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Editorial

Enzyme Engineering—The Core of Biocatalysis

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Biocatalysis has emerged as a powerful approach for sustainably synthesizing pharmaceuticals, fine chemicals, and value-added materials, owing to the catalytic efficiency, selectivity, and functional diversity of enzymes [1,2]. However, the properties of naturally occurring enzymes are not always ideally suited to the requirements of practical applications [3]. Enzyme engineering therefore is essential in modern biocatalysis, providing a powerful means of tailoring catalytic activity, stability, selectivity, substrate scope, and compatibility with reaction conditions [4]. Importantly, contemporary enzyme engineering is increasingly integrating mechanistic enzymology, structural biology, computational modeling, directed evolution, protein design, and reaction engineering [3,5,6]. The contributions collected in this Special Issue, entitled "Enzyme Engineering—the Core of Biocatalysis," illustrate this broad and rapidly evolving landscape, ranging from molecular engineering, mechanistic investigations, and enzyme immobilization to applications in biomanufacturing and environmental biotechnology.

A major development in the field is the increasing convergence of experimental and computational approaches for enzyme engineering. Advances in structural biology, high-throughput screening, sequence analysis, and artificial intelligence have substantially expanded the ability to identify sequence–structure–function relationships and to design enzymes with improved or entirely new properties [3,7–9]. In particular, protein language models, structure prediction, molecular simulation, and machine-learning-guided design are increasingly being integrated with directed evolution and rational engineering, enabling more-efficient exploration of the vast sequence and functional space of proteins [10–13]. These approaches are not only accelerating the optimization of established biocatalysts but are also opening opportunities for the creation of catalysts with altered substrate specificity, higher catalytic efficiency, enhanced robustness, and new-to-nature activities [14,15]. Additionally, mechanistic understanding remains essential for translating computational predictions and evolutionary trajectories into reliable engineering strategies [10,12].

Beyond improving individual enzymes, enzyme engineering is increasingly being considered within the broader context of catalytic systems and biomanufacturing processes. Enzyme performance in practical applications depends on not only intrinsic catalytic properties but also enzyme expression, cofactor regeneration, substrate and product transport, enzyme stability under process conditions, immobilization, reaction integration, and the physicochemical environment of the overall system [1,16,17]. Accordingly, the future development of biocatalysis will increasingly rely on the integration of molecular-level enzyme engineering with reaction engineering, synthetic biology, metabolic engineering, and process development [18–20]. Such interdisciplinary strategies have the potential to transform enzyme engineering from an optimization tool into a platform for constructing efficient and sustainable catalytic systems [12]. Against this backdrop, the contributions in this

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Special Issue highlight the fundamental principles and practical opportunities of enzyme engineering, providing perspectives on how advances in molecular understanding, computational technologies, and experimental innovation can continue to expand the capabilities of biocatalysis.

A central theme emerging from this collection is that engineering the enzyme microenvironment provides an effective route for enhancing catalytic performance. In contribution 1, Yan et al. demonstrate this concept: they covalently conjugated SmDHFR with a superfolder green fluorescent protein mutant carrying 30 negatively charged surface residues. Modulating the enzyme microenvironment through this strategy resulted in a 2.16-fold increase in tetrahydrofolate production, reaching a final concentration of 4223.4 μM. Furthermore, the authors incorporated the engineered enzyme into a dual-enzyme cascade incorporating pseFDH to recycle NADPH. The study illustrates that enzyme engineering need not be restricted to modifications directly surrounding the active site. Instead, engineering the broader protein environment and coupling catalytic steps can provide additional opportunities to improve enzyme performance and process efficiency.

The catalytic versatility of cytochrome P450 enzymes provides another important example of the need for systematic enzyme engineering. In contribution 2, Liu and Chen summarize three major strategies for P450 engineering: rational design, semi-rational design, and directed evolution. Rational approaches combine structural information, computational modeling, site-directed mutagenesis, and biochemical characterization to introduce targeted modifications. Semi-rational approaches exploit structural and mechanistic knowledge to identify promising positions before experimentally exploring sequence space, whereas directed evolution uses iterative mutation and screening to discover variants with desirable properties. Numerous examples demonstrate how these strategies have expanded the catalytic repertoire of P450s, including C–H amination, oxidative demethylation, hydroxylation, oxidation, carbene transfer, fluoroalkylation, and even cleavage of Si–C bonds. The review also highlights persistent challenges, including heterologous expression, the trade-off between activity and stability, cost and scalability, and the development of efficient high-throughput screening platforms. Together, these issues emphasize that successful enzyme engineering requires not only the identification of beneficial mutations but also their integration into an experimentally and industrially viable system.

In addition to individual enzymes, engineering and biochemical characterization can be applied to complex enzymatic machineries. In contribution 3, Li et al. describe the development of a tandem affinity purification strategy for the COMPASS complex from *Saccharomyces cerevisiae*, an enzyme complex responsible for histone H3 lysine 4 methylation. Using recombinant nucleosomes as substrates, they characterized the enzymatic activity of the purified complex and demonstrated its dependence on H2B ubiquitination. The study highlights an important dimension of enzyme engineering and biocatalysis: understanding how enzymes function within their native molecular context. Such knowledge can provide a foundation for reconstructing, engineering, and ultimately redesigning complex biological catalytic systems.

A particularly important theme emerging from this Special Issue is the role of mechanistic enzymology and quantitative kinetic analysis in guiding and evaluating enzyme engineering. In contribution 4, Atampugbire et al. discuss how mechanistic enzymology informs industrial biocatalysis by elucidating fundamental catalytic parameters, including $k$cat and $k$cat/Km, and by identifying potential bottlenecks associated with the chemical step, product release, and overall catalytic efficiency. The authors also highlight common misconceptions that lead to inappropriate strategies for enzyme design and optimization and discuss kinetic solvent viscosity effects (KSVEs) as a useful tool for probing catalytic mechanisms. Complementing this mechanistic perspective, in contribution 10, Rodríguez-

Enríquez et al. compare *k*cat values from mutagenesis studies of laccases employing rational design, semi-rational design, and directed evolution. Although *K*m is frequently used together with *k*cat to assess catalytic efficiency, the analysis revealed no consistent directional trend in *K*m among engineered variants, whereas changes in *k*cat provided a more direct measure of improvements in catalytic turnover. Together, these contributions emphasize that successful enzyme engineering requires not only the generation of improved variants but also a mechanistic understanding of which kinetic step has been altered and how individual kinetic parameters should be interpreted. Such quantitative analysis helps distinguish genuine improvements in catalytic performance from changes in substrate binding or other aspects of the catalytic cycle, thereby providing a more rational basis for enzyme optimization.

The importance of mechanistic analysis is further illustrated by Paz-Ramirez et al., who investigated the mechanism of chloroperoxidase-mediated dye decolorization using LC/MS and density functional theory calculations (contribution 5). Their results indicate that C–N bond cleavage is a preferential initial step in the oxidative degradation pathway, providing insight into how the enzyme transforms complex dye molecules. Such mechanistic information is valuable not only for understanding enzymatic catalysis but also for evaluating the potential of chloroperoxidase as a biocatalyst for industrial and environmental applications. Combining experimental product analysis with computational chemistry provides a powerful framework for connecting enzyme structure and catalytic function.

Enzyme engineering also extends beyond the modification of amino acid sequences. In contribution 6, Garcia et al. review *Aspergillus* as an expression platform for enzymes used in industrial biocatalysis, including engineered enzymes for kinetic resolution. They also discuss how protein engineering, enzyme immobilization, and reaction-medium optimization can be integrated to improve catalytic performance. Their analysis highlights an important principle of applied biocatalysis: enzyme properties and process conditions cannot always be optimized independently. Immobilization can increase operational stability and facilitate enzyme recovery, whereas modification of the reaction medium can influence activity, selectivity, and substrate accessibility. Nevertheless, challenges in yield, scalability, and enzyme discovery remain. Addressing these challenges will require an integrated strategy encompassing molecular engineering, enzyme formulation, and process development.

The application-oriented scope of enzyme engineering is further demonstrated by Nie et al., who employed *Bacillus subtilis* to express urease and investigated its effects on serpentine weathering and secondary mineral formation (contribution 7). Their results show that urease overexpression enhances Mg^{2+} release and promotes the formation of magnesium-containing calcite. Although the study differs from conventional enzyme engineering for synthetic chemistry, the authors illustrate the broader potential of engineered microorganisms and enzymes in manipulating geochemical processes. Such applications expand the concept of biocatalysis beyond the synthesis of small molecules toward environmental biotechnology and biomineralization.

Two contributions by Ertürk and Gulcin, and Özden et al. further highlight the broad applicability of biochemical and computational approaches in evaluating enzyme–small-molecule interactions (contributions 8 and 9). The researchers examined the antioxidant properties of luteolin and its glycosides, as well as coniferyl alcohol and coniferyl aldehyde, using multiple antioxidant assays. The compounds were evaluated for their inhibitory effects against carbonic anhydrase I and II, acetylcholinesterase, butyrylcholinesterase, and α-glycosidase. Molecular docking simulations were used to investigate the potential interactions between the compounds and enzyme active sites. These studies illustrate how biochemical assays combined with molecular modeling can provide insight into enzyme–

ligand recognition and may inform future strategies for designing or optimizing bioactive molecules and enzyme inhibitors.

Taken together, the ten contributions demonstrate that enzyme engineering is no longer confined to the traditional paradigm of introducing mutations and screening for improved activity. Instead, modern enzyme engineering is increasingly a multidimensional discipline, encompassing manipulation of the protein microenvironment, rational and computational design, semi-rational engineering, directed evolution, mechanistic enzymology, enzyme immobilization, reaction-medium optimization, metabolic and microbial engineering, and computational analysis of enzyme–ligand interactions. Equally important, these approaches are increasingly becoming interconnected. Structural and mechanistic knowledge can guide protein engineering; engineered enzymes can be incorporated into multienzyme cascades; computational methods can accelerate the identification of promising variants; process-level optimization can translate molecular improvements into practical catalytic systems.

Despite these advances, several challenges remain. Predicting the effects of mutations from sequence and structure alone remains difficult, particularly when multiple mutations interact through epistasis [21]. Improvements in catalytic activity may come at the expense of stability, expression, selectivity, or substrate tolerance [22]. Efficiently exploring sequence space continues to require powerful screening and selection methods, whereas laboratory-scale improvements must ultimately be translated into robust, economical, and scalable processes. Emerging technologies—including artificial intelligence, machine learning, protein structure prediction, generative protein design, high-throughput experimentation, and automated directed evolution—are beginning to address these challenges and may fundamentally change how enzymes are engineered.

The contributions in this Special Issue collectively reinforce a simple but fundamental concept: the future of biocatalysis depends on our ability to understand, engineer, and control enzymes at multiple levels, from individual catalytic steps and molecular interactions to whole reaction systems and biological processes. By bringing together mechanistic insights, engineering strategies, computational tools, and diverse applications, this Special Issue provides a snapshot of the rapidly evolving field of enzyme engineering and highlights its central role in transforming biocatalysis from a naturally occurring phenomenon into a predictable and programmable technology.

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