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Multidisciplinary approaches for enzyme biocatalysis in pharmaceuticals: protein engineering, computational biology, and nanoarchitectonics

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Enzyme biocatalysis is reshaping pharmaceutical synthesis, offering sustainable and efficient pathways for drug discovery and production. This paradigm shift towards eco-friendly methodologies addresses concerns inherent in traditional chemical synthesis. Enzymes, celebrated for their precision and adaptability to mild conditions, are poised as ideal candidates for pharmaceutical applications. Their versatility facilitates the synthesis of diverse pharmaceutical compounds, ensuring precise drug design and minimizing environmental impact. The integration of multidisciplinary approaches, including protein engineering, computational biology, and nanoarchitectonics, holds the potential to propel enzyme biocatalysis even further. Protein engineering utilizes directed evolution and rational design to customize enzymes, enhancing their stability and efficacy. Computational biology aids in deciphering enzymatic mechanisms, while nanoarchitectonics introduces innovative enzyme integration strategies into continuous flow systems. This comprehensive review explores how these multidisciplinary approaches can revolutionize pharmaceutical research and production. The synergy among these disciplines promises to expedite pharmaceutical processes, promote sustainability, optimize efficiency, and elevate precision—aligning perfectly with the evolving requirements of the pharmaceutical industry.

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Broader context

Enzyme biocatalysis is propelling a significant revolution in pharmaceutical synthesis, offering eco-friendly and highly efficient pathways for drug discovery and production. This departure from traditional chemical methods not only aligns with sustainability goals but also reflects a broader global trend towards greener practices. Enzymes, renowned for their precision and adaptability under mild conditions, are poised to play a pivotal role in shaping the pharmaceutical industry's future. Their inherent versatility allows for the precise synthesis of a wide spectrum of pharmaceutical compounds, simultaneously reducing the environmental impact—a critical consideration in contemporary research and development. In this evolving landscape, multidisciplinary approaches, reminiscent of recent catalyst development strategies, hold the key to unlocking the full potential of enzyme biocatalysis. These approaches encompass protein engineering, computational biology, and nanoarchitectonics, which collectively contribute to enhancing enzyme stability, deciphering complex enzymatic processes, and introducing innovative integration methods within continuous flow systems. This comprehensive review not only underscores the remarkable impact of multidisciplinary approaches but also highlights their potential to streamline pharmaceutical processes, promote sustainability, optimize efficiency, and elevate precision—a paradigm shift aligned with the evolving demands of the pharmaceutical industry and broader environmental objectives.

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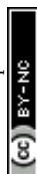
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1. Introduction

Enzyme biocatalysis, encompassing the utilization of microorganisms or enzyme preparations to catalyze chemical transformations, has arisen as a pivotal force in the field of pharmaceutical synthesis, providing a sustainable and efficient pathway for drug discovery and production.^{1–5} In an era characterized by unprecedented demands for speed, sustainability, cost-efficiency, and precision in drug development, the pharmaceutical industry is undergoing a profound shift catalyzed by the rise of enzyme biocatalysis.^{6,7} This paradigm shift toward environmentally friendly and more precise methodologies addresses both environmental concerns and efficacy issues associated with conventional chemical synthesis approaches.^{8,9} Historically, the pharmaceutical industry heavily relied on chemical synthesis methods to produce therapeutic compounds.¹⁰ While these approaches undeniably accelerated drug development and production, they also introduced inherent challenges. For example, the employment of hazardous reagents, significant energy consumption, and the generation of substantial waste posed significant environmental and economic concerns.¹¹

The call for more sustainable alternatives has grown louder in recent years, and in response, enzyme biocatalysis has emerged as a compelling solution.

Enzymes, often referred to as the catalysts of life, have evolved over millions of years to orchestrate precise chemical transformations within living organisms.¹² Their intrinsic specificity, combined with compatibility with mild reaction conditions, positions enzymes as ideal candidates for pharmaceutical applications.^{5,12} Harnessing enzymes in drug development and synthesis not only enhances the selectivity of reactions but also aligns pharmaceutical processes with the principles of green chemistry, mitigating their environmental footprint.¹³ Enzyme biocatalysis in pharmaceuticals is no longer confined to the realm of theoretical promise, it has evolved into a practical, advantageous reality with discernible benefits. Globally, researchers have harnessed enzymes' catalytic potency to execute a diverse range of transformations, encompassing small-molecule drug synthesis and the production of intricate biological therapeutics.¹⁴ For example, enzymes such as lipases and hydrolases have played a fundamental role in optimizing the synthesis of enantiopure chiral intermediates—a



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formidable challenge frequently confronted in the pharmaceutical chemistry.¹⁵ This progress underscores the central role of enzyme biocatalysis in modern pharmaceutical research and manufacturing.

Indeed, enzyme biocatalysis in pharmaceuticals has offered a multitude of advantages that have an impact on various facets of drug development and production. Firstly, enzymes have played a crucial role in the assembly of complex peptide and oligonucleotide therapeutics, elevating the precision of drug design.^{16–19} By selectively and efficiently catalyzing specific chemical reactions, enzymes enable pharmaceutical scientists to fine-tune the molecular structures of drugs, optimizing their efficacy and safety profiles. This newfound precision in drug synthesis carries profound implications, extending beyond the creation of novel therapeutic agents to encompass the optimization of existing medications.²⁰ The ability to finely manipulate molecular structures through enzymatic catalysis not only facilitates the design of innovative drugs but also opens avenues for enhancing the efficacy, safety, and targeted delivery of established pharmaceuticals.²¹

Additionally, one of the outstanding features that distinguishes enzyme biocatalysis in pharmaceuticals is its inherent compatibility with aqueous environments and mild operational conditions.^{7,12,22,23} Unlike conventional chemical catalysts, enzymes thrive in water-based solutions and typically function at relatively low temperatures. These favorable conditions not only diminish the energy requirements of pharmaceutical processes but also elevate safety standards by minimizing potential hazards linked to high-temperature reactions and the use of hazardous reagents.^{9,24} Furthermore, enzymes frequently showcase an exceptional level of substrate versatility, demonstrating the capacity to accommodate a wide range of substrates.^{2,24,25} This adaptability paves the way for innovative approaches in synthesizing a wide spectrum of pharmaceutical compounds. Such versatility has notable importance for addressing the dynamic requirements of the pharmaceutical sector, characterized by a continual demand for complex and highly targeted drug molecules.

The evolutionary journey of enzyme biocatalysis from a promising concept to an indispensable tool in pharmaceutical research and production can further be driven by multidisciplinary approaches. This convergence of disciplines encompasses three central pillars: protein engineering, computational biology, and nanoarchitectonics. Protein engineering, through directed evolution and rational design, has empowered researchers to tailor enzymes for specific reactions and enhance their stability, paving the way to novel applications in pharmaceutical synthesis.^{12,22,26,27} Computational biology has augmented our understanding of enzymatic mechanisms and facilitated the rational design of enzymes with enhanced catalytic activity.^{28–32} Additionally, computational tools have streamlined the prediction of enzyme–substrate interactions, expediting the development and optimization of enzymatic processes.^{33,34} Meanwhile, nanoarchitectonics has introduced innovative strategies for enzyme immobilization and encapsulation within custom-designed nanomaterials.^{35–37} These advances not only enhance

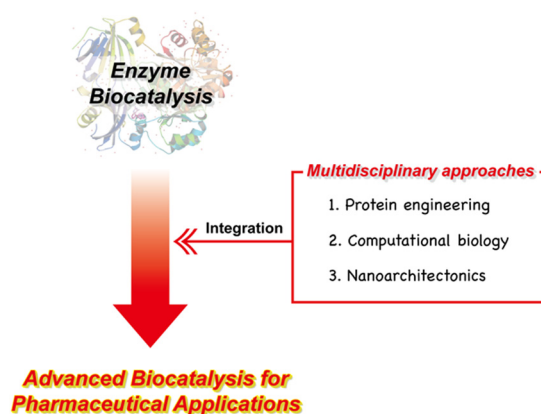


Fig. 1 Outline of the multidisciplinary approaches to advanced enzyme biocatalysis for pharmaceutical applications.

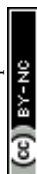
enzyme stability and reusability but also enable their seamless integration into continuous flow processes, a crucial requirement for large-scale pharmaceutical production.

This review paper presents a comprehensive examination of enzyme biocatalysis within the realm of pharmaceutical applications, with a primary focus on introducing multidisciplinary approaches to advance the field of enzyme biocatalysis (Fig. 1). We begin by providing a concise overview of the background and advantages of utilizing enzyme biocatalysis in pharmaceutical contexts. Following this, we introduce the key factors enabling the development of advanced enzymes for pharmaceutical applications. Within the context of multidisciplinary approaches, we extensively explore the significant contributions made by disciplines such as protein engineering, computational biology, and nanoarchitectonics in advancing the field of enzyme biocatalysis. Additionally, we address the persistent challenges that remain and offer insights into the prospects for these multidisciplinary strategies. As we explore this transformative landscape, it becomes evident that the synergy among these multidisciplinary facets holds great potential to have a significant impact on the evolution of pharmaceutical research and production. This potential contribution revolves around the core principles of speed, sustainability, efficiency, and precision, which are increasingly important in today's pharmaceutical landscape.

2. Enzyme biocatalysis as a potential solution for pharmaceutical applications

2.1 Definition of enzyme biocatalysis

Enzyme biocatalysis, situated at the crossroads of biology and chemistry, represents a cutting-edge discipline with the power to reshape how we approach chemical reactions and synthesis processes.^{12,38} At its core, this transformative field harnesses the remarkable catalytic properties of enzymes—biological macromolecules finely honed by evolution over millions of years to function as molecular machinery within living organisms.^{8,12} These enzymes exhibit an exceptional blend of



specificity and efficiency, unrivalled in their capacity to orchestrate complex chemical transformations.

Enzyme biocatalysis, in essence, can be defined as the application of enzymes as catalysts to drive and accelerate chemical reactions.¹ These biological catalysts possess the unique ability to enhance the speed and precision of chemical transformations under mild conditions, making them invaluable tools in various scientific and industrial applications.^{2,12} Central to the concept of enzyme biocatalysis is the idea of catalysis itself. In chemical reactions, catalysts are substances that facilitate the conversion of reactants into products without undergoing any permanent change themselves.³⁹ Enzymes excel in this role, lowering the activation energy required for reactions and thus effectively expediting chemical transformations. Their catalytic action is highly specific, with each enzyme tailored to interact with a particular substrate or class of molecules, ensuring precision in the reactions that they catalyze.^{2,40}

Furthermore, enzyme biocatalysis offers a sustainable and environmentally friendly approach to chemical processes.^{5,9} Enzymes typically operate in aqueous environments and under relatively mild temperatures and pH conditions, which reduces energy consumption and minimizes the production of hazardous byproducts.²³ These characteristics align with the principles of green chemistry, making enzyme biocatalysis an attractive choice for industries seeking eco-conscious and economically viable solutions.^{41,42} Ultimately, enzyme biocatalysis represents a convergence of nature's biochemical marvels with human ingenuity, opening doors to a wide range of applications across fields as diverse as pharmaceuticals, biotechnology, food production, and more. It holds the potential to transform the way we approach chemical synthesis and catalysis, offering a greener, more efficient, and more precise alternative to traditional chemical methods.

2.2 Beneficial properties of enzyme biocatalysis in pharmaceutical applications

Enzyme biocatalysis has emerged as a game-changer in the realm of pharmaceuticals, offering a plethora of highly advantageous properties that are transforming the drug development and manufacturing landscape. Within this section, we will explore several key advantageous properties that define the potential of enzyme biocatalysis, with a specific focus on its applications in the pharmaceutical sector. These properties encapsulate the versatility and sustainability that make enzyme biocatalysis an indispensable tool in modern pharmaceutical research and production.

2.2.1 Precise and selective. Enzymes, often referred to as nature's molecular machines, exert an unmatched degree of precision and selectivity when catalyzing chemical reactions.¹³ This excellent specificity ensures that enzymes interact exclusively with their target substrates, minimizing the formation of unwanted byproducts. In the pharmaceutical industry, where the synthesis of complex and highly specific therapeutic molecules is critical, this precision is invaluable. Enzyme biocatalysis empowers researchers to fine-tune the synthesis of drugs and

therapeutic compounds, significantly enhancing their purity and efficacy.

2.2.2 Sustainable and compatible with mild conditions. Enzyme biocatalysis seamlessly aligns with the principles and metrics of green chemistry and sustainable development, solidifying its status as a robust and environmentally responsible technology.⁴³ Operating under mild reaction conditions, typically at moderate temperatures and in aqueous environments, enzymes significantly reduce energy consumption and curtail the generation of hazardous waste, establishing themselves as a sustainable choice for pharmaceutical production. As the global focus on environmental responsibility intensifies, enzyme biocatalysis has emerged as a pivotal strategy for shrinking the carbon footprint of the pharmaceutical industry. In addition, the mild reaction conditions preserve the structural integrity of delicate biomolecules and prevent chemical degradation, guaranteeing the production of high-quality pharmaceuticals.⁴³ This capability to function under such conditions facilitates the synthesis of intricate peptides, oligonucleotides, and biologics with minimal risk of denaturation or deterioration.

2.2.3 Fast and cost-effective. Enzyme biocatalysis offers unique advantages in pharmaceutical applications, particularly in terms of speed and cost-effectiveness.³⁹ Compared to traditional chemical catalysts, enzymes demonstrate exceptional efficiency and rapid catalytic activity while consuming less energy. Their capacity to catalyze reactions at lower temperatures not only conserves energy but also enhances safety by minimizing the risk of high-temperature reactions. This rapid and energy-efficient characteristic translates into substantial cost savings in pharmaceutical manufacturing, reinforcing the economic viability of enzyme biocatalysis.

2.2.4 Robust and biocompatible. Enzymes often exhibit broad substrate tolerance, enabling them to work with a wide range of molecules.¹³ This versatility is particularly valuable in pharmaceutical applications, where the synthesis of diverse compounds is common. Enzymes can be adapted and tailored to accept various substrates, making them versatile tools for the production of a wide array of pharmaceuticals, ranging from small-molecule drugs to complex biologics. Furthermore, enzymes are inherently biocompatible, making them ideal for applications involving biologics and biomolecules.⁴⁴ Their compatibility with living organisms and biomimetic reactions is pivotal in drug delivery systems, bioconjugation processes, and the synthesis of biopharmaceuticals.²¹ Enzyme-based approaches can facilitate the conjugation of therapeutic agents with targeting ligands, enabling precise drug delivery to specific cells or tissues.

2.2.5 Stereochemical control. Enzymes offer exquisite control over the stereochemistry of reactions, a critical factor in pharmaceutical chemistry.^{8,45} Their application in biocatalysis enables the synthesis of enantiomerically pure compounds, meeting the exacting demands for chiral purity in drug development. This proficiency simplifies subsequent purification procedures and elevates the overall efficacy of pharmaceutical compounds, streamlining the drug development process.



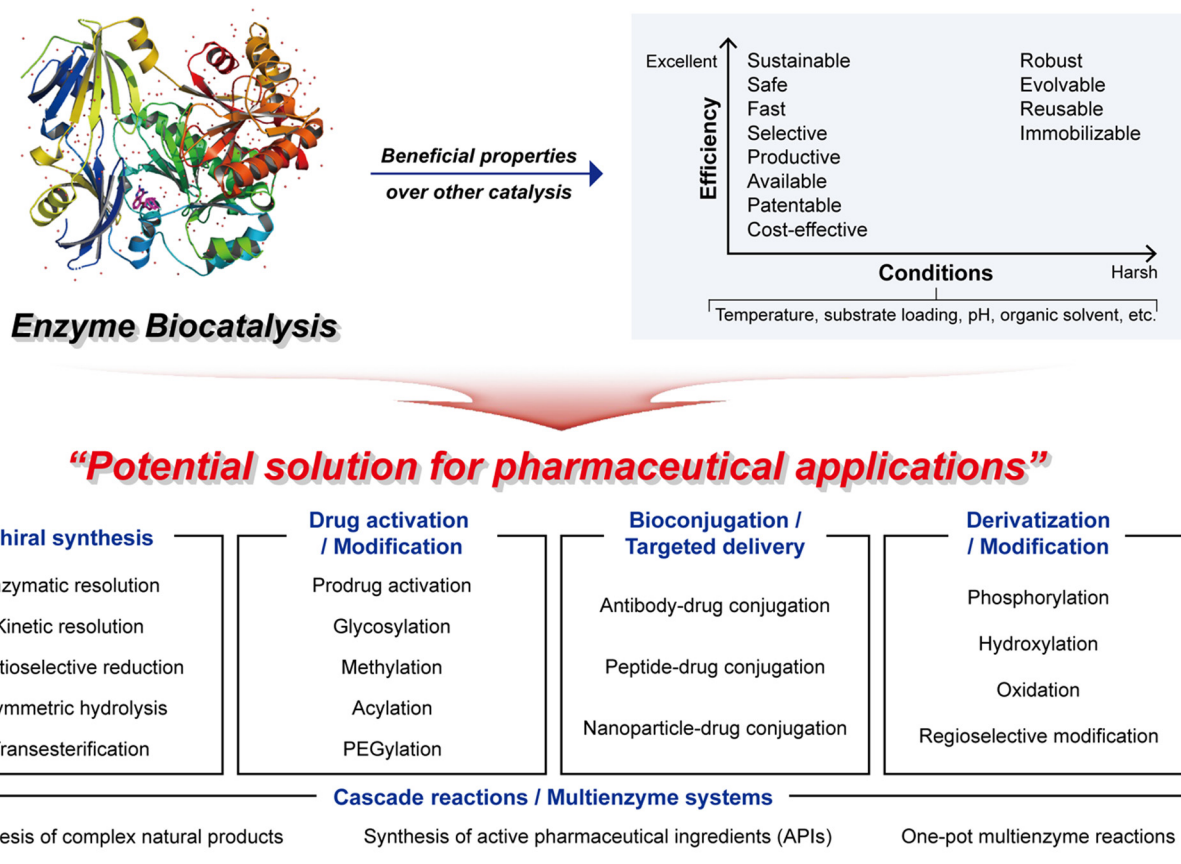


Fig. 2 Enzyme biocatalysis as a potential solution for pharmaceutical applications.

2.2.6 Productive and available. Enzyme biocatalysis often leads to streamlined and more efficient processes. Enzymes can catalyze complex reactions in a single step, reducing the number of reaction stages and minimizing the need for additional reagents. This efficiency translates to shorter production times and reduced costs, making enzyme biocatalysis an economically viable choice for pharmaceutical manufacturing.⁴⁶ Moreover, the specificity of enzymes ensures the production of high-purity pharmaceutical compounds. Contaminants and impurities are minimized, reducing the need for extensive purification steps. The resulting purity enhances the safety and efficacy of pharmaceutical products while simplifying quality control processes.

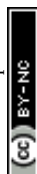
In conclusion, enzyme biocatalysis in pharmaceuticals embodies a host of advantageous properties that have positioned it at the forefront of drug discovery and production (Fig. 2). The plot (top right) highlights the most desirable characteristics of enzyme biocatalysis. In harsh industrial settings, attributes such as robustness and tolerance to mutations, which enhance stability, often become imperative. Conversely, whether operating under mild or more challenging conditions, the pharmaceutical industry consistently seeks biocatalyst properties that offer flexibility and efficiency.¹² These advantageous attributes can be synergistically harnessed to yield high-value pharmaceutical compounds, even in the most taxing industrial scenarios, while

concurrently achieving commendable economic and environmental performance.

2.3 Main applications of enzyme biocatalysis in pharmaceutical applications

Enzyme biocatalysis has become a significant factor behind pharmaceutical advances, offering a diverse set of tools and techniques that enhance speed, precision, efficiency, and sustainability. As a result, it finds widespread utility in the pharmaceutical industry, covering various applications, from chiral synthesis to targeted drug delivery and cascade reactions (Fig. 2). These applications leverage the exceptional catalytic capabilities of enzymes, allowing pharmaceutical researchers to address challenges, refine drug development processes, and create innovative therapeutic solutions. In this section, we explore the primary applications of enzyme biocatalysis in pharmaceuticals, each representing a foundational step towards greener, more effective, and more precisely tailored pharmaceuticals.

2.3.1 Chiral synthesis. Enzyme biocatalysis has revolutionized pharmaceutical chemistry, particularly in the field of chiral synthesis.^{13,47,48} Chirality, or handedness, is a critical aspect of many drugs, as different enantiomers (mirror-image isomers) can exhibit distinct pharmacological effects, making precise stereochemistry essential in drug development.⁴⁹



Enzymes such as lipases and hydrolases have emerged as invaluable tools in this context. They catalyze asymmetric reactions with remarkable selectivity, enabling the production of enantiopure compounds.⁵⁰ For example, lipase from *Candida antarctica* is a valuable biocatalyst for kinetic resolution in chiral synthesis due to its high enantioselectivity and ability to selectively transform one enantiomer in a racemic mixture into a pure, pharmaceutical-grade compound.⁵¹ This enzyme contributes to the production of enantiomerically pure compounds with high efficiency, thus enhancing the quality and efficacy of pharmaceutical products. In addition, ketoreductases are highly efficient biocatalysts for the asymmetric reduction in chiral synthesis, leading to the production of chiral alcohols with high selectivity and purity.⁵² Their role in the pharmaceutical industry significantly contributes to the synthesis of key intermediates and compounds required for drug development and manufacturing.⁵²

This capability eliminates the need for cumbersome and expensive separation methods, which are traditionally required to isolate desired enantiomers from racemic mixtures.⁵³ By harnessing the power of enzyme biocatalysis, pharmaceutical scientists can confidently synthesize single enantiomers, thus ensuring both the safety and the efficacy of pharmaceutical compounds. This transformative aspect of enzyme biocatalysis has led to the development of safer and more effective chiral pharmaceuticals, offering enhanced therapeutic outcomes.

2.3.2 Drug activation and modification. In the pharmaceutical domain, enzyme biocatalysis assumes a crucial role in drug activation and modification. Prodrugs, which are initially inactive compounds that transform into their active forms within the body, are extensively used to improve drug bioavailability and mitigate side effects. Enzymes serve as the conductors of this precisely controlled activation, ensuring the targeted delivery of drugs.^{54,55} Moreover, enzymes exhibit remarkable proficiency in the modification of existing drug molecules.⁵⁶ This capacity paves the way to the optimization of drug pharmacokinetics, the enhancement of stability, and the alteration of the drug's mode of action.⁵⁷ For example, glucuronosyltransferase contributes by conjugating drugs and xenobiotics with glucuronic acid, thereby increasing their water solubility, facilitating elimination, and ensuring the safety and efficacy of pharmaceutical products. Additionally, it plays a critical role in the body's defense against toxic substances.⁵⁸ In addition, sulfotransferases contribute to sulfation for drug metabolism and bioactivation by conjugating drugs and xenobiotics with sulfate groups, thereby modifying their chemical structures and properties.⁵⁹ These modifications can lead to the activation of prodrugs, modulation of drug activity, enhancement of water solubility, and detoxification, all of which are essential for ensuring the safety and efficacy of pharmaceutical products. Utilizing enzyme biocatalysis for these modifications empowers pharmaceutical scientists to precisely tailor drug properties, leading to the attainment of desired therapeutic outcomes. This capacity equips the pharmaceutical industry with a versatile toolkit to enhance drug effectiveness while mitigating undesirable side effects.

2.3.3 Bioconjugation and targeted delivery. The precision inherent in enzyme biocatalysis finds another valuable application in bioconjugation and targeted drug delivery.^{21,60} Through the incorporation of specific enzyme recognition sites into drug molecules, researchers can craft prodrugs that selectively activate in particular tissues or cells, a strategy known as targeted drug delivery. This approach minimizes off-target effects and amplifies the therapeutic potential of drugs, which is particularly beneficial in fields such as oncology, where precision is paramount. Moreover, enzymes facilitate the conjugation of therapeutic compounds with targeting ligands, antibodies, or nanoparticles, a process termed bioconjugation.²¹ This bioconjugation capability enables the development of precision medicine strategies that enhance drug localization while reducing systemic toxicity. For example, sortases play a crucial role in pharmaceutical applications by enabling site-specific protein labeling and bioconjugation. This technology is instrumental in bioconjugation for targeted drug delivery, allowing the attachment of various molecules to specific proteins, thereby enhancing the specificity and efficacy of drug delivery systems.⁶¹ Additionally, lipoic acid ligase and similar lipidation enzymes are instrumental in pharmaceutical applications by enabling protein modification for targeted drug delivery. Through the covalent attachment of lipid moieties to proteins, these enzymes enhance membrane association, enable precise targeting, and improve the stability of drug delivery systems, ultimately enhancing the effectiveness of pharmaceutical products in targeted drug delivery and bioconjugation.⁶²

By harnessing enzyme biocatalysis for bioconjugation, pharmaceutical scientists can tailor drug delivery systems to suit the unique requirements of diverse diseases and patient populations, thus advancing the field of personalized medicine.

2.3.4 Derivation and modification. Enzyme biocatalysis extends its influence to the derivation and modification of pharmaceutical compounds.⁶³ In this capacity, enzymes can be strategically employed to introduce or remove specific functional groups from drug molecules, thereby facilitating the synthesis of derivatives with modified pharmacological properties.⁶⁴ This capability has proved instrumental in the optimization of drug candidates and the development of analogs characterized by enhanced efficacy or reduced toxicity. Furthermore, enzymes serve as indispensable assets in pharmaceutical manufacturing processes, where they execute specific modifications crucial for ensuring the consistency and purity of drug products. For example, glycosyltransferases enable enzymatic glycosylation to synthesize glycosylated compounds with a wide range of derivatization and modification options. The resulting glycosylated products find utility in drug development, biotechnology, and other pharmaceutical endeavors, contributing to the enhanced efficacy, stability, and targeted delivery of pharmaceutical products.⁶⁵ Additionally, halogenases allow for the selective halogenation of organic molecules. This process contributes to derivatization and modification by introducing halogen atoms into substrates, thereby enhancing their chemical properties for use in drug development.⁶⁶



By harnessing enzyme biocatalysis in these manufacturing procedures, pharmaceutical manufacturers can attain a heightened level of control over drug production, yielding pharmaceuticals that are more dependable, uniform, and ultimately beneficial for patients.

2.3.5 Cascade and multienzyme reactions. A notable characteristic of enzyme biocatalysis in pharmaceutical synthesis lies in its adept utilization of cascade and multienzyme reactions. In cascades, enzymes are meticulously organized in a sequential fashion, where each enzyme adeptly catalyzes a specific step in a complex reaction sequence.^{27,67,68} This approach significantly streamlines the synthesis of intricate pharmaceutical compounds, effectively reducing the number of intermediates required and minimizing the need for extensive purification steps. The cascade strategy proves particularly advantageous when crafting complex molecules adorned with multiple functional groups.^{69,70} Representative examples include taxadiene synthase, taxadiene-5- α -hydroxylase, and acetyltransferase for taxol biosynthesis cascade,⁷¹ amorpha-4,14-diene synthase, CYP71AV1, and ALDH1 for artemisinin biosynthesis multienzyme system,⁷² ACVS, IPNS, and IAT for penicillin G biosynthesis cascade,⁷³ and HMG-CoA synthase, HMG-CoA reductase, and mevalonate kinase for statin synthesis multienzyme network.⁷⁴

Concurrently, multienzyme systems, often featuring engineered enzymes, unlock the potential for highly customized and efficient reactions that would present formidable challenges or even impossibilities, using traditional chemical methods.^{75,76} These intricate enzymatic networks bestow upon pharmaceutical scientists an unprecedented level of control and precision. Consequently, they elevate the versatility and efficiency of drug synthesis, ultimately culminating in more streamlined and cost-effective pharmaceutical production processes.

In summary, enzyme biocatalysis in pharmaceutical applications encompasses a broad range of vital functions, extending from chiral synthesis to drug activation, bioconjugation, derivation, and cascade reactions. These applications collectively empower pharmaceutical scientists to craft safer, more effective, and more precisely targeted therapies, all while streamlining drug synthesis and production processes. Enzyme biocatalysis remains a driving force in the evolution of the pharmaceutical industry, propelling innovation and extending the boundaries of drug discovery and development. For a detailed overview of various enzyme biocatalysis applications, please refer to Table 1.^{51,52,58,59,61,62,65,66,71–74,77–93}

3. Key factors enabling enzyme biocatalysis in pharmaceutical applications

Enzyme biocatalysis has emerged as a vital facet of contemporary organic synthesis, exerting substantial influence across academic research and the chemical and pharmaceutical industries. Originally, during the early 2000s, its primary

application lay in the production of optically active intermediates.⁹⁴ The field of biocatalysis has undergone a transformative evolution, however, expanding its domain to encompass a diverse array of applications, with profound implications for the synthesis of chiral compounds integral to pharmaceuticals. This transformation has engendered the imperative adoption of a cyclical and iterative approach, characterized by three pivotal phases: design, build, and optimize (Fig. 3).⁴ These phases have become indispensable in harnessing the immense potential of enzyme biocatalysis, enabling pharmaceutical scientists to judiciously engineer enzymes, fabricate novel biocatalysts, and systematically refine their performance.⁹⁵ In this manner, they drive innovation and enhance efficiency in both drug discovery and production.

Design: the process commences with a rigorous design phase, marked by the pursuit of high efficacy, enantioselectivity, and specificity in enzyme biocatalysis tailored for pharmaceutical applications. It necessitates the meticulous selection of target substrates and the systematic delineation of requisite enzyme engineering strategies essential for achieving precise substrate binding.

Build: following the design phase, the spotlight shifts to the efficient production of biocatalysts. The fabrication of biocatalysts for industrial pharmaceutical use demands productivity, practicality, and cost-effectiveness. This pivotal phase serves as the bridge between conceptual enzyme design and its tangible application.

Optimize: once biocatalysts are assembled, they undergo comprehensive testing to ascertain their capacity for effective biocatalysis. The development of a rigorous examination system assumes paramount importance in the progression of pharmaceutical biocatalysis, ensuring reliability and efficiency in the synthesis of pharmaceutical compounds.

In this section, we embark on a comprehensive exploration of these three critical phases that underpin the success of enzyme biocatalysis in pharmaceutical applications. By exploring the intricacies of design, production, and optimization, we aim to provide a thorough scientific elucidation of the dynamic field of enzymatic biocatalysis and its seminal role in shaping the future of pharmaceutical science.

3.1 Design: precision at the molecular level

Protein design in the context of enzymes involves creating or modifying proteins to enhance their catalytic activity, substrate specificity, stability, and other properties for various biocatalytic applications.⁹⁶ A comprehensive understanding of protein structure and function to engineer enzymes makes it possible to improve their performance. Designing enzymes for high efficacy, enantioselectivity, and specificity is the starting point in developing enzyme biocatalysis for pharmaceutical applications.⁹⁷ Selecting a target substrate and uncovering which enzyme engineering is required for specific substrate binding are fundamental aspects of this process.

Rational design particularly involves making specific changes to an enzyme's amino acid sequence or structure based on our understanding of its catalytic mechanism. Unlike





Table 1 Selected examples of enzyme biocatalysis in pharmaceutical applications

Applications	Used enzyme	Biocatalysis	Key features	Ref.
Chiral synthesis	Lipase from <i>Candida antarctica</i>	Kinetic resolution for the synthesis of enantiomerically pure compounds	• Stereoselective hydrolysis of racemic mixtures	51
	Transaminase	Asymmetric amination for chiral amine synthesis	• High enantioselectivity • Broad substrate scope • Enantioselective conversion of ketones/aldehydes to chiral amines	77 and 78
	Hydantoinase	Enantioselective hydrolysis of hydantoin for chiral amino acid synthesis	• Mild reaction conditions • Compatibility with diverse substrates • Regio- and stereoselective hydrolysis	79
	Ketoreductase	Asymmetric reduction for the synthesis of chiral alcohols	• High enantioselectivity • Biocatalytic resolution of racemic hydantoin • Stereoselective reduction of ketones	52, 80 and 81
Drug activation and modification	Glucuronosyltransferase	Glucuronidation for drug metabolism and detoxification	• Selective conjugation of glucuronic acid to drugs, facilitating elimination and enhancing water solubility	58 and 82
	Sulfotransferase	Sulfation for drug metabolism and bioactivation	• Selective sulfonation of xenobiotics, modulating pharmacokinetics and biological activity	59
	Thiopurine S-methyltransferase	Methylation of thiopurine drugs for pharmacogenetic applications	• Methylation of thiopurine drugs, influencing efficacy and toxicity in personalized medicine	83 and 84
	Cytochrome P450 3A4	Drug metabolism and activation	• Oxidative metabolism of diverse drugs, influencing efficacy and toxicity profiles	85 and 86
Bioconjugation and targeted drug delivery	Sortase	Site-specific protein labeling and bioconjugation	• Selective ligation of peptides or proteins, facilitating protein engineering and bioconjugate synthesis	61 and 87
	Transglutaminase	Protein cross-linking for drug delivery systems	• Site-specific conjugation of proteins, forming stable and controlled drug delivery vehicles	88
	Lipoic acid ligase	Protein modification for targeted drug delivery	• Site-specific attachment of lipoic acid to proteins, enabling targeted drug delivery and imaging	62 and 89
	Protein kinase	Phosphorylation for targeted drug delivery and protein engineering	• Site-specific phosphorylation of proteins, modulating activity and interactions	90 and 91
Derivatization and modification	Glycosyltransferase	Enzymatic glycosylation for the synthesis of glycosylated compounds	• Regio- and stereoselective glycosylation	65
	Methyltransferase	Methylation for the synthesis of methylated compounds	• Versatile donor and acceptor substrate specificity • Selective transfer of methyl groups to substrates, enabling site-specific modification	92
	Halogenase	Halogenation for the synthesis of halogenated compounds	• Regio- and stereoselective halogenation, introducing halogen atoms into organic molecules	66
	Decarboxylase	Decarboxylation for the synthesis of bioactive compounds	• Conversion of carboxylic acids to corresponding amines or aldehydes, enabling diversification of chemical space	93
Cascade reactions and multienzyme systems	Taxadiene synthase, taxadiene-5- α -hydroxylase, and acetyltransferase	Taxol biosynthesis cascade	• Sustainable production of the anticancer drug taxol from simple precursors, showcasing the potential of enzymatic synthesis for pharmaceutical compounds	71
	Amorphadiene synthase, CYP71AV1, and ALDH1	Artemisinin biosynthesis multienzyme system	• Multienzyme system for artemisinin biosynthesis illustrating the power of enzymatic pathways in producing vital antimalarial drugs efficiently	72
	ACVS, IPNS, and IAT	Penicillin G biosynthesis cascade	• Penicillin G biosynthesis cascade that can efficiently produce antibiotics, revolutionizing pharmaceutical manufacturing	73
	HMG-CoA synthase, HMG-CoA reductase, and mevalonate kinase	Statin synthesis multienzyme network	• Multienzyme system for statin synthesis to produce cholesterol-lowering drugs with precision	74

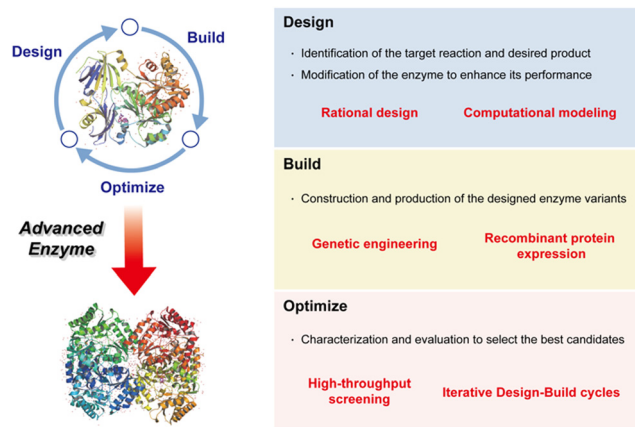


Fig. 3 Key factors enabling the development of an advanced enzyme for pharmaceutical applications.

random or empirical methods, rational design relies on scientific principles, computational tools, and biochemical knowledge to make precise changes to a protein's amino acid sequence or structure, with the goal of improving or customizing its properties. Because rational design starts with knowledge of the protein's three-dimensional structure, technology such as X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, or cryogenic electron microscopy (cryo-EM) is important for understanding the structure of the desired enzyme and provides insights into its active site, substrate-binding regions, and overall conformation.

Rational design focuses on the enzyme's active site, where chemical reactions take place. Researchers can introduce mutations or structural alterations to optimize the active site's geometry, electrostatics, or binding affinity towards substrates. Additionally, by altering key residues in the active site, researchers can make the enzyme preferentially recognize different substrates. Rational design can also enhance protein stability and solubility, making the enzyme more robust under varying conditions, and create enzymes with high specificity for a particular substrate or to improve selectivity.

Furthermore, computational molecular modeling of enzymes is a valuable tool for gaining insights into enzyme structure and function, elucidating reaction mechanisms, and facilitating the design of novel enzymes or engineered enzymes for various applications in pharmaceuticals. Computational tools and molecular modeling techniques can predict how changes to the protein's structure, such as mutations or structural modifications, will impact its function. Among these tools, molecular dynamics (MD) simulations and energy calculations provide dynamic information on enzyme behavior, including conformational changes, flexibility, and the simulation of enzyme-substrate interactions in a realistic environment.

The integration of rational design principles and computational techniques has revolutionized the field of enzyme biocatalysis, enabling the precise engineering of enzymes for pharmaceutical applications. In the subsequent sections of this review, we will explore the crucial phases of building and

optimizing these designed enzymes, culminating in their impactful role in pharmaceutical synthesis.

3.2 Build: crafting custom biocatalysis

Following the meticulous design phase, the focus shifts to the efficient production of biocatalysts—a phase that stands as a critical bridge between conceptual enzyme design and its tangible application. While some well-known enzymes such as oxidoreductases, transferases, and lyases are readily available through chemical vendors, the development of designed enzymes requires a more tailored approach.

To obtain a designed enzyme with a known amino acid sequence, researchers typically turn to gene synthesis. This process entails the creation of a synthetic gene encoding the enzyme engineered through rational design and computational molecular modeling to enhance its catalytic activity. Once the synthetic gene is prepared, it undergoes cloning using techniques such as the polymerase chain reaction (PCR) to amplify the gene. The cloned gene is subsequently inserted into a suitable host organism or expressed using cell-free protein synthesis systems.

The choice of expression system depends on various factors, including the nature of the enzyme and the intended application. Common expression hosts encompass bacteria, yeast, mammalian cells, or even cell-free systems. For living organisms, optimizing cultivation conditions, such as temperature and nutrient supply, becomes essential to ensure efficient enzyme production.

Upon successful expression, the next crucial step involves the harvesting and purification of the protein from the expression system. This purification process typically commences with cell lysis to release the protein, followed by various purification techniques such as chromatography or filtration. Importantly, the purified protein undergoes rigorous characterization to ensure it aligns with the desired specifications. Techniques such as mass spectrometry and gel electrophoresis are important in verifying the protein's identity and purity.

Moreover, if necessary, the production process can be further optimized to achieve higher yields and can be scaled up to generate larger quantities of the protein suitable for pharmaceutical applications. The specific details of each step may vary based on the protein, the chosen host organism, and the intended application. Genetic engineering, through the precise control and manipulation of genes, offers the versatility needed to produce a wide range of proteins for diverse purposes.

In essence, the efficient production of biocatalysts represents a pivotal phase that culminates in the transformation of conceptual enzyme design into tangible solutions for pharmaceutical applications. It is at this juncture that the engineered enzymes become the tools of choice for driving innovation and efficiency in drug discovery and production.

3.3 Optimize: continuous refinement and enhancement

Optimizing the engineered enzyme is a systematic approach that involves testing numerous enzyme variants and making



incremental improvements through high-throughput screening (HTS) and iterative design-build cycles. The goal of optimization can encompass various aspects, such as improved activity, substrate specificity, and improved stability, depending on the defined purpose of enzyme enhancement. To assess enzymatic activity efficiently and accurately, a robust screening method is crucial.

HTS, a good example, serves as a valuable tool in this regard, enabling the rapid and systematic testing of a large library of enzyme variants.⁹⁸ HTS, a method widely utilized across various scientific and industrial disciplines, including drug discovery, materials science, and enzyme engineering, automates the screening process for enhanced efficiency. HTS allows researchers to identify enzyme variants with improved properties, whether they are enhanced catalytic activity, altered substrate specificity, or increased stability. This method accelerates the identification of promising enzyme candidates from the vast pool of variants. Analysis of HTS screening data further refines the selection process, pinpointing the most promising enzyme variants. These selected variants often become the foundation for the next generation of enzyme enhancements, where targeted mutations or modifications are introduced to fine-tune their performance to meet specific application requirements.

The design-build-optimize process will not reach its conclusion in just one iteration. With each successive cycle, enzymes become increasingly customized, more efficient, and more sustainable. In practice, the iterative process described here need not rigidly adhere to a predetermined sequence and

exhaustively cover all three phases. Researchers, particularly those with potent design strategies, can have the flexibility to focus their efforts primarily on the build and optimize phases, persisting until the most favourable candidate is identified. However, it is worth noting that there are instances where, prior to advancing to the optimize phase, a reassessment of the design phase may be warranted, especially when certain enzymes have not been adequately constructed or produced.

This iterative design-build-optimize methodology, as elucidated, underscores the key role of precision, adaptability, and optimization in the quest for innovative pharmaceuticals and therapeutic solutions. It emphasizes the dynamic nature of the biocatalyst development process, acknowledging that the sequence of phases may vary based on the unique requirements and challenges of each biocatalyst design.

4. Multidisciplinary approaches for advanced enzyme biocatalysis

The necessity of introducing multidisciplinary approaches in the realm of enzyme biocatalysis for pharmaceutical applications is intimately tied to the field's continuous quest for optimization and advancement (Fig. 4). Enzyme biocatalysis, while already a transformative force in drug development and manufacturing, is far from reaching its full potential. To harness the full power of enzyme biocatalysis and overcome the multifaceted challenges presented by modern pharmaceutical demands, a more defined

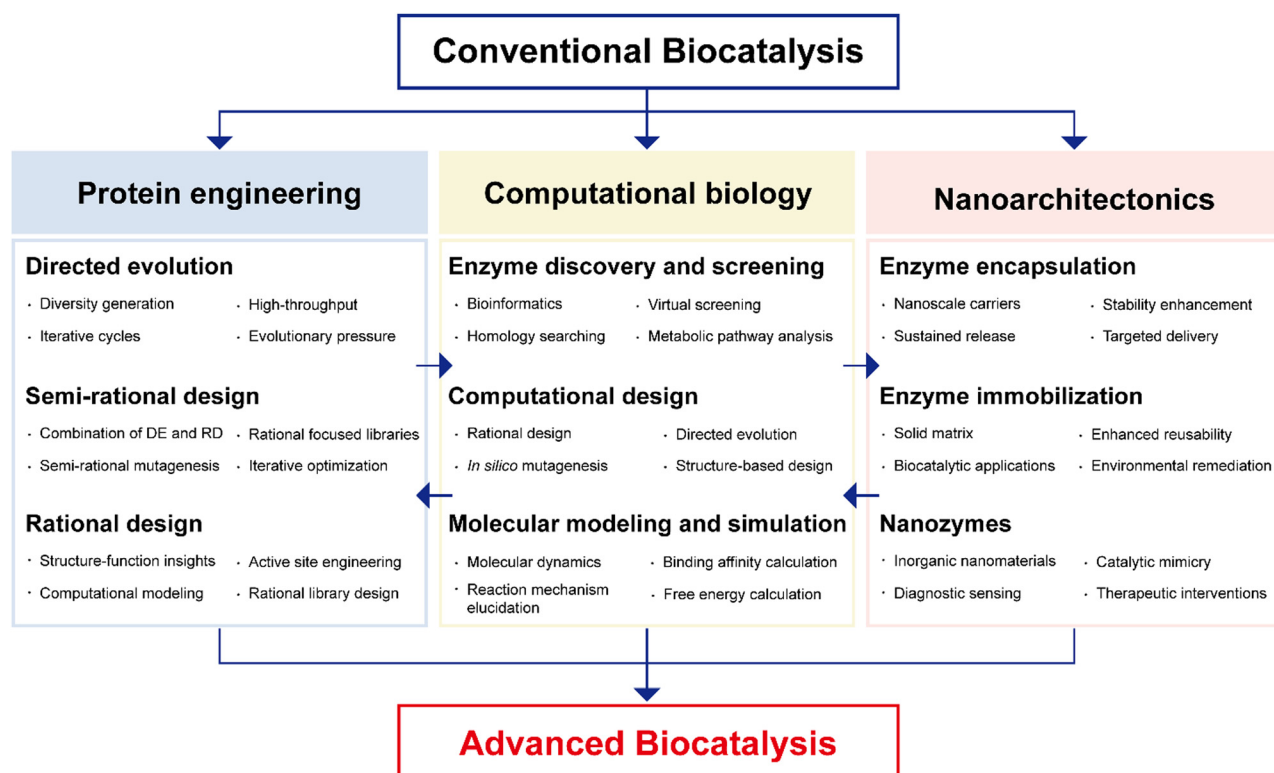
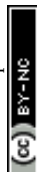


Fig. 4 Multidisciplinary approaches to advanced enzyme biocatalysis in the pharmaceutical industry. DE: directed evolution; RD: rational design.



and advanced approach is required. Enzymes, as Nature's molecular machines, possess remarkable catalytic capabilities, yet there is room for improvement.^{6,99,100} To tailor enzymes for specific pharmaceutical reactions, enhance their stability, and broaden their substrate scope, protein engineering emerges as a pivotal multidisciplinary approach.^{22,25,26,101,102} Directed evolution, semi-rational design, and rational design techniques offer sophisticated strategies for fine-tuning enzymes. These advances are not only driven by biology, but are heavily reliant on computational biology.^{103–109} The marriage of computational modeling and biological understanding empowers researchers to predict, manipulate, and optimize enzyme behavior at a molecular level. Furthermore, the integration of nanoarchitectonics into enzyme biocatalysis brings novel strategies for enzyme immobilization, encapsulation, and deployment within custom-designed nanomaterials.^{35–37,110} These innovations enhance enzyme stability, reusability, and their seamless integration into continuous flow processes—critical requirements for large-scale pharmaceutical production.

It will be the convergence of multidisciplinary approaches: protein engineering, computational biology, and nanoarchitectonics that promises to unlock the full potential of enzyme biocatalysis, making it an indispensable tool in pharmaceutical research and production (Fig. 4). This synergy not only enhances efficiency but also facilitates more sustainable and precise pharmaceutical processes, solidifying the significance of enzyme biocatalysis in shaping the future of the pharmaceutical industry.

4.1 Protein engineering for advanced enzyme biocatalysis

In recent decades, the field of protein engineering has witnessed significant advances, driven by breakthroughs in molecular

biology, crystallography, and computational methods.¹¹¹ These innovations have equipped researchers with powerful tools to enhance existing biocatalysts and tailor them for novel substrates. Beginning with the emergence of molecular biology in the late 1970s, the discipline of protein engineering has seen remarkable growth.¹¹² In the context of biotechnology and industrial applications, the exploitation of enzymes as biocatalysts holds immense promise. Enzymes, with their exquisite specificity and catalytic efficiency, are the biological marvels at the heart of this endeavor. To unlock their full potential, scientists have developed a repertoire of protein engineering techniques, prominently including directed evolution, semi-rational design, and rational design (Fig. 5).³⁸ Directed evolution emulates the forces of natural selection in the laboratory, enabling the creation of enzyme variants with enhanced traits through controlled mutation and selection processes. Semi-rational design integrates empirical methods with structural insights, allowing researchers to strategically introduce mutations to improve enzyme properties. Rational design leverages a deep understanding of enzyme structure–activity relationships, enabling precise modification for targeted applications. These methodologies have ushered in a new era, opening exciting possibilities for tailoring enzymes with augmented catalytic activity, stability, and substrate specificity. These properties are of paramount significance, serving as the bedrock for advances in enzyme biocatalysis, underpinning achievements in biotechnology, pharmaceuticals, and green chemistry.¹¹³

4.1.1 Directed evolution. Directed evolution is a robust and widely adopted approach in the field of protein engineering, enabling the systematic improvement of enzymes.¹¹⁴

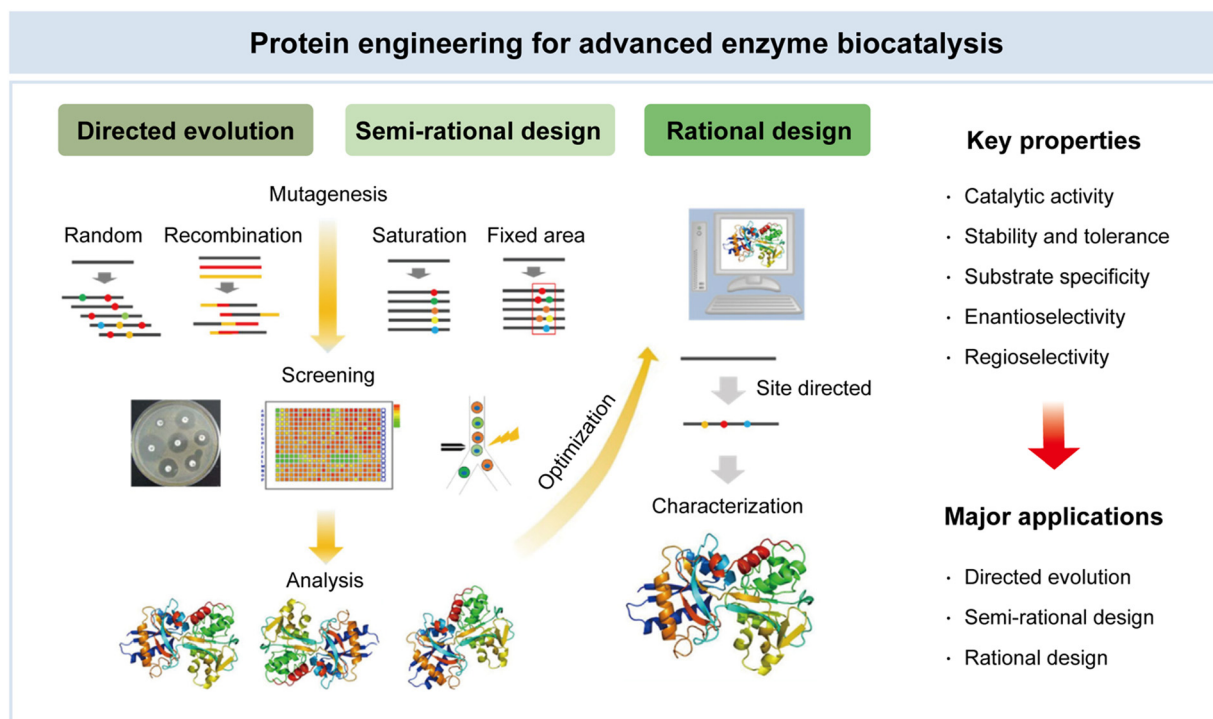
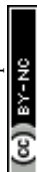


Fig. 5 Protein engineering for advanced enzyme biocatalysis in pharmaceuticals. Reproduced with permission from ref. 95. Copyright 2023, Springer Nature.



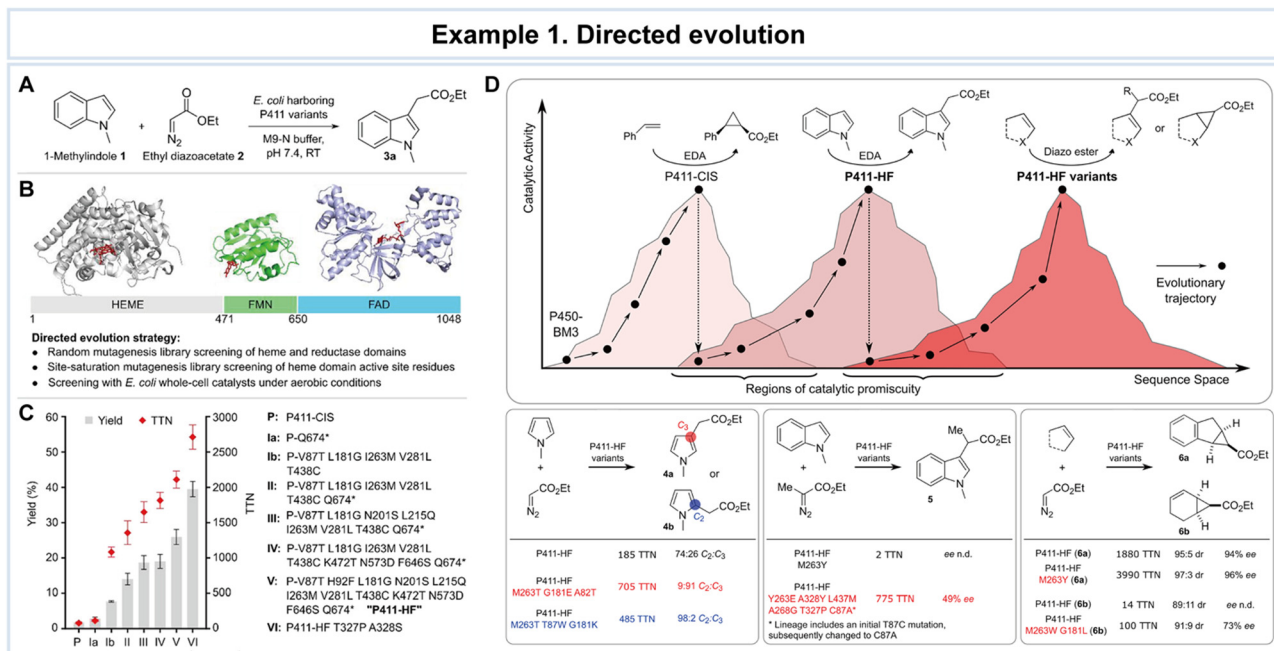


Fig. 6 Representative example of directed evolution for advanced enzyme biocatalysis. Directed evolution of P411-CIS for 1-methylindole C3-alkylation. (A) Screening reaction of P411 variants for higher indole alkylation activity. (B) Domain structure of P450-BM3. Individual domain structures with approximate domain boundaries were used to construct the P450-BM3 protein structure. (C) Evolutionary trajectory from P411-CIS to P411-HF T327P A328S. (D) Evolving P411-HF for selective (hetero)cycle functionalization reactions. Schematic representation of the relation between protein sequence and catalytic function. A given enzyme sequence may exhibit additional promiscuous functions that can be optimized by directed evolution. Reproduced with permission from ref. 116. Copyright 2019, American Chemical Society.

This multifaceted strategy entails iterative cycles of random mutagenesis and stringent selection to engender enzyme variants with enhanced characteristics.¹¹⁵ It offers scientists the means to traverse vast sequence spaces, thereby affording enzymes the capacity to function optimally under specified conditions. Directed evolution, *via* the application of selective pressures, can yield enzymes boasting augmented catalytic activity, bolstered stability, and an expanded repertoire of substrates.

In a notable example, Brandenburg *et al.* directed their attention towards cytochrome P450 variants, particularly P411-CIS, employing the formidable tools of directed evolution to enhance the selective functionalization of cyclic compounds (Fig. 6).¹¹⁶ The outcome was nothing short of astonishing, as the engineered enzyme variant exhibited remarkable prowess, catalyzing the C3-alkylation of 1-methylindole with a remarkable 14% yield and an astonishing turnover number (TTN) of 690. The intrigue deepened as the resulting alkylated product showcased a pronounced red-shift in ultraviolet (UV) absorbance compared to its parent compound (Fig. 6A). P411-CIS, an intricate 1048-residue protein, comprises a heme domain encompassing the active site (residues 1–470) and a reductase domain responsible for electron transport from nicotinamide adenine dinucleotide phosphate (NADPH) to the heme domain. This electron-shuttling mechanism is essential for the native oxo-transfer chemistry of P450-BM3, with residues 471–1048 subdivided into flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) domains (Fig. 6B). Through an iterative

sequence of directed evolution rounds, the research team culminated in the emergence of P411-HF (where HF stands for heterocycle functionalization; Fig. 6C). The quest continued with three compelling ‘case studies’ on P411-HF, delving into regioselective pyrrole alkylation, enantioselective indole alkylation employing an α -disubstituted carbene precursor, and the complex domain of cyclopropanetrone involving cyclic alkenes (Fig. 6D). As the research unfolded, it became evident that P411-HF bore more than its designated function. It exhibited additional ‘promiscuous’ activities, thus revealing a gateway to further evolutionary adaptations. This versatility was harnessed in the context of regioselective 1-methylpyrrole alkylation, enantioselective 1-methylindole alkylation with ethyl 2-diazopropanoate, and stereoselective cyclic alkene cyclopropanation. The significance of this study lies in its profound demonstration of precise control over regioselectivity, enantioselectivity, and catalytic activity. The remarkable adaptability and tunability of P411-HF underscore its potential as a catalyst in a diverse array of challenging reactions.

4.1.2 Semi-rational design. Semi-rational design combines empirical methods with a deeper understanding of the target enzyme’s structure and function.¹¹⁷ In this strategy, specific amino acids are strategically mutated based on insights obtained from structural data or a comprehensive knowledge of enzyme mechanisms.¹¹⁸ This approach allows for the precise modulation of substrate specificity, enantioselectivity, and regioselectivity, making it an efficient strategy, especially when prior knowledge of the enzyme is available.

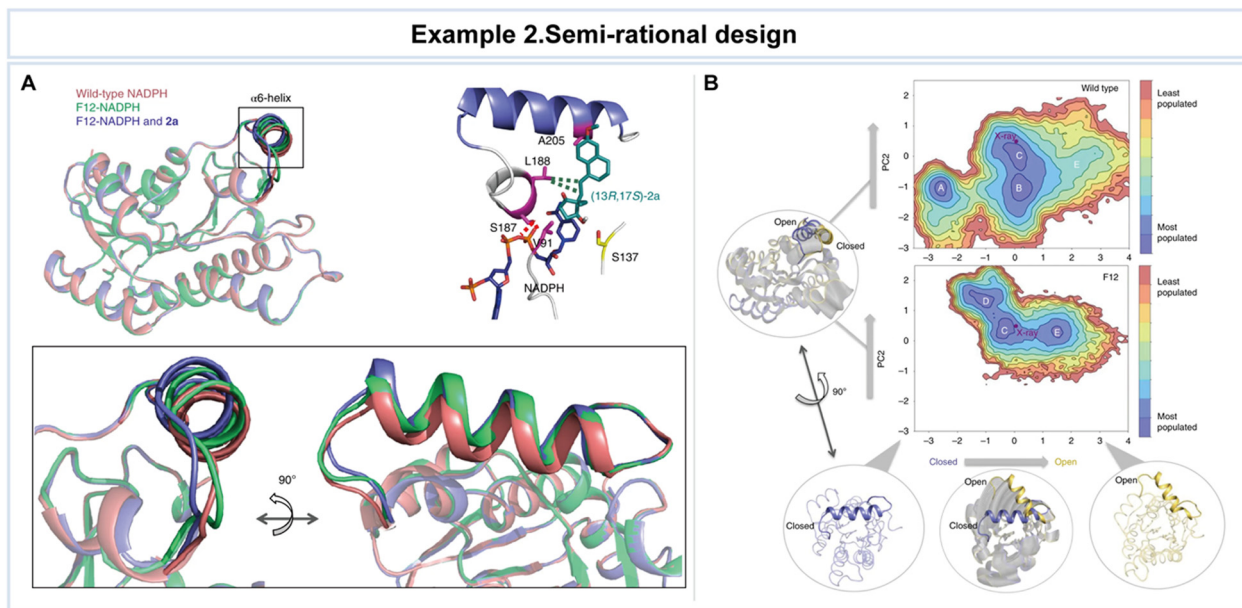


Fig. 7 Representative example of semi-rational design for advanced enzyme biocatalysis. (A) Crystal structures of wild-type NADPH and mutant F12-NADPH with or without **2a**. (B) Conformational population analyses. The two most important principal components (PC1 and PC2) that are based on C α contacts for the RasADH wild-type enzyme and the F12 variant were analyzed. In the conformational landscape represented, the most populated conformations are colored in blue, whereas the least populated are in red. Reproduced with permission from ref. 119. Copyright 2019, Springer Nature.

In the work by Chen *et al.*, the application of semi-rational design to the *Ralstonia* sp. alcohol dehydrogenase (RasADH) was aimed at overcoming challenges associated with the reductive desymmetrization of cyclic diketones by engineering an enzyme, designated as F12, which exhibited exceptional efficiency in catalyzing the reductive desymmetrization of **1a**, as well as other 2,2-disubstituted-1,3-cyclopentanones (Fig. 7).¹¹⁹ To gain a comprehensive understanding of the molecular basis underlying the remarkable enhancements in catalytic activity and stereocontrol achieved through semi-rational design, the researchers conducted a combination of crystal structural studies and MD simulations. These investigations involved a detailed comparison of the wild-type and mutant enzymes in the presence of the co-factor NADPH and the substrate, (13*R*,17*S*)-ethyl secol (**2a**). While an overall structural similarity was observed in the presence of NADPH for both the wild-type and mutant enzymes, subtle but significant differences were noted in the conformation of the α 6-helix of the mutant enzyme when bound to **2a** (Fig. 7A).

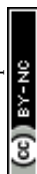
A meticulous analysis of these structural findings unveiled critical interactions that take a lead in enhancing the enzyme's activity and stereocontrol. For example, amino acid S187 was found to interact indirectly with (13*R*,17*S*)-**2a**, forming a crucial hydrogen bond with the phosphate group of NADPH. While the precise influence of the α 6-helix and the factors responsible for the improved activity and stereoselectivity remained elusive solely through structural analyses, computational assessments were undertaken to provide valuable insights. MD simulations unveiled dynamic transitions of the α 6-helix, with the enzyme switching between open, partially closed, and closed conformations at the entrance of the active site (Fig. 7B). Notably, these

conformational changes, especially the transition to an open state, were notably more pronounced in the F12 variant compared to the wild type. The combination of structural insights and computational analyses clarifies the intricate conformational dynamics that govern enzyme–substrate interactions and catalytic outcomes, providing valuable guidance for future enzyme engineering endeavors. This resulted in high yields of the desired (13*R*,17*S*)-stereoisomer of ketol products, along with favorable diastereomeric ratio values.

4.1.3 Rational design. Rational design, on the other hand, leverages a comprehensive understanding of the enzyme's structure–activity relationships.¹²⁰ By pinpointing critical residues and their roles in catalysis, stability, and substrate binding, rational design enables the deliberate engineering of enzymes with tailored properties. This approach is particularly valuable when atomic-level details of the enzyme's structure are known.

In a study conducted by Son *et al.*, rational design was employed to enhance the thermal stability of IsPETase (Fig. 8).¹²¹ Key structural issues affecting thermal stability were identified, including a disrupted central β -sheet caused by an abnormal β 6 strand conformation and the flexibility of the β 6– β 7 connecting loop in the enzyme (Fig. 8A). To address these challenges, the researchers developed the IsPETaseP181A variant to restore the disrupted β -sheet. Furthermore, the flexibility of the β 6– β 7 connecting loop in IsPETase was found to be critical for stability, as revealed through a comparison with the more stable TfcUT2 enzyme (Fig. 8B). To improve loop stability, the IsPETaseS121D/D186H variant was created by introducing a hydrogen bond.

The impact of these mutations on thermal stability was assessed by comparing the melting temperatures (T_m) that IsPETaseP181A and IsPETaseS121D/D186H exhibited T_m values



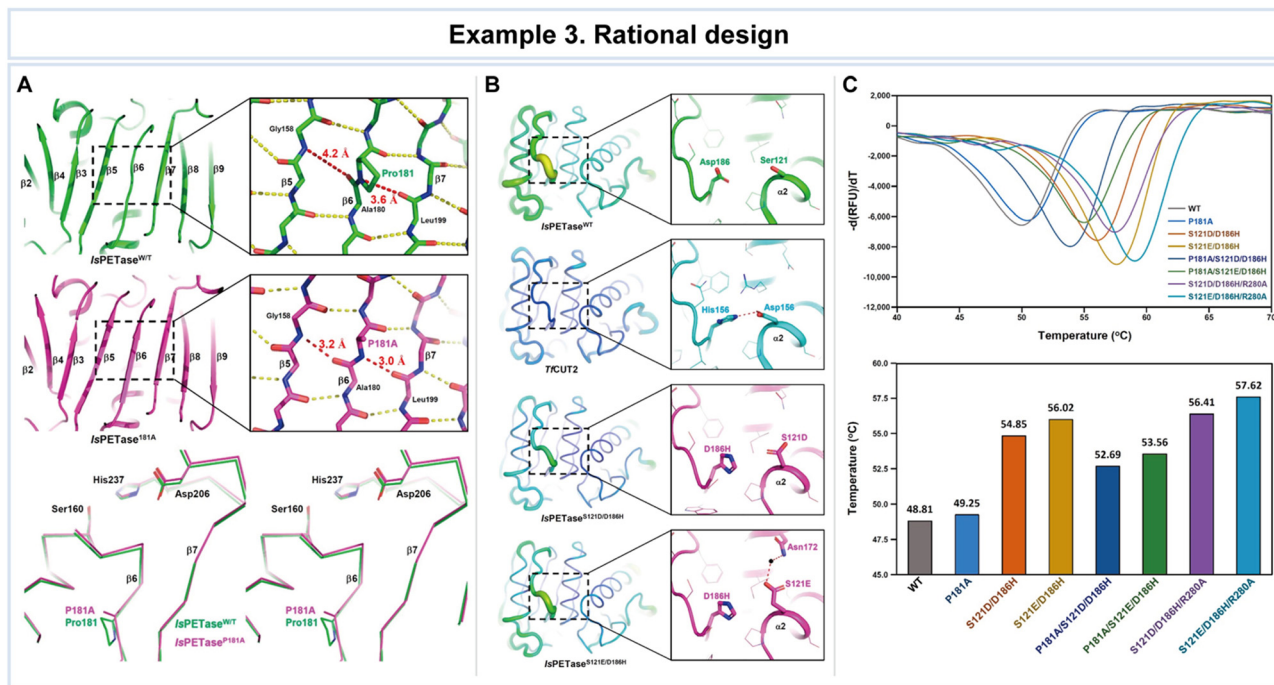


Fig. 8 Representative example of rational design for advanced enzyme biocatalysis. (A) Structural comparison of IsPETaseWT with IsPETaseP181A variant: the central mixed β -sheets of IsPETaseWT and IsPETaseP181A variant; stereoview of superimposed structures of IsPETaseWT and IsPETaseP181A variant. (B) The β 6– β 7 connecting loops of IsPETaseWT, TfCUT2, IsPETaseS121D/D186H, and IsPETaseS121E/D186H. (C) Thermal stability comparison of IsPETaseWT and variants. Reproduced with permission from ref. 121. Copyright 2019, American Chemical Society.

of 49.25 °C and 54.85 °C, respectively, representing increases of 0.5 °C and 6 °C compared to IsPETaseWT at 48.81 °C (Fig. 8C). The IsPETaseP181A variant successfully restored the disrupted β -sheet, while the IsPETaseS121D/D186H variant mitigated flexibility in the β 6– β 7 connecting loop (Fig. 8B). Remarkably, the IsPETaseS121E/D186H variant displayed an impressive T_m of 56.02 °C, surpassing IsPETaseWT by 7.21 °C (Fig. 8C). Unexpectedly, enhanced thermal stability in this variant was attributed to a water-mediated hydrogen bond between Glu121 and Asn172. IsPETaseS121D and IsPETaseS121E variants exhibited slightly increased polyethylene terephthalate (PET) degradation ability at 30 °C, however, but demonstrated lower T_m values and decreased activity at 40 °C.

In summary, rational design has proven to be a potent approach for enhancing enzyme properties, as demonstrated in the context of improving thermal stability. Structural insights and targeted mutations guided the development of variants with enhanced thermal resilience, albeit with some trade-offs in activity at higher temperatures.

The combination of directed evolution, semi-rational design, and rational design represents an impact shift in the field of enzyme biocatalysis. These methodologies have emerged as indispensable tools for the precise tailoring of enzymes, conferring upon them exceptional properties that are paramount for pushing the boundaries of biocatalysis. Further exemplification of enzyme engineering can be found in Table 2, where additional instances are provided.^{122–140} Enhanced catalytic activity accelerates reactions, stability ensures that enzymes can endure challenging environments,

and refined substrate specificity opens doors to a plethora of novel applications. However, these methods still have several limitations, for example, directed evolution can be time-consuming and resource-intensive and may not always yield enzymes with the desired properties. The accuracy of semi-rational design heavily depends on the available structural data and the quality of computational algorithms. Rational design requires in-depth knowledge of protein structure and biochemistry, which may not be available for all enzymes. While these methods have undeniably pushed the boundaries of enzyme biocatalysis, their full potential is yet to be realized. Continued research to address their limitations will be crucial in maximizing the practical benefits of directed evolution, semi-rational design, and rational design in enzyme engineering.

4.2 Computational biology for advanced enzyme biocatalysis

Within the domain of advanced enzyme biocatalysis, computational biology emerges as a significant discipline, catalyzing groundbreaking advances at the intersection of biology and computation (Fig. 9).^{12,141,142} Computational biology, broadly defined, is the application of computational techniques and mathematical modeling to solve complex biological problems.^{143–145} Its significance lies in its ability to harness the power of algorithms and high-performance computing to decipher intricate biological processes, predict molecular interactions, and optimize enzymatic properties.^{146,147} Key among these properties are the prediction of enzyme properties, including catalytic activity and stability, substrate specificity, docking and elucidation of binding mechanisms, and the



Table 2 Selected examples of protein engineering for advanced enzyme biocatalysis

Example	Applications	Enzyme	Key features	Detailed information	Ref.
(a) Directed evolution					
Fucosyltransferase	Fucosylated glycoconjugates	α -1,3-Fucosyltransferase	- Improved activity - Enhanced substrate specificity	- Engineered variants with improved activity and substrate specificity - Enabling the efficient synthesis of key intermediates for the production of pharmaceutical compounds	122
Transaminases	Synthesis of chiral amines	ω -Transaminase (ω -TA)	- Synthesis of key intermediates - Expanded substrate scope	- Engineered variants with expanded substrate scope and improved activity - Enabling efficient synthesis of diverse chiral amines	123
Esterases	Enantioselective synthesis of chiral compounds	Bacillus subtilis esterase	- Improved activity - Synthesis of diverse chiral amines - Enhanced stability	- Engineered variants with enhanced stability and activity towards specific ester substrates - Efficient hydrolysis of ester prodrugs, contributing to drug activation and improving drug delivery	124
Nitrilases	Nitrile hydrolysis, synthesis	Rhodococcus rhodochrous nitrilase	- Improved activity - Hydrolysis of ester prodrugs - Drug activation - Improved substrate specificity	- Engineered nitrilase variants with improved substrate specificity and resistance to inhibitory compounds - Conversion of nitrile-containing precursors into valuable pharmaceutical intermediates, contributing to the synthesis of bioactive compounds	125
Glycosidases	Glycosylation, glycoside synthesis	β -Glucosidase	- Resistance to inhibitory compounds - Conversion of nitrile precursors - Synthesis of drug intermediates - Enhanced thermostability - Substrate tolerance - Enzymatic hydrolysis - Drug activation	- Engineered variants with enhanced thermostability and substrate tolerance - Efficient enzymatic hydrolysis of glycosidic bonds in drug glycosides, facilitating drug activation and improving bioavailability	126
(b) Semi-rational design					
Proteases	Drug synthesis, enzymatic modifications	Engineered subtilisin E	Altered substrate specificity	Semi-rational design strategies to modify specific amino acid residues in the active site for broadened substrate specificity towards non-natural amino acids	127
Kinases	Drug discovery, screening, activation	Engineered protein kinase	- Enhanced catalytic efficiency - Improved selectivity - Enhanced substrate specificity	Semi-rational design approaches to modify the active site of a protein kinase, enhancing its substrate specificity towards specific peptide sequences relevant to drug targets	128
Isomerases	Drug synthesis, pharmaceutical production, bioconversion	Engineered xylose isomerase	- Improved binding affinity - Improved thermostability - Enhanced catalytic activity	Modification of active site to enhance catalytic activity and stability during bioconversion through semi-rational design	129
Ligases	DNA manipulation, protein production, diagnostics	Engineered DNA ligase	- Improved stability - Increased conversion efficiency - Enhanced fidelity in DNA ligation - Improved efficiency - Reduced side reactions	Semi-rational design to enhance the fidelity and efficiency for precise DNA manipulation in pharmaceutical research and development	130

Table 2 (continued)

Example	Applications	Enzyme	Key features	Detailed information	Ref.
Dehalogenases	Detoxification, remediation, drug production	Engineered haloalkane dehalogenase	- Altered substrate specificity - Enhanced catalytic efficiency - Increased detoxification efficacy	Semi-rational design techniques to modify the active site, enhancing its substrate specificity towards specific halogenated compounds encountered in pharmaceutical manufacturing processes	131 140
(c) Rational design Oxidases	Stereoselective oxidation, drug synthesis	Engineered laccase variant T2/T3 site mutants	- Enhanced activity towards specific substrates - Improved pH stability	- Rational design to introduce mutations at the T2 and T3 copper-binding sites in laccase - Computational analysis and MD simulations to identify key residues for targeted mutagenesis	132 and 133
Transferases	Drug synthesis, enzymatic cascade	Engineered transaminase variant with optimized active site residues	Altered substrate specificity	Rational design to optimize active site residues	134
Esterases	Drug synthesis, chiral resolution	Engineered esterase variant with altered binding pocket residues	- Increased thermostability - Enhanced AHL-degrading activity	- Computational analysis and protein structure modeling to identify key amino acids for modification - Rational design to modify binding pocket residues	135 and 136
Glycosidases	Glycosylation, drug synthesis	Engineered glycosidase variant with altered active site residues	- Improved binding affinity with substrate - Increased thermostability	- Computational modeling and substrate docking studies to select key amino acids for mutation - Rational design strategies to modify active site residues in glycosidases	137
Oxidoreductases	Precursor synthesis, oxidation	Engineered oxidoreductase variant with expanded substrate	- Improved glycosylation efficiency - Enhanced substrate promiscuity - Catalytic efficiency	- Computational modeling and MD simulations to select key amino acids for mutation - Rational design to expand the substrate binding pocket in oxidoreductases - Computational analysis and protein–ligand docking studies to select key amino acids for modification	138 and 139



Computational biology for advanced enzyme biocatalysis

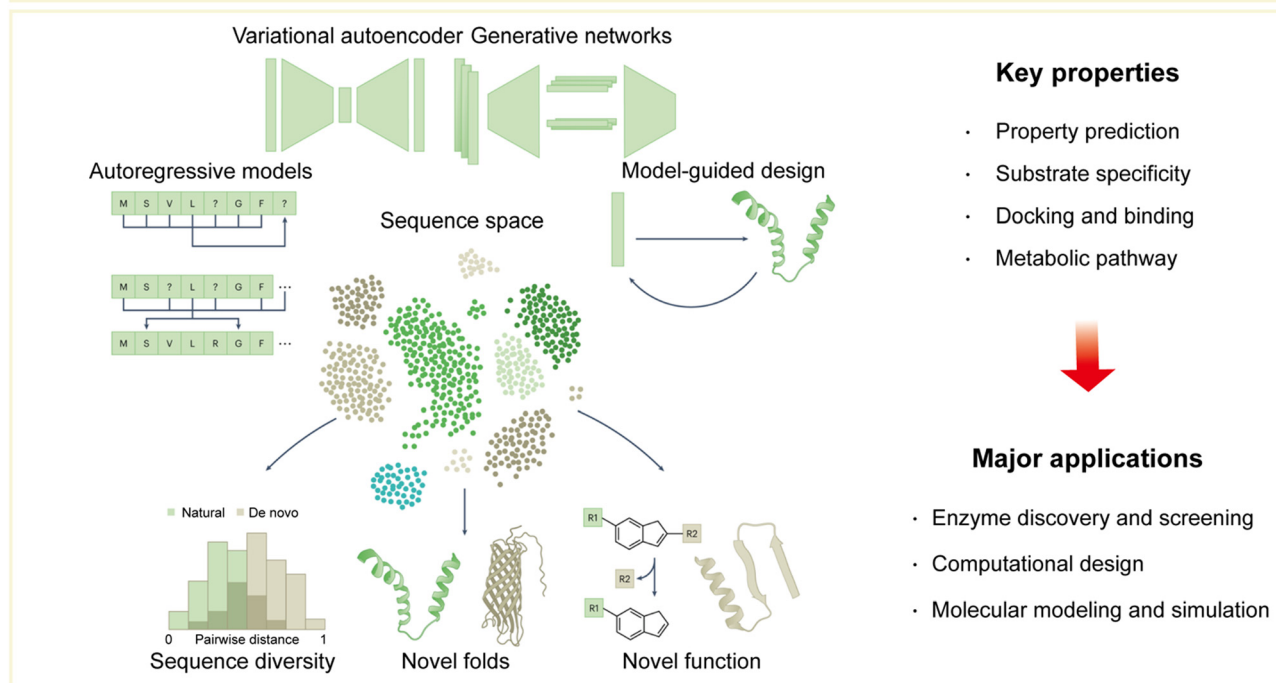


Fig. 9 Computational biology for advanced enzyme biocatalysis in pharmaceuticals. Reproduced with permission from ref. 173. Copyright 2023, Springer Nature.

mapping of intricate metabolic pathways.^{28,30,34,106,141} These predictive capabilities are indispensable in the rational design and engineering of enzymes, ultimately driving the evolution of enzyme biocatalysis.

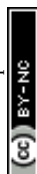
Foremost among these applications is enzyme discovery and screening.^{108,148} By employing computational tools, researchers can efficiently sift through vast databases of potential enzymes, identifying candidates with the desired catalytic properties for specific reactions. This accelerates the process of enzyme selection, streamlining the search for novel biocatalysts. Another notable application lies in computational enzyme design.^{28,34,104,108,109,149} Here, computational biology enables the tailored engineering of enzymes with enhanced catalytic activity and selectivity. Researchers can iteratively model enzyme structures and interactions to predict the effects of mutations, ultimately guiding the development of enzymes optimized for specific reactions. This approach revolutionizes the field by expediting the creation of tailor-made biocatalysts for pharmaceutical applications. Molecular modelling and simulation constitute yet another critical facet of computational biology in enzyme biocatalysis.^{140,150,151} These techniques enable researchers to simulate enzyme–substrate interactions, providing essential insights into reaction mechanisms, binding affinities, and conformational changes. By unraveling these intricacies at the molecular level, computational biology empowers scientists to fine-tune enzymatic reactions with unparalleled precision, ensuring the development of efficient and sustainable bioprocesses.

In summary, computational biology serves as an indispensable partner in the pursuit of advanced enzyme biocatalysis.

Its predictive prowess in property assessment, combined with its major applications in enzyme discovery, design, and molecular modeling, shapes a landscape of innovation that continues to redefine the boundaries of biocatalytic research and applications (Fig. 9).

4.2.1 Enzyme discovery and screening. Enzyme discovery and screening have been revolutionized by computational biology. Traditional methods for enzyme discovery were often laborious and time-consuming, involving extensive experimentation.^{152,153} Computational approaches now play a critical role in accelerating this process. By leveraging computational tools and databases, researchers can predict potential enzymes with desired properties, shortening the time required to identify promising candidates for biocatalysis.

In a study conducted by Vanacek *et al.*, computational methodologies proved highly effective in facilitating the exploration of a vast pool of protein sequences (Fig. 10).¹⁵⁴ This capability addressed a critical need in the postgenomic era, characterized by an exponential surge in available protein sequences, representing an untapped source of diverse enzyme catalysts. Despite the immense potential for biological and biotechnological discoveries, only a small fraction of these sequences has undergone experimental characterization.¹⁵⁵ To efficiently harness this genomic wealth stored in public databases, the research team devised an integrated system comprising an automated *in silico* screening protocol and experimental procedures. This system aimed to unlock the structural and functional diversity of entire enzyme families



Example 1. Enzyme discovery and screening

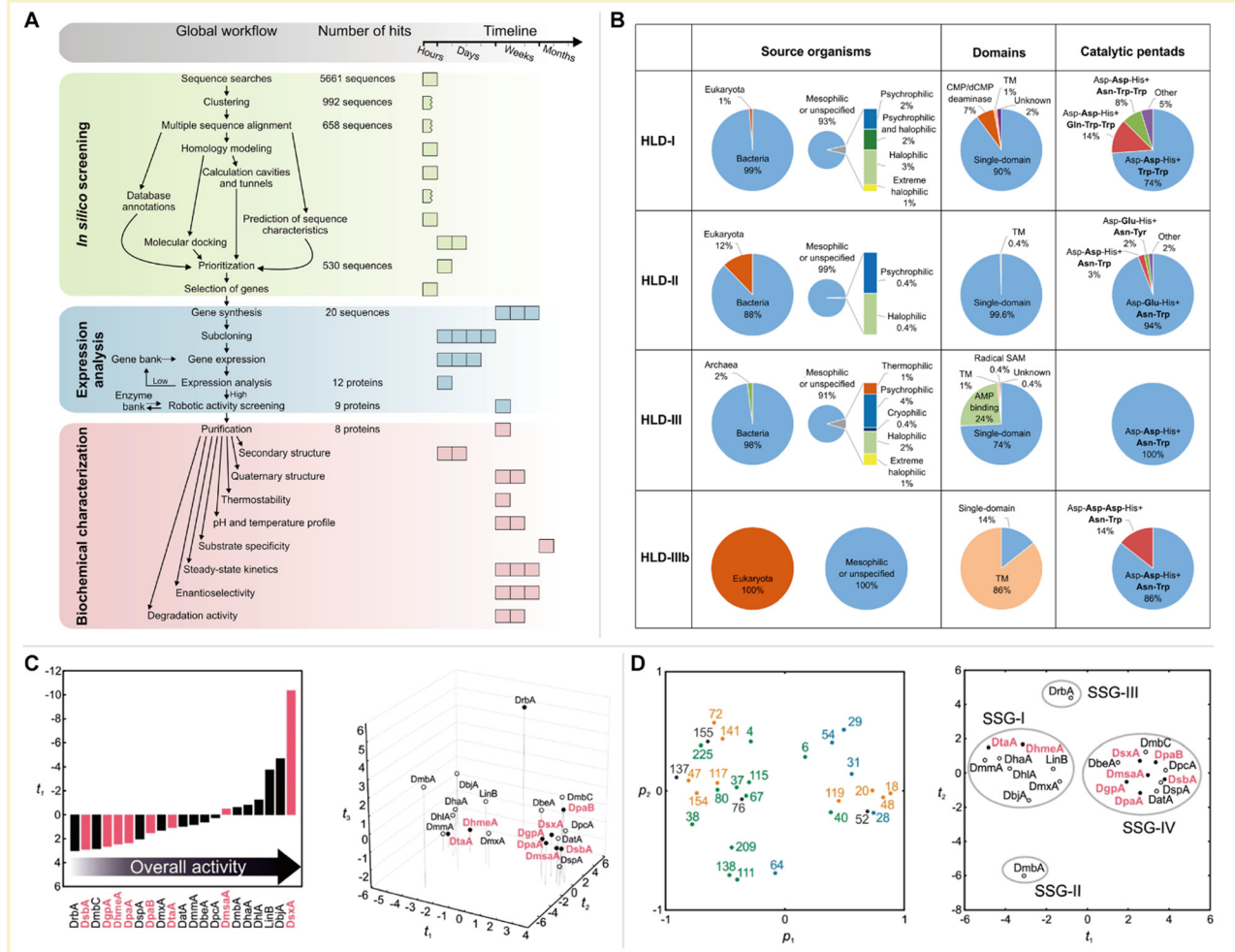


Fig. 10 Representative example of enzyme discovery and screening for advanced enzyme biocatalysis. (A) Workflow of an integrated system for the exploitation of enzyme structural and functional diversity. (B) Overview of putative haloalkane dehalogenases (HLDs) identified. (C) Comparison of the substrate specificities and overall catalytic activities of novel HLDs with previously characterized enzymes using multivariate statistics. (D) The loading plot p_1/p_2 and the corresponding score plot t_1/t_2 from principal component analysis (PCA) of the transformed data set. Reproduced with permission from ref. 154. Copyright 2023, Springer Nature.

(Fig. 10A). As a proof of concept, the study focused on exploring the diversity within the microbial enzyme group known as haloalkane dehalogenases (EC 3.8.1.5, HLDs).

The workflow of this integrated system encompassed three distinct phases: (1) automated sequence and structural bioinformatics, (2) protein production and robotic activity screening, and (3) biochemical characterization. Beginning with a staggering 5661 sequences, the researchers successfully identified 20 sequences corresponding to putative HLDs. These sequences provided valuable information about their source organisms, catalytic pentads, and domain compositions (Fig. 10B). Following expression analysis and robotic activity screening, a mere eight proteins emerged as candidates for comprehensive biochemical and biophysical characterization. By comparing the substrate specificities and overall catalytic activities of these novel HLDs with previously characterized enzymes using

advanced multivariate statistics (Fig. 10C and D), the team achieved significant milestones. These included the discovery of the most catalytically proficient native HLD to date ($k_{\text{cat}}/K_{0.5} = 96.8 \text{ mM}^{-1} \text{ s}^{-1}$, where k_{cat} is the catalytic constant and $K_{0.5}$ is the substrate concentration that leads to half of the maximal velocity), the identification of the most thermostable enzyme boasting a melting temperature of 71°C , the characterization of three distinct cold-adapted enzymes exhibiting dehalogenase activity at near-zero temperatures, and the development of a biocatalyst capable of degrading the warfare chemical sulfur mustard. This study exemplifies the vast potential of computational approaches in addressing the big data challenge presented by the postgenomics era.

4.2.2 Computational design. Computational design, or computation-aided enzyme engineering, has emerged as a transformative approach for precisely tailoring enzymes

to specific tasks. This method involves the *in silico* manipulation of enzyme structures and active sites to enhance their catalytic efficiency or expand their substrate specificity. By harnessing computational insights, researchers can predict with remarkable accuracy how modifications at the molecular level will impact enzyme function. This enables the creation of customized biocatalysts with greatly improved properties, exemplifying the power of computational biology in advancing enzyme biocatalysis for pharmaceutical applications.^{105,156,157}

In a groundbreaking study led by Lu *et al.*, computational methodologies emerged as a potent tool for the engineering of PET hydrolases, enabling them to exhibit robustness across a wide pH and temperature range, accelerated reaction rates, and the unique ability to directly degrade untreated postconsumer plastics (Fig. 11).¹⁵⁸ To achieve this remarkable feat, the research team harnessed the power of a structure-based machine learning algorithm, applying it to engineer a highly robust and active PET hydrolase. Specifically, the researchers employed a cutting-edge three-dimensional self-supervised convolutional neural network, known as MutCompute,¹⁵⁹ to pinpoint stabilizing mutations within the enzyme (Fig. 11A). This innovative approach allowed them to identify positions within the enzyme's crystal structure where the original amino acid residues were suboptimal compared to potential substitutions, and these positions were ranked based on predicted probabilities (Fig. 11B). Subsequently, the team uncovered four pivotal mutations (S121E, T140D, R224Q, and N233K) that held the key to enhancing the PET-hydrolytic activity and expanding its operational temperature range. Notably, the machine-learning-guided predictions led to a significant enhancement in enzymatic activity across all tested conditions, spanning temperatures from 30 to 60 °C (Fig. 11C).

To gain further insights into the enhanced stability and catalytic prowess of the engineered enzyme, crystal structure analysis was conducted on the top-performing variant, aptly named FAST-PETase (PDB 7SH6, where PDB stands for Protein Data Bank), which contained a total of five mutations, including those identified through predictions (N233K/R224Q/S121E) and scaffold-derived mutations (D186H/R280A). The structural analysis revealed the formation of favorable residue interactions that contributed to FAST-PETase's superior PET-hydrolytic activity compared to both the wild-type enzyme and previously engineered variants (Fig. 11D).

This groundbreaking study illustrates how the integration of computational biology can revolutionize enzyme engineering, resulting in enzymes with enhanced functionalities that were previously unattainable through traditional experimental methods. Such an approach holds immense promise not only in the realm of sustainable plastic degradation but also in diverse practical applications, including the pharmaceutical industry. The ability to tailor enzymes with precision and efficiency opens new avenues for addressing complex challenges in drug discovery, development, and manufacturing.

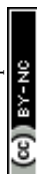
4.2.3 Molecular modelling and simulation. Molecular modeling and simulation have become indispensable tools for gaining insights into enzyme behavior at the atomic level.

These techniques allow researchers to visualize enzyme–substrate interactions, study reaction mechanisms, and predict enzyme stability under different conditions.^{151,160,161} MD simulations provide a particularly dynamic view of enzyme dynamics and conformational changes during catalysis, offering valuable information for enzyme engineering and optimization.^{162,163} In a study conducted by Chen *et al.*, computational methodologies emerged as powerful tools for unraveling the molecular mechanisms underpinning enzyme promiscuity (Fig. 12).¹⁶⁴ The research focused on the aromatic prenyltransferase (aPTase) AtaPT, which displayed an unprecedented level of substrate promiscuity, accommodating a wide range of aromatic acceptors and prenyl donors (C₅–C₂₀), including dimethylallyl, geranyl, farnesyl, geranylgeranyl, and phytyl pyrophosphates. To decipher the molecular basis of AtaPT's remarkable promiscuity, the team embarked on a multifaceted investigation, including three-dimensional (3D) molecular structure determination through crystallography (Fig. 12A, PDB 4LD7). Their comparative analysis of the apo structure with substrate-binding pocket complex structures revealed dynamic conformational changes in the tyrosine shield and various acceptor binding sites of AtaPT (Fig. 12B). These insights, combined with molecular simulation studies, shed light on the enzyme's multiple states and substantial fluctuations, particularly in the loop 1 and tyrosine shield regions, offering valuable clues regarding its promiscuous enzymatic activity.

Furthermore, the research team conducted mutagenesis experiments to pinpoint the residues responsible for prenylation promiscuity (Fig. 12C). Armed with a profound understanding of AtaPT's molecular mechanisms governing prenylations, it became feasible to employ structure-guided mutagenesis to fine-tune both the prenyl donor and the prenylation selectivity of this enzyme. Such precision engineering has the potential to enhance the utility of AtaPT by synthesizing novel prenylated derivatives, opening new avenues in drug discovery programs and beyond. This study exemplifies how computational methodologies can provide invaluable insights into enzyme behavior, offering a strategic advantage in various scientific and industrial applications.

In summary, computational biology has revolutionized enzyme biocatalysis in pharmaceutical applications by enabling efficient enzyme discovery and screening, precise computational design, and insightful molecular modeling and simulation. These computational approaches have accelerated the identification of novel enzymes, facilitated tailored biocatalyst engineering, and deepened our understanding of enzymatic behaviour. Further exemplification of enzyme engineering can be found in Table 3, where additional instances are provided.^{52,103,165–180} As the pharmaceutical industry seeks sustainable, efficient, and precise solutions, computational biology emerges as a transformative tool, bridging the gap between theory and experiment and propelling innovation in drug development and production.

The critical point for computational biology is that the validation of computational predictions through rigorous



Example 2. Computational design

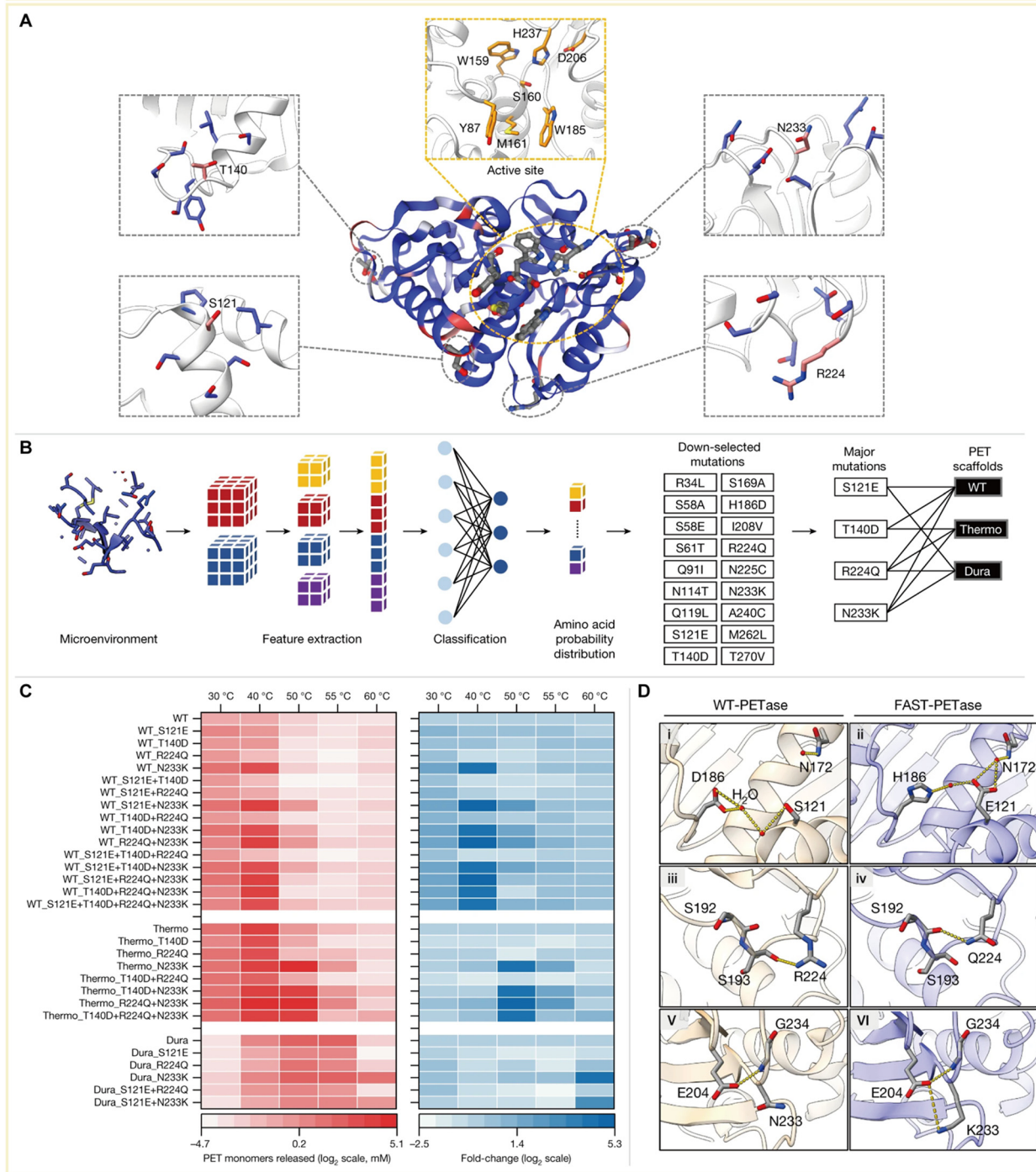


Fig. 11 Representative example of computational design for advanced enzyme biocatalysis. (A) WT PETase protein structure rendered by the output of MutCompute. (B) Predictions based on both WT PETase and ThermoPETase were ranked by the fold change in the probabilities between the predicted and the WT amino acid. (C) The red heatmap shows the PET-hydrolytic activity of the resulting variants, and the blue heatmap shows the fold change of activity over their respective scaffolds based on the total PET monomers from the hydrolysis of circular gf-PET films by the PETase variants. (D) Predicted mutations from the neural network algorithm to stabilize FAST-PETase. Reproduced with permission from ref. 158. Copyright 2022, Springer Nature.

experimental testing is imperative to establish the trustworthiness of designed enzymes. Reliable experimental validation not only verifies the accuracy of computational models but also provides valuable feedback loops for refining these models. As

the complexity of biological systems increases, it becomes crucial to address the limitations of current computational methodologies. Factors such as protein dynamics, solvent effects, and long-range interactions pose significant challenges



Example 3. Molecular modeling and simulation

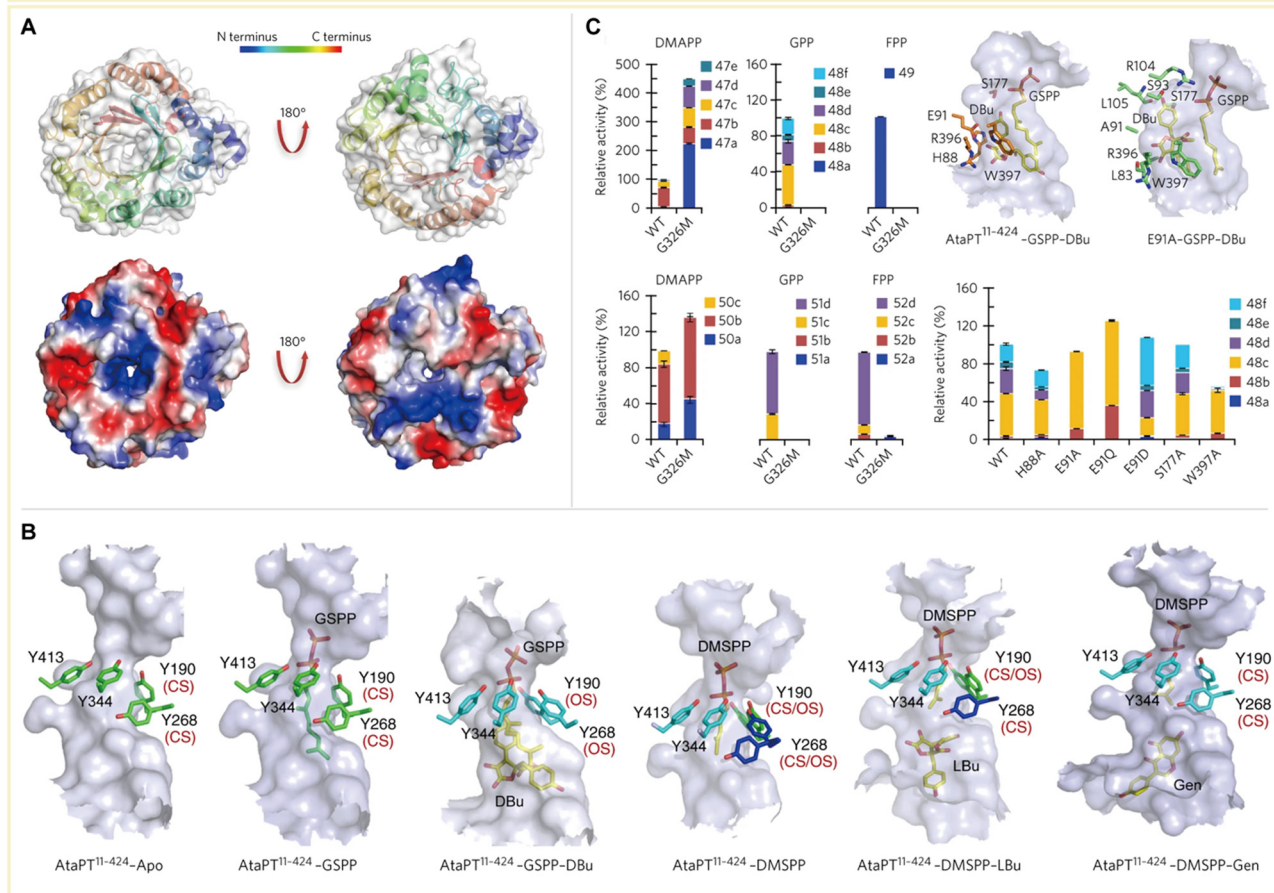


Fig. 12 Representative example of molecular modeling and simulation for advanced enzyme biocatalysis. (A) Crystal structure of AtaPT and its catalytic chamber. (B) Conformational dynamics of the tyrosine shield and various acceptor binding sites of AtaPT. (C) Structure-guided mutagenesis of AtaPT to alter its substrate promiscuity. Reproduced with permission from ref. 164. Copyright 2017, Springer Nature.

to accurate predictions. Understanding these complexities and developing computational approaches that can capture them will be essential for advancing the field.

4.3 Nanoarchitectonics for advanced enzyme biocatalysis

Nanoarchitectonics is an interdisciplinary technological paradigm, initially proposed by Masakazu Aono.¹⁸¹ This innovative concept revolves around the meticulous design and construction of structures and materials on the nanoscale, where precision and organization are important. Nanoarchitectonics is driven by synthesis techniques, such as chemical synthesis, molecule manipulation, chemical nano-manipulation, self-assembly, and self-organization, to craft functional materials from fundamental building blocks at the nanoscale.^{182,183}

This versatile concept extends its range into various fields and applications. Notably, it has been employed in the design and synthesis of catalysts spanning a diverse spectrum, including photocatalysts, environmental catalysts, electrochemical catalysts, and biocatalysts. Beyond catalysis, the applications of nanoarchitectonics extend to material production, structural regulation, device fabrication, sensor development, energy

harnessing, environmental science, and even the biological and biomedical realms.³⁵

In particular, nanoarchitectonics is widely considered for sensing various biomolecules. Recently, microRNA (miRNA) has emerged as prominent biomolecule for next generation of diagnostic and prognostic biomarkers. Numerous RNA detection techniques based on engineered nanomaterials-based electrochemical biosensing strategies, such as gold nanoparticle, carbon-based nanomaterials, quantum dots (QDs), metal-organic frameworks (MOF) show huge potential for cancer management.¹⁸⁴

One of the distinguishing facets of the nanoarchitectonics concept is its dynamic and harmonized nature.^{185,186} At the nanoscale, there are uncertainties, including thermal fluctuations, static distributions, and quantum effects, so a balanced harmonization incorporating dynamism becomes crucial within the framework of nanoarchitectonics. The dynamics bears a striking similarity to the intricacies observed in biological systems, where functional components dynamically cooperate and harmonize their operations.¹⁸⁷ Consequently, nanoarchitectonics appears exceptionally promising in catalyst design that is tailored for biological functions.

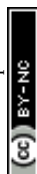




Table 3 Selected examples of computational biology for advanced enzyme biocatalysis

Example	Applications	Enzyme	Key features	Detailed information	Ref.
(a) Enzyme discovery and screening Substrate-specific enzyme design	Peptide synthesis, drug discovery, protein engineering	Subtilisin Carlsberg	• Improved enantioselectivity and stability • Virtual screening and molecular docking	• <i>In silico</i> screening methods to design enzymes with improved substrate specificity • Specific mutations to enhance affinity and selectivity through substrate screening and computational analysis of binding interactions	165
	Lipid modification, drug synthesis	Candida rugosa lipase	• Expanded substrate specificity and catalytic efficiency • <i>In silico</i> screening and molecular docking	• <i>In silico</i> screening and molecular docking to engineer the substrate binding pocket • Mutations to enhance substrate specificity and catalytic efficiency for drug synthesis through virtual screening and computational analysis	166
	Cancer treatment, drug discovery	Dihydrofolate reductase (DHFR)	• High affinity and specificity • <i>In silico</i> screening and molecular docking	• <i>In silico</i> screening and molecular docking to design DHFR inhibitors for pharmaceutical applications • Identification of potential inhibitors through virtual screening against target enzyme and binding interaction analysis	167
	Drug synthesis, biotransformation	Alcaligenes faecalis nitrilase	• Understanding substrate preferences and promiscuity • <i>In silico</i> screening and molecular docking	• <i>In silico</i> screening and molecular docking to analyze the substrate promiscuity of enzymes • Substrate preferences and promiscuity study by virtual screening and computational analysis of binding interactions for pharmaceutical synthesis	168
Substrate docking and reactivity analysis	Drug metabolism, pharmacokinetics, personalized medicine	Human cytochrome P450 2D6 (CYP2D6)	• Understanding substrate binding and metabolism • <i>In silico</i> docking simulations and reactivity analysis	• <i>In silico</i> docking and reactivity analysis to study the binding and metabolism of substrates by enzymes • Investigation of binding affinity and regioselectivity through virtual docking simulations and computational analysis for insights into drug metabolism and drug-drug interactions	169 and 170
(b) Computational design					
Engineered lipase	Chiral drug synthesis, enzymatic resolution	Candida antarctica lipase B	• Improved substrate specificity and catalytic activity	• Prediction and introduction of specific amino acid substitutions in the active site of the enzyme using computational modeling and MD simulations • Enhanced activity and selectivity towards a range of pharmaceutical substrates, enabling efficient synthesis of enantiopure drug intermediates	171 and 172
Engineered oxidoreductase	Chiral synthesis, pharmaceutical intermediate production	Ketoreductase ChKRED12	• Altered co-factor specificity • Improved catalytic efficiency	• Computational analysis of active site and molecular docking simulations to modify co-factor binding and enhance catalytic efficiency • Improved activity and selectivity of chiral alcohol synthesis for pharmaceutical applications, facilitating more efficient and sustainable routes	52
Engineered esterase	Ester hydrolysis for drug synthesis	Pseudomonas fluorescens esterase	• Enhanced substrate specificity and stability • Increased enantioselectivity	• Computational methods, including homology modeling and MD simulations, to explore the active site and predict mutations • Enhanced catalytic activity and selectivity towards specific substrates, enabling efficient enzymatic synthesis of pharmaceutical intermediates	173
Engineered halogenase	Halogenated compound synthesis, drug lead optimization	CYP450	• Altered substrate specificity and regioselectivity	• Computational modeling and MD simulations to study the active site and predict mutations for altering substrate specificity and regioselectivity • Improved activity and selectivity in halogenation reactions, enabling the synthesis of halogenated compounds for drug development	103
Engineered decarboxylase	Aromatic compound, drug precursor production	O-Succinylbenzoate synthase	• Altered substrate specificity • Improved catalytic efficiency	• Computational methods to study active site and identify key residues in substrate binding and catalysis • Improved activity in synthesis of valuable aromatic compounds for pharmaceutical applications	174



Table 3 (continued)

Example	Applications	Enzyme	Key features	Detailed information	Ref.
(c) Molecular modeling for Antibiotic resistance studies, antibiotic design	Molecular modeling and simulation Antibiotic resistance studies, antibiotic design, optimization	Beta-lactamase	• Molecular modeling and simulation for substrate specificity analysis	• Computational modeling and simulation methods to investigate the binding interactions with different beta-lactam antibiotics	175 and 176
Understanding chitin substrate interactions in drug delivery	Chitin degradation, drug delivery systems	Chitinase	• MD simulations for substrate binding and catalytic mechanism analysis	• Analysis for insights into the enzyme's substrate specificity and molecular determinants governing substrate recognition and binding • MD simulations to study the substrate binding process and catalytic mechanism	177
Computational analysis for neurological disorders	Alzheimer's disease treatment, drug discovery for neurological disorders	Acetylcholinesterase	• Molecular docking and binding free energy calculations for substrate binding analysis	• Insights into dynamics of substrate binding, role of key residues in catalysis, and binding energy landscape of different chitin substrates • Computational methods, including molecular docking and binding free energy calculations, to investigate binding with substrates or inhibitors	178
Enzyme design for pharmaceutical synthesis	Glycosylation reactions, drug conjugation	Glycosyltransferase	• Homology modeling and MD simulations for substrate binding analysis	• Analysis for insights into enzyme-substrate/inhibitor binding modes and key residues in binding affinity • Homology modeling and MD simulations to investigate substrate binding process	179
Virtual screening for drug metabolism studies	Drug metabolism, pharmacokinetics studies, drug design and optimization	Cytochrome P450	• Virtual screening and molecular docking for substrate identification and binding analysis	• Analysis for insights into binding with various donor and acceptor substrates, elucidating the structural basis for substrate recognition and specificity • Virtual screening techniques and molecular docking simulations to identify potential substrates for enzymes and predict their binding modes • Analysis for insights into substrate preference and binding interactions, enabling the identification of novel drug metabolites or optimization of activities	180

Nanoarchitectonics for advanced enzyme biocatalysis

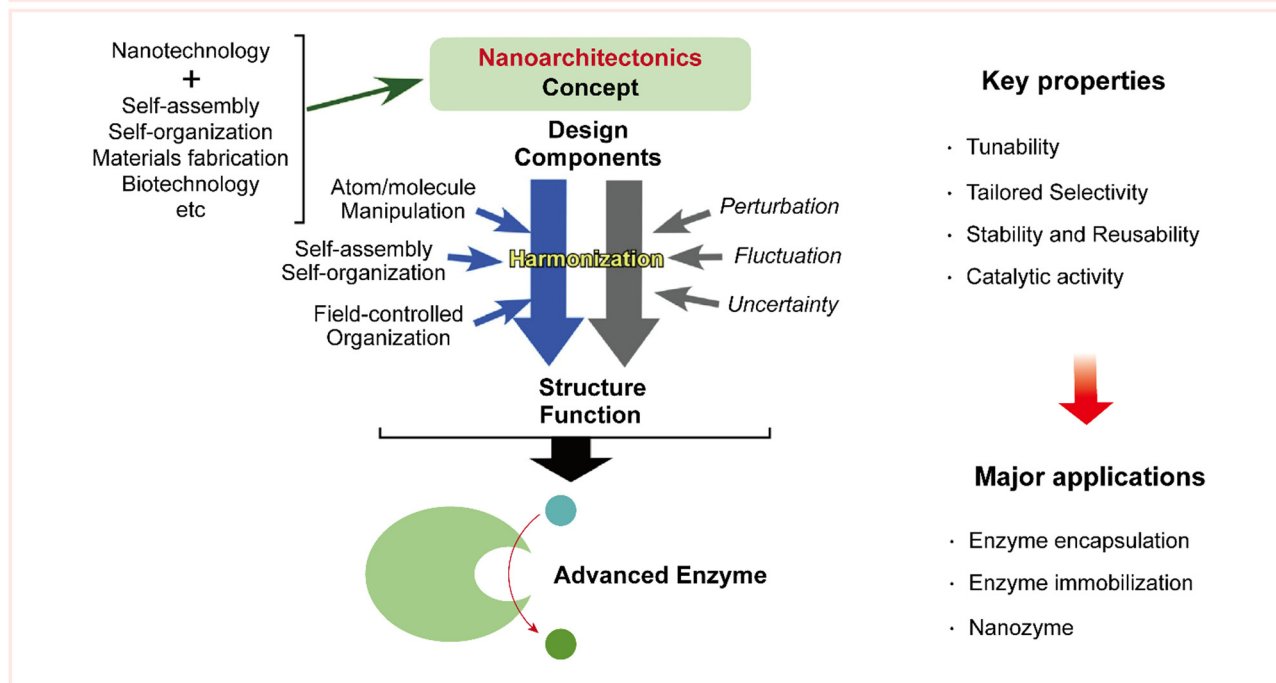


Fig. 13 Illustration of the nanoarchitectonics concept for advanced enzyme biocatalysis. Reproduced with permission from ref. 35. Copyright 2019, Elsevier.

Example 1. Enzyme encapsulation

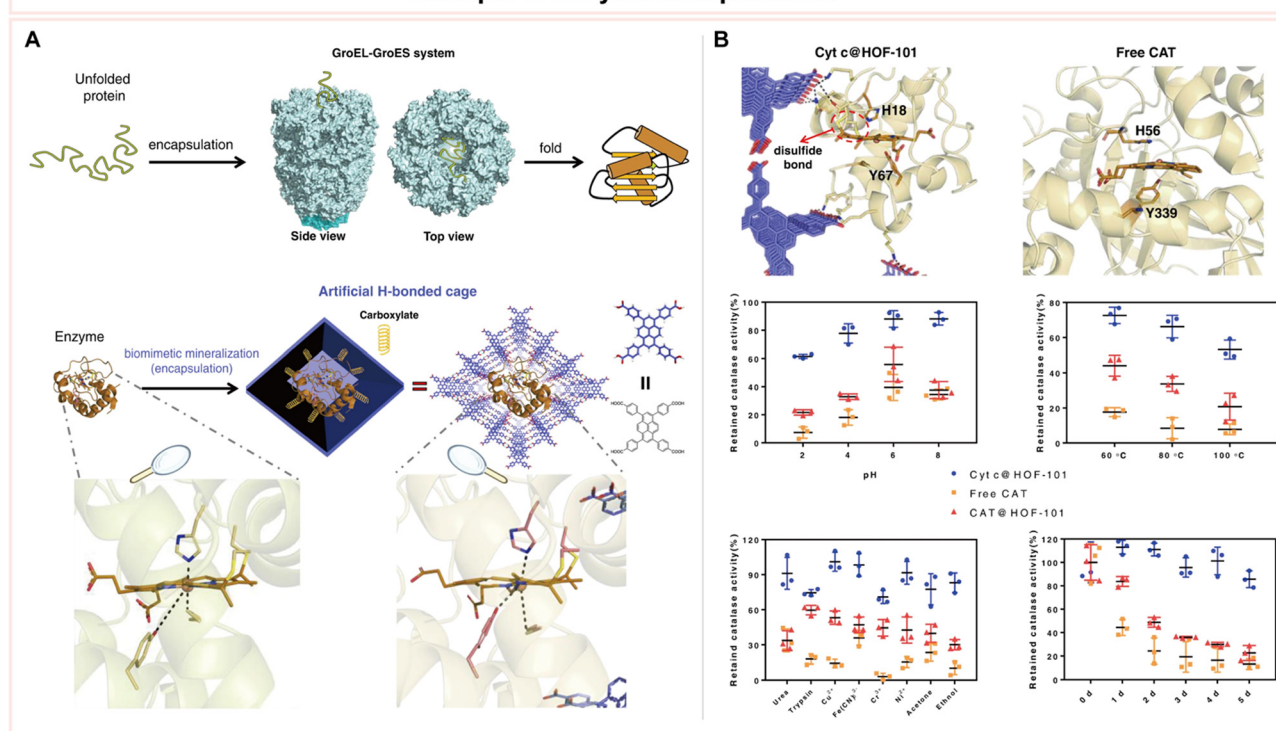


Fig. 14 Representative example of enzyme encapsulation for advanced enzyme biocatalysis. (A) Schematic illustration of the technique for modulating the conformation of an enzyme by H-bonded cage encapsulation. (B) Structure of the heme macrocycle of Cyt c@HOF-101 based on MD simulation and its catalase-like biocatalysis performance. Reproduced with permission from ref. 190. Copyright 2022, Springer Nature.

For instance, in nanoarchitectonics, integration of hydrogel into common biosensing platforms creates soft structures that are both physically and chemically regulated. These structures boast superior biocompatibility, improved immobilization of biomolecules, and the ability to design biosensors that are specific and highly sensitive. The physical and chemical properties of 3D hydrogel structures can be modified by integrating with nanostructures, increasing their sensitivity to various stimuli like mechanical, optical, thermal, magnetic, and electric forces.¹⁸⁸

Porous materials derived from coordination compounds like metal-organic frameworks (MOFs) and porous coordination polymers (PCPs) possess well-defined pore structures and promising properties. As these coordination compounds, Prussian blue (PB) and its analogues (PBA) exhibit excellent physical and chemical properties, making them versatile nanoarchitectonics for a wide range of applications including sensing, batteries, biomedicine, and imaging.¹⁸⁹

In the context of enhancing enzyme biocatalysis, nanoarchitectonics has emerged as a dynamic fusion of nanotechnology,

materials science, and enzymology (Fig. 13).³⁵ It offers a compelling route to not only amplify catalytic activity but also strengthen enzyme stability, bolster reusability, and enhance versatility. In this part, we will describe in more detail the three central strategies encompassed by nanoarchitectonics for advanced enzyme biocatalysis: enzyme encapsulation, enzyme immobilization, and nanozymes. These strategies harness the principles of nanoarchitectonics to revolutionize the world of biocatalysis and hold immense promise for a multitude of applications across diverse industries.

4.3.1 Enzyme encapsulation. Enzyme encapsulation for advanced enzyme biocatalysis refers to the process of enclosing or trapping enzymes within protective materials, typically on the nanoscale, to enhance their stability, activity, and suitability for various catalytic applications. This technique offers several advantages for enzyme-based applications in various fields such as pharmaceuticals, the food industry, and diagnostics.

Chen *et al.* designed an external hydrogen-bonded organic framework that could modulate the shape of the enzyme cytochrome *c* (Cyt *c*) (Fig. 14).¹⁹⁰ This structural alteration

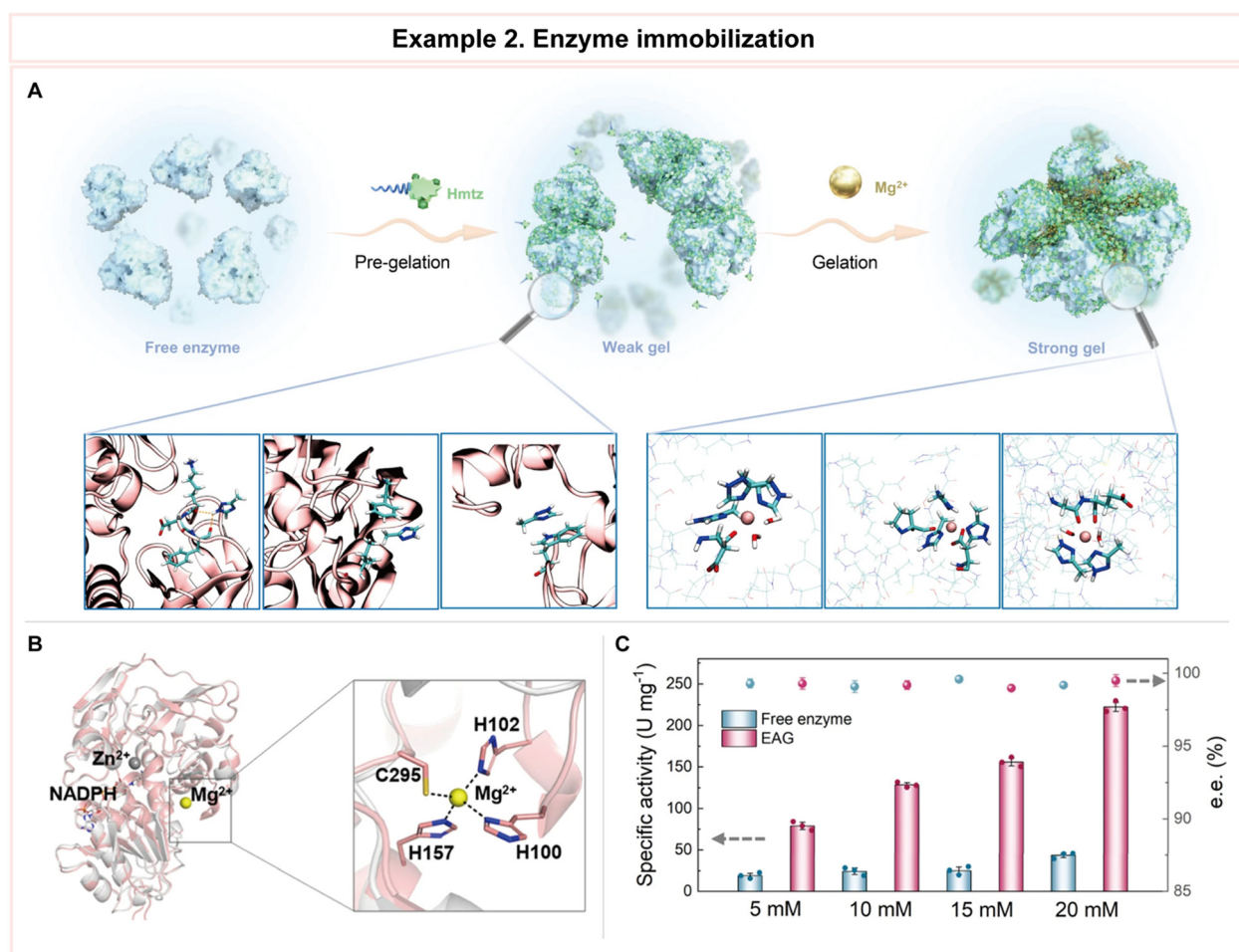


Fig. 15 Representative example of enzyme immobilization for advanced biocatalysis. (A) Schematic illustration of enzyme assembled hydrogel (EAG) preparation using 1H-3-methyl-1,2,4-triazole (Hmtz) and Mg²⁺. (B) Overlay of the 7XY9 (salmon) and 1YKF (gray) structure showing the coordination of the Mg²⁺ ion with the adjacent residues. (C) Specific activity and chiral selectivity values of the free enzyme and EAG when catalyzing the conversion of acetophenone with concentrations of 5–20 nM. Reproduced with permission from ref. 191. Copyright 2019, Springer Nature.



enables the enzyme to exhibit functions that it could not naturally perform. A hydrogen-bonded organic framework, which is sturdy and has carboxylate molecules arranged in a specific way, is introduced onto the native Cyt *c* (Fig. 14A). The resultant hydrogen bonded nano-biointerface causes Cyt *c* to adopt a catalase-like conformation, which it typically could not take on when interacting with porous organic frameworks. The heme macrocycle of the Cyt *c* nanosystem in this paper is more structurally stable than those in other heme enzymes such as catalase (CAT). Also, this hydrogen-bonded organic framework continues to stabilize the enzyme, ensuring its structural integrity and activity (Fig. 14B).

This research introduces a novel nanotechnological concept, which involves controlling the flexible shapes of enzymes. It demonstrates how artificial hydrogen-bonded frameworks can

be used to modify enzyme activity, highlighting the benefits of using such scaffolds to modulate enzyme behavior. In essence, the study explores a new way to manipulate the structure and function of enzymes for various practical applications.

4.3.2 Enzyme immobilization. Enzyme immobilization for advanced enzyme biocatalysis is a technique that involves fixing or anchoring enzymes to a solid support or within a matrix to enhance their stability, activity, and reusability in various catalytic processes. This method plays an essential role in biotechnology, pharmaceuticals, and industrial applications where enzymes are used as catalysts for specific reactions.

In an important example, Chen *et al.* presented a feasible approach that leverages the combined action of triazoles and metal ions to trigger the creation of porous hydrogels

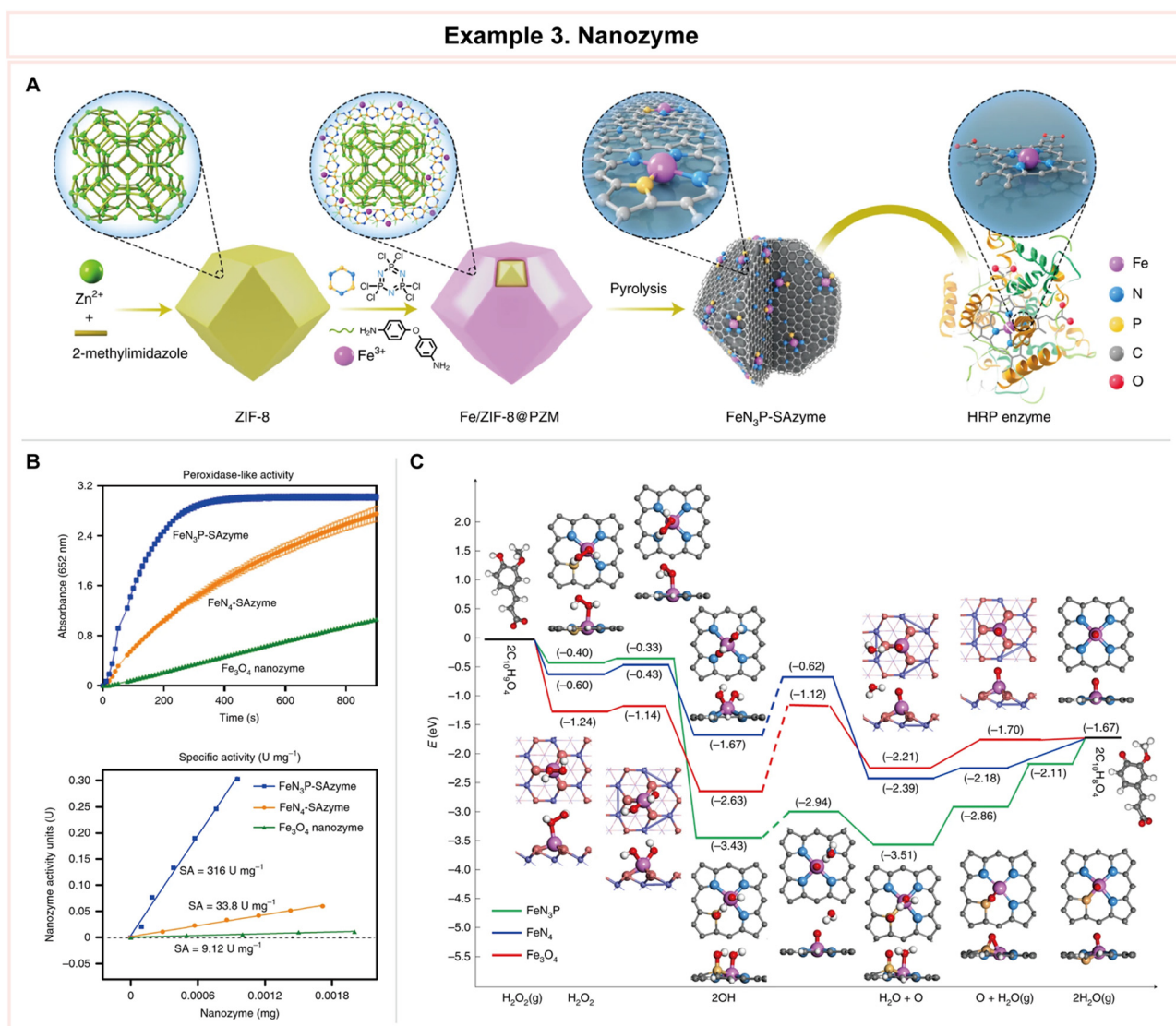


Fig. 16 Representative example of nanozyme for advanced enzyme biocatalysis. (A) Schematic illustration of the preparation process for FeN₃P-SAzyme. (B) Reaction-time curves of the colorimetric reaction and comparison of the specific activities of FeN₃P-SAzyme, FeN₄-SAzyme, and Fe₃O₄ nanozyme. (C) Density functional theory (DFT) studies and energy profile of FeN₃P-SAzyme, FeN₄-SAzyme and Fe₃O₄ nanozyme. Reproduced with permission from ref. 192. Copyright 2021, Springer Nature.



Table 4 Selected examples of nanoarchitectonics for advanced enzyme biocatalysis

Example	Applications	Enzyme	Key features	Detailed information	Ref.
(a) Enzyme encapsulation Metal-organic framework (MOF)	Pharmaceuticals, gas storage	Cytochrome <i>c</i> (Cyt <i>c</i>)	• A porous, crystalline material composed of inorganic nodes and organic linkers • MOF could be also used as solid supports for enzyme immobilization	• Common approaches: surface adsorption, covalent attachment, precipitation & biomimetic mineralization, diffusion into MOF pores/channels and • Extremely high Brunauer–Emmett–Teller (BET) surface areas compared with other porous materials up to 7000 m² g^{−1} • Glucose oxidase (GOx) loaded liposome for starvation therapy shows high enzymatic activity • Cyclic RCD (cRGD) functionalized liposome encapsulated with uronidase for thrombolysis	194 and 195
Liposomal vesicle	Pharmaceuticals, food industry	α -Amylase	• Encapsulation efficiency is influenced by liposome number and size • Hydrophilic, amphiphilic, and hydrophobic enzymes can all be loaded into liposomes • In non-aqueous environments, hydrogels are essential for creating the favorable environment required by most enzymes	• Insulinase entrapped in calcium alginate–gelatin beads exhibits better thermal stability and reusability	196 and 197
Hydrogel	Diagnostics, biomedical engineering	Horseshoe peroxidase (HRP)	• Most of natural polymers for hydrogels, are biocompatible, biodegradable, mechanically flexible, and renewable • Various strategies inspired by viral capsid assembly and genome loading mechanisms have been developed to achieve greater and more controlled enzyme loading • Encapsulation method: passive, electrostatic interaction, RNA packing signal, scaffold protein (SP), covalent conjugation	• Lacase with synthetic polymer hydrogel composed of HEMA, ITA, and ECAA for wastewater treatment • GFP fused with positively charged tag is directed to inside of the AaLS-neg cage <i>via</i> electrostatic interaction • Co-encapsulation of SP-tagged alcohol dehydrogenase (AdhD) and wild-type SP, with controlled stoichiometry is achieved by modulating the input ratio	198 and 199
Viral capsid/virus like particle (VLP)	Pharmaceuticals, diagnostics	<i>Candida antarctica</i> lipase B (CALB)	• Biomimetic synthesis of silica nanoparticles is inspired by bio mineralization in marine organisms, such as diatoms • Size and morphology of the particle could be tuned by controlling the initiator molecular weight and the precipitation conditions	• Cys-R5 peptide <i>via</i> a disulfide bond are incorporated into silica particles. • Co-precipitation by means of physically mixing the protein with the R5 peptide	200 and 201
Biomimetic silica	Pharmaceuticals, diagnostics	Protease	• Physical adsorption on hydrophobic media is most often used to immobilize lipase • Lipase immobilization for biofuel production in mesoporous silica nanoparticles increases thermal and mechanical stability • Various proteases immobilized in alginate–chitosan beads exhibits reasonable stability and good activity • Catalase embedded into single crystalline ZIF-90 crystals shows activity in hydrogen peroxide	• Physical adsorption on hydrophobic media is most often used to immobilize lipase • Lipase immobilization for biofuel production in mesoporous silica nanoparticles increases thermal and mechanical stability • Various proteases immobilized in alginate–chitosan beads exhibits reasonable stability and good activity • Catalase embedded into single crystalline ZIF-90 crystals shows activity in hydrogen peroxide	204 and 205
(b) Enzyme immobilization Immobilization of lipase	Pharmaceuticals, food industry	<i>Candida antarctica</i> lipase B (CALB)	• Reusability • Regeneration • Enhanced stability • Reduced autolysis • Tailored selectivity • Enhanced stability	• Immobilization of deoxyadenosine kinase from dictyostelium discoideum (DddAK) by ionic interaction on an aminated epoxy functionalized support enhances stability under high pH and temperature conditions • A gold-binding polypeptide (GBP)-linked kinase immobilized on gold surface shows kinase assay sensitivity	206 and 207
Immobilization of protease	Pharmaceuticals, detergents, food industry	Protease from <i>Bacillus subtilis</i>	• Reusability • High selectivity • Enhanced stability	• Recombinant NstCII esterase adsorbed on accurel MP1000 exhibits high immobilization efficiency and storage stability under various conditions • Est684, a cold adapted esterase, immobilization enhances thermostability and reusability for bioremediation	208 and 209
Immobilization of kinase	Pharmaceuticals, diagnostics	Protein kinase A (PKA)	• Reusability • High selectivity • Enhanced stability	• Recombinant NstCII esterase adsorbed on accurel MP1000 exhibits high immobilization efficiency and storage stability under various conditions • Est684, a cold adapted esterase, immobilization enhances thermostability and reusability for bioremediation	210 and 211
Immobilization of esterase	Pharmaceuticals, bioremediation, paper industry	Carboxylesterase (CEs)	• Reusability • High activity	• Recombinant NstCII esterase adsorbed on accurel MP1000 exhibits high immobilization efficiency and storage stability under various conditions • Est684, a cold adapted esterase, immobilization enhances thermostability and reusability for bioremediation	212 and 213





Table 4 (continued)

Example	Applications	Enzyme	Key features	Detailed information	Ref.
Immobilization of aldolases	Bioremediation, diagnostics, organic synthesis	2-Deoxy-D-ribose-5-phosphate aldolase (DERA)	- Enhanced stability - Longer lifespan - Cost effective	- Threonine aldolase immobilization on different supports for productive, cost-efficient enzymatic microreactors - Fixation of DERA-bearing thin film on a polymeric membrane support to gain atorvastatin efficiently	211 and 212
(c) Nanozymes					
Gold nanozymes	Pharmaceuticals, diagnostics	Peroxidase, catalase, reductase	- Tunability - Biocompatibility	- A simple, sensitive and selective label-free colorimetric sensing of malathion using palladium-gold nanorod as nanozyme - A mesoporous carbon-gold hybrid nanoprobes for real-time imaging, photothermal/photodynamic and nanozyme oxidative therapy	213 and 214
Iron oxide nanozymes	Pharmaceuticals, diagnostics	Peroxidase, catalase	- Cost effectiveness - Non-toxic and allergenic - High stability	- Yan's group discovered that iron oxide nanoparticles have an intrinsic peroxidase-like activity and could be used as a natural enzyme substitute - Gu's group showed that iron oxide nanozymes perform peroxidase-like activity under acidic pH and catalase-like activity under neutral pH	215
Magnetic nanozymes	Pharmaceuticals, diagnostics	Peroxidase, catalase, oxidase	- Tunability - High stability - Cost-effective	- Cobalt(II,III) oxide (Co₃O₄) nanotubes, prepared by electrospinning, show oxidase-like activity, peroxidase-like activity, and catalase-like activity - An aptamer conjugated PtCo bimetallic nanoparticle has improved oxidase-like catalytic activity for cancer-cell detection without the destructive H₂O₂	216 and 217
Quantum dot (QD) nanozymes	Pharmaceuticals, diagnostics	Peroxidase, catalase, oxidase	- High activity - High stability - Low toxicity	- Mo, S co-doped carbon quantum dots (Mo-CQDs) as a peroxidase mimic detects cholesterol with excellent selectivity and high sensitivity in the range of 0.01–1.0 mM - Graphene oxide (GO) nanosheets, GO quantum dots (GOQDs), as nanozymes, efficiently reduce reactive oxygen species (ROS) and H₂O₂ in 1-methyl-4-phenyl-pyridinium ion (MPP⁺)-induced PC12 cells	218 and 219
MOF nanozymes	Pharmaceuticals, diagnostics	Peroxidase, catalase, oxidase, hydrolases	- High activity - High stability - High stability - Low toxicity	- Hierarchically structured and highly dispersible MOF nanozymes showed enhanced peroxidase-like activity for persistent chemiluminescence immunoassay - Ultrasensitive detection of pathogenic bacteria using a 2D MOF nanozyme with peroxidase-like activity	220 and 221

assembled with enzymes (Fig. 15).¹⁹¹ They developed an enzyme-assembled hydrogel (EAG) by adding 1*H*-3-methyl-1,2,4-triazole (Hmtz) and Mg^{2+} ions to the enzyme solution before the pre-gelation and gelation processes, respectively (Fig. 15A). Specifically, when they tested the catalytic efficiency of EAG in reducing acetophenone, it was an impressive 6.3 times higher than that of the free enzyme. Furthermore, they showed the reusability of this hydrogel by demonstrating its sustained high catalytic activity even after undergoing 12 cycles of use. To gain a deeper understanding of the structure of the enzyme within the hydrogel, they employed cryo-EM, achieving a near-atomic resolution of 2.1 Å (PDB: 7XY9). With these results, they analyzed the differences in enzyme activity and stability, before (1YKF) and after (7XY9) gelation. An overlay of the 7XY9 (salmon) and 1YKF (gray) structures shows the coordination of the Mg^{2+} ion with the adjacent residues (Fig. 15B). Also, EAG exhibited a higher specific activity than that of the free enzyme in various concentrations of acetophenone, and its enantioselectivity remained above 99% without any decrease (Fig. 15C).

Additionally, this paper was able to shed light on the mechanism behind the formation of these hydrogels.¹⁹¹ The team discovered that both triazoles and metal ions play essential roles in this process. Armed with this knowledge, they extended our approach to utilize two other enzymes to create enzyme-assembled hydrogels, ensuring their excellent reusability. In conclusion, the strategy that they have described holds significant promise for advancing the development of practical catalytic biomaterials and immobilized biocatalysts. This approach

represents a notable step forward in creating efficient and reusable biocatalytic materials with potential applications in various industries and processes.

4.3.3 Nanozymes. Nanozymes are nanomaterials with intrinsic enzyme-like catalytic activity. These nanomaterials mimic the catalytic functions of natural enzymes and can be tailored for specific applications in biocatalysis and other fields. Nanozymes have gained significant attention for their potential to enhance the efficiency, stability, and versatility of enzymatic reactions. One approach to achieving this goal is by developing single-atom catalysts with precisely defined atomic structures and electronic coordination, allowing them to closely mimic the behavior of natural enzymes.

In 2021, Ji *et al.* published their results on their single-atom iron nanozyme (Fig. 16).¹⁹² They prepared their FeN_3P -single-atom nanozyme (SAzyme) based on zeolitic imidazolate framework-8 (ZIF-8) as a raw material followed by a coating process that involved polymerization and pyrolysis at 950 °C for 3 hours under flowing nitrogen (Fig. 16A). The engineered FeN_3P -SAzyme exhibited peroxidase-like catalytic activity that is comparable to that of natural enzymes. In this study, they quantitatively determined the specific activity values by measuring the absorption intensity of the nanozyme-catalyzed colorimetric reactions. FeN_3P -SAzyme showed much higher catalytic activity compared to the most widely used Fe_3O_4 nanozyme or FeN_4 -SAzyme without P coordination. The measured specific activity of the FeN_3P -SAzyme was more than 30-fold higher than that of the Fe_3O_4 nanozyme and almost 10-fold higher than that of the FeN_4 -SAzyme (Fig. 16B). Among

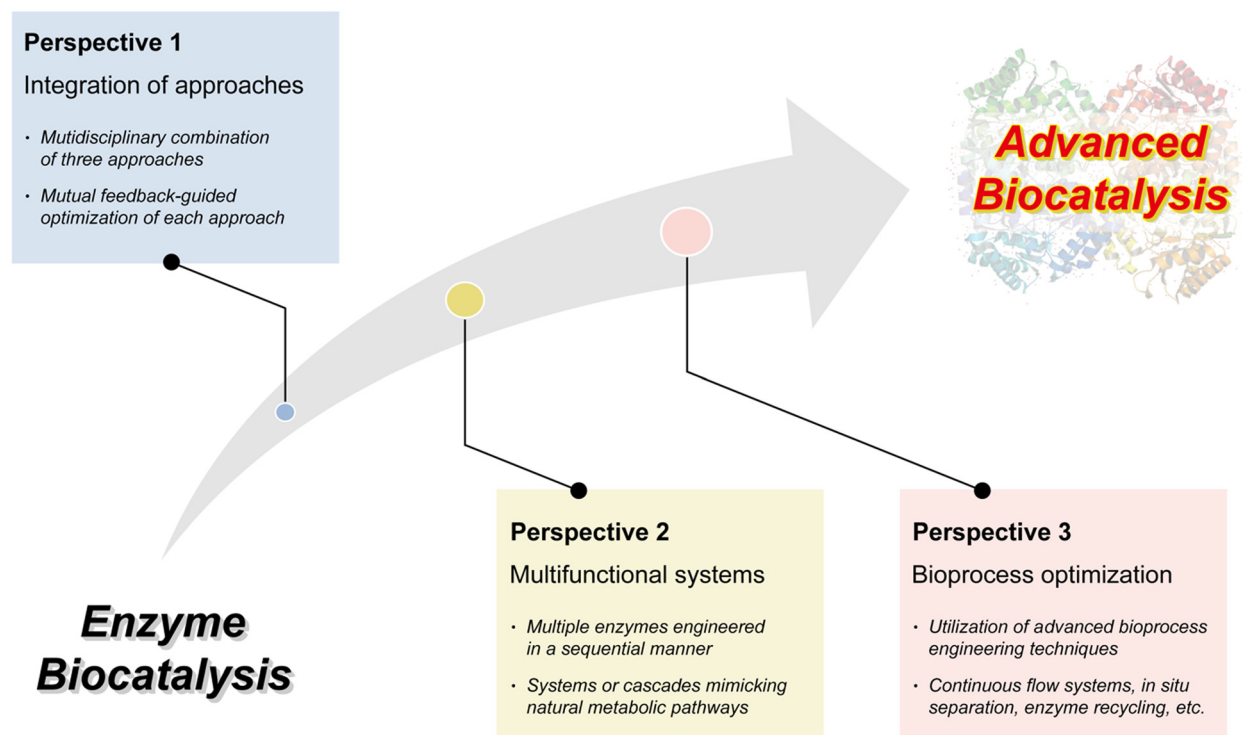


Fig. 17 Perspectives on advanced enzyme biocatalysis for optimal pharmaceutical applications.



these three catalysts, adsorbed H_2O_2 will be easily dissociated into two surface OH species ($\text{H}_2\text{O}_2 \rightarrow 2\text{OH}$) with very low barriers of 0.07, 0.17, and 0.10 eV on the three models of FeN_3P -SAzyme, FeN_4 -SAzyme, and Fe_3O_4 (Fig. 16C).

To understand the basis for its high enzyme-like activity, they conducted density functional theory (DFT) calculations, providing insights into the underlying mechanisms. Importantly, they demonstrated that FeN_3P -SAzyme with its superior peroxidase-like activity can serve as an effective therapeutic strategy for inhibiting the growth of tumor cells both in laboratory experiments and within living organisms. This shows the promising potential of SAzymes, such as FeN_3P -SAzyme, for creating artificial enzymes that replicate the catalytic kinetics of natural enzymes. In summary, this research presents a significant step forward in the quest to develop artificial enzymes that rival the catalytic capabilities of their natural counterparts. The precise coordination of single atoms, as demonstrated by FeN_3P -SAzyme, offers exciting prospects for future applications in biomedicine.

In summary, nanoarchitectonics for advanced enzyme biocatalysis is a cutting-edge field that harnesses nanotechnology to optimize the performance of enzymes in various applications. This multidisciplinary approach involves the precise design and construction of nanoscale structures and materials to enhance catalytic activity, enzyme stability, reusability, and versatility. Three primary strategies within this field are enzyme encapsulation, enzyme immobilization, and the development of nanozymes. However, these three strategies are not fully separated, for example, enzyme encapsulation and enzyme immobilization within hydrogel could be considered as both strategies. Also, when nanozymes, nanomaterials with intrinsic enzyme-like catalytic activity, are immobilized or encapsulated, several challenges such as loss of activity and specificity could be solved.¹⁹³ So, by leveraging nanoscale structures and materials, researchers can optimize enzymes for a wide range of applications, including pharmaceuticals, biocatalysis, environmental remediation, and diagnostics. More research examples of nanoarchitectonics for advanced enzyme biocatalysis are described in Table 4.^{194–221}

In conclusion, multidisciplinary approaches in enzyme biocatalysis mark a critical moment in the evolution of pharmaceutical research and production. By the precision of protein engineering, the computational prowess of computational biology, and the innovative strategies of nanoarchitectonics, scientists are poised to unlock the full potential of enzymes as molecular tools. These methods not only promise to address the challenges posed by modern pharmaceutical demands but also lay the groundwork for a more sustainable and efficient future in drug development and manufacturing. As stated in the following sections, the intricate interplay of these multidisciplinary techniques will shed light on the transformative impact they have on enzyme biocatalysis. Through collaborative efforts and continuous advancements, the pharmaceutical industry is on the brink of a new era, one where enzyme biocatalysis stands as an indispensable cornerstone, shaping the landscape of pharmaceutical innovation for years to come.

Looking ahead, for industrial applications, further exploration, and consideration of additional modifications in biocatalysis for long-term use, such as enzyme cross-linking,²²² chemical modification,²²³ and formulation environment control,²²⁴ hold the potential to revolutionize the efficiency and scalability of enzyme-driven processes.

5. Conclusions

5.1 Summary

In the ever-evolving realm of pharmaceutical research and production, the utilization of enzyme biocatalysis stands as a beacon of innovation and sustainability. Throughout this comprehensive exploration, we have emphasized the significance of enzyme biocatalysis in advancing pharmaceutical applications, with particular attention to the introduction of multidisciplinary approaches as catalysts for progress. The convergence of multidisciplinary approaches, encompassing protein engineering, computational biology, and nanoarchitectonics, has heralded a new era for enzyme biocatalysis. This interdisciplinary synergy has proven to be greater than the sum of its parts, unlocking novel opportunities and redefining the boundaries of what is achievable in pharmaceutical research and production.

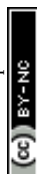
This transformative technology offers a wealth of advantages, from the precision and selectivity of enzyme-catalyzed reactions to their eco-friendly and sustainable attributes. Significant progress lies in the field of computational biology. This burgeoning field has emerged as a groundbreaking approach for optimizing enzyme biocatalysis, and its computational prowess empowers pharmaceutical scientists to optimize drug candidates, improve pharmacokinetics, and reduce toxicity, all with a level of precision that was once unimaginable. In the field of nanoarchitectonics, innovative strategies for enzyme encapsulation and immobilization within tailor-designed nanomaterials have enhanced enzyme stability and reusability.

5.2 Perspectives

As we conclude this comprehensive review, it is crucial to cast our gaze towards the future of advanced enzyme biocatalysis in pharmaceutical applications. There are three key perspectives that hold immense promise (Fig. 17):

5.2.1 Integration of multiple approaches. The integration of multidisciplinary approaches, including protein engineering, computational biology, and nanoarchitectonics, stands as a fundamental imperative. The multidisciplinary combination of these approaches, coupled with mutual-feedback-guided optimization of each facet, will create a synergistic environment that propels enzyme biocatalysis to unprecedented heights.

5.2.2 Multifunctional integrated systems. The trajectory of progress in enzyme biocatalysis is expected to include the emergence of multifunctional systems as a defining feature. This evolution encompasses the strategic engineering of multiple enzymes in a sequential fashion, mirroring the intricacy of natural metabolic pathways. Furthermore, the establishment of systems or cascades that emulate these inherent pathways will



play a pivotal role in attaining multifunctional enzymatic capabilities.

5.2.3 Bioprocess optimization. Future strides in enzyme biocatalysis will prominently feature bioprocess optimization as a central pillar. Leveraging cutting-edge bioprocess engineering techniques and embracing continuous flow systems, *in situ* separation, enzyme recycling, and other avant-garde methodologies will fortify the position of enzyme biocatalysis as a vanguard in sustainable and efficient pharmaceutical production.

In summary, the evolution of enzyme biocatalysis in pharmaceutical applications continues to unfold. The integration of multidisciplinary approaches, the advancement of multifunctional systems, and the continued pursuit of bioprocess optimization beckon as uncharted territories brimming with untapped potential. As researchers embark on this next phase, their vision encompasses a pharmaceutical domain marked by heightened speed, sustainability, efficiency, and precision. Through collaborative efforts and innovative strides, researchers are poised to expand the boundaries of what can be accomplished, confirming enzyme biocatalysis as an indispensable cornerstone of pharmaceutical research and production for the foreseeable future.

Author contributions

S. Kim: conceptualization, visualization, writing – original draft, writing – review & editing. S. Ga: writing – original draft. H. Bae: writing – original draft. R. Sluyter: writing – review & editing. K. Konstantinov: writing – review & editing. L. K. Shrestha: writing – review & editing. Y. H. Kim: supervision, funding acquisition, writing – review & editing. J. H. Kim: supervision, project administration, writing – review & editing. K. Ariga: supervision, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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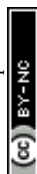
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