Broadening the scope of biocatalysis engineering by tailoring enzyme microenvironment – A review

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Abstract

The rational design of catalysts that fine-tune/mimics the enzyme microenvironment remains the subject of supreme interest. Several strategies moving from traditional to

technologically advanced methods have been proposed and deployed to develop high efficacy enzymes. There is a plethora of literature on simple enzyme immobilization through different materials as support carriers, even at the micro- and nanoscale. Regardless of extensive strategic efforts, the existing literature lacks deep insight into tailoring the microenvironment surrounding the target enzyme molecules and can sophisticatedly integrate the bio-catalysis for multipurpose applications. The ongoing advancement in the industrial sector also demands catalysts with unique features. For instance, catalytic turnover, substrate affinity, stability, specificity, selectivity, resistivity against reaction impurities or inhibitors, prevention of subunit dissociation, ease in recovery, and reusability are highly requisite features. This review spotlight state-of-the-art protein engineering approaches that facilitate the redesigning of robust catalysts or fine-tuning the catalytic microenvironment of enzymes. The entire work critically focuses on protein engineering approaches, i.e., regulating pH microenvironment, creating a water-like microenvironment, activating enzyme catalysis in organic solvents and gas phase, tuning reaction kinetics (K_M and k_{cat}), engineering substrate specificity, reaction promiscuity, computational design, and structure-guided biocatalyst engineering. This study unveils the advanced insights of enzyme microenvironment engineering, which can also be considered catalytic yield enhancement strategies to green the future bio-catalysis research for industrial bioprocesses.

Keywords: Enzyme engineering; Bio-catalysis; Yield enhancement strategies; Protein engineering; Microenvironment engineering; Computational modeling

Introduction

Enzymes are biocatalysts that are likely to restructure traditional chemical processes eco-friendlier and more efficient [1-3]. Although genetic engineering strategies hold promise for the discovery, mutation, and de novo design [4, 5] of catalysts with high stability and bioactivity, chemical modification. Immobilization imparts enzymes with unique catalytic properties for practical exploitation, such as improved biocatalytic performance, mechanical strength, aggrandized stability, and repeated useability [6, 7]. In addition, providing a distinct microenvironment to the enzymes is a noteworthy characteristic of immobilization. Furthermore, engineering a biocompatible microenvironment for catalysts

is valuable for optimizing enzyme-catalyzed reactions [6]. The microenvironment is the micro-and nanoscale local physicochemical atmosphere surrounding the enzyme molecules or a cluster of enzymes at their catalytic region. The physicochemical parameters, i.e., temperature, pH, and reactants concentrations in the microenvironment, vary greatly from the bulk environments because of carrier's immobilization, entrapment in micro- or nanoarchitectures, polymers conjugation, and aggregation into complexes. Microenvironment can help to stabilize enzymes by protecting them from harsh conditions, regulating reaction kinetics, governing enzyme activity concentrating or excluding cofactors and substrates, augmenting cascade reactions efficacy by compartmentalization, and speeding up reactions by an increased local temperature.

Considering the rapid progress in enzyme catalysis, we provide detailed and comprehensive insights into the microenvironmental engineering of enzymes in this review. This work focuses on new and/or advanced protein engineering approaches, i.e., regulating microenvironmental pH, creating a water-like microenvironment, activating enzyme catalysis in organic solvents and gas phase, tuning reaction kinetics (K_M and k_{cat}), engineering substrate specificity, reaction promiscuity, computational design, and structure-guided biocatalyst engineering (Figure 1). We highlight four mechanisms: localizing and excluding reactants and regulators, regulating microenvironmental pH, creating a water-like microenvironment, and increasing the local temperature. These effects can be exploited to regulate the apparent enzyme performance without requiring engineering of the intrinsic enzyme properties.

Strategies to tailor enzyme microenvironment

Regulation of microenvironmental pH

The biocatalytic efficiency of most of the enzymes is highly dependent on pH. The inherent pH dependencies of the enzymes are usually conquered by protonation/deprotonation of critical amino acid residues in the catalytic region at various pH [8]. Site-directed mutations at the substrate-binding pocket might change the pH requirement of a specific enzyme but is likely to result in a substantial reduction in the biocatalytic performance [9, 10]. On the contrary, manipulating buffer pH for a particular enzyme appears to be a much more reasonable approach than mutagenesis. Thus, intended adjustment of the enzyme dependence on pH seldom becomes an applied issue.

Nevertheless, it represents considerable importance for one-pot multi-enzymatic catalytic reactions that implicates biocatalysts with different optimum pH because the optimal output cannot be attained in the same buffering solution.

Immobilization offers a potential alternative strategy for modifying the working pH environment of an enzyme because the immobilization process often alters the pH-activity profile of the enzymes. This displacement in pH is predictable and significant if the biocatalysts are bound to ionic macromolecules or polyelectrolyte support materials. [11] determined that the pH profile of different enzymes, including papain, chymotrypsin, trypsin, and subtilopeptidase A were displaced toward more basic pH values following immobilization on polyanionic carriers. Likewise, attachment to polycationic supports caused a shift toward acidic pH than native counterparts. They inferred that the highly charged support matrices retained a more alkaline (polycationic matrix) or acidic (polyanionic matrix) microenvironment surrounding the immobilized enzymes than the bulk solution. Many researchers have substantiated this effect with various polymeric carriers and enzymes [12-14].

Therefore, immobilizing enzymes onto polyelectrolyte support matrices might be a general strategy for manipulating the pH dependence of enzymes. A proof of concept was confirmed with D-amino acid oxidase (DAAO), and cytochrome c (Cyt c) catalyzed cascade reaction [15]. Though DAAO showed its optimum performance at alkaline pH and Cyt c was more vigorous at acidic pH, conjugation of negatively charged poly(methacrylic acid) with Cyt c could effectually change its optimum pH towards alkaline and natural environments, thereby facilitating both enzymes to function at an efficient way at pH 8.0 compared to free counterpart (Figure 2) [15]. Rational manipulation of micro-environmental pH surrounding the multienzymes can considerably increase the biosynthesis of cell-free multienzyme-based bioprocesses and high-value chemicals.

Tailoring microenvironment for enzyme catalysis in water-poor systems

It is well known that water plays an invaluable contribution to the structure, dynamics, function, and stability of enzyme molecules. However, it remains a long-existing question of how much hydration level ensures adequate catalysis. The last several years have demonstrated that the catalytical activity in non-aqueous systems, like supercritical fluids, organic liquids, and gas phases, expands their application prospects to reactions that are

not likely to occur in aqueous media [16]. Nevertheless, the catalytic performance of enzymes in non-aqueous media profoundly relies on the medium water activity and significantly less than the bioactivity of their complements in an aqueous medium. These several orders of magnitude reduced might be attributed to the solvent's intolerance, inflexible enzyme configurations, and inadequate access to substrates. Though some limitations arise in using non-aqueous media compared to the aqueous phase, the selection of optimal conditions may help achieve possible enzyme activity [17]. Therefore, the favorable creation of a microenvironment represents the highly effective and most general strategy to enhance the stability and activity of enzymes.

Boosting enzyme performance in organic solvents

The discovery that enzymes can work in many nearly anhydrous organic solvent suspensions opened an interesting horizon of enzyme catalysis for poorly soluble compounds in water or reactions unlikely to occur in an aqueous system [18]. The enzyme-based bio-catalysis (in the lyophilized form) was supposed to achieve an appropriate hydration level for maintaining its conformational flexibility and native conformation [19]. An increase in water level can vividly accelerate the enzymatic performance in organic suspensions [20]. Non-polar solvents that usually have a low water activity can enhance the water cluster formation attached to the enzyme's surface, whereas the poor enzyme solubility results in conformational rigid and low activity but elevated thermal steadiness. On the contrary, polar solvents can replace the bound water molecules, causing a dramatic reduction in the stability and activity of the enzyme [21, 22]. Though tolerance to organic solvents differs from enzyme to enzyme, the utilization of non-polar organic mixtures is preferred over polar solvents because of ensuring a more hydrous micro-environment [23]. Controlling the hydration level to equilibrate the stability and structural flexibility is crucial for superior biocatalytic activity in organic systems.

Adding sugars, polyols, polysaccharides, polyethylene glycol, and inorganic salts to an enzyme mixture before lyophilization can enhance organic solvents' enzymatic and biocatalytic performance. For instance, four oxidative enzymes co-lyophilized with polyethylene glycol and polyols exhibited 10-to 100-fold high bioactivity in unfavorable solvents such as acetone (97%) [24]. These hydrophilic additives protect the enzyme

confirmation by replacing surface-bound water molecules. During lyophilization, the loss of water molecules without additives results in the inactivation of the enzyme.

Though the use of additives plays a role in engineering a water-like microenvironment for the enzymes, the additives tend to lose in reaction solvents because of their small size during the catalytic bioprocesses. Thus, their removal from the reaction products is challenging. Thus, enzyme encapsulation or immobilization in a support matrix offers practical advantages by protecting the microenvironment. Bruns and Tiller, [25] fabricated sponge-like microarchitectures for enzyme entrapment in its hydrophilic network. The immobilized chloroperoxidase and HRP presented a significantly greater catalytic performance and augmented stability than that of the soluble form of the enzyme in n-heptane. Confinement of HRP via in-situ polymerization in a hydrophilic polyacrylamide nano-matrix substantially improved the temperature resistance and stability in various organic solvents [26]. The nanogel-immobilized nanobiocatalyst maintained 80% of its original performance in the mixed aqueous-organic solutions (15 vol% tetrahydrofurans, dioxane, and methanol) at a high temperature of 60 °C for 10 min. At the same time, the native enzyme dropped its activity under the same conditions. Nanogel-encapsulated Candida rugosa lipase retained its original transesterification efficiency in anhydrous DMSO for 10 days at 60 °C [27].

Triggering enzyme catalysis in the gas phase

Enzymes are also able to carry out catalytic reactions in the gas phase. Yagi and coworkers first reported the lyophilized hydrogenase catalyzed gas-phase conversion of ortho-H_2 to para-H_2 in 1969 [28]. Later on, Barzana et al. [29] investigated that DEAE-cellulose adsorbed dehydrated alcohol oxidase can mediate the ethanol vapors oxidation by adding oxygen. These dry enzymes often demonstrate hydration-mediated activity and significantly improved thermal tolerance. Even though some researchers proposed an extent of hydration threshold for enzymes to perform in the gas phase, Dunn and Daniel, [30] negated this concept and highlighted the enzyme operation at a very low hydration degree. They suggested that the hydration activation might instigate improved enzyme flexibility in the presence of high moisture, and hydration is not necessary for enzyme catalytic activity [31]. Like biocatalysis in the organic media, incorporating additives, i.e., sucrose and glycerol could promote the stability and activity of enzymes that act on

gaseous substrates [32, 33]. Badieyan et al. [34] carried out the covalent immobilization of haloalkane dehalogenase on the surface exhibiting poly(sorbitol methacrylate) chains that furnished a hydrophilic microenvironment for the attached biocatalyst (Figure 3). At ambient humidity, the immobilized biocatalyst catalyzed superior gas-phase dehalogenation of 1-bromopropane, which was 40-fold greater than the equivalent lyophilized preparation activity under identical conditions. In the dry state, the supported enzyme maintained its secondary structure without the effect of humidity ranging from 26-80%. Xu et al. [35] fabricated an aerogel from hydroxyl-rich graphene oxide (GO) and lipase B from Candida Antarctica (CALB) via gelation followed by freeze-drying. The as-synthesized CALB-coupled GO aerogel showed its utmost transesterification performance at a water activity of 0.34, retaining over 65% of the maximal activity in anhydrous conditions. The number of bound water molecules essential for maintaining the enzyme active conformation was reduced by hydrogen bonding between GO and CALB [36]. Although some reports have demonstrated the exploitation of enzyme's catalytic performance in non-aqueous environments, the real-time applicability of enzyme for industrial production is still rare. Additional efforts should be directed on the development of alternative enzyme-based prototypes for replacing traditional organochemical methods. The industrial deployment of engineered enzymes also needs to be improved in terms of mechanical strength, facile synthesis, thermal stability, reuse, and recyclability.

Tuning reaction kinetics (K_M and k_{cat}) - boosting activity and stability

Enzyme kinetics is a critical factor that should be considered for tuning the reaction environment and boosting the activity and stability. Herein, a particular emphasis is given to understand the fundamental and critical aspects of enzyme reaction kinetics (K_M and k_{cat}) that will allow to tune or process of the catalytic rate at a highly desired and requisite level. In comparison, the catalytic rate, also known as catalytic velocity, significantly relies on a particular reaction time that an enzyme molecule typically takes to convert substrate molecules into the end-product magnificently [37-39]. Therefore, to successfully transform the substrate into products, traditional enzyme engineering deals by targeting the residues that exist inside the functional pockets or under close proximity to the active sites. This, in turn, changes the imperative sequence of available amino acids to

adjust/modify the enzyme-substrate recognition or binding affinity, selectivity, specificity, reactivity via conformational changes, catalytic reaction rate, catalytic turnover, catalytic yield, and end-product release [38-41]. Under these circumstances, the entire catalytic process usually involves traditionally defined transformation steps, i.e., chemical, biochemical, physical, or biophysical steps. The substrate diffusion rate, enzyme-substrate complex affinity, catalytic breakdown, and product release steps are integral elements of a transnational catalytic process [38, 42]. Herein, we spotlighted some important examples where enzymes were alerted or modified to enhance the reaction kinetics by considering the microscale structural aspects and enzyme microenvironment. This ultimately results in an evident improvement in enzyme kinetic parameters, i.e., K_M and k_{cat} [40].

The key aspects that our group has extensively explored and reviewed are the rational design of enzyme immobilization, engineering nano-constructs for enzyme immobilization as a paradigm shift in bio-catalysis, enzyme microenvironment, and modifying bio-catalytic properties and reaction kinetics of enzymes for efficient bio-catalysis with applied perspectives [38, 41, 43-46]. Working with model enzymes like laccase [47-50], lignin peroxidase [51], manganese peroxidase [52, 53], and horseradish peroxidase [54-59]. We have engineered catalytic constructs with improved catalytic efficiency, thermo-stability, and reaction specificity with effectively lowered Michaelis-Menten constant (K_M) and high turnover (k_{cat}), driving higher catalytic rates using various substrates concentrations for different purposes. For instance, a highly thermostable laccase obtained from an indigenous white-rot fungal strain *Trametes versicolor* IBL-04 was immobilized using alginate-chitosan beads. The reaction specificity was recorded via Lineweaver–Burk double reciprocal models kinetics. The Michaelis–Menten constant (K_M) and the maximum reaction rate (V_{max}) of the free and immobilized laccases were 0.14 mM, and 780 µmol/mL and 0.10 mM and 814 µmol/mL, respectively [48]. The apparent KM(app) reduction affirms an increased affinity of immobilized laccase for the substrate molecule. Furthermore, compared to the free enzyme molecule, the Vmax of engineered laccase was increased from 0.10 mM and 814 µmol/mL, which suggests an efficient carrier-bonded laccase could catalyze the reaction much faster than its free counterpart. Likewise, Qamar et al. [60] used a rational Ca-alginate entrapment strategy to improve

the catalytic kinetics of alkaline protease obtained from *Bacillus brevis*. The catalytic measures were taken using 0.1-1.0 mM casein as a substrate. The recorded Kinetic data revealed lower K_M (0.09 µM) and higher V_{max} (454.5 U/mL) for Ca-alginate immobilized protease constructs than pristine counterparts, i.e., *Vmax* 333.3 U/mL and K_M 0.16 µM. Again, these results confirm a higher enzyme-substrate recognition/affinity for immobilized constructs than free protease in the solution [60].

Considering the above discussed K_M-engineered case studies using different enzymes, a phenomenal catalytic mechanism offers to control or alter the reaction kinetics. The enzyme-substrate binding interactions or even covalent interactions of cofactor molecules in close proximity to the enzyme molecule results in an increased catalytic reaction rate and catalytic turnover, i.e., K_M and k_{cat} [40]. However, a precise mechanism of enhanced catalytic turnover is unclear and needs further investigation. Several other aspects are involved in the whole catalysis reaction (some of which are discussed in different sections of the work).

Engineering substrate specificity by targeting enzyme active site

Alteration in enzyme-substrate specificity and selectivity is an important modification in biocatalysts engineering. Native enzyme molecules do not present high catalytic performance for various substrates or a vast range of substrate acceptability. Likewise, they show high specificity towards the nature of their substrate and the type of catalytic reaction. Therefore, modification of enzyme selectivity to extend their substrate acceptance is regarded as the most significant industrial requirement [61]. The substate modification process can be categorized into three main types: narrowing, expanding substrate specificity (Figure 4) [51], and complete substitution of the specificity towards new kinds of substrates [62]. *In-silico* analyses can accurately predict enzyme hotspot regions for tailoring unique targeted attributes. These advanced analyses have facilitated the assessment of prophesied hotspots and the determination of the most appropriate amino acids for replacement [63]. Furthermore, many investigations have revealed that engineering a targeted enzyme binding pocket by site-directed mutagenesis is beneficial [64].

Many reports have demonstrated the successful improvement or change in numerous enzyme specificity by amino acid manipulation surrounding the pocket binding sites of the

enzyme. For instance, changing the Asp36, Asp55, Tyr4, Tyr29, Tyr45, and Tyr95 lining the pocket binding site substantially (6-folds) enhanced the regio- and enantioselectivity of *Pseudomonas cepacia* lipase toward p-NPP [65]. The specificity of phospholipase D from *Streptomyces antibioticus* was improved toward phosphatidylinositol by saturation mutagenesis of four residues in the receptor-binding regions [66]. Jan et al. [67] reported a dramatic improvement in the catalytic activity of acyltransferase from *Pseudozyma antarctica* by targeted manipulation of the substrate attachment site. In another study, targeting the CRL access channel by replacing small amino acid residues with a site-saturation mutagenic strategy with bulky ones has swung the chain length specificity of lipase toward shorter chains [68]. Some other reports have revealed that replacing the enzyme lid region alters their catalytic activity, stability, and enantioselectivity [69].

Researchers have also demonstrated some other microenvironmental engineering approaches. In a series of studies, amino-acid-coated gold nanoscale particles were used to create a charged local environment for chymotrypsin [70, 71]. After chymotrypsin binding to particles glutamic acid residues decorated nanoparticles, the catalytic activity of this enzyme was 3-fold improved towards cationic substrates. On the other hand, the activity was diminished by 95% towards the negatively charged substrate while reduced to half for neutral enzyme substrates. The experimental analysis concluded that the modifications by glutamic acid had influenced the substrate diffusion to the nanoparticle-coupled enzyme. Cationic substrate molecules could easily reach the activity of immobilized biocatalysts, whereas a significant restriction was speculated in diffusing anionic peptides to the local environment.

Controlling access to the active region by conditional steric interference is an alternative approach for tailoring substrate specificity. Recently, this notion has been exploited using a light-induced conformational switch attached close to the catalytic site of a lipolytic enzyme from *B. thermocathenolatus*. Two small molecules that can undergo light-activated structural modifications were separately attached to five diverse residues near the active site. Cis and trans transitions of azobenzene under UV and visible light, while ring-closing and opening of iodoacetate-spiropyran undergo the identical light switching pattern. These systems have shown the capability to alter and control lipase's inherent enantioselectivity from *B. thermocathenolatus* [72].

Huber et al. [73] developed a novel biorthogonal approach for methyltransferases (MTs) and AdoMet-utilizing enzymes by evaluating 38 human methionine adenosyltransferase II-α (hMAT2A) variants combined with 14 non-native methionine equivalents for the identification of appropriate bio-orthogonal mutants (Figure 5). By implementing a hMAT2A high-throughput strategy, hMAT2A K289L afforded 160-folds inverted hMAT2A selectivity for a non-native methionine analog compared to native substrate L-methionine. This work described for the first time the exchange of L-Met terminal amine/carboxylate recognition elements within the hMAT2A active-region for the utilization of non-native bio-orthogonal substrate. Several developed hMAT2A variants and synthesized AdoMet analog products showed high stability. In conclusion, this new engineering platform is likely to decipher an array of AdoMet-utilizing enzymes.

Tailoring Reaction promiscuity

Serval enzymes drive many mechanistically different transformations and chemical reactions. Their catalytic efficiencies are influenced by the enzyme tunnels that govern the accessibility of the substrate and cofactors or solvent to the active region. By modification of three residues at a water tunnel and one residue in the binding pocket, David et al. [74] reported that the agarase enzyme from *Zobellia galactanivorans* lost its hydrolase activity, however, trans-glycosylase activity was enhanced. They speculated that the water molecules must generate a hydrogen-bonding-based network for anchoring enzyme molecules for hydrolytic activity, which is impeded by elevated water dynamics in the catalytic region.

Hydrolase Mhg is another example of a promiscuous enzyme that exhibits perhydrolase as well as γ-lactamase activities. Nevertheless, this enzyme has presented any esterase activity despite an identical catalytic triad and high structural resemblance with esterase from *P. fluorescens*. Substitutions of the entry tunnel with one or two amino acid residues yielded five esterase Mhg mutants that showed broader substrate specificity and can hydrolyze *p*-nitrophenol esters and an array of various other chiral esters [75]. Noteworthy, variants with L233G mutation demonstrated a particular esterase activity without per-hydrolase and any γ-lactamase activities. The findings revealed the entrance tunnel engineering as a promising approach for regulating catalytic aptitudes of the enzyme. An ingenious method was employed for engineering a catalytically robust triterpene cyclase

from *A. acidocaldarius* [76]. Interference with the water channels by Trp point alterations (originally Phe or Ser) 100-fold elevated K_{cat}/K_M values were observed for pentacyclic formation. Results revealed a significant contribution of entropy for rate improvement by releasing water molecules via particular channels. Disrupting one of these water channels by a single point mutation caused a reduced entropy contribution to bio-catalysis by 60 kcal mol^{-1}.

Computational design for protein engineering

The ongoing technological hike at the micro-and nanoscale has further revolutionized protein engineering to construct robust catalysts. For instance, computational-based rational design and modeling progressively support the catalytic features of enzymes. Such as enzyme selectivity, specificity, activity, substrate preference, and stability are noteworthy examples of expanding applications of engineered bio-catalysis. Before running an experimental setup followed by extensive analytical characterization, *in-silico* tools and molecular dynamics (MD) simulations significantly support the identification of potential regions for manipulations to mimic the catalytic characteristics [41]. So far, various computational-based studies related to efficient catalysis have been made to comprehend the outstanding performance of enzymes by designing robust biocatalysts. Besides, a directed evolution strategy further enhances a computationally designed enzyme [77]. However, regardless of the successful exploitation of protein engineering, the de-novo synthesis that mimics natural enzymes' affinity and specificity remains one of the Holy Grails of chemistry [78], along with other essential factors that affect the designed enzyme activity. The examples include but are not limited to the reaction dynamics effects, entropic effects, electrostatic effects, and others, which are not easy to incorporate into the computational-based enzyme design [77].

Gordon et al. [79] used a computational protein design tool, i.e., Rosetta Software Suite, to re-engineer to redesign the substrate specificity of an α-Gliadin Peptidase (Figure 6) [79]. The redesigned enzyme construct displayed a k_{cat}/K_M of 568 $M^{-1}s^{-1}$, which affirmed a 116-fold greater proteolytic activity for a model gluten tetrapeptide than the native template enzyme. In addition, the re-engineered enzyme also exhibits an over 800-fold switch in the substrate specificity toward immunogenic portions of gluten peptides [79]. By computational-guided protein engineering approach together with cofactor binding

energy calculations, Khoury et al. [80] attained nicotinamide adenine dinucleotide (NAD) + hydrogen (H), collectively abbreviated as (NADH) utilizing 10 variants of xylose reductase, as a substitute of the native cofactor nicotinamide adenine dinucleotide phosphate (NADPH). Investigational results revealed a specific behavior of some variants for NADH, while the rest unveiled dual substrate preference. In summary, besides activity enhancement, the enzyme stability can also be improved by harnessing the power of the computational design and methods. Computational optimization of unstable residues, known as important to the thermal unfolding of Bcx, based on thermal fluctuation analysis, was performed by Joo et al. [81]. More specifically, they used computational modeling as a substitute for random mutagenesis to optimize unstable residues of *Bacillus circulans* xylanase (Bcx) [81]. The MD simulations were completed at 300 K and 330 K to recognize promising residues for the said purpose. Likewise, Joo et al. [82] elected flexible residues on the protein surface, followed by the characterization of residue flexibility using the Framework Rigidity Optimized Dynamics Algorithm (FRODA) to alleviate the thermal resistance profile xylanase Bcx from *Bacillus circulans*, as a model enzyme. The adopted surface-cavity design strategy presented that flexible surface residues tolerant to mutations are valid targets for thermo-stabilization with no catalytic activity reduction. In comparison, the local-interaction stabilization of cavity-lining residues using the computational method could be an exceedingly adequate substitute to the conventional cavity-filling method [82].

Structure-guided biocatalyst engineering

Most protein or enzyme structures are solved or prophesied by modeling, rational design, and focused libraries. Furthermore, the directed evolution screening could be replaced with highly informative, structure-assisted combinatorial strategies, likely to reduce the total number of variants screened [83]. Oligonucleotides designed through structure-guided engineering are used to create modifications in the regions envisioned to exhibit a remarkable effect on substrate recognition or biocatalytic performance. Oligonucleotides are often designed so that the target mutated sites encode all or a subset of possible amino acids [84]. For example, Cytochrome P450-cam was rationally engineered to tailor site-selectivity for oxidative hydroxylation of (+)-a-pinene [85]. Various point mutations were created, i.e., F87W, V247L, F87A, and Y96F, and their pair was

assessed. Among the combination generated, the variant F87W/Y96F/L247A presented a high catalytic activity to produce (+)-cisverbenol with superior regioselectivity of over 85%.

Concluding note and futuristic views

Enzymes constitute a green and eco-friendly biocatalytic system for diverse biotechnological processes, and therefore, the global market for specialty enzymes is continuously growing. Nonetheless, the widespread deployment of biocatalysts in their natural states is often impeded by insufficient stability, lower biocatalytic performance, and inadequate specificity in different industrial settings. Thanks to the sophisticated and state-of-the-art technologies, significant progress has recently been achieved in engineering enzymes to meet the ever-growing requirements for economical, robust, and high-performance catalysts. Considering the rapid advancement in enzyme catalysis, we provide in this review detailed and comprehensive insights into the microenvironmental engineering of enzymes, such as regulating microenvironmental pH, tailoring a water-like environment for ensuring non-aqueous catalysis, tuning reaction kinetics, manipulating substrate specificity, reaction promiscuity, as well as structure-guided and computational designs. Recent literature has substantiated that engineering a biocompatible microenvironment for catalysts helps optimize enzyme-catalyzed reactions. The microenvironment can stabilize enzymes by protecting from harsh conditions, regulating reaction kinetics, governing enzyme activity by concentrating or excluding cofactors and substrates, augmenting cascade reactions efficacy by compartmentalization, and speeding up reactions. Innovative advances in enzyme engineering strategies may provide exceptional opportunities to address the drawbacks associated with the utilization of wild-type enzymes. The ongoing technological hike at the micro and nanoscale microenvironmental engineering and active sites implies that enzyme catalysis is speculated to expand further in an array of advanced biotechnological purposes. The incredible functional performance of engineered enzymes in water-poor systems is another step closer to achieving the ultimate objective. However, it is of paramount importance to optimize industrial bio-transformations from an ecological and economic standpoint. Combining the potency of microenvironmental engineering, enzyme tailoring,

stabilization, and computational-aided rational design and modeling are likely to play a notable role in accomplishing a cleaner, greener, and innovative bio-catalysis.

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Conflict of interests

The author(s) declare no conflicting interests.

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Figure captions

Figure 1 Strategies to tailor enzyme microenvironment for efficient catalysis.

Figure 2 The influence of microenvironmental pH on single enzymes and cascade enzymes. Reprinted from Ref. [15] with permission from the American Chemical Society.

Figure 3 Mimicking a water-like environment for non-aqueous enzymatic reactions. Reprinted from Ref. [34] with permission from the American Chemical Society.

Figure 4 A simplified schematic illustration of substrate engineering that assist to manipulate substrate specificity of the enzyme. Reprinted from Ref. [51] with permission from Springer Nature.

Figure 5 Corresponding approaches to AdoMet-producing/utilizing enzyme bioorthogonal platform development. Reprinted from Ref. [73] with permission from the American Chemical Society.

Figure 6 Computational Design of an α-Gliadin Peptidase. Reprinted from Ref. [79] with permission from the American Chemical Society.

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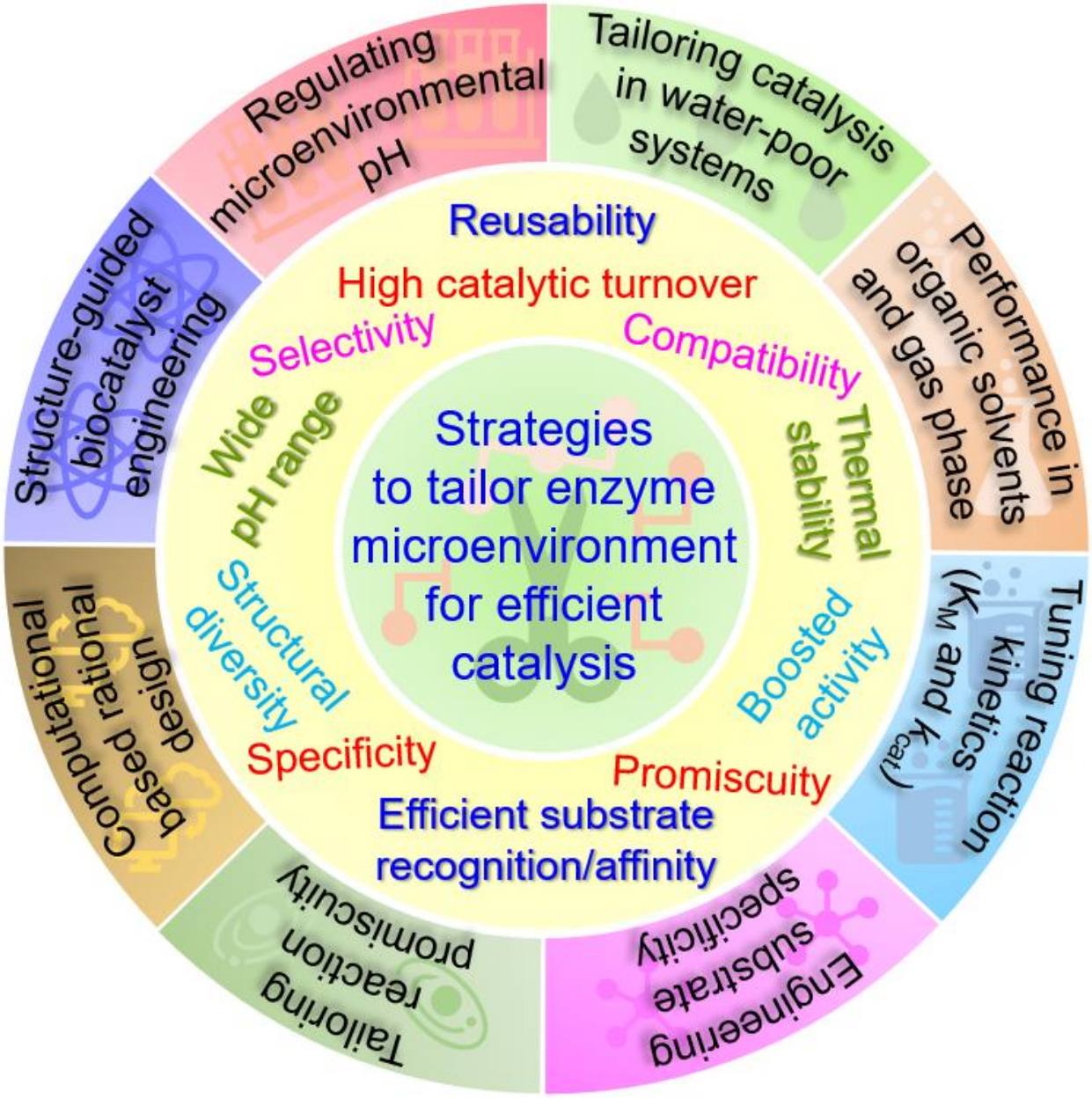
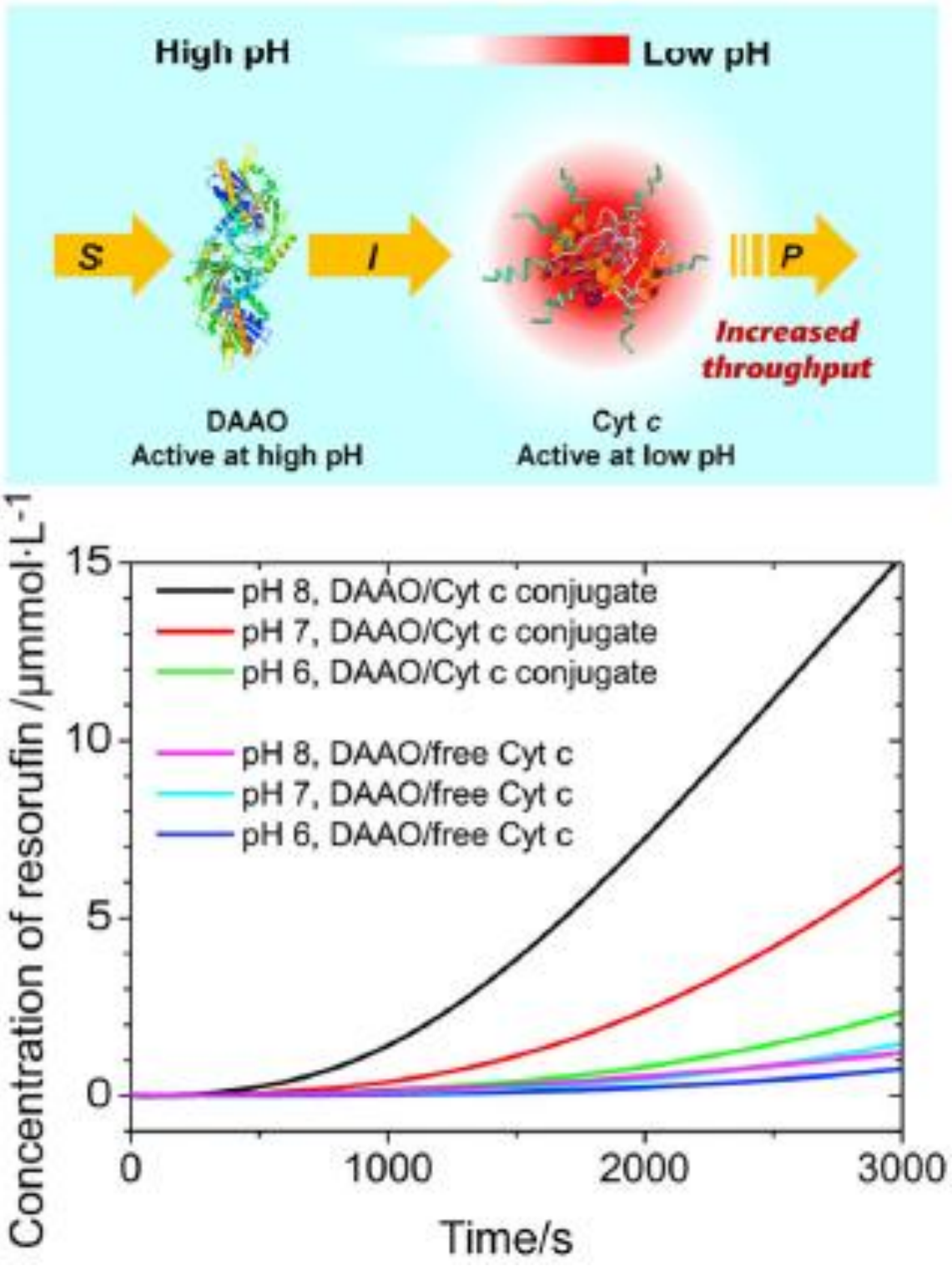


Figure 1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2000
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Figure 2

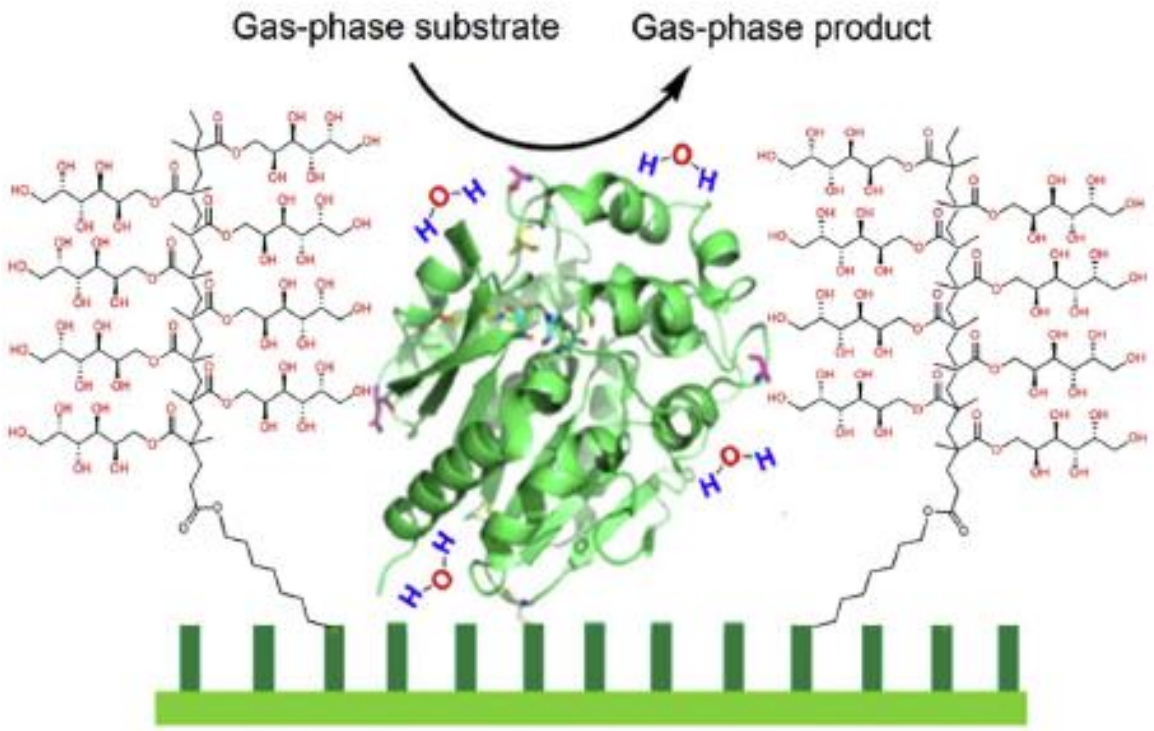
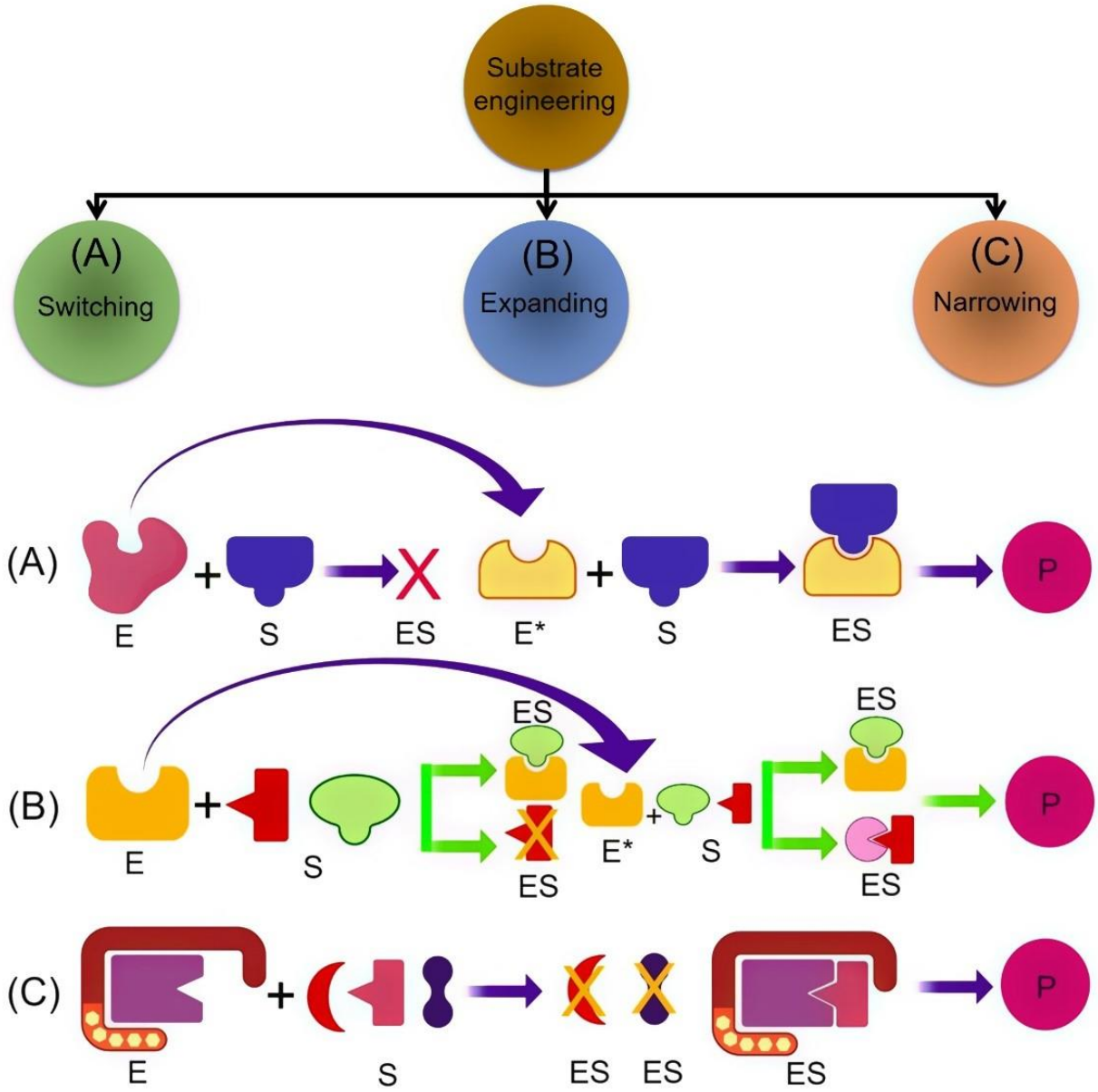


Figure 3



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795 **Figure 4**

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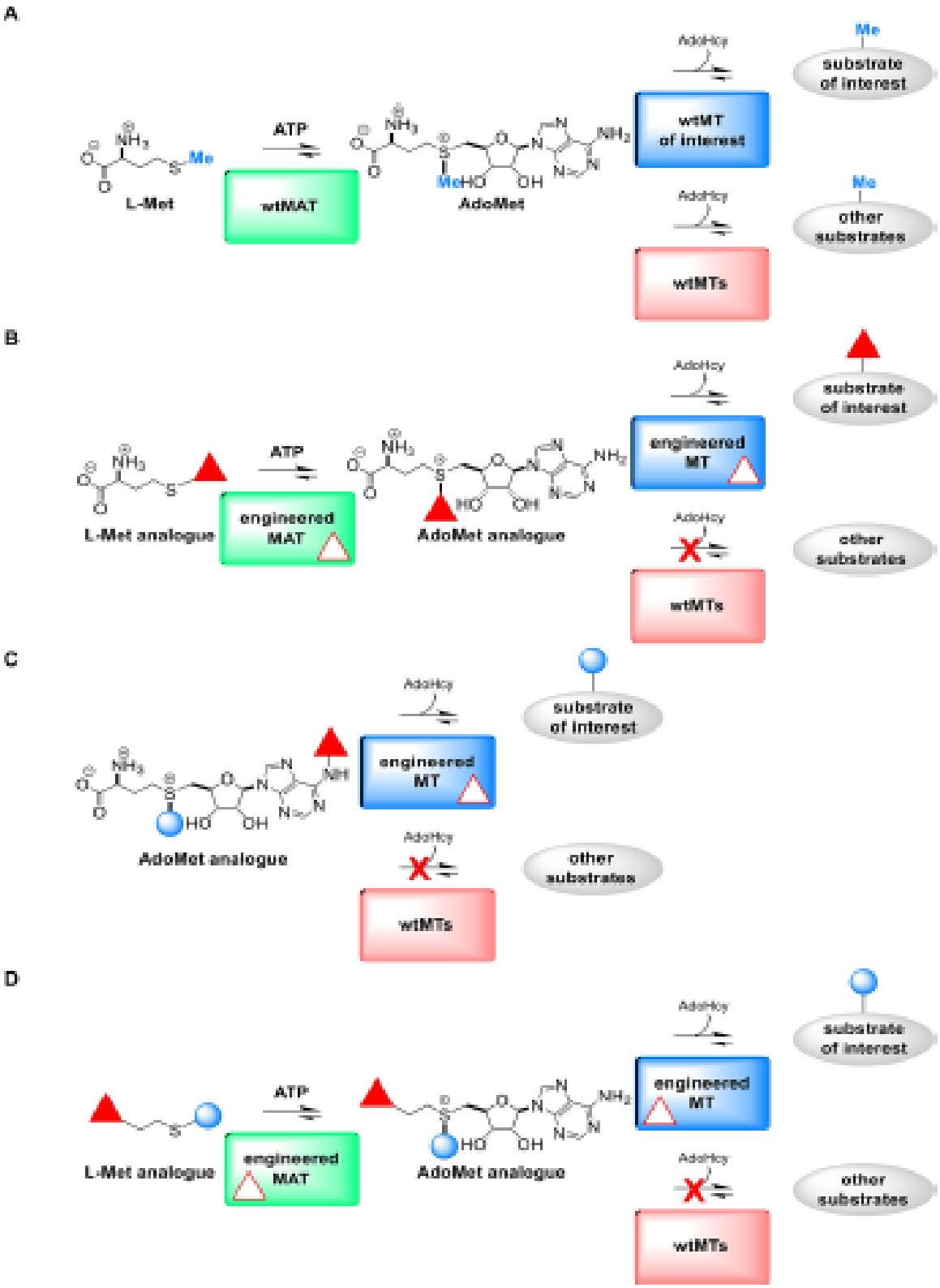

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804 **Figure 5**

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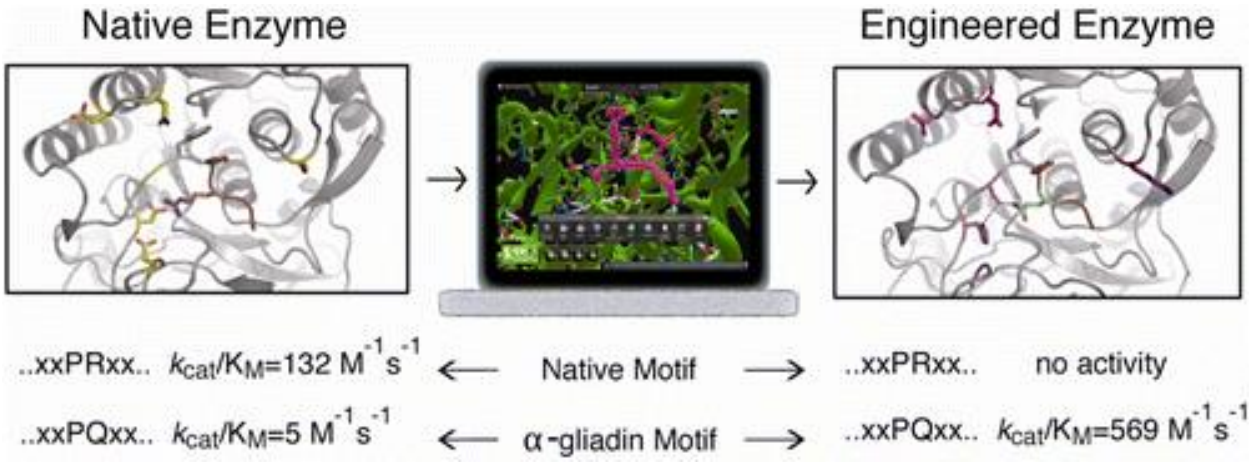
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Figure 6