and minimizes energy consumption. The limitation in the use of enzymes for the detergent formulation is stability (pH and temperature) and the ability to degrade different substrates. To overcome these limitations, site-directed mutagenesis was combined with directed evolution procedure to generate mutant Pro9Ser/Lys27Gln and Pro9Ser/Thr162IIe from alkaline serine protease derived from *Bacillus pumilus* BA06 [186]. This mutation brought about 5-fold increases in chitinolytic activity, and no decrease in thermostability was observed. On the other hand, lipases needed to be alkaline pH resistible and thermostable before it could be applied for removal of difficult oil grease and stain in fabric. To achieve this aim, Khan et al. made use of a rational approach to generate mutant bsl-the3, which had higher pH stability maintaining 65 % activity when incubated for 24 h at pH 9 [16]. Also, its optimal temperature was 55 °C, and it showed better thermostability than the wild type. This result was observed when bsl-the3 was incubated in several surfactants during industrial applications.

10.5. Food and animal feed industry

Starch and other classes of food are the chief source of energy for non-photosynthetic organisms and plants. These classes of food could be processed either chemically or enzymatically into varieties of products in food industries and animal feed industries, as the

Table 4
Engineered enzymes employed in biofuel production.

Enzyme	sources	Enzyme engineering approach	Uses	Objective	Modification made	References
Lipase	*Streptomyces* sp. (W007 strain)	overlap extension approach of Site-directed mutagenesis	Used in fat/oil modification to manufacturing of biofuel	Improve thermostability and activity	Phe153Ala; His108Ala; Val233Ala	[18]
Xylanase	*Penicillium janthinellum* (MA21601)	Site-directed mutagenesis	Saccharification of xylan, applied in biofuel production	Increase catalytic efficiency and thermostability	DB-s3s4: Ser27Cys/Ser186Cys; DB-s1s4: Ser27Cys/Ser39Cys; DB-s1s3: Ser39Cys/Ser186Cys	[179]
β-1,4-Endoglucanase	*Chaetomium thermophilum*	Site-directed mutagenesis	biodegradation of cellulose	Improve thermostability and activity	Tyr173Phe; Tyr30Phe; Tyr30Phe/Tyr173Phe	[184]
Xylose reductase	*Pichia stipites*	Site-directed mutagenesis	Fermentation of xylose to ethanol	Change in specificity coenzyme	2-2C12: Ser271Gly, Asn272Pro, Lys270Ser, and Arg276Phe	[185]
Xylanase	*Geobacillus stearothermophilus*	error-prone PCR Directed evolution	Saccharification of xylan, applied in biofuel production	Enhance xylose tolerance	Leu131Pro, Leu133Val, and Met116Ile,	[25]
Xylanase	*Penicillium canescens*	Site-directed mutagenesis	Saccharification of xylan, applied in biofuel production	Enhance thermostability	Leu18Phe	[178]

case may be. Based on the complexity of these molecules used as raw materials, they require a combination of various enzymes for proper breakdown into small components. Engineering of these enzymes used in this process could help force a reaction to proceed in the desired direction, enhance their selectivity or specificity, and finally stabilize its activity towards reaction conditions that are required for large-scale production or synthesis. Pullulanase from *Bacillus deramificans* was engineered by Duan et al., by substituting Asp437 to His437 and Asp503 to Tyr503, and this mutation led to an increasing in the thermostability of the enzyme, which is applied in the production of glucose syrup and fructose syrup [17].

On the other hand, Gao et al., made use of a molecular cyclization technique to convert mesophilic mannose extracted from *Bacillus subtilis* to the thermophilic enzyme [20]. Moreover, the spy tag/spy catcher system was employed in the engineering of a more stable mannose-xylanase bifunctional chimeric employed in the degradation of several non-starch polysaccharides and lignocellulosic biomass degradation industries.

11. Conclusion and recommendation

This review has expatiated the economic viability of enzymes with suitable biochemical properties in various industrial applications. It has also explained in details various challenges encountered in the production of these enzymes and possible advanced biotechnological enzyme engineering process to overcome those challenges. The rationale approach as discussed in this review is one of the effective procedures that could be employed in discovering of novel enzyme with improved biochemical features for enzyme that possesses a well-known and resolved protein structure. Directed evolution approach on the other hand could be more efficient in the discovery of sporadic novel enzyme due to its ability to screen wide range of sequences from unknown protein structure. Combination of these two approaches (semi-rationale) with high-throughput screening of generated smart libraries had resulted in production of novel enzymes with enhanced biochemical properties than utilizing either rationale approach or directed evolution approach. Finally, the application of machine learning procedure has also help to overcome most limitation that are being encountered in utilizing the three different approaches (rationale, directed evolution and semi-rationale) as discussed in this review. This process had led to discovery of novel drugs and other biocatalysts that are utilized in food, biofuel, detergent formulation and bioremediation processes. However, the untapped potential of machine learning in the design of biocatalysts has not yet been completely explored. The community still confronts numerous issues. Some of the most urgent challenges today include the absence of uniform and reliable high-quality data sets for training and validation, traditional imbalances and biases in data, the inherent multidisciplinary nature of the approach, and the complexities involved in explaining, interpreting, and comparing the results of predictors. These issues are currently receiving more recognition and attention as a result of the rising needs and the growing population of scientists involved in the fascinating field of enzyme engineering. Advanced experimental techniques such as next-generation sequencing, high-throughput screening, deep mutational scanning, and microfluidics enable the collection of bigger quantities of data with improved quality and consistency. With the accumulation of additional data, the utilization of increasingly sophisticated machine learning techniques, such as deep learning, will become prevalent. This will need the effective utilization of computational resources and allocation of memory. The latest advancements in interpretable topologies of artificial neural networks and feature significance scores offer valuable insights into the underlying principles that contribute to improved prediction. Dependable machine learning tools will offer the most optimal initial positions for enzyme engineering. In addition, these changes will facilitate additional research endeavors aimed at elucidating derived models, deciphering their parameters, and comprehending the underlying molecular mechanisms. Ultimately, this will contribute to a more comprehensive understanding of the links between the structure and function of enzymes.

CRediT authorship contribution statement

Obinna Giles Ndochinwa: Writing – original draft, Conceptualization. **Qing-Yan Wang:** Methodology. **Oyetugo Chioma Amadi:** Supervision. **Tochukwu Nwamaka Nwagu:** Validation. **Chukwudi Innocent Nnamchi:** Validation. **Emmanuel Sunday Okeke:** Writing – review & editing, Writing – original draft. **Anene Nwabu Moneke:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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