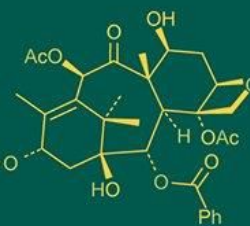


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Next-generation biocatalysis: Integrating enzyme engineering, synthetic biology, and information technologies for industrial applications

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Abstract

Biocatalysis has rapidly evolved into a vital part of modern industrial processes that are enabled by technology and sustainable methods when compared to legacy chemical catalysis. Enzymes achieve remarkable chemo-, regio-, and stereoselectivity, yet their utility in varied industrial processes is frequently limited due to restrictions and weaknesses related to: (1) instability under reaction conditions, (2) limited substrate specificity, and (3) challenges with recovery and reuse. Recent innovations towards producing biocatalysts performance enhancements through enzyme engineering, stabilization, and immobilization developments have contributed to the transformational capacities biocatalysis systems. These developments are both theoretical and practical, with advances in protein engineering, e.g., rational design, directed evolution, chimeric, bifunctional, and semi-synthetic enzymes, representing the development of natural enzymes into novel catalytically-active proteins. In addition, immobilization technologies of state-of-the-art carriers, cross-linked enzyme aggregates, and co-factor recycling of enzymes improved enzyme stability and active life. Synthetic biology has developed ways to establish optimized microbial hosts, and artificial pathways for high-yielding enzyme production, along with cell-free systems for high scale biotransformation's, and biotransformation's of high-value products and processes. Finally, unprecedented tools of information technologies, e.g., molecular modeling, artificial intelligence, machine learning, bioinformatics, etc., are rationalizing enzyme discovery and design and predictive optimization. In total, their improvements will produce next-generation biocatalysts suitable for wide-ranging industrial uses, including pharmaceuticals, food and beverages, biofuels, textiles, and environmental clean-up. This review summarizes current advances, industrial relevance, and future outlooks of enzyme-based biocatalysis for sustainable industrial biotechnology.

Keywords: Enzyme engineering, immobilization, chimeric enzymes, semi-synthetic enzymes, industrial biocatalysis, protein engineering, artificial intelligence, green biotechnology

Introduction

Biocatalysis, or the use of natural and engineered enzymes to perform chemical transformations, has emerged as one of the cornerstones of biotechnology (Bornscheuer *et al.*, 2012; Fryszkowska *et al.*, 2020) [9, 35]. Enzymes are proteinous biocatalyzers that control the rate of biological reactions as well as complex metabolic pathways and other cellular processes (McLure *et al.*, 2022; Du *et al.*, 2023; Brandenburg *et al.*, 2019) [10, 27, 76]. It is well known that enzymes are highly effective and versatile biocatalysts. When enzymes are present, reactions happen far more quickly than when they are lacking (Dotsenko *et al.*, 2023) [25]. These biological molecules are extremely specific in identifying the chemicals that need to be transformed. Enzymes have developed independently over time, resulting in novel biocatalysts with superior properties and a variety of biological uses (Musil *et al.*, 2018; Edison *et al.*, 2022; Kroll *et al.*, 2023) [30, 57, 80]. Enzymes provide unique catalytic performance, regioselectivity, and stereoselectivity at mild conditions for biocatalysis, some of which are applicable for sustainable production in industry. Enzymes operate at physiological temperature, pressure, and pH, unlike most chemical catalysts that require extreme conditions for activity. As a result, biocatalysis can reduce energy consumption and environmental hazards. Aside from biocatalysis,

enzymes have developed in industrial biotechnology scope to include pharmaceuticals, food and beverages, agriculture, biofuels, textiles, waste, and remediation (Vidya *et al.*, 2017; Woodley, 2018) [121, 123].

Nevertheless, natural enzymes present substantial disadvantages that limit immediate application at scale. Natural produced enzymes generally lack stability against extreme process conditions, have limited substrate range, efficiency recovering and reusing the enzyme, and costly production, posing significant challenges for commercialization (Hailes *et al.*, 2007) [43]. To mitigate these challenges, the biocatalysis field has transitioned swiftly from historical enzymes utilized at either natural or altered levels, moving toward next generation concepts, merging disciplines of protein engineering, synthetic biology, enzyme immobilization, and computational software (Khan *et al.*, 2019; Zhao *et al.*, 2018; Gao *et al.*, 2019) [38, 55, 137].

Recent advancements in protein engineering have permitted the emergence of stable enzymes with improved thermostability, solvent tolerance, and increased substrate specificity. Directed evolution and rational design methods enable us to modify the structure of an enzyme to provide an industrial need. In addition, the creation of chimeric or bifunctional enzymes has generated multifunctional catalysts capable of complex reactions within one reaction system.

Simultaneously, synthetic biology has sparked a revolution in enzyme production and pathway optimization. Through reprogramming microbial hosts and creating artificial metabolic circuits, synthetic biology offers scalable strategies for enzyme synthesis and utilization that are more sustainable (Gao *et al.*, 2007) [39]. In conjunction with immobilization technologies that improve stability and reusability, these strategies are practically beneficial for continuous and large-scale bioprocessing.

Enzymes are perfect candidates for pharmaceutical applications because of their inherent specificity and compatibility with mild reaction conditions (Yi *et al.*, 2021) [128]. In addition to improving reaction selectivity, using enzymes in drug discovery and synthesis aligns pharmaceutical operations with green chemistry concepts, reducing their environmental impact (Bell *et al.*, 2021) [4].

The use of natural or artificial enzymes to speed up chemical reactions is known as biocatalysis, and it has become a potent and sustainable substitute for conventional chemical catalysis. Protein engineering has enabled scientists to modify enzymes for certain processes and improve their stability through directed evolution and rational design, opening the door to new uses in pharmaceutical synthesis (McIntosh *et al.*, 2021; Chen *et al.*, 2020; Sheldon *et al.*, 2017) [16, 75, 108]. The rational design of enzymes with increased catalytic activity has been made easier by computational biology, which has also improved our comprehension of enzymatic operations. Enzymes are very appealing in industrial biotechnology because of their special capacity to offer strong chemo-, regio-, and stereoselectivity. Enzymes normally function in mild reaction conditions, such as ambient temperature, air pressure, and aqueous solutions, in contrast to harsh chemical catalysts that frequently ask for high temperatures, extreme pressures, or hazardous solvents. In addition to lowering energy use and the production of hazardous waste, this is in line with the global movement toward sustainable

manufacturing methods and green chemistry (Leresche *et al.*, 2006; Pollard *et al.*, 2007) [62, 87].

Over the past thirty years, the use of enzymes in pharmaceuticals, agrochemicals, food and drink, detergents, textiles, bioenergy, and environmental bioremediation have grown dramatically. These days, industrial biocatalysis is essential to the synthesis of chiral intermediates, high-fructose syrups, bioethanol, eco-friendly detergents, and active pharmaceutical ingredients (APIs). In order to provide a solid basis for their later incorporation into various industrial processes, early research focused on isolating these specialized enzymes and describing their enzymatic characteristics (Tatineni *et al.*, 2008; Zhang *et al.*, 2016) [113, 134].

They are currently employed in many different industries. For example, in agriculture, keratin-rich waste is converted into protein-rich animal feed and nutrient-dense biofertilizers (Saeed *et al.*, 2024; Xu *et al.*, 2022) [99, 127], encouraging resource recycling and lowering pollution levels. Additionally, waste management systems use enzymatic recycling to decrease the environmental impact of conventional disposal methods like landfilling and incineration (Jagadeesan *et al.*, 2020; Srivastava *et al.*, 2020) [53, 111]. The global enzyme business is still expanding because of advancements in enzyme engineering technology and rising demand for sustainable processes. Despite these developments, there are still certain limitations on the industrial use of enzymes.

Therefore, next-generation biocatalysis is an amalgam of cutting-edge disciplines like computational sciences and synthetic biology with conventional enzymology. In addition to discussing the revolutionary significance of information technology, this study attempts to give a thorough summary of recent developments in enzyme stabilization, regeneration, protein engineering, immobilization, and synthetic biology-driven biocatalyst design. Particular attention is paid to their usefulness in a variety of industries, as well as the difficulties and potential paths for making biocatalysis the foundation of environmentally friendly and technologically advanced industrial processes.

Challenges of Native Enzymes in Industrial Applications:

The direct use of natural enzymes often fails to meet the rigorous demands of large-scale processes. Native enzymes are typically evolved to function under physiological conditions within living organisms, which are markedly different from industrial environments. Challenges include:

- **Limited stability:** Most enzymes lose activity under high temperatures, extreme pH, or in the presence of organic solvents commonly used in industrial reactions.
- **Narrow substrate specificity:** Natural enzymes often act on a limited range of substrates, restricting their applicability for diverse chemical transformations.
- **Cofactor dependence:** Many industrially relevant enzymes require cofactors (e.g., NADH, NADPH, FAD), which are expensive and unstable, making continuous processes uneconomical.
- **Recovery and reuse difficulties:** Enzymes used in homogeneous systems are difficult to separate and recycle, increasing production costs.
- **Short shelf-life:** Storage and operational stability limit long-term industrial deployment.

These challenges have necessitated a shift from the direct use of natural enzymes toward next-generation biocatalyst systems, which combine molecular biology, synthetic biology, materials science, and computational approaches.

Evolution of Next-Generation Biocatalysis

Advances in protein engineering, enzyme immobilization, synthetic biology, and computational technologies have revolutionized the field of biocatalysis. Rational layout and directed evolution strategies permit researchers to introduce centered mutations that enhance enzyme thermostability, catalytic performance, or substrate scope. Chimeric and bifunctional enzymes are designed to carry out multi-step reactions inside a unmarried protein, disposing of the want for a couple of enzymes. Semi-synthetic enzymes, incorporating unnatural amino acids or artificial cofactors, enlarge the chemical reactivity of natural enzymes into new domains.

Meanwhile, immobilization techniques together with entrapment, covalent binding, adsorption, and cross-related enzyme aggregates (CLEAs) offer answers for enzyme balance and reuse, enabling continuous-flow approaches at business scale. Synthetic biology strategies similarly aid these improvements by way of supplying engineered microbial structures for massive-scale enzyme production, cofactor regeneration, and artificial metabolic pathways.

Crucially, the integration of modern facts technologies has multiplied enzyme discovery and optimization. Molecular modeling, bioinformatics, synthetic intelligence (AI), and machine gaining knowledge of (ML) equipment now are expecting enzyme-substrate interactions, simulate catalytic mechanisms, and manual rational engineering with minimum experimental attempt. These advances together represent a paradigm shift, remodeling enzymes from fragile herbal catalysts into sturdy, flexible, and sustainable industrial biocatalysts.

Scope and Objectives of This Review

This review aims to provide a comprehensive overview of recent advances in enzyme-based biocatalysis, with a focus on their stabilization, engineering, and application in technology-driven processes. The objectives are:

1. To critically analyze the advantages and disadvantages of biocatalysis in industrial biotechnology.
2. To examine strategies for stabilization and regeneration of enzyme systems, including immobilization, cofactor recycling, and material-assisted stabilization.
3. To discuss advances in protein engineering, including rational design, directed evolution, chimeric and bifunctional enzyme construction, and semi-synthetic enzyme development.
4. To highlight the role of modern information technologies AI, machine learning, molecular modeling, and bioinformatics in accelerating enzyme discovery and design.

5. To explore practical industrial applications across pharmaceuticals, food, bioenergy, textiles, and environmental remediation.
6. To present future perspectives on integrating enzyme engineering with synthetic biology and computational platforms for sustainable industrial transformation.

Through this framework, the review positions next-generation biocatalysis as a driving force in shaping the future of industrial biotechnology. By emphasizing both technological innovation and practical significance, it bridges the gap between academic research and real-world applications, offering valuable insights for researchers, industry practitioners, and policymakers.

Enzyme Stabilization and Regeneration Strategies

The low stability of biocatalysts in chemical conditions is one of the main obstacles to their practical use (Woodley *et al.*, 2019) ^[124]. According to Rigoldi *et al.* (2018) ^[95], thermostability is a critical issue that is controlled by numerous parameters and nearly always necessitates modifications to the peptide chain of amino acid residues that make up the protein. We changed the potentially important serine residues found in structure and sequence comparisons with the thermophilic homologous CelA from *Clostridium thermocellum* in order to increase the thermostability of chitosanase-CsnTS. This engineered CsnTS can be used in the industrial scale-up production of chitoooligosaccharides (Zhou *et al.*, 2021) ^[138].

Stabilization and regeneration of enzymes are critical for industrial application of biocatalysis. While enzymes confer great specificity and efficiency, they evolved to function optimally at mild physiological conditions and when subjected to industrial conditions, especially extremes in temperature, pH, solvents, and mechanical stress. Enzymes tend to denature, aggregate or be inactivated very quickly. Thus, the economic value of enzyme-based processes rests heavily on the stabilization, reusability and operational lifespan of enzymes. Many physical, chemical, biological, and engineering-based strategies have been developed to overcome these challenges.

Enzyme Immobilization

To allow to improve the catalytic activity, stability, and reusability of soluble enzymes, enzyme immobilization is defined as limiting the mobility of the enzyme in a specific area of space (Kujawa *et al.*, 2021; Rodrigues *et al.*, 2021; Bolivar *et al.*, 2023) ^[8, 58, 96]. They can be retrieved, reused, and limited to a specific area of space with retained catalytic activity (Das *et al.*, 2023) ^[121]. According to Zhang *et al.* (2023) ^[133], an insoluble enzyme is immobilized. This type of heterogeneous biocatalysis is distinct. An immobilized enzyme affects some features, including catalytic activity, thermal stability, and storage stability (Abdel-Hameed *et al.*, 2022) ^[2].

Table 1: Parameters influencing the activity of immobilized enzymes (Hwang *et al.*, 2013) ^[51]

Factors related to enzymes	Factors related to the carriers	Specific factors related to the reaction system
Size of the enzyme	Organic or inorganic	Reaction medium
Conformational flexibility required by the mechanism	Hydrophobic or hydrophilic	Diffusion limitations
Isoelectric point	Surface charges	Enzyme inhibition
Surface functional groups/charge density	Surface functionalization	Precipitation of products
Glycosylation	Chemical and mechanical stability	Viscosity of the mixture
Stability under immobilization conditions	Surface area	Reaction thermodynamics

Presence of hydrophobic regions	Porosity	Non-specific solute-support interactions
Presence of hydrophilic regions	Particle size	
Additives in the enzymatic preparation		

Adsorption, cross-linking or covalent bonding, entrapment, and/or encapsulation are the three ways to immobilize the enzyme on a support material (Sampaio *et al.*, 2021) ^[100]. The enzyme, a carrier material, and the attachment technique are the three primary parts of an immobilized enzyme system (Cowan *et al.*, 2011) ^[20]. Because the immobilization procedure has a significant impact on the biocatalyst's characteristics, the immobilization technique will have an impact on the stability and catalytic activity of the biocatalyst (Tang *et al.*, 2022) ^[112].

Adsorption

Adsorption is the mechanism whereby an enzyme builds up on a solid support surface through intermolecular interactions (Ramalho *et al.*, 2023; Kharazmi *et al.*, 2023) ^[56, 92]. It also eliminates the need to alter the surface and linkers like glutaraldehyde and cysteine for cross-linking or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide for covalent bonding (Kumar *et al.*, 2020; Smith *et al.*, 2020) ^[60, 109]. Hydrogen bonds and electrostatic interactions are involved in surface adsorption interactions with enzymes (Feng *et al.*, 2023) ^[33]. The adsorption process involves the interaction of enzymes with support surfaces. Therefore, in order to ensure high and repeatable enzyme coverage on a support to accomplish typically mild, easy, economical, rapid, good performance, and good reusability, the polarity and charge of the enzyme will be crucial attributes (Aghabeigi *et al.*, 2023) ^[3]. Nevertheless, adsorption-based enzyme immobilization has drawbacks, such as enzyme leaching and possibly lower enzyme binding to the support than covalent binding (Nageib *et al.*, 2022) ^[81].

Cross-Linking

The act of creating a covalent bond between two or more molecules is known as cross-linking (Moosavi *et al.*, 2022; Paiva *et al.*, 2024; Feng *et al.*, 2022) ^[34, 79, 85]. One commonly used technique for immobilizing enzymes is the use of cross-linked enzyme aggregates. It frequently results from an activation or surface modification process (Narayanan *et al.*, 2022; Hu *et al.*, 2023; Jaafar *et al.*, 2022) ^[48, 52, 82]. The most popular method of initial surface modification of inorganic substrates is silanization, which is the creation of organic functional groups on a surface using an organofunctional silane reagent. Surfaces having natural amino groups or those coated with p-nitrobenzoyl chloride can be treated to produce aldehyde or arylamine groups. (Shaarani and others, 2016) ^[104]. Purification from an aqueous buffer and cross-linking the resulting physical aggregate of enzyme molecules are the only steps in this straightforward process, which is easily optimized (Liu *et al.*, 2021) ^[69]. Cross-linking produces a stable, immobilized enzyme that can be used as a biocatalyst. However, the main disadvantages of gel-like cross-linked enzymes are frequently poor activity retention, challenges with reuse, low mechanical stability, and general handling problems (Li *et al.*, 2020) ^[63].

Entrapment or Encapsulation

Enzymes that are caged with covalent or non-covalent bonds are said to be immobilized through entrapment. These

matrices preserve the biocatalyst's characteristics while minimizing conformation change (Reshmy *et al.*, 2021; Tavernini *et al.*, 2020; Xing *et al.*, 2022) ^[94, 114, 126]. The enzyme is retained while allowing substrates and products to move via a network of polymers (Abbasi *et al.*, 2023) ^[1]. After entrapment, enzymes are not bound to the polymeric matrix, but their diffusion is constrained. Compared to an enzyme that is physically adsorbed, an entrapped enzyme is more stable. Although enzyme activity is either maintained or increased, entrapment immobilization is easier to achieve than covalent bonding (Rashid *et al.*, 2022; Ganonyan *et al.*, 2020; v *et al.*, 2022) ^[37, 93].

Enzyme immobilization through physical entrapment has many uses and might not interfere as much with the characteristics of the native enzyme (Rivero Berti *et al.*, 2021) ^[5]. To guarantee that the substrate and product can diffuse into and out of the polymer matrices, encapsulation techniques must consider the chemical conditions of the polymerization matrices, the pore diameter and volume, and the compatibility of enzymes with the pore matrices. Since many enzyme encapsulations do not cause a major reduction in enzyme activity following immobilization, catalytic activity retention is rather common (Datta *et al.*, 2021) ^[22].

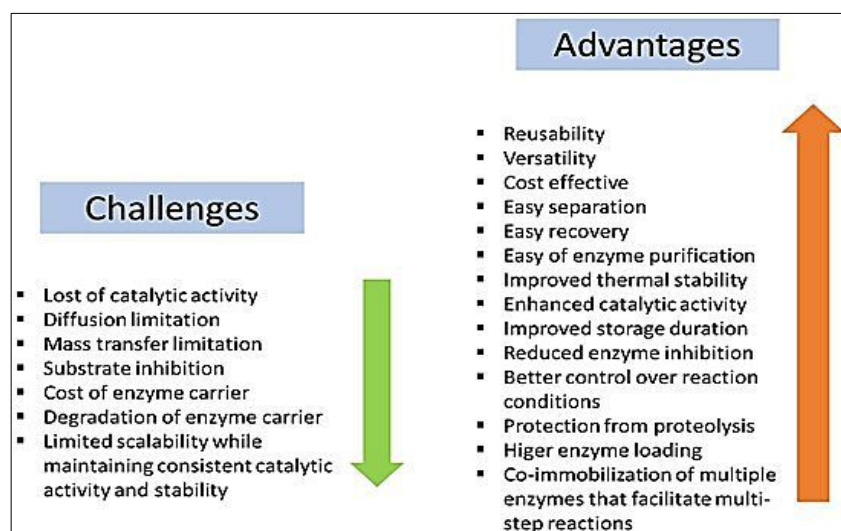
Covalent Bonding

The formation of covalent bonds between each molecule of an immobilized enzyme and the support creates a section of the enzyme where the residues attached to the support stay in place during any movement or dislocation of the enzyme molecule caused by hazardous conditions like heat, extremely high or low pH, and organic solvents (Meng *et al.*, 2023) ^[77]. The highly rigidized region can be relatively large if the support's structure is sufficiently compatible with the enzyme's surface (for example, a flat support structure and a globular protein that is moderately flexible). This establishes the essential stabilization of the enzyme molecule as protection against any harmful conditions, such as extremely high acidic or alkaline conditions and temperature (Guisan *et al.*, 2022) ^[42].

Parmegiani Marcucci *et al.* (2022) ^[73] used the SBA-15, which was modified with tin (SnS8 and SnS23) and had varying pore diameters (S8 with 9.1 nm and S23 with 23 nm), to study the immobilization of Burkholderia cepacia lipase via covalent bonding. The immobilized enzyme had the highest specific activity on the support with the largest pore size (25 nm). A strong link between lipases and modified silicas was demonstrated by the absence of lipase in the buffer solution used for lipase desorption during the leaching tests. The successful production of PNGase F with a glutamine tag in Escherichia coli and the site-specific covalent immobilization of PNGase F with this unusual tag via microbial transglutaminase (MTG) are reported by Zhang *et al.* (2023) ^[133]. Using MTG, PNGase F was immobilized from the glutamine tag and covalently and site-specifically transformed into primary amine-containing magnetic particles. Immobilized PNGase F may deglycosylate substrates and exhibit strong thermal and reusability capacity with enzymatic efficiency similar to that of its soluble counterpart (Tvorynska *et al.*, 2022) ^[118].

Table 2: Summary of the methods and materials used in enzyme immobilization together with its advantages and disadvantages.

Method	Materials	Advantages	Disadvantages	References
Synthesising magnetic-cross-linked enzyme aggregates (CLEAs) or trapping the CLEAs in particles with superior mechanical qualities.	Glutaraldehyde as a linker; Ammonium sulfate as a precipitating agent; Bovine serum albumin (BSA) and 3-aminopropyltriethoxysilane (APTES) as additive.	It is suggested to tackle substrate diffusion issues by generating more porous CLEAs, among other things.	Diffusion limitation.	Sampaio <i>et al.</i> , 2022 ^[100]
Immobilisation of protease via covalent bonding on chitosan.	Chitosan as a carrier; glutaraldehyde and ethylenediamine as modification agents and cross-linkers.	The catalytic activity of immobilised enzymes is equivalent to that of free enzyme (pH 9 and 60 °C); however, the immobilisation procedure broadened the optimum temperature range of enzyme activity (50-70 °C).	After three cycles of use, the immobilised enzyme only maintained 47.08% of its initial activity.	Ramvalho <i>et al.</i> , 2023 ^[92]
Laccase was immobilised on a low-cost, nanosized magnetic biochar (L-MBC) via adsorption, precipitation, and cross-linking.	Bagasse biochar as a carrier; ammonium sulfate as a precipitation agent; glutaraldehyde as a cross-linker.	The magnetic biochar could immobilise a substantial quantity of enzymes with increased catalytic activity (2.251 U per mg MBC), better stability, improved storage stability, pH tolerance, and thermal stability over free laccase.	The catalytic activity of immobilised laccase decreased more than 50% of its initial activity at pH 5.5 and 6.	Zhang <i>et al.</i> , 2020 ^[136]
Encapsulation of laccase using alginate, alginate-silica and silica sol-gel.	Tetraethyl orthosilicate (TEOS) and alginate as carriers.	The experimental results showed that incorporating silica into alginate resulted in a better (70%) encapsulation efficiency (EE) for the laccase extract than for the alginate alone (59%). Furthermore, encapsulating the laccase extract in sol-gel resulted in an increase in its catalytic activity, as well as a 90% rise in the EE. The alginate and sol-gel matrices also improved laccase catalytic efficiency compared to free laccase, with kcat values of 89.9 (alginate), 63.7 (alginate-silica), and 56.9 min ⁻¹ (silica sol-gel), respectively.	After three reaction cycles, the catalytic activity of immobilised laccase with alginate-silica was decreased by 50%.	Gill <i>et al.</i> , 2018 ^[40]
New enzyme immobilisation ideas via multipoint covalent attachment on support surfaces.	Conventional supports such as cyanates, tosyl chloride and N-hydroxy-succinimide esters as carriers. Polyethylene glycol as modification agent.	The formation of several bonds between each molecule of an immobilised enzyme and the support creates a region of the enzyme in which the residues attached to the support cannot change their position during any distortion of the enzyme molecule caused by heat and organic cosolvents.	Difficult to achieve desired results.	Guisan <i>et al.</i> , 2022 ^[42]

**Fig 1:** Advantages and challenges of enzyme immobilization (Mohidem *et al.*, 2023) ^[78].

Protein Engineering-Based Stabilization

Enzyme robustness can be directly increased through genetic manipulation. Examples of employing protein engineering to control plant immunology and energy conversion have been documented, but there are very few instances of secondary plant metabolic regulation based on protein engineering. It was established that the modified rice RGA5 could specifically recognize and bind the pathogen effector protein AVR-PikD, which is not recognized by the original RGA5, extending the crop's resistance to disease by engineering the heavy metal-associated (HMA) structural domain of rice RGA5 (Maqbool *et al.*, 2015) [72]. RbcS is a tiny subunit of Rubisco. In rice Rubisco, eliminating the OsRbcS (rice RbcS) multigene family and substituting OsRbcS entirely with SbRbcS (sorghum RbcS) increased the rate of photosynthetic CO₂ assimilation and photosynthetic efficiency (Matsumura *et al.*, 2020) [74], which can significantly increase plant growth and productivity. Protein engineering offers a foundation for the development of new enzymes that can create a quick system for protein screening and modification *in vivo*, which offers the potential to reshape the secondary metabolic network in plants. CRISPR/Cas9-assisted engineering now offers technical support for the replacement and removal of pathway enzymes.

Protein Engineering of Enzymes

Rational Design, Directed Evolution, Chimeric and Semi-Synthetic Enzymes

Thus, enabling the development of customized catalysts with enhanced activity, stability, and substrate selectivity, enzyme engineering has transformed contemporary biotechnology. Even though natural enzymes are quite effective in physiological settings, they frequently exhibit limitations in industrial settings with high temperatures, severe pH, organic solvents, or high substrate concentrations. Protein engineering offers answers by altering the structures and sequences of enzymes to achieve the appropriate stability and catalytic properties. Protein engineering techniques can be broadly classified into two groups: directed evolution and rational design. Hybrid approaches, such as chimeric proteins and semi-synthetic enzymes, are becoming more and more significant.

Rational Design of Enzymes

After altering the natural metabolic network of plants, metabolic engineering seeks to improve plant resilience and produce a value-added chemical, among other beneficial functional traits (Lynch *et al.*, 2021) [70]. The most successful method of metabolic engineering is now changing the synthetic pathways in plants using genetic engineering techniques (Fu *et al.*, 2018) [36]. With improved stability, increased catalytic activity, and most importantly a broader range of protein applications, protein engineering methods and approaches have made it a reality quickly and easily, in contrast to genetic engineering techniques that obtain targets through mass disorderly screening (Cai *et al.*, 2015) [12]. This offered a great target for modifying the structure and/or content of lignin in plants. A better phenotype can be obtained in plants by substituting enzymes with a higher catalytic rate for those with a low catalytic rate and weak affinity and specificity. The sensing of arbuscular mycorrhizal fungus in rice is mediated by the LysM receptor heteromer OsMYR1/OsLYK2 and OsCERK1. The

recognition of nodulation factors in rice was greatly enhanced by substituting the ectodomains of OsMYR1 and OsCERK1 with those from the homologous Nod factor receptors MtNFP and MtLYK3 of *Medicago truncatula*. This also increased calcium oscillations in response to Nod factors (He *et al.*, 2019) [45].

- Introduction of disulfide bonds, salt bridges, and hydrophobic residues improves thermostability.
- Alteration of surface charges enhances solvent tolerance.
- Computational modeling and molecular dynamics predict stabilizing mutations prior to laboratory testing.

Rational design is a knowledge-driven approach that relies on structural, biochemical, and computational data to predict beneficial mutations.

Principles of rational design

- Based on three-dimensional structures obtained via X-ray crystallography, cryo-EM, or homology modeling.
- Involves identifying catalytic residues, flexible regions, and stability determinants.
- Uses computational tools such as molecular dynamics simulations, docking, and energy minimization.

Strategies in rational design

- **Active-site redesign:** Altering residues around the catalytic pocket to expand substrate specificity (e.g., redesigning lipases for biodiesel synthesis).
- **Stability enhancement:** Introducing disulfide bonds, salt bridges, or proline residues to increase rigidity.
- **Surface charge modification:** Enhancing tolerance to pH or ionic strength by altering surface amino acids.
- **Cofactor switching:** Engineering enzymes to use alternative, cheaper cofactors (e.g., switching from NADPH to NADH).

Advantages and limitations

- Precise and predictable when sufficient structural data is available.
- However, limited by the accuracy of computational models and incomplete understanding of protein folding dynamics.

Directed Evolution of Enzymes

This is a potent method for creating efficient enzyme catalysts that can carry out a range of biocatalytic tasks in different sectors. In contrast to rational design, directed evolution allows for relatively quick enzyme modification and does not require in-depth knowledge of the structure-function correlations. Additionally, it is similar to the process of natural evolution (Zeymer and Hilvert, 2018) [131]. The general process of directed evolution is quite straightforward: a library of mutants is created, and those that possess the desired trait are identified using an appropriate selection or screening procedure. The biosynthetic machinery of the cell is then utilized in an *in vitro* approach or engineering in a heterologous or homologous host organism to produce the comparable enzyme changes. Until an enzyme with the desired characteristics is obtained, the process of mutant creation and selection is repeated multiple times. Although useful, thorough prior knowledge of the enzyme is not required because directed evolution does not require any foresight

regarding the mutation locations, which is an advantage over rational design.

Frances H. Arnold's guided evolution of enzymes earned her the 2018 Chemistry Nobel Prize. George P. Smith and Sir Gregory P. Winter split the prize for their phage display of peptides and antibodies. The directed evolution approach is a reliable protein engineering technology that produces random mutations in a target gene and then quickly selects protein variants based on advantageous characteristics for certain uses (Vidal *et al.*, 2023) ^[120].

An experimental, iterative method that simulates natural selection in a lab setting is called directed evolution. It entails creating genetic diversity and screening for desired features rather than depending on past structural knowledge. To create DNA libraries, mutations are made to the coding gene of the biocatalyst that needs to be modified. By the end of the procedure, enzymes with better qualities are found by screening and selection, and the polymerase chain reaction is then applied to the corresponding genes (Zeng *et al.*, 2020) ^[130].

- Iterative random mutagenesis and selection have yielded enzymes that remain active at temperatures above 100 °C, tolerate organic solvents, or resist high substrate/product concentrations.
- Industrial lipases, cellulases, and oxidoreductases have been optimized using this method.

Methodology

- Library generation: Random mutagenesis (error-prone PCR, chemical mutagens) or recombination (DNA shuffling).
- Screening/selection: High-throughput assays identify improved variants.
- Iteration: Multiple cycles lead to cumulative improvements.

Applications

- Development of thermostable cellulases for biofuel production.
- Engineering of oxidoreductases for synthesis of pharmaceutical intermediates.
- Evolution of esterases and proteases with resistance to organic solvents.

Advantages

- Powerful for improving properties without structural knowledge.
- Requires robust screening platforms; may generate “hidden” beneficial mutations not predicted by rational design.
- Often combined with computational methods for semi-rational approaches.

De-novo design: De novo enzyme design is the process of creating a catalytic activity based on the exact residues of amino acids necessary for the stability of the transition state, as determined by the score derived from the configuring atoms. Both the transition state activation energy and the biocatalysis activation energy are decreased by the denovo design. Active site design is the first step in the de novo design process. In order to create new active sites for a certain transition state in a reaction, all of these designs rely on complex computational calculations. Generally speaking, the initial step is to develop a catalytic mechanism for the desired reaction. For transition state stability, essential residues that constitute a minimum active site are then added (Quin and Schmidt-Dannert, 2011) ^[90]. The active site supporting protein backbones are then found using a similarity searching technique like the Rosetta Match hashing method (Zanghellini *et al.*, 2006) ^[129]. The geometry of catalysis and the binding energy of the transition state are analyzed using in silico screening (Smith *et al.*, 2014) ^[109]. Table 3 contains a list of computer-aided designed enzymes. Recently, Lapidoth *et al.* proposed an effective automated method for building enzymes by combinatorial backbone assembly. Although catalytic residues are conserved, this method uses a variety of homologous but structurally distinct enzymes, assembles novel protein backbone combinations, and uses Rosetta software to arrange and optimize the amino acid residues. This method was used to design 43 and 34 proteins, respectively, for two distinct families of enzymes with TIM-barrel folds: GH10 xylanases and phosphotriesterase-like lactonases. Seven PLL designs and 21 GH10 designs were quite active. Accordingly, combinatorial-backbone assembly may be a suitable option for creating stable and active enzymes for industrial processes (Lapidoth *et al.*, 2018) ^[61]. Figure 2 illustrates the main approaches of enzyme engineering.

Table 3: Computational tools assisted enzyme engineering.

Study Focus	Approach / Method Used	Key Findings	Reference
Ser-specific domain of NRPS from <i>E. coli</i>	IPRO exploration and site-directed mutagenesis	Identified new Ser-specific domains	Throckmorton <i>et al.</i> , 2019 ^[116]
β-barrel scaffold redesign (<i>E. coli</i> OmpF)	PoreDesigner	Successfully redesigned scaffold	Chowdhury <i>et al.</i> , 2018 ^[19]
Acyl-ACP thioesterase engineering	Computational engineering	27 mutant variants with high C8-production titers developed	Grisewood <i>et al.</i> , 2017 ^[41]
Highly active Acyl-ACP synthesis	Computational modeling	~1.7 g/L C8 titers with >90% specificity toward C8	Hernandez-Lozada <i>et al.</i> , 2018
Esterase from <i>Pseudomonas fluorescens</i>	3DM analysis + site-saturation mutagenesis	~20-fold higher enantioselectivity and 200-fold activity enhancement	Jochens & Bornscheuer, 2010 ^[9, 54]
<i>S. capsulata</i> prolyl endopeptidase	Multiple sequence alignment	200-fold increased protease resistance	Ehren <i>et al.</i> , 2008 ^[31]
Serine esterase engineering	Quantum mechanical calculation + point mutations	Substantial lytic activity toward p-nitrophenyl acetate	Li <i>et al.</i> , 2020 ^[63]

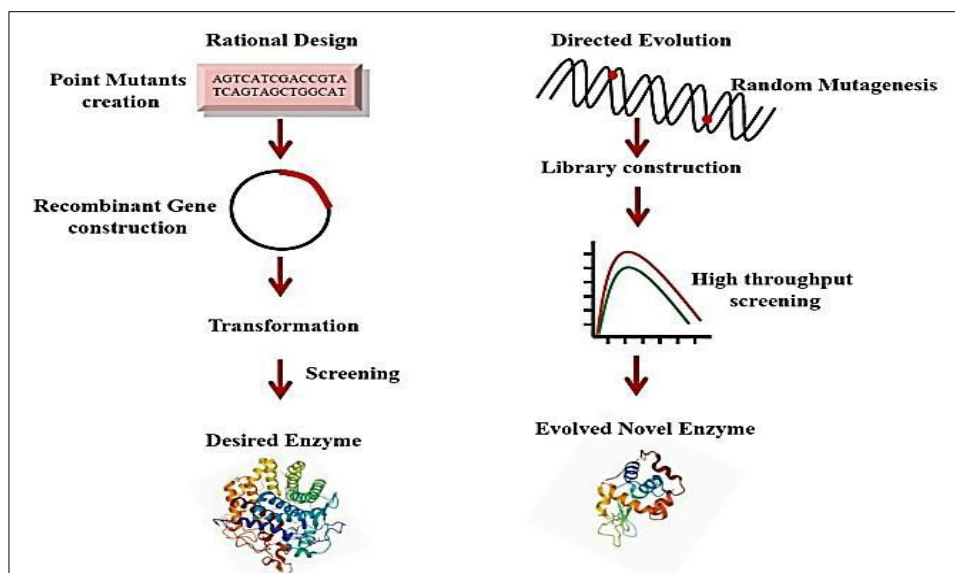


Fig 2: Strategies of enzyme engineering (Madhavan *et al.*, 2021) ^[70].

Chimeric Enzymes

Chimeric enzymes are generated by fusing domains from different proteins to create hybrid catalysts with novel functionalities.

Development of chimeric proteins with subdomains of other proteins is another way to improve thermostability. In this manner, positive traits from various parents might be blended. For instance, subdomains from Baeyer-Villiger monooxygenases (BVMOs) with varying stabilities were combined to create chimeric versions of BVMOs. While maintaining or even enhancing the substrate selectivity of a thermolabile BVMO, certain chimeric enzymes were 10-15 °C more stable than their thermolabile parent enzymes (van Beek *et al.*, 2012) ^[119]. A number of stabilizing chimeras between various xylanases and between xylanases and glucanases have been produced (Liu *et al.*, 2012) ^[67]. A chimera produced by grafting loops from an archeal enzyme onto a human aldo-keto reductase demonstrated that advantageous characteristics of archeal and human proteins might be combined, in this case stability and activity (Campbell *et al.*, 2013) ^[13]. By merging the domains of two mesostable polyol dehydrogenases, it is even possible to create such a stabilized chimera without utilizing a thermostable variation (Hulf *et al.*, 2012). Compared to its parents, the resulting chimera was 12 °C more stable. Lastly, it was demonstrated that proteins with minimal sequence similarity (<30%) can also form chimeric enzymes by substituting portions of a ($\beta\alpha$)8-barrel protein with portions of flavodoxin-like proteins (Shanmugaratnam *et al.*, 2012) ^[106]. Because the structure of flavodoxin-like proteins resembled a portion of the ($\beta\alpha$)8-barrel, the replacement was feasible.

Another study used an active learning algorithm in conjunction with the SCHEMA approach to predict a huge collection of stabilizing chimeras by analyzing data from a training set of chimeras (Romero *et al.*, 2012) ^[98]. Enzymes with improved long-term stability were created using this technique, which showed a negative association between a chimeric enzyme's isoelectric point and its long-term stability. A chimeric P450 enzyme that was 14.5 °C more stable than its parent was recently developed (Hwang *et al.*, 2013) ^[51].

Design principles

- **Domain shuffling:** Combining catalytic domains from one enzyme with stability-enhancing domains from another.
- **Fusion with affinity tags:** Enhancing immobilization or recovery (e.g., His-tags, cellulose-binding domains).
- **Multifunctional chimeras:** Engineering bifunctional enzymes capable of catalyzing two sequential reactions.

Examples

- Fusion of cellulase catalytic domains with carbohydrate-binding modules (CBMs) for improved cellulose degradation.
- Chimeric lipases fused with hydrophobins to increase activity at water-oil interfaces.
- Enzyme fusions in metabolic engineering to create artificial metabolic pathways.

4. Semi-Synthetic Enzymes

The lack of an enzyme with the required catalytic activity or substrate selectivity, as well as their instability in biotechnological processes, frequently limit the growing demand for enzymes as highly selective, mild, and environmentally benign catalysts. These issues included various classes of enzyme mimics as well as genetically modified enzymes. Here, we outline chemical enzyme engineering's potential: Native enzymes can be altered using simple tools and chemical processes to create "semisynthetic" enzymes. Thus, the catalytic adaptability of a synthetic active site is paired with the great substrate selectivity of the enzymatic peptide framework. We use the idea of the semisynthetic peroxidase seleno-subtilisin to demonstrate the possibilities of chemically modified enzymes. Subtilisin, a serine endoprotease, was first crystallized and cross-linked with glutaraldehyde to produce cross-linked enzyme crystals that were shown to be extremely stable and insoluble in organic solvents or water (Feng *et al.*, 2022) ^[34]. Second, an oxidized derivative of selenocysteine (Enz-SeO₂H) was produced chemically from serine 221 in the active site (Enz-OH). Consequently, the hydroperoxide reduction was catalyzed by the former

proteolytic enzyme, which also acquired peroxidase activity. Given the well-known selectivity of subtilisin, the substrate selectivity of seleno-subtilisin was anticipated because the binding sites of semisynthetic peroxidase and protease were identical (Häring *et al.*, 1999) [44].

Design Approaches

- 1. Unnatural amino acid incorporation:** Provides novel reactivity or enhanced stability.
- 2. Artificial metalloenzymes:** Insertion of synthetic metal complexes into enzyme frameworks for unique catalytic reactions.

- 3. Polymer conjugation:** Attaching polymers or nanoparticles to enzymes for improved solubility, stability, and recyclability.

Industrial Applications

- Semi-synthetic peroxidases for oxidative degradation of pollutants.
- Artificial metalloenzymes in enantioselective chemical synthesis.
- Hybrid enzyme systems for green chemical manufacturing and bioremediation.
- Artificial metalloenzymes used in enantioselective hydrogenation and C-C bond formation.

Table 4: Comparative Evaluation of Approaches

Approach	Basis	Strengths	Limitations	Industrial Examples
Rational design	Structural and computational data	Precise, targeted	Requires detailed structural info	Lipase redesign for biodiesel
Directed evolution	Random mutagenesis and screening	Powerful, no prior knowledge needed	Screening-intensive	Thermostable cellulases
Chimeric enzymes	Domain fusion	Multifunctional, modular	Risk of misfolding	CBM-cellulase hybrids
Semi-synthetic enzymes	Synthetic modification, unnatural amino acids	Access to new chemistries	Complex, costly	Artificial metalloenzymes

Enzyme cofactor specificity engineering

An enzyme's cofactor specificity engineering may help increase the enzyme's potential applications in commercial biocatalysis or be very successful in achieving redox balance in metabolic designed pathways. This is primarily used in the synthesis of chiral intermediates for use in the pharmaceutical industry and in the manufacturing of biofuels (Wang *et al.*, 2013). Scrutton *et al.* (1990) [103, 122] found the amino acid residues in the Rossmann fold that give glutathione reductase selectivity for NADP⁺ over NAD⁺. When Ehsani *et al.* (2009) [32] compared the structure and sequence of NADP (H)-dependent yeast alcohol dehydrogenase to that of NAD(H)-dependent Bdh1, they found three alterations in the amino acid sequence that resulted in a variant of Bdh1 with a shift toward NAPH cofactor specificity. Alcohol dehydrogenase D is a member of the aldo-keto reductase (AKR) protein family and is an extremely effective and temperature-tolerant alcohol dehydrogenase. Several researchers expanded the selectivity of aldo-keto reductase to cofactor by introducing mutations in the cofactor's binding pocket (Sanli, 2004; Campbell *et al.*, 2010) [15, 101]. This enzyme's steady state kinetic research provided fresh insights into the conformational shifts and ensuing biocatalyst performance. A biofuel cell was created using this enzyme-cofactor pair when the maximal dynamic mutant catalyst simultaneously gained activity with a non-native cofactor, nicotinamide mononucleotide, or NMN(H) (Campbell *et al.*, 2012) [14]. By adding the active site loop region from human aldose reductase, the thermostable AdhD enzyme's substrate selectivity was altered. Both substrate specificity and cofactor specificity were altered by this engineering strategy. *Clostridium thermocellum* is a powerful candidate microbe in integrated bioprocessing for lignocellulosic conversion to ethanol. It is an obligatory anaerobe and a highly thermophilic bacterium. The primary economic disadvantage is intolerance to ethanol. According to genome sequencing, this bacteria's mutant bifunctional acetaldehyde-CoA/alcohol dehydrogenase gene (*adhE*) is responsible for its ethanol tolerance (Brown *et al.*, 2011) [11].

Application of Enzyme Engineering in Food, Pharmaceuticals, Biofuels, and Environmental Biotechnology:

Enzyme engineering has transformed industrial biotechnology by enabling tailored biocatalysts with improved efficiency, stability, and substrate specificity. These engineered enzymes are now pivotal in sectors such as food processing, pharmaceuticals, biofuels, and environmental remediation, where they offer greener, safer, and more cost-effective alternatives to conventional chemical processes.

Future developments are likely to integrate artificial intelligence (AI), machine learning, and synthetic biology to accelerate enzyme design. Computational prediction of stabilizing mutations combined with high-throughput directed evolution will lead to faster discovery of optimized biocatalysts. Furthermore, hybrid enzymes that merge natural biocatalysis with synthetic catalytic capabilities are expected to open entirely new avenues for green and sustainable chemistry.

Food Industry Applications

Starch and other classes of food are the chief source of energy for non-photosynthetic organisms and plants. Depending on the situation, these food classes could be processed chemically or enzymatically to create a variety of products for the food and animal feed industries. Because of their complexity, these molecules used as raw materials need a variety of enzymes to properly break them down into smaller components. By engineering these enzymes used in this process, it may be possible to improve their selectivity or specificity, push a reaction in the desired direction, and ultimately stabilize their activity toward reaction conditions necessary for large-scale synthesis or production.

Duan *et al.* (2013) [29] modified pullulanase from *Bacillus deramificans* by changing Asp437 to His437 and Asp503 to Tyr503. This mutation increased the thermostability of the enzyme, which is used to make fructose and glucose syrup (Duan *et al.*, 2013) [29]. However, Gao *et al.* transformed mesophilic mannose isolated from *Bacillus subtilis* into the

thermophilic enzyme using a molecular cyclization method (Gao *et al.*, 2019) [38].

Medical and pharmaceutical applications

The production of the antidiabetic sitagliptin by Codexis and Merck is a very promising example of protein engineering (Choi *et al.*, 2015; v *et al.*, 2011; Savile *et al.*, 2010) [18, 102]. R-selective transaminase, an enzyme created through directed evolution, was used in the procedure. This enzyme produced sitagliptin with a 99.95% enantiopurity by asymmetrically aminating pro-sitagliptin ketone (Savile *et al.*, 2010) [102]. A modified monoamine oxidase is used to create the chiral amine boceprevir. It is used to treat chronic hepatitis C. Researchers found that water consumption decreased by 40% and process yield rose by 150% (Li *et al.*, 2012). The reduction of a ketone intermediate into a chiral alcohol is catalyzed by *LactoBacillus* kefir ketoreductases (KRED). Intermediates for a variety of medications, such as montelukast, atorvastatin, duloxetine, ezetimibe, crizotinib, and phenylephrine, are produced by ketoreductase-mediated mechanisms (Huisman *et al.*, 2010; Huisman and Collier,

2013) [49, 50]. Techniques like directed evolution and process optimization are examples of potential KRED improvements. With a value exceeding 99.55%, KRED exhibits remarkable enantioselectivity (Huisman *et al.*, 2010) [50]. By saturating the active site of a KRED enzyme, Zhang *et al.* created two mutants (P170H/L174Y and P170R/L174Y) that made it easier to reduce 3-(dimethylamino)-1-phenylpropan-1-one and 3-(dimethylamino)-1-(2-thienyl)-propan-1-one to their (R)- γ -amino alcohols with a maximum enantiomeric excess of 95% (Zhang *et al.*, 2015). Chiral γ -amino alcohols are essential for the synthesis of certain serotonin reuptake medications, such as fluoxetine and tomoxetine (Zhang *et al.*, 2015) [132]. Because enantiomers of the same drug may exhibit different biological activities and toxicities, there is an increasing need for chiral compounds (Patel *et al.*, 2018) [86]. Pharmaceutical and medical research is paying attention to the creation of diagnostic enzymes. An engineered fructosyl peptide oxidase (FPOX) was used by Shahbazmohammadi *et al.* as a diagnostic enzyme for diabetes mellitus (Shahbazmohammadi *et al.*, 2019) [105].

Table 5: Engineered enzymes with medical and pharmaceutical uses.

Enzyme Source	Enzyme Engineering Approach	Uses	Objective	Modification Made	References
<i>Eupenicillium terrenum</i>	Site-directed mutagenesis	Diagnosis of diabetes mellitus	Enhanced specificity	Tyr261Trp	Shahbazmohammadi <i>et al.</i> , 2019 [105]
<i>LactoBacillus kefir</i> (Keto-reductases, KRED)	Quantum mechanical calculations and molecular dynamics simulations	Enantioselective reduction of 3-triacyclopentanone and 3-oxacyclopentone	Enhanced enantioselectivity	Glu145 and Ala94Phe	Noey <i>et al.</i> , 2015 [83]
<i>Sporobolomyces salmonicolor</i> AKU4429 (Carbonyl reductases, KRED)	Directed evolution	β -Amino ketones enantioselective reduction	Enhanced activity and improved enantioselectivity	Pro170His-Leu174Tyr and Pro170Arg-Leu174Tyr	Zhang <i>et al.</i> , 2015 [132]
Herpes simplex virus type 1 (Thymidine kinase, TICKLE switch)	Directed evolution	Cancer treatment	Enhanced specificity	HSV-TK/CH1 fusion	Shelat <i>et al.</i> , 2017 [107]

Biofuel and Bioenergy Applications

In recent years, the demand for energy has been rising significantly worldwide. Investigating more ecologically benign and potentially renewable alternative energy sources is essential as fossil fuels cannot be replenished. The production of biofuels is becoming more and more popular. Enzymes are useful for producing biofuel because they can break down a variety of substrates to produce ethanol and biodiesel. However, many enzymes do not possess the intrinsic robustness needed for commercial use (Prajapati *et al.*, 2018; Otte and Hauer, 2015) [84, 88]. Enzymes called xylanases are widely used in the generation of biofuel. The main component of plant biomass that causes hemicellulose degradation, xylan, can be broken down by these enzymes. Therefore, xylanases can use the agroindustrial wastes that the industry normally discards as substrates (Denisenko *et al.*, 2017) [23]. Teng *et al.* exploited site-directed mutagenesis to create two disulfide linkages into recombinant xylanases generated from *Penicillium janthinellum* MA21601, as part of their xylanase engineering project (Teng *et al.*, 2019). The mutant DB-s1s3 displayed a considerable increase in the optimum temperature range, increasing from 50 °C to 70 °C. Furthermore, compared to the wild-type xylanase, it showed a 4.76-fold increase in specific activity (Teng *et al.*, 2019) [115]. The main byproduct of xylan breakdown, xylose,

has inhibitory effects that limit the large-scale production of xylanase. Hegazy *et al.* used directed evolution with error-prone PCR to create genetically engineered xylanases that are xylose-tolerant in order to overcome this limitation (Hegazy *et al.*, 2019). Three alterations were discovered in the variants: Met116Ile, Leu131Pro, and Leu133Val. The most effective mutation showed a 3.5-fold increase in the inhibition constant (k_i) and a threefold increase in catalytic activity (Hegazy *et al.*, 2019) [46].

Additionally, lipases help hydrolyze triacylglycerides into glycerol and free fatty acids. A class of enzymes known as lipases is widely used in the synthesis of biofuels. In the field of oleochemistry, fatty acids are very helpful chemicals, especially for uses like making biodiesel (Dror *et al.*, 2014; Zorn *et al.*, 2018) [26, 139]. Dror *et al.* created mutations in a lipase generated from *Geobacillus stearothermophilus* T6 using two techniques: structure-guided consensus and error-prone PCR random mutagenesis (Dror *et al.*, 2014) [26]. Their objective was to produce a lipase with increased stability, methanol tolerance, and transesterification activity all of which are essential characteristics in the manufacture of biodiesel. In a solution containing 70% methanol, both engineering approaches might produce enzymes with a longer half-life.

Table 6: Engineered enzymes employed in biofuel production

Enzyme Source	Enzyme Engineering Approach	Uses	Objective	Modification Made	References
<i>Streptomyces</i> sp. (W007 strain) Lipase	Overlap extension approach of site-directed mutagenesis	Fat/oil modification and biofuel manufacturing	Improved thermostability and activity	Phe153Ala; His108Ala; Val233Ala	Zhao <i>et al.</i> , 2018 ^[137]
<i>Penicillium janthinellum</i> (MA21601) Xylanase	Site-directed mutagenesis	Saccharification of xylan for biofuel production	Increased catalytic efficiency and thermostability	DB-s3s4: Ser27Cys/Ser186Cys; DB-s1s4: Ser27Cys/Ser39Cys; DB-s1s3: Ser39Cys/Ser186Cys	Teng <i>et al.</i> , 2019 ^[115]
<i>Chaetomium thermophilum</i> β -1,4-Endoglucanase	Site-directed mutagenesis	Biodegradation of cellulose	Improved thermostability and activity	Tyr173Phe; Tyr30Phe; Tyr30Phe/Tyr173Phe	Chen <i>et al.</i> , 2018 ^[17]
<i>Pichia stipitis</i> Xylose reductase	Site-directed mutagenesis	Fermentation of xylose to ethanol	Changed coenzyme specificity	2-2C12: Ser271Gly, Asn272Pro, Lys270Ser, Arg276Phe	Liang <i>et al.</i> , 2007 ^[66]
<i>GeoBacillus stearothermophilus</i> Xylanase	Error-prone PCR (Directed evolution)	Saccharification of xylan for biofuel production	Enhanced xylose tolerance	Leu131Pro; Leu133Val; Met116Ile	Hegazy <i>et al.</i> , 2019 ^[46]
<i>Penicillium canescens</i> Xylanase	Site-directed mutagenesis	Saccharification of xylan for biofuel production	Enhanced thermostability	Leu18Phe	Denisenko <i>et al.</i> , 2017 ^[23]

Environmental Biotechnology Applications

Enzyme engineering is crucial in environmental bioremediation, where enzymes are used to degrade pollutants and treat industrial waste. Synthetic pollutants, such as plastics, pesticides, and industrial dyes, are highly resistant to natural degradation processes. Engineered enzymes, particularly laccases and peroxidases, are designed to withstand extreme pH and temperature conditions, making them suitable for breaking down these persistent contaminants (Tuan *et al.*, 2018; Prasad and roy, 2018) ^[89, 117]. For instance, laccases have been modified to function in alkaline environments and are effective in degrading azo dyes commonly found in textile industry wastewater. Enhanced stability and catalytic efficiency of these engineered enzymes allow them to reduce pollutant levels significantly, improving wastewater quality before discharge (Bhandari *et al.*, 2021) ^[6]. Several successful applications of engineered enzymes in bioremediation highlight their environmental impact. A notable case is the use of laccases for dye degradation in wastewater treatment. Engineered laccases that operate in alkaline conditions have been shown to break down synthetic dyes by cleaving azo bonds, reducing water toxicity by up to 90% and facilitating safer disposal (Bilal *et al.*, 2018; Kumar *et al.*, 2022) ^[7, 59]. Another impactful case is the application of engineered cutinases in plastic degradation. These enzymes have been modified to hydrolyze polyethylene terephthalate (PET), a major component of plastic waste. Engineered cutinases can depolymerize PET under industrial conditions, providing a potential solution for recycling plastic waste and reducing environmental pollution. Such examples underscore the value of enzyme engineering in addressing pressing environmental issues (Radley *et al.*, 2023) ^[91].

Practical Significance, Challenges, and Future Perspectives of Engineered Industrial Enzymes

Engineered enzymes have emerged as pivotal tools in industrial biotechnology, offering eco-friendly, efficient, and highly selective alternatives to conventional chemical catalysts. By combining advanced protein engineering, immobilization, semi-synthetic modifications, and modern information technologies, industries have been able to optimize reaction rates, improve product quality, and reduce

environmental footprints. Despite these advancements, practical implementation still faces certain challenges that require careful consideration.

Practical Significance

Engineered enzymes play a vital role in industrial biotechnology by offering eco-friendly, highly specific, and energy-efficient alternatives to traditional chemical catalysts. Their applications span food processing, pharmaceuticals, biofuels, textiles, and environmental remediation, where they enhance productivity while reducing waste and hazardous by-products. The ability to tailor enzymes through protein engineering, immobilization, and synthetic biology has significantly improved catalytic efficiency, substrate specificity, and operational stability, making them indispensable for green chemistry and sustainable manufacturing.

Challenges

Despite their promise, several challenges limit the widespread adoption of engineered enzymes:

- **Stability Issues:** Enzymes often lose activity under harsh industrial conditions such as extreme pH, temperature, or solvent environments.
- **High Production Costs:** Large-scale enzyme production and purification remain expensive compared to chemical catalysts.
- **Scale-up Limitations:** Laboratory successes may not always translate effectively into industrial-scale operations.
- **Regulatory and Safety Concerns:** Particularly in pharmaceuticals and food applications, approval processes can be lengthy and complex.

Future Perspectives

The future of enzyme engineering is closely linked with artificial intelligence, machine learning, and synthetic biology, which will accelerate predictive design and optimization. Nanotechnology and smart immobilization matrices will enhance enzyme stability and reusability, while hybrid catalytic systems combining natural and synthetic elements will broaden reaction possibilities.

Furthermore, the development of dynamic enzyme cascades, artificial cofactors, and modular biocatalytic platforms is expected to open new frontiers in industrial biotechnology. Ultimately, these innovations will help overcome current limitations, leading to cost-effective, scalable, and sustainable enzyme-based solutions at the heart of the green industrial revolution.

Conclusions

Engineered enzymes have emerged as pivotal tools in shaping the future of industrial biotechnology, providing sustainable, selective, and efficient alternatives to traditional chemical processes. The convergence of protein engineering, immobilization strategies, synthetic biology, and advanced computational approaches has not only expanded their applicability but also addressed key challenges of performance and scalability. With the integration of modern information technologies such as AI, machine learning, and bioinformatics, enzyme engineering is shifting toward a predictive, rational, and high-throughput discipline. Looking ahead, advancements in nanotechnology, artificial cofactors, and hybrid catalytic systems are expected to further enhance efficiency and broaden industrial applications. Collectively, these developments position engineered enzymes as central drivers of the green industrial revolution, enabling eco-friendly, cost-effective, and innovative solutions across diverse sectors including food, pharmaceuticals, biofuels, and environmental sustainability.

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Conflict of Interest

Regarding the publication of this review, the authors affirm that they have no conflicts of interest.

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