Chapter

Biocatalysis and Strategies for Enzyme Improvement

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Abstract

Biotransformation with the help of enzymes can greatly improve the rate and stereospecificity of reactions in organic chemistry. However, the use of organic solvents and harsh conditions in biotechnological applications often correlates with enzyme deactivation or a dramatic drop in catalytic activity. Detailed molecular understanding of the protein structure and conformational dynamics allows us to address such limitations and to finely tune catalytic activity by modifying the solvent, the support, or the active site of the enzyme. Along with physico-chemical methods of enzyme stabilization, such as additive approach, chemical modification, and immobilization of enzymes, approaches of enzyme engineering based on DNA recombination can be used to enhance the performance of biocatalysts. Since successful synthetic and industrial applications of biocatalysts require systems that are not only stable and active, but can also be reused in a continuous flow process reducing the production cost, the goal of this chapter is to introduce the reader to the vast scope of techniques available for enzyme improvement, highlighting their opportunities and limitations for the real-world technological processes.

Keywords: biocatalysis, enzymes, additive approach, PEGylation, enzyme immobilization, adsorption, entrapment, encapsulation, cross-linking, CLECs, CLEAs, nano-biocatalysis, enzyme engineering, rational redesign, site directed mutagenesis directed evolution, DNA shuffling, error prone PCR, saturation mutagenesis, CASTing, *de novo* enzyme design

1. Introduction

Enzymes are biological catalysts that are believed to be the cornerstones of life. They assure metabolic needs of cells and assist in a great range of life-sustaining biochemical reactions. The majority of natural enzymes are highly efficient and can increase the rate of biotransformation up to 10^{17} fold [1]. Enzymes can carry their functions at ambient temperatures and pressures, with a minimum of by-products and waste, leading to the specific product of interest in a single catalyzed step, whereas synthesis of the same product with the means of organic chemistry may require many steps and produce a mixture of undesired isomeric, epimeric, or rearranged compounds [2]. The field of biotechnology strives to exploit isolated enzymes and whole cell cultures as biocatalysts capable of accelerating and refining complex chemical transformations of organic compounds for industrial and synthetic use [3]. Well known examples of such biocatalysts include microbial

lipases that are used to synthesize cost-effective biopolymers, biodiesel, pharmaceuticals, and agrochemicals from renewable natural sources, *b*-glycosidases employed in industrial plant biomass saccharification [4, 5] and fungal oxidoreductases that have a potential to become biocatalysts in a bio-based (circular) economy by converting biomass into renewable building blocks for manufacturing biodegradable materials [6]. Unfortunately, the scope of natural enzymes is limited, and certain challenges have to be overcome before we can rely on biocatalysts for efficient, low-cost industrial transformations and greener synthetic chemistry. Such challenges include instability of enzymes *in vitro* (denaturation in high temperatures or extreme pH), low selectivity, product and substrate inhibition, and low reaction yield in non-aqueous solvents [2, 7]. Four general approaches exist to address the above mentioned limitations: additive approach, chemical modification, enzyme immobilization, and protein engineering [8]. While protein engineering is concerned with modifying functional properties of the enzyme at the genetic level, the other three approaches are focused on physico-chemical alterations of the media, enzymatic surface residues, or support material for biocatalyst stability [9].

Using **Figure 1** as a guide, we will review both physico-chemical and functional modification strategies for enzyme improvement, starting with the earliest methods to address solvent-dependent limitations and leading to the most recent technologies, like *de novo* and computational enzyme design [10, 11] (**Figure 1**).

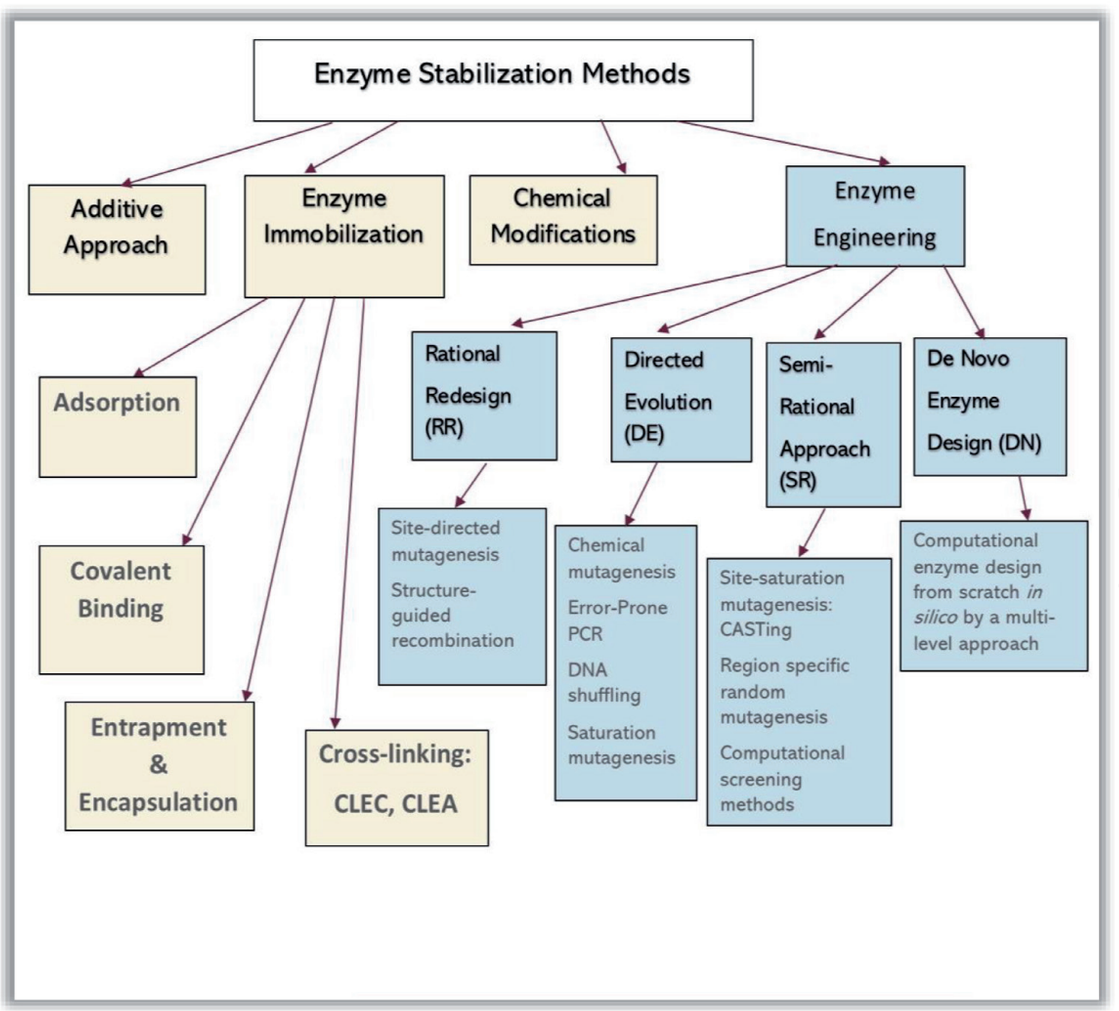

Figure 1.
Enzyme stabilization methods available for improvement of physico-chemical (yellow) and functional (blue) properties of biocatalysts.

2. Improving biocatalysis in organic solvents by additive approach

Enzymes may be remarkable catalysts in biological systems where water is ubiquitous, but they are less suited for biotechnology where organic solvents are largely employed for chemical transformations. For example, the activity of intestinal proteases subtilisin and *a*-chymotrypsin is reduced 10^4–10^5 times if the enzymes are transferred from aqueous to anhydrous octane media [12]. Knowing that water is essential for structural integrity of many biomolecules and seeing vast experimental evidence of decreased catalytic activity in non-aqueous solvents, scientists have been skeptical about using enzymes as industrial biocatalysts or about using water as a solvent for industrial applications due to undesired hydrolytic side-reactions [3]. However, these challenges were proved to be surmountable when new, improved properties of the enzymes in organic and ionic solvents were discovered several decades ago. In many cases, enzymes that had been stripped of their folded structure in a non-aqueous solution not only became more thermostable and easier to store (due to higher melting points), but also became capable of catalyzing new reactions, impossible in aqueous media. For instance, hydrolases, such as subtilisin, routinely accelerate hydrolysis in aqueous conditions, but in anhydrous solvent, they are capable of catalyzing transesterification reactions [12]. The possibility of using novel, industrially favored substrates as well as the possibility of controlling enzymatic activity and selectivity by finely tuned modifications of the solvent lead to the discovery of numerous approaches to stabilize the enzyme in a non-aqueous solvent [3]. Early trial and error experiments with lyophilized (freeze dried) enzyme powders and solvent additives resulted in the development of empirical strategies like addition of water or water-mimicking solvents and addition of salts for stabilizing biocatalysts. The additive approach currently employs the addition of wide variety of lyophilized chemical substances, also known as lyoprotectants, to the media and still appeals to scientists, as it incorporates the simplicity of use and high efficiency [13, 14].

2.1 Addition of water

The lubricating effects of water on biocatalyst flexibility in organic systems were highlighted in multiple studies [12, 15–19]. For instance, in chymotrypsin activity trials, the amount of residual water retained on the enzyme after the addition of organic component correlated with the catalytic activity of the enzyme [12]. It was later determined that the addition of trace amounts of water, even if the enzyme have been unfolded in organic media, can remediate some of the activity loss: in the experiments with subtilisin Carlsberg suspended in organic solvents, increase in water content from 0 to 1% resulted in the increase of reaction rate 11-fold in isooctane and 50-fold in THF [18]. Moreover, hydration of organic solvent does not prevent the enzyme from acquiring novel properties valuable for synthetic and industrial applications. For example, adding 1% water to glycerol helped to retain the secondary structure of *a*-chymotrypsin similar to that in aqueous solvent, however, the enzyme stability at high pH was still much greater in 99% organic solvent over that in water [19]. More importantly, while the enzyme suspended in water was fully denatured after 1 min at 100°C, *a*-chymotrypsin in 99% glycerol retained 80% of its catalytic activity after incubation at 100°C for 10 h [19].

In recent years, water addition strategy has benefitted many promiscuous biocatalyzed synthesis reactions, such as Henry reaction, Michael addition, Mannich reaction, asymmetric aldol reactions, and others [14]. You can refer to the excellent review by Liang and Lin for the empirical data on yield increase in these reactions due to hydration.

It is important to note that while too few water molecules may be not enough to activate biocatalyst in organic solvent, too many water molecules may result in reduced substrate solubility or hydrolytic reactions side product [14].

2.2 Addition of water mimics and lyoprotectants

Just like water that is thought to lubricate the enzyme enhancing protein flexibility with its multiple hydrogen bonds, water-mimicking substances, such as glycerol, formamide, ethylene glycol and formic acid can provide similar hydrogen bonding, while avoiding unintended hydrolysis product [14, 20]. One of the early water mimic studies concluded that adding 0.1% ethylene glycol to the solvents with optimal water content of 0.2% can increase the activity and stereo-selectivity of *Candida cylindracea* lipase [21].

Since enzymes often have to be freeze-dried, it is important to ensure their stability during the long-term storage or temperature changes associated with thawing. The most common lyoprotectant up to date is trehalose sugar that helps to preserve enzyme structure and allows for industrial storage of biocatalysts [18].

Most recent reviews also list organic bases, crown ethers, surfactants, and salts as possible additives used to improve catalytic activity of enzymes in chemical synthesis [14]. For the purpose of this section, we will only cover the addition of salts and the role of ionic interactions in biocatalyst enhancement.

2.3 Addition of salt

In 1994, Khmelnitsky et al. discovered that lyophilization of an enzyme in a salty matrix prior to its suspension in organic media lead to a dramatic enhancement in the rate of catalyzed reaction [22]. In this study, 3750-fold increase in activity of subtilisin Carlsberg was documented when 98% w/w KCl-containing lyophilized enzyme powder was added to hexane, as opposed to salt-less enzyme preparation [22]. The authors explained this phenomenon by the protective ability of the salt that was able to prevent direct contact between enzyme molecules and the organic solvent; however, more recent findings using electron spin resonance spectroscopy suggest that salt-induced ionization stabilizes the charged transition state and thus, increases the polarity of the active site [23]. It is also known that while adding certain ions to enzyme preparation or sometimes directly into the solvent can improve both the reaction rate and enantioselectivity, other ions improve only the rate of the reaction or have no effect on the catalysis [14]. Empirical evidence suggests that only kosmotropic (increasing viscosity of water) salts can stabilize catalysts due to preferential hydration effect that addition of Ca ions is more activating than the addition of Ba, Sr, or Mg divalent metal salts [24, 25], and that by using aqueous solutions of smaller alkali metals or alkaline earth metals rather than hydrating the enzyme with water alone we can markedly increase enantioselectivity of the reaction [26].

Even though additive approach for biocatalysis improvement has offered many successful results, several disadvantages limit its use and call for exploring other methods of enzyme stabilization. Such disadvantages include the fact that the effect of molecular additives varies widely from case to case, depending on the enzymes used, desired substrates and reactions. Since the majority of successful additive methods were discovered by accident, there are no general protocols developed for this approach, and only few stabilizing additives are researched enough for us to clearly understand the molecular mechanisms behind their role in catalysis [14].

3. Chemical modifications to stabilize enzymes

Chemical modification of enzymes is a very common stabilization strategy. In fact, covalent modification by the cross-linking with glutaraldehyde reagent can stabilize almost any enzyme, protecting it from denaturing and other effects of the new solvent [27]. This finding led to the development of carrier-free enzyme immobilization methods (to be discussed in detail in Section 4).

Another popular chemical method involves covalent conjugation with an amphiphilic polymer polyethylene glycol (PEG) and is often referred to as enzyme PEGylation [28]. PEGylating permits binding of specific polymeric functional groups to the free amino groups on the enzyme, creating PEGylated biocatalyst soluble in organic solvents [19]. This method is especially useful for preparation of biopharmaceuticals with high stability and low antigenicity [29]. Additionally, reagent methoxymethyl-PEG (mPEG) bound to the enzyme horseradish peroxidase (HRP) can protect the protein from pH extremes and high temperatures, making HRP particularly useful in industrial and clinical biosensors [30].

Chemical alterations of the enzyme can introduce a new functional group for a covalent attachment or modify one of the reactive side chains. For instance, the treatment of *Candida rugosa* lipase with diethyl *p*-nitrophenyl phosphate modified one of the two reactive functionalities and resulted in a more selective lipase catalyzing a single reaction [31].

Chemical modification makes it possible to introduce and attach a new cofactor, which can in turn induce novel enzyme functions [32]. Lastly, some post-translational enzyme modifications either *in vivo* or *in vitro* have been linked to an enhanced stability of enzymes [33]. For example, when DNA ligase from *Thermus scotoductus* was chemically adenylated, new irreversible covalent binding of the cofactor resulted in structural changes within the active site and overall protein compaction. As the result of this cofactor-induced conformational change, the enzyme gained increased resistance to thermoinactivation [34, 35].

As strategies for enzyme improvement continue to evolve, chemical modification has been rediscovered to become a robust complimentary approach to both protein engineering and immobilization [36].

4. Enzyme immobilization

It is clear that while additive or chemical approaches represent a simple and attractive route for a small-scale chemical synthesis, they have to be supplemented or substituted with other enzyme-stabilizing approaches when applied to complex enzyme systems or whole-cell catalysts in biotechnology.

Much like in a living organism, where enzymes are associated with a membrane or a cell structure that ensures their stability, in industrial setting, it is often necessary to anchor the enzyme to a certain area of a reactor in order to stabilize and reuse the same catalytic device over and over [8]. Enzyme immobilization approach usually achieves this goal by constraining enzymes to a more stable support (a carrier), thus, creating insoluble heterogeneous catalyst of native conformation with reduced flexibility [37]. Not only immobilized catalyst is less likely to be deactivated in organic solvent, is not perturbed by lyophilization [38], is more resistant to sheer stress and high temperatures [39, 40], but it is also less costly, as it is recycled in a continuous fixed-bed process and allows for an efficient enzyme purification and recovery with the help of selective adsorbents [41, 42].

Biocompatible carrier can be chosen from almost any organic polymer or inorganic material that is inert [43]. The requirements to the ideal carrier also include: affordability (the cost of the enzyme and the carrier should not be more than a few percent of the total production cost), stability, physical strength, regeneration ability, as well as the ability to aid in catalytic functions [38, 44], promoting enzyme specificity and reducing product inhibition and non-specific interactions [45]. Moreover, the carrier has to match certain surface properties of the enzyme (e.g. polar groups of amino acids or apolar surface areas), have high superficial density of reactive groups [46], and have a large surface area (e.g. contain high number of small-sized particles or large dimensional pores) [47]. In some cases, where stronger covalent binding with the enzyme is desired, reactive functional groups have to be chemically introduced as monomers in a polymeric carrier matrix [48]. All the above requirements for the carrier are not easy to meet, and sometimes carrier-less approach is used, where enzyme molecules are cross-linked to each other, providing support [38]. Fundamental strategies of biocatalyst immobilization include adsorption, covalent binding, cross-linking, and entrapment/encapsulation [8, 47, 49] (**Figure 2**). In this section, we will review these approaches and their successful combinations, as well as some promising "smart" enzyme immobilization techniques with possible applications.

4.1 Adsorption

More than a century ago, it was empirically proven that invertase enzyme can be adsorbed onto a solid support like charcoal or aluminum hydroxide with its catalytic activity remaining similar to that in its free soluble state [50]. Since then, the methods of adsorption of enzymes onto carriers may have evolved, but the principle has remained the same: intermolecular forces between the enzyme and support result in protein accumulation on a solid surface [8]. Adsorption forces are rather mild and generally involve non-specific van der Waals, hydrophobic, hydrogen bond (H-bond) and ionic interactions [42]; however, high coverage and stronger adherence can be achieved by choosing the right carrier based on enzyme's surface

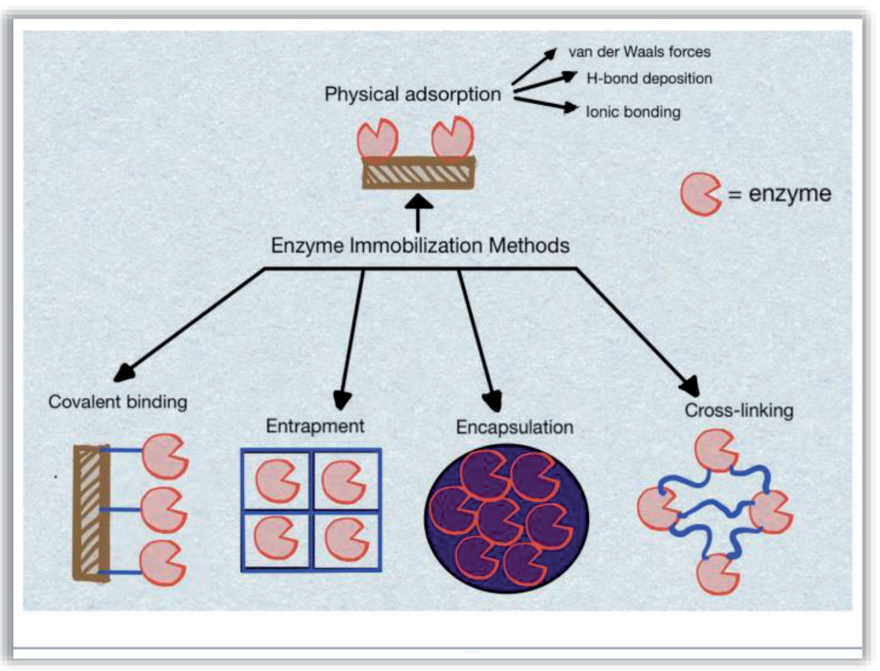

Figure 2.
Fundamental methods of enzyme immobilization.

charge and polarity [51]. For instance, a hydrophobic carrier will work well with the enzyme of large lipophilic surface area due to entropy and hydrophobic effect [47]. Alternatively, H-bonding to hydrophilic carriers (e.g. cellulose, porous glass, silica gel, Avicel, or Celite) is used if the enzyme has hydrophilic surface residues, especially, if the enzyme is glycosylated [47].

The main advantage of adsorption method is its simplicity and low cost [52, 53]. The enzyme can be simply added to the active adsorbent's surface [43], or deposited onto the carrier via evaporation of aqueous phase [54], there is no need to previously modify the ligand [55], or to wash off the enzyme that has not been adsorbed [43]. Typically, mild, reversible interactions of physical adsorption are preferred for immobilization, since they do not change the native structure of the catalyst and do not disturb its active enzymatic sites [53]. The disadvantages of adsorption include the fact that it only proceeds in organic solvents due to intrinsic solubility of enzymes in such media; otherwise, enzyme leaching is unavoidable either as a result of H-bonds with water or due to ion exchange for the enzymes immobilized by ionic forces [47]. Apart from enzyme leaching as a function of time [8], in a physical process such as adsorption, active site blockage due to nonspecific interaction with the carrier can greatly reduce the activity of immobilized catalyst, especially if the substrate is large [49, 56].

In order to improve the stability and activity outcomes of non-specific adsorption, multiple specific adsorption methods were proposed in recent years, including biospecific adsorption that involves immobilized antibodies, affinity adsorption that uses carrier-bound immobilized dyes as ligands, coordination adsorption where immobilized transition metal ions interact with amino acid residues on the enzyme, and others [57].

4.2 Covalent binding

It was discovered in 1960s that certain pre-functionalized carriers covalently bound to the enzyme (native or modified) can act as a scaffold for enzyme stabilization, while substantially improving its performance as a biocatalyst [42]. Covalent forces between the functional groups of the support matrix (whether it is an inner wall of a bioreactor, or a packed-bed industrial reactor filled with glass or biopolymeric beads [8] and reactive active amino acid residues on the enzyme surface can create extremely strong linkages [58]. For example, pre-treatment of glucose oxidase from *Aspergillus niger* with periodic acid helped to activate its carbohydrate residues for new covalent linkages to a hydrophobic polymer *p*-aminostyrene. As a result, immobilized glucose oxidase retained its full activity and even gained higher thermostability at 60°C compared to that of a native soluble enzyme [59]. Unlike adsorption, covalent binding can be performed in any solvent; however, it is a method of choice for immobilizing enzymes in aqueous solvents or under denaturing conditions [47]. The rigid covalent binding prevents enzyme leaching caused by non-specific interactions with water and locks the enzyme in its native conformation, resisting thermoinactivation [60]. Similar to physical adsorption, both hydrophilic and hydrophobic carriers can be used for immobilization [47]. Carrier activation may also require long or short spacer molecules between the carrier and the enzyme, in order to provide more accessible catalytic sites and to attach desired reactive groups to the matrix [55]. Most commonly introduced reactive groups include aldehydes or epoxides to be attacked by nucleophilic amino groups on the protein, which is followed by instant reduction of the product and irreversible attachment of the enzyme to the carrier [42, 48]. Nevertheless, such chemical modifications can be harsh for the three-dimensional protein structure, especially, if chemical microenvironment of the enzyme (including possible storage additives)

is interfering. The main disadvantages of immobilization by covalent binding include non-uniform attachment if the bond density is too low [61], or, if the bond density is too high, the risk of immobilized catalyst being irreversibly deactivated and rendered unusable [37].

4.3 Entrapment and encapsulation

Entrapment and encapsulation within the polymeric matrix are immobilization techniques that, similar to physical adsorption, employ non-covalent interactions [49]. Unlike in surface adsorption, the support matrix is not pre-fabricated but is synthesized at the same time as the enzyme is being entrapped or encapsulated *in situ* [38].

In the entrapment method, a catalyst (soluble or insoluble) is dispersed in a solution of a monomer or polymer of low molecular weight, and later becomes entrapped in a matrix formed by hydrolytic polymerization [42]. Inside the polymer lattice, almost every side chain on the enzyme surface physically interacts with the support material around it, while allowing small substrate product molecules move in and out of the complex through the pores [43]. As an attempt to modulate the porosity and diffusion pattern, silica sol–gels of varying densities (e.g. xerogels, ambigels, aquagels, or aerogels) with additives to create hydrophilic or hydrophobic surfaces are commonly used [62]. When xerogels of high density entrap one or more enzymes by hydrolytic polymerization, the substrate selectivity of the extremely small pores can be particularly useful for the development of biosensors [63, 64]. Entrapment matrices with hydrophobic surfaces can activate lipases, the most used enzymes in synthetic organic chemistry [65]. Moreover, catalytic activity of *Candida antarctica* lipase entrapped in a hydrophobic sol-gel can be improved 2–8-fold compared to that of non-immobilized freeze-dried lipase powder [66]. Nonetheless, the practical application of entrapment is limited because of the requirements, like small substrate size and delicate balance between physical properties of the matrix and enzymatic activity [43]. Insufficient substrate interaction and leakage of enzymes due to continuous use are major disadvantages of the entrapment method [67].

Alternatively, enzymes can be encapsulated within a semi-permeable polymeric membrane [43]. Immobilization by encapsulation occurs when microcapsule walls are formed around enzymes as a result of polymer desolvation [68]. The capsule can provide desired chemical microenvironment for a specific enzyme in terms of pH, temperature and solvent stability, very similar to the microenvironment in the living cell [69]. Depending on the material used, the membrane can be permanent or non-permanent. Non-permanent membranes can be formed by liquid surfactants, while permanent membranes are often made of polystyrene, cellulose, gliadin, nylon, and other materials used to encapsulate pharmaceuticals, food, cosmetics, and chemicals [68, 70].

Although high enzyme concentrations and large surface areas of encapsulated biocatalyst can ensure faster reaction rates [71], similar to enzyme entrapment, traditional method of microencapsulation can only be applied to a limited number of enzymes with small substrates [62]. In recent years, the structure of microcapsule from a hollow bead evolved to a complex multilevel three-dimensional sphere. Such biomimetic capsule design often allows to adjust permeability in favor of mass transfer of substrate and product, while dramatically improving catalytic activity of encapsulated enzymes [72].

4.4 Carrier-free immobilization by cross-linking

Carrier-free immobilization method was developed as a way to address the issue of inevitable activity drop associated with the carrier attachment. The non-catalytic

part often represents 90–99% of the immobilized catalyst [37], and although it provides the catalytic element with enhanced stability and longer half-life, it is also responsible for unwanted mass-transfer effects, low product yields and higher production cost [73].

It was first reported by Yale scientists in 1964 that di-functional reagent glutaraldehyde, normally used as a fixative for electron microscopy slide preparation, can form irreversible covalent linkages between enzyme molecules. When carboxypeptidase-*a* enzyme crystals treated with glutaraldehyde were tested by diffraction techniques, cell dimensions of the enzyme remained very similar to that of its native state, even after mechanical stress and changing the solvent; moreover, the enzyme tested retained most of its catalytic activity [27]. Therefore, pure enzyme in almost any form (solubilized, crystallized, aggregated or atomized) now can be chemically modified to act as its own carrier [74]. The mechanism of this reaction involves the formation of Schiff's bases between carbonyls on the cross-linker and free amino groups on the enzyme's lysine residues together with a pH-dependent Michael 1,4-addition to *a,b*-unsaturated aldehydes [75].

Two most popular carrier-less preparations that can be used in industrial and pharmaceutical synthesis are cross-linked enzyme crystals (CLEC) and aggregates (CLEA) [76]. CLECs, obtained by crystallization of the pure enzyme prior to cross-linking [77], according to the patent of 1997, are able to retain at least 91% of enzyme's initial catalytic activity if incubated with a protease for 3 h, compared to unmodified enzyme that loses at least 94% of the activity under the analogous conditions [78]. Resistance to proteases also allows to administer CLEC biocatalyst orally as a longer-acting drug [79]. As for the industrial uses, advantages of CLECs, like possibility of indefinite storage at room temperature, their near-maximum catalytic activity in harsh conditions, and high tolerance to organic solvents [80], allow for various potential applications, including microporous phase for chromatography and environmental toxicity biosensors [81, 82]. And yet, CLEC method requires highly purified enzyme for crystallization, which translates into higher expense and renders CLEC superseded by closely related technique CLEA [37].

CLEAs, first described in 2000, are prepared by aggregating enzymes with precipitants, like acetone, ethanol, or ammonium sulfate, and then by cross-linking the aggregates with glutaraldehyde or dextran polyaldehyde [38, 83]. After the protein is cross-linked with the reagent, sometimes protective reagents and other additives can be used to create a "tailor-made" biocatalyst [47]. For instance, knowing that surfactants, amines, and crown ethers can activate lipases, one study prepared CLEAs of seven microbial lipases by co-precipitation with different additives. As a result, two of the lipases were hyper-activated (had 2–3 times higher activity than that of a native enzyme) in the presence of SDS surfactant, demonstrating that precipitation with the right additive can lock the catalyst in the preferred conformation [84].

Apart from broad applicability, high stability, and possibility of catalytic hyperactivation by chemical optimization, the CLEA technology eliminates laborious enzyme purification and crystallization steps required for CLECs as well as the expense of using a carrier required for other immobilization methods [85]. This makes CLEA protocols reproducible in almost any lab that has a relatively crude enzyme sample [49]. Interestingly, crude samples containing several enzymes can be used to make combi-CLEAs that recreate multistep, multi-enzyme cascade processes for biotechnological or clinical applications [85].

4.5 Combinatory physicochemical approaches

Rational combinations of immobilization methods described above together with chemical enzyme modifications (see Section 3) often have synergistic effect [33].

Therefore, combinatory approaches have been increasingly applied with the goal of designing an improved, robust catalyst from the native enzyme by physicochemical means at a low cost. For instance, physical adsorption, a simple and less expensive immobilization technique [52], would be more commercially viable if it could avoid desorption of enzyme immobilized by weak, non-specific forces. It was reported that entrapment of *Candida* lipase prior to its co-adsorption with lipase-activating chemical additives not only protected the enzyme from desorption, but also was a cheaper and more efficient method of biodiesel production from waste cooking oil, compared to immobilization methods described in previous studies [57]. Combination of enzyme cross-linking prior to entrapment in sol–gel beads was determined to be a viable technique for stabilizing enzymes in glucose biosensors [67]. In some cases, filtration-enabled reusability of CLEAs may be problematic due to similar size of the particles and the substrate. To ensure easy enzyme separation, it was proposed to encapsulate the enzyme solution in a soft porous membrane and thus, create particles of desired size prior to their aggregation and cross-linking [44].

With a number of successful techniques available and so many variables involved in the trade-off between stability and activity of immobilized enzyme, choosing the most efficient approach for a less-studied enzyme can be a problem. In this case, combination of computational analysis with experimental methods, known as structure-based immobilization, can be of assistance [47]. For instance, GRID computational analysis of the functional groups on the enzyme can help to locate nucleophilic amino groups on the lysine residues that would be involved in the covalent binding. If such groups are present in the close proximity of the active site, non-covalent techniques, like adsorption, entrapment, or encapsulation should be considered. Additionally, hydrophobic and hydrophilic surfaces of the enzyme can be established in order to choose the appropriate support material [86].

According to bibliometric analysis of 2019, new trends in enzyme immobilization research can be seen from the top-50 author-keywords list, based on the works published globally in the last 5 years. The top directive terms included: gold nanoparticles, meso-porous silica, magnetic nanoparticles, response surface methodology, glucose biosensor, and cross-linked enzyme aggregates (CLEAs) [74]. It is evident, that immobilization on nano-particles, nano-fibers, and nano-gels is a field of special interest, due to high adaptability, high retention of activity, and effortless enzyme separation and recycling [49, 87]. The main advantage of nanostructures is their high surface to volume ratio, where decreasing size of the carrier allows progressive exposure of the enzyme to reaction media, making nano-immobilization a method of choice for development of powerful enzyme-based fuel cells [88]. Magnetic nanoparticles (e.g. mCLEA) that can be functionalized for enzyme isolation by magnetic decantation or used in magnetically stabilized reactor beds are part of a new, promising approach, known as "smart" enzyme immobilization [89]. Finally, there is a possible turn of interest to a matrix algebra- and statistics-based response surface method (RSM) that can be used for optimizing operational conditions of immobilized biocatalysts [90].

5. Enzyme engineering

"What I cannot create, I do not understand"
Richard Feynman

Enzyme engineering approach originated as an ultimate challenge to test our understanding of protein structure and function in early 1980s [91]. Since then, the

field of biocatalysis have been revolutionized by the advances of recombinant DNA technology, use of computational tools, and modern bioengineering. Alterations of the primary protein structure can be tailored not only to stabilize the enzyme, but also to broaden the substrate range, optimize catalytic performance, and obtain products of high value for various biotechnological applications. Enzyme designers can modify or develop a novel biocatalyst with a new primary function [3] by using one of the following approaches: rational redesign (RR), directed evolution (DE), semi-rational redesign (SR), or *de-novo* enzyme design (DN) [92]. Enzyme engineering methods are laborious and all have the following pre-requisites in common: enzyme encoding gene of interest, microbial or yeast expression system, and sensible screening tools for mutant detection [93]. In this section, we will briefly review these methods and their possible applications in fine chemical synthesis.

5.1 Rational redesign (RR)

RR strategies for enzyme improvement genetically modify the existing biocatalyst based on known structural criteria. To begin with, different pieces of data, like protein structure obtained by X-ray diffraction techniques, molecular models based on computational algorithms, and biochemical specifics, like locations of interacting ligand residues obtained by NMR-analysis, must be evaluated to propose rational genetic alterations [94]. The method of choice for introducing specific alterations is site-directed mutagenesis (SDM) [2]. Most commonly, beneficial mutations are induced with the goal of changing catalytic mechanism, reinforcing the promiscuous reaction, altering substrate/cofactor specificity and improving the overall stability of the biocatalyst [93]. To ensure the specificity of mutations, the majority of RR techniques employ polymerase chain reaction (PCR) with the primers (short sequences of synthetic DNA complementary to the template gene) that have been modified using a known mutant codon or codons [2].

Two common methods of inserting/deleting specific amino acids into/from the target gene by SDM are overlap extension and whole plasmid single round PCR [10]. The former method uses two pairs of primers with one of the primers in each pair containing a modified (mutant) codon, which results in a PCR-produced heteroduplex plasmid with overlapping breaks. The later method is simplified by using a "QuikChange Site-Directed Mutagenesis Kit" patented by Stratagene and adding two primers with desired mutations that are complementary to the opposite strands of the DNA template. The mutant plasmid obtained by PCR is then nicked by a specific restriction enzyme to be repaired upon transformation into competent cells [95]. A more efficient one-step version of QuikChange method suitable for single or multiple-site insertions and deletions was described in [96]. Successful applications of SDM include the induction of acid-resistance and 16.7-fold higher catalytic activity in *a*-amylase used for industrial sugar and detergent production [97], thermostability enhancement in sucrose isomerase producing a non-cariogenic, nutritional sugar isomaltulose that slows down the rate of insulin release in diabetes management [98], and even attempts to design high efficiency H_2-producing cyanobacterial cells to make biofuels [94]. At the same time, numerous attempts of RR fail due to insufficient knowledge of mechanisms responsible for the specific structure–function relationships. The process of SDM is often too tedious and expensive, requiring mutant enzyme confirmation by sequencing and purification [99].

5.2 Directed evolution (DE)

In contrast to RR, the methods of DE can be applied to the enzymes even in the absence of existing structural and mechanistic data. DE relies on accelerating

Darwinian evolution in laboratory with the means of mutation and random recombination, followed by multiple rounds of selective "molecular breeding" [100]. Three steps common for all DE methods are: (1) construction of mutant library by the means of random mutagenesis or *in-vitro* recombination, (2) screening/selection of mutants with the desired properties via high throughput assays, and (3) improved protein gene isolation [49, 92]. Initial generation of molecular diversity can be achieved by methods like chemical mutagenesis, error-prone PCR (epPCR), gene site saturation mutagenesis, and DNA shuffling [2, 49].

Chemical mutagenesis is used extensively for a "food grade" enzyme improvement: avoiding the introduction of heterologous DNA, it applies chemical agents like ethyl methyl sulfonate or nitrous acid to bacterial strains lacking DNA repair mechanisms, generating random mutations [101].

EpPCR method introduces random changes in a catalyst encoding gene by using error-prone Taq DNA polymerase for the PCR process. Unlike in chemical mutagenesis, the rate of mutation can be controlled by modifying PCR conditions. For instance, it is possible to introduce an average of one amino acid substitution per PCR cycle, and 3–7 cycles are usually enough to improve the thermostability and enantioselectivity of the enzyme [2, 102].

DNA shuffling method relies on in vitro DNA recombination of closely related parental genes either obtained from different organisms or produced by epPCR [92]. The genes are digested with DNaseI or with a mixture of restriction endonucleases to yield random, small fragments that will be purified and reassembled by epPCR, where fragments cross-prime each other [95]. Therefore, genes from multiple parents, including different species, can be shuffled in a single step, sometimes resulting in a hybrid DNA with unique, novel traits not expressed in either parent [103]. Nevertheless, DNA shuffling cannot induce drastic functional changes, as these are known to require considerable evolutionary changes in polypeptide backbone [99].

The technique known as gene site saturation mutagenesis can be used for the replacement of each amino acid of a protein with each of the other 19 amino acids occurring in nature [2]. For instance, PCR amplification with a mixture of 64 different forward and 64 different reverse primers would be necessary to randomize one codon in the enzyme gene (based on 4 letters of genetic alphabet and 3 nucleotides in one codon), but one could eliminate stop codons by restricting the third nucleotide to G or C and use a mixture of 32 forward and reverse degenerate primers. Statistically, the size of the mutant library obtained by this method can be calculated as 20^n, where n is the number of amino acid residues in the protein of interest [2]. Hence, the biggest limitation of DE methods is the requirement of an efficient high throughput screening process for the mutant libraries that even with millions of variants, still sample only a very small fraction of the enzyme sequence [104]. Nevertheless, the field of DE is actively developing, and recent publications report achievements, such as induction of pH-, temperature- and oxidation-tolerance in a catalyst [105, 106], including the development of a novel enzyme structure as a result of a new function acquired by DE [107].

5.3 Semi-rational (SR) and *de novo* (DN) design approaches

Addressing major limitations of both DE and RR, SR design creates smaller, manageable mutant libraries based on the structural/biochemical data or computational predictive algorithms [104]. In other words, if the 3-D structure of an enzyme is available, random mutagenesis can be focused on a specific site (usually within the active site) in the protein sequence, leading to higher probability of identifying the key amino acids, randomly replacing them or reinforcing them via cumulative effects [3].

Site saturation mutagenesis rationally applied to a specific codon or codons is an example of a semi-rational approach. In the method known as CASTing (combinatorial active site saturation test) several amino acids of the active site are targeted and mutated one by one or in combination [108]. Combinatorial saturation of residues can produce mutants improved by synergistic effects. However, these vast multiple-site saturation libraries are almost impossible to screen with traditional DE means. In this case, computational algorithms are used to virtually screen the library, eliminating the mutants that have been misfolded due to unfavorable amino acid interactions [109].

"Region specific random mutagenesis" is another semi-rational method that employs SDM performed with 64 different forward and reverse primers targeting a single codon to be randomized based on its importance for enzyme structure and function [95].

There is no doubt that the fine-tuning of engineered enzymes benefits from the use of combinatorial approaches. SR design has a great potential to create specialized biocatalysts for the industrial use. For example, improved uronate dehydrogenase enzyme of high thermostability can be used to catalyze the production of glycaric acids (top-value chemical precursors for greener and less expensive biofuel synthesis) [110]. Nevertheless, even with reduced mutant libraries, SR approaches are not straight forward. Sometimes, molecular interactions and cascade effects far away from the active site are responsible for certain catalytic effects [3].

In cases when enzyme structures do not appear to be optimized for a specific chemical transformation, synthesizing enzyme of a completely different structure *de novo* may be the most reasonable solution. In 2008, DN engineering of artificial enzyme Kemp eliminase from scratch provided the evidence that it is possible to create a new functional enzyme using a multi-level approach that involves quantum mechanical (QM) calculation, computational algorithms, and directed evolution [111]. Generalized DN workflow starts with the engineering of a minimal ideal active site containing catalytic machinery and interaction residues, optimized by QM into a model structure called "theozyme". The theozyme is then matched to a pre-existing protein scaffold by a hashing algorithm. After the scaffold-theozyme structure is stabilized, the enzyme is ranked *in silico* based on its geometry and binding energy and can be empirically tested. If tested active, but inefficient, the catalytic activity can be improved by several rounds of DE [11]. DN development would not have been possible without computational tools, like METAL SEARCH, DEZYMER, ORBIT, ROSETTA match, ROSETTA design, and MODELER (see [49, 109] for details).

Although it is able to provide predictive frameworks for rational *in silico* engineering and generate more focused, "smart" libraries, computational enzymology is still in its infancy. Combinatorial strategy where directed evolution is integrated with rational optimization remains a method of choice for protein engineering [104].

6. Conclusions

While enzymes have been involved in commercial production processes for centuries, their vast potential for a large scale chemical synthesis and industrial applications was not fully realized until better empirical models and methods of biocatalyst stabilization were developed using a trial and error approach. In this chapter, we reviewed fundamental strategies for enzyme improvement, such as chemical modification, additive approach, enzyme immobilization, and protein engineering. It appears that enzyme immobilization is currently considered to be the most promising strategy for obtaining industrial biocatalysts with controlled,

more specific substrate interactions, resistance to denaturation, and high product yield at low cost [74]. At the same time, enzyme engineering methods recently made numerous successful advances to redesign existing enzymes on the level of their primary structure using targeted random mutagenesis, *in vitro* recombination, and various computational tools. Although there is high demand for such specialized, robust biocatalysts, they are generally produced as soluble enzymes, not reusable in the industrial synthesis. Therefore, integration of physico-chemical methods and protein engineering is possibly the most efficient strategy for creating a powerful, recyclable biocatalyst fit for the real-world biotechnological processes.

Conflict of interest

To our knowledge, there has been no conflict of interest.

Notes/thanks/other declarations

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