Trends in lipase engineering for enhanced biocatalysis

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Abstract

Lipases, also known as triacylglycerol hydrolases (E.C.No. 3.1.1.3), are considered as leading biocatalysts in the lipid modification business. With properties like ease of availability, capability to work in heterogeneous media, stability in organic solvents, property of catalyzing at the lipid–water interface and even in nonaqueous conditions, have made them a versatile choice for applications in the food, flavor, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries [1]. The increasing alertness toward sustainable technologies, lesser waste generation and solvent usage and minimization of energy input has brought light toward the production and usage of recombinant/improved lipases. For example, Novozym 435, a broadly used recombinant lipase isolated from *Candida antarctica*, dominates the lipase industry and has even created a supplier bias in the market. This shows that there is a desperate need for novel, low-cost lipases with better properties. For this, mining of existing extremophilic genomes seems more rewarding. But considering the diversity of industrial requirements such as types of solvents used or carrier systems employed for enzyme immobilization, tailor-designed enzymes are an unrealized pressing priority. Therefore, protein engineering strategies in collaboration with the discovery of new lipases can serve as a vital tool to obtain tailor-made enzymes with specific characteristics. © 2021 International Union of Biochemistry and Molecular Biology, Inc. Volume 69, Number 1, Pages 265–272, 2022

Keywords: *lipase, protein engineering, rational design, directed evolution, industrial application*

1. Introduction

As compared with other chemically derived catalysts, lipases are ecofriendly and sustainable and thus used in numerous sectors of industry ranging from textile, paper, food, bioenergy, chemical, and detergent industry [2]. With the vast assortment of the utilization of lipases, it still faces some shortcomings. The cost of lipase production is the main sticking point to the commercialization of the lipase-catalyzed processes [3]. Thus, the current supply of lipases falls short to match the escalating demand of the bio-based industry. The usage of free state lipases is limited to commercial applications dealing with low pH, temperature, and solvent tolerance [4]. Similarly, the processes in which the separation of products from the reaction mixture is crucial, it gets difficult and tedious while using free enzymes. Therefore, better separation methods are required to prevent contamination of the product. Even after immobilization, enzyme leaching is a common problem, resulting in low selectivities, activities, and volumetric productivities. Hence, regardless of lipase-catalyzed processes having incredible business importance, the utilization of these catalysts is not broad, due to their low reproducibility, low yields, and the not-constantly ideal execution in their inherent structure. This has created an immediate need for better and stable enzymes [5].

In search of better enzymes with enhanced properties from those existing in the market, researchers have propositioned various techniques. Using the method of metagenomics screening, a plethora of unique enzymes with lipolytic activity have been taken from diverse environmental sources [6, 7]. With the growing knowledge of the machinery for the modulation of gene expression, functional improvement of the expression systems is currently in progress, and new improved strains are regularly emerging. Overproduction of engineered enzymes is commercially beneficial, not only to achieve a boost in volumetric productivity, but also for a reduction in cost for downstream purifications and resulting in a purer crude enzyme. The exploitation of new or modified expression hosts is being done for better expression of these enzymes with rapid screening done by high-throughput (HTP) screening

Abbreviations: *EC, enzyme comission; HTS, high throughput system.*

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methods. Also, lipases can be modified to have high selectivities at the high substrate and low enzyme loadings. Similarly, drawbacks of the free enzymes can be undermined through enzyme entrapment techniques like immobilization, protein-based scaffolds, fusion proteins, and so on. The present review demonstrates the latest approaches in enzyme modification and engineering used to produce tailored lipases. We also highlight recent technologies developed for enzyme engineering that have a huge scope for transforming lipases, yet these remain underexplored.

Highlights

Methods for cost-effective industrial production of lipase
Tools to obtain designer-enzymes for use in green processes
Spatially organized enzymes for cascade biocatalysis
Microenvironment engineering is the future of biocatalysts

2. Discovery of Lipolytic Enzymes and Protein Engineering

Protein engineering, through modification/altering of existing genes or creating novel ones, has been an encouraging tool for the designing and production of proteins having desired properties as per the industry requirements. Figure 1 highlights the various techniques that have been used for lipase engineering. It shows how the engineering mechanism has evolved (following the third wave of biocatalysis) [8] and what new techniques have a great chance for lipase engineering. Following are the commonly used strategies for transforming proteins with enhanced capabilities.

2.1. Rational design

It works on the principle of enzyme structure studies with mechanistic evidence and uses molecular biology techniques such as site-directed mutagenesis [9]. This creates small focused libraries (less than 100 variants) that are conveniently screenable. These along with recent immobilization techniques on a specific support can be used to generate immobilized biomolecules for application as biosensors. Advancements in support design and broader expertise of the procedures of enzyme-support interactions have enabled the exploration of newer and better possibilities [10]. Also, molecular and structural simulation tools have supported projections of point mutations on the characteristics of lipases, for example, as in the case of thermostability of *Bacillus subtilis* lipase [11] where its six thermostable mutants have been created by point mutations. Similarly, in a case where structural data of a specific lipase is unavailable, the structure of its homolog can aid the modification process [12]. Rational design (RD) has also helped in the generation of various lipase variants via modification of the lid-domain region in their structure [13]. For most lipases, entry to the active site comprising of a catalytic triad is protected by a lid structure that consists of α-helices, joined by a loop to the body of a lipase. Many lipase variants have been generated by methods (Table I), like insertion of a disulfide bridge in the hinge region of the lid, lid swapping [14], and so on, altering its substrate specificity, activity profile, and thermostability (Fig. 2). Therefore, this mobile lid region contributes to the stability, temperature, and activity of the lipase and can be regarded as the "potential hot spot" for producing designer lipases.

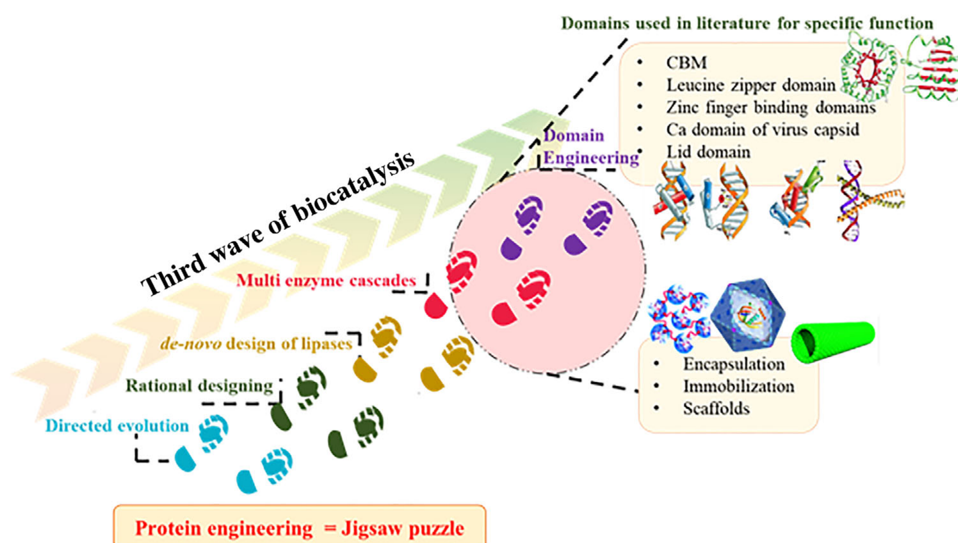

FIG 1 *Protein engineering methods used to improve lipase properties.*

TABLE 1 *Protein engineering techniques used to alter specific targets in industrial enzymes*

Target	*Enzyme*	*Mutation methods*	*Reference*
Thermostability	*Candida Antarctica lipase B*	Directed evolution (site saturation mutagenesis)	[23]
	Aspergillus niger	Directed evolution (iterative saturation mutagenesis)	[24]
	Bacillus sp. 37	Directed evolution (ep-PCR)	[25]
	Candida Antarctica lipase B	Rational design	[26]
	Geobacillus sp. r03Lip	Directed evolution (iterative saturation mutagenesis) and ep-PCR	[27]
	Geobacillus stearothermophilus lipase T6	Directed evolution (structure guided consensus)	[28]
Substrate selectivity	*Candida Antarctica* lipase B	Rational design	[29]
	Rhizopus delemar	Rational design	[30]
Proteolytic resistance	*Bacillus subtilis* Lip A	Rational design (loop scanning and site-saturation mutagenesis)	[31]
Substrate tolerance	*Thermoanaerobacter thermohydrosulfuricus* TTL	Rational design (genetic code engineering)	[32]
Increased production	*Burkholderia glumae* lip AB	Directed evolution (random mutagenesis)	[33]
Protein activity	*Bacillus pumilus* lipase BpL5	Point mutation	[34]
	Serratia marcescens lipase A	Rational design	[35]
Surface hydrophobicity	*Bacillus thermocatenulatus* BTL2	Rational design (lid domain engineering)	[36]
Catalytic activity	*Bacillus thermocatenulatus*	Rational design (lid domain engineering)	[37]
	Candida rugosa LIP4	Rational design	[38]

2.2. Directed evolution

Unlike the RD method, this does not require a deep study of the protein structure. The commonly used approaches for carrying out directed evolution are error-prone PCR and/or DNA shuffling [15]. Therefore, it mainly steps (Fig. 3) as (i) random mutagenesis library generation with 10^4–10^5 variants from a parent gene; (ii) expression of the genes in a competent host; (iii) and screening of the created mutant library to spot improved variants using HTP techniques [16]. Here, development in library generation techniques like recombination, mutant screening efficiency, DNA synthesis, and computational studies is foundational for multifaceted commercial use [17]. The libraries are then screened/selected using methods like enzyme activity assays; microtiter plate screening using chromogenic assay method with substrates like p-nitrophenyl (pNP) esters, α-naphtyl acetate or laurate, Fast Blue B or RR, and so on; agar plate screening by halo formation that use Victoria Blue polyvinyl alcohol-pNP-myristate (pNP-C14)-agarose (VPMA) plates, and so on [18,19]. Many HTP screening methods also use microtiter plates coated with purified triacylglycerols where conjugated diene/triene can be measured by UV absorption spectra at 272 nm [20]. These High Throughput system (HTS) methods vary from the general wet-laboratory biochemical and spectrophotometric plate assays to high-end mass spectrophotometry. Researchers are also working on logic-gates genetic circuits as an advanced HTP method to create a promising engineering approach [21]. Therefore, with the continual advancement in HTP screening methodologies [22], directed evolution should continue as the primary choice for the generation of enzymes best suited for commercial biocatalysis. Various

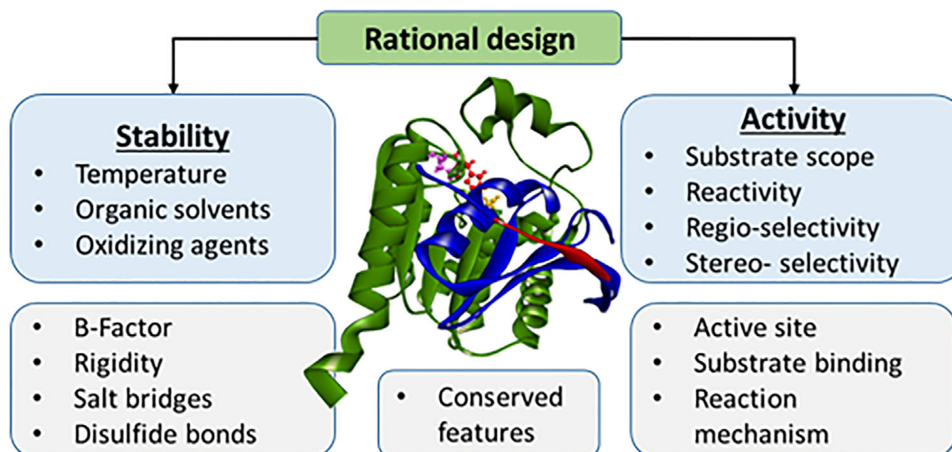

FIG 2 *Targets for rational design depending on the property desired to be altered.*

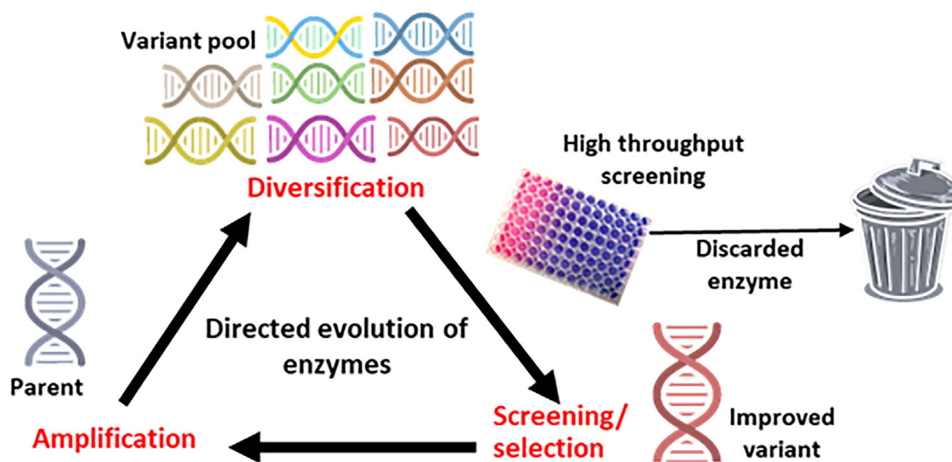

FIG 3 *Workflow for the directed evolution of lipases.*

strategies in molecular engineering were either used to find the catalytic process or to boost the properties of lipolytic enzymes, as shown in Table 1. The target for engineering has primarily been thermostability, solvent tolerance, and various catalytic properties.

2.3. Semi-rational protein design

This approach utilizes the already existing knowledge about the sequence, structure, and function of the proteins of interest in the public database and builds a smaller but higher quality, an expertise-based library using predictive algorithms. To limit the screening efforts, various strategies are used, such as (i) structure-based combinatorial protein engineering, which uses structure-based data and DNA manipulation strategies already available [39]; (ii) site-saturation mutagenesis for designing integrative-mutational libraries [40]. It also uses a computer-based enzyme designing strategies for generating small-sized functional libraries using The Hot-Spot Wizard server (http://loschmidt.chemi.muni.cz/hotspotwizard) that combines data from the sequence and structural database and then constructs a mutation-probability map for the protein [41]. Similarly, the commercially available 3DM database (http://3dm.bio-prodict.com) interprets the sequence and structure data from GenBank and the PDB to create alignments of protein super families [42]. Using these techniques, a mutant of lipase was produced from *Proteus mirabili* having 30-fold enhancement in thermotolerance and the 50-fold rise in methanol tolerance [43]. From a practical point of view, this method clearly enhances the efficiency of designed biocatalyst as it reduces the desire for automation/robotic-based costly screening methods. The schematics of semi-rational protein design are shown in Fig. 4.

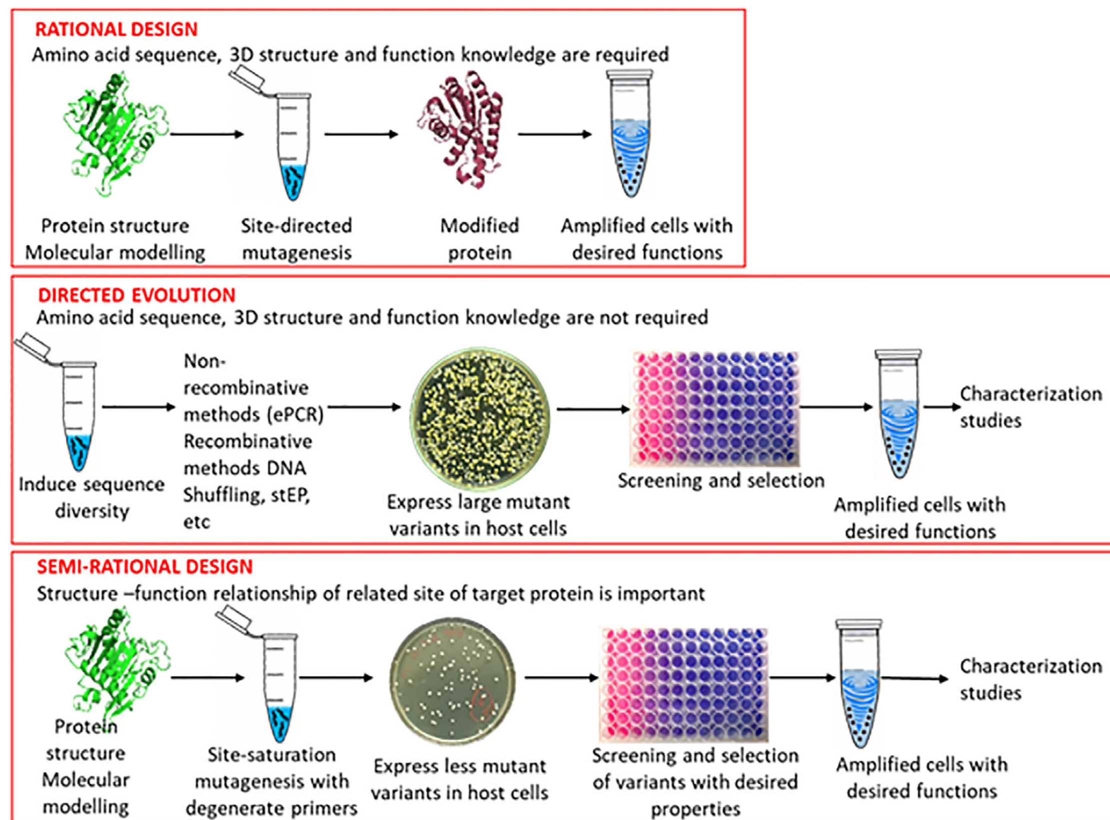

FIG 4 *Schematics of semi-rational design: a combined approach from rational design and directed evolution.*

2.4. *De novo* designing

This has developed as a tool for producing designer-made biocatalysts from scratch. The computational methodology has reached a point where researchers can easily achieve the production of new proteins with sequences unrelated to those in nature based on physical principles. The very high stability of proteins designed by this method makes them robust starting points for creating new functions [44]. Considering enzyme stability, various cutting-edge studies to generate novel and functionally active biocatalysts have been conducted. *In silico* tools like RosettaVIP [45] and WISDOM [46], apart from molecular modeling and protein design, can also be used for data analysis. Certainly, with the latest computational tools, powerful computer hardware, and HTP screening, the number and utility of these protein catalysts will rise. This will eventually lead to an improved success rate for the design and modification of enzyme stability, in order to match the ever-increasing industrial needs.

3. Latest Enzyme Engineering Trends

Following the biocatalysis roadmap (Fig. 5), it is conceivable that both biocatalytic and synthetic methods will be synergistically used in the generation of chemicals and products of industrial importance, but such advanced biomanufacturing processes currently face several major challenges. Enzymes can be spatially organized using various approaches, known as cascade biocatalysis. By bringing enzymes in close vicinity, various factors like reaction efficiency between cascade partners, loss of intermediates by diffusion, channeling of substrates and intermediates between active sites, and so on get affected [47]. These are discussed in further sections in ascending order of complexity: protein-based; carrier-free coimmobilization; two- and three-dimensional scaffolding of enzymes; and enzyme encapsulation within containers, and reprocessing of cellular organelles for biosynthesis [48].

3.1. Fusion proteins and protein-based scaffolds

By introducing a short linker peptide conjoining the biocatalyst, covalent fusions facilitating direct channeling of the substrate between enzyme active sites have been genetically encoded (Fig. 5A). This approach has been successful in creating independent enzymes with coherent cofactor reusing components. For example, use of leucine zipper fusions to create enzymatic protein building blocks that self-assemble into enzymatic hydrogels [49]. Many researchers have engineered proteins with tunable scaffolds like β-barrel, β-sandwich, cellulose binding modules, and so on [50]. There are the reports showcasing the successful engineering of hydrolases using α-leucine zipper domains [51] and β-roll [52] into hydrolytic-hydrogels. This

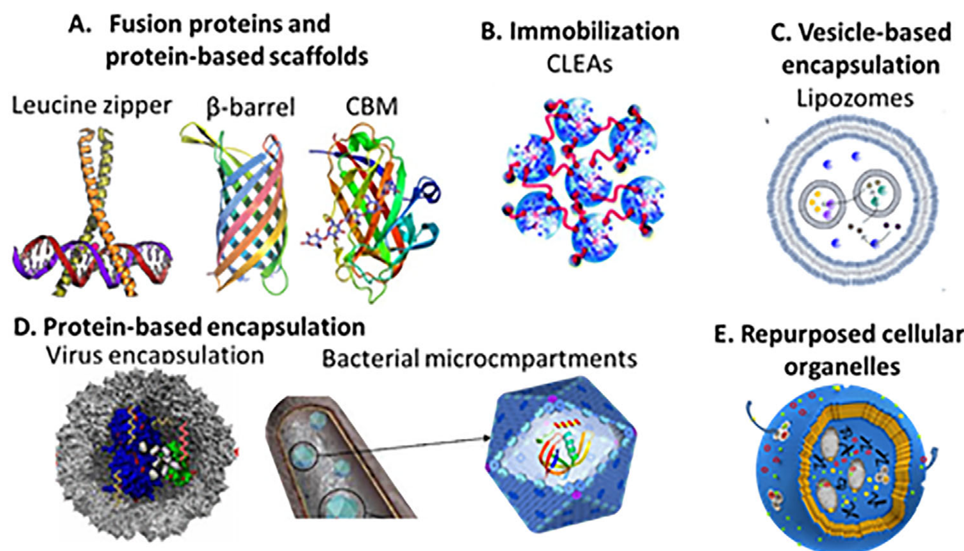

FIG 5 *Strategies for multienzyme cascades. (A) Fusion protein and protein-based scaffolds. (B) Immobilization of enzyme in the form of cross-linked enzyme aggregates (CLEAs). (C) Vesicle-based encapsulation in liposomes. (D) Protein-based encapsulation in virus and bacterial microcompartments. (E) Encapsulation of multienzyme pathways in cellular organelles. (For figure references, follow references in the text.)*

methodology has the potential for designing protein-based self-assembling lipolytic hydrogels in applications for separations, recycling enzymes, use in biphasic systems, and enzyme transformations.

3.2. Immobilization

Many carrier-free coimmobilized enzymes have been produced using the technology of cross-linked enzyme aggregates (CLEAs) formation [53]. Recently, a low-cost CLEA lipase (Fig. 5B) from cocoa pod husk was used in the manufacture of low-cost biodiesel [54]. Also, a mixture of lipase CLEAs are being used for efficient ethanolysis of soybean oil [55].

3.3. Vesicle-based encapsulation

The capability of lipid-based amphiphilic block copolymers to self-organize in specific solvents has been widely investigated in academia and exploited for commercial products. These can encapsulate single- or multienzyme cascades as done with *Candida rugosa* lipase [56] (Fig. 5C). Besides liposomes (from natural phospholipids), polymersomes (made of synthetic block copolymers) are considered more versatile. Their selection criteria depend on specific physicochemical properties, such as permeability, stability, and chemical reactivity [57]. These are unexplored self-fueled microreactor droplets that can be programmed for standalone functions [58].

3.4. Protein-based encapsulation

A lot of hard work has been invested in developing stable protein-based systems for encapsulation of proteins. Virus-like particles have been widely used for encapsulation of proteins owing to their regular dimensions, high stability in vitro, and the skill to self-assemble from minimal components [59]. Apart from viruses, another system is the bacterial microcompartments (BMCs) that are famous for encapsulating metabolic pathways in bacteria [60]. The encapsulated colocalization of heterologous enzymes is being considered as an attractive method for synthesis of multienzymatic biocatalysts. BMCs and nanotubes (Fig. 5D) have been subsequently used for heterologous expression by directed loading of cargo proteins within shells using short N/C-terminal peptide [61–63], thereby increasing the yield by minimizing cellular toxicity and competitive metabolic pathways. The active expression of the rationally designed BMCs containing the lipase and other enzymes has already been studied [64]. Thus, their use as enzyme carriers can be considered as a stepping stone toward green synthesis of products of industrial importance.

3.5. Repurposed cellular organelles

The organelles and vesicles of commercially exploited eukaryotes (*Aspergillus* and *Trichoderma*) are being used for compartmentalization of enzyme cascades [47]. Here, metabolic pathway enzymes are being targeted to different organelles such that they are not affected by the inhibitory or toxic compounds. Researchers are further investigating on how to harness this subcellular compartmentalization for multienzyme biosynthesis (Fig. 5E). Several examples show that mitochondria [65] and peroxisomes [66] can be successfully repurposed for the production of various compounds.

4. Conclusions

Considering the diversity of industrial requirements, tailor-designed enzymes are an unmet industrial need. This mini-review provides light to the lipase-mediated biotechnology with a view of broadening its scope according to the requirements and successful use in respective industrial applications. Therefore, protein engineering approaches to modify proteins at the molecular level serve as a vital tool to obtain enzymes with desired intrinsic characteristics for use in green processes. This can be done by increasing cross-disciplinary research with collaborative efforts in biological engineering, biochemistry, chemistry, and process engineering. Thus, when combined with the power of traditional RD and combinatorial enzyme engineering approaches, microenvironment engineering proves to have a significant impact on the next generation of biocatalysts.

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The author declares no conflict of interest.

6. Ethical Approval

Studies dealing with human participants or animal trials were not performed by the author for this manuscript.

7. Author Contributions

S.S. conceived, designed, and wrote the manuscript

8. References

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