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Protein Engineering of Substrate Specificity toward Nitrilases: Strategies and Challenges

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ABSTRACT: Nitrilase is extensively applied across diverse sectors owing to its unique catalytic properties. Nevertheless, in industrial production, nitrilases often face issues such as low catalytic efficiency, limited substrate range, suboptimal selectivity, and side reaction products, which have garnered heightened attention. With the widespread recognition that the structure of enzymes has a direct impact on their catalytic properties, an increasing number of researchers are beginning to optimize the functional characteristics of nitrilases by modifying their structures, in order to meet specific industrial or biotechnology application needs. Particularly in the artificial intelligence era, the innovative application of computer-aided design in enzyme engineering offers remarkable opportunities to tailor nitrilases for the widespread production of high-value products. In this discussion, we will briefly examine the structural mechanism of nitrilase. An overview of the protein engineering strategies of substrate preference, regioselectivity and stereoselectivity are explored combined with some representative examples recently in terms of the substrate specificity of enzyme. The future research trends in this field are also prospected.

KEYWORDS: *Nitrilase, substrate specificity, protein engineering, regioselectivity and stereoselectivity, mechanistic analysis*

1. INTRODUCTION

Nitrilases (EC 3.5.5.1) can facilitate the one-step hydrolysis of nitrile substrates into corresponding carboxylic acids, showcasing immense potential in regioselectivity, stereoselectivity and enantioselectivity.[1] These enzymes are of significant interest due to their wide-ranging applications in biocatalysis, particularly in the degradation of toxic nitrile compounds, the synthesis of valuable intermediates, and their powerful performance in the field of green chemistry.[2] However, limitations such as a narrow substrate range, suboptimal catalytic activity and stability, and poor selectivity toward non-natural substrates beyond the natural ones remain huge barriers to the development of nitrilases. This makes them an attractive target for engineering efforts aimed at optimizing their substrate specificity for industrial applications. Therefore, it has become a major challenge to rationally design nitrilases to selectively hydrolyze specific nitriles.

The substrate specificity of nitrilases is limited by a number of factors such as the active site of the enzyme and the shape and size of the substrate channel,[3] which vary among different nitrilase families. Advances in protein engineering have provided promising strategies for modifying the specificity of enzyme. Early studies on nitrilases were dominated by the traditional directed evolution technology,[4−7] which bypasses the need to consider the spatial structure and catalytic mechanism of enzymes. By simulating natural evolutionary processes, researchers can modify enzyme genes outside the body and purposefully select mutated enzymes with targeted properties. However, this process is often lengthy, labor-intensive and inefficient, making it challenging to tailor engineered enzyme preparations with ideal functionality. The development of bioinformatics has helped researchers to delve deeper into the physicochemical properties, three-dimensional structures, structure−activity relationships, and catalytic mechanisms of nitrilases, leading to the exploration of a suite of innovative computer-aided enzyme design methods.[8,9] Computer-aided design transformations employ structural simulation and energy calculations, allowing not only for the modification of substrate specificity and thermal stability[10] but also for the de novo design of artificial enzymes with specific functions.[11] In recent years, advancements in artificial intelligence technologies, such as machine learning, have consistently yielded breakthroughs in predicting protein structures and functions,[12] and these techniques are also being applied to enzyme modification and design, opening up new possibilities for the research and application of biocatalytic reactions.

Several reviews have already explored the broad spectrum of approaches for engineering the specificity of nitrilases, including the application of directed evolution, site-directed mutagenesis, and rational design.[13−15] For instance, studies have focused on enhancing the catalytic efficiency and substrate range of nitrilases through single-site mutations or by expanding the active site to accommodate larger substrates.[16] Others have explored the use of computational

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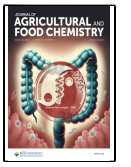

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methods to predict the effects of mutations on enzyme−substrate interactions and enzyme stability.[17] However, there remains a lack of comprehensive reviews that specifically address the strategies and challenges associated with the protein engineering of nitrilases for improved substrate specificity. The aim of this review is to provide a systematic overview of the biological background and the current state of research on the protein engineering of nitrilases with a focus on strategies to improve substrate specificity and explain their underlying mechanisms in terms of substrate preference, regioselectivity and stereoselectivity. Additionally, we will discuss the challenges faced by researchers in this area. By summarizing the existing literature and identifying gaps in current knowledge, this review will lay a foundation for future research on the engineering of nitrilases with tailored substrate specificity.

2. NITRILASES

2.1. Source and Substrate Diversity of Nitrilases. The first discovered synthetase of indoleacetic acid, a plant hormone, was isolated from the leaves of barley.[18,19] Due to its high selectivity in converting nitriles to carboxylic acids, it was later named nitrilase.[20] To date, nitrilases have been found in various organisms, spanning archaea, bacteria, fungi, plants and animals.[21] Nitrilases derived from bacteria and fungi are particularly important in industrial applications, such as *Pseudomonas*, *Streptomyces*, and *Saccharomyces cerevisiae*, which have diverse catalytic properties and are widely used in the synthesis and separation of compounds.[22−25]

With the rapid advancements in modern molecular biology technology, more and more nitrilase genes have been cloned and heterologously expressed, enabling the industrial application of nitrilases from different strains.[26] This can be further demonstrated by the publication of articles and patents related to nitrilase research that the enzymes being mined and utilized are becoming increasingly abundant over the past 30 years (Figure 1). For example, Qiu et al.[27] cloned a nitrilase from *Sphingomonas wittichii* RW1 and expressed it in *Escherichia coli* BL21 (DE3), exhibiting high enantioselectivity in the asymmetric synthesis of (*R*)-phenylglycine. Using resting cells as catalysts, the final yield of (*R*)-phenylglycine was 46%, with an *ee* value of up to 95%. In addition, Wang et al.[28]

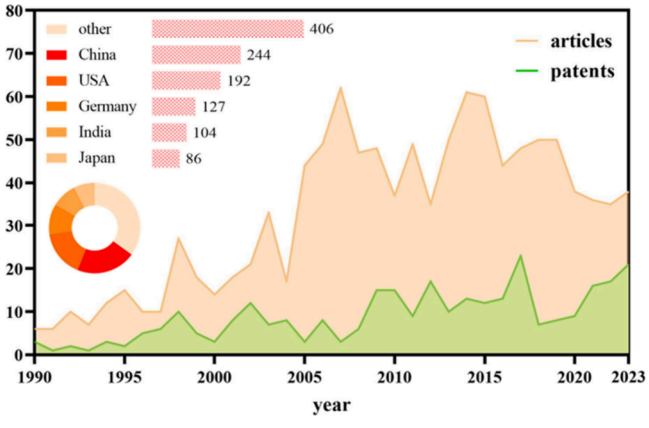

Figure 1. Statistics on the number of publications on nitrilase research and the total number of articles published in the top five countries. Articles were assessed according to Web of Science; patents according to Espacenet search conducted at the end of 2023.

cloned and overexpressed a novel nitrilase from *Rhodobacter sphaeroides* LHS-305 in *E. coli* Rosetta-gami(DE3), which can regioselectively hydrolyze aliphatic dinitriles to produce cyanide carboxylic acids. These studies not only demonstrate the specificity of nitrilases in different sources, but also demonstrate the enormous potential of genetic and protein engineering in optimizing the performance of nitrilases for applications.

Nitrilases also play a key role in the plant kingdom, especially in plants containing glucosinolates.[29] Glucosinolates are mainly found in plants of the *Brassicaceae* family, of which the model plant *Arabidopsis thaliana* and some other crucifers (e.g., *Brassicas*) contain multiple nitrilase genes. In addition, nitrilase activities have been found in some plants such as *Sinapis*. They are not only involved in detoxification and defense, but also play important functions in nutrient recycling and hormone regulation.[30−32] Although nitrilases have been less well studied in animals, some studies have found the presence of nitrilases in certain arthropods such as millipedes, which catalyze the decomposition of mandelonitrile, releasing hydrocyanic acid and benzaldehyde that have toxic effects on predators.[33]

The substrate spectrum of nitrilase is very broad, capable of hydrolyzing a variety of substrates, including aliphatic, aromatic and acylacetonitrile compounds. Aromatic nitrilases primarily include those from bacteria, the majority of fungi and plants, such as *Bacillus pallidus* Dac521,[34] *Rhodococcus* ATCC39484,[35] *Aspergillus niger* K10,[36] and *Arabidopsis thaliana*.[37] They show high catalytic activity for aromatic and heterocyclic nitriles, but less effective for aliphatic nitriles. Arylacetonitrilases from *Alcaligenes faecalis* JM3[38] and *Pseudomonas putida*[39] show a preference for phenylacetonitrile and its para-substituted derivatives; aliphatic nitrilases from *Comamonas testosterone*[40] and *Rhodococcus rhodococcus* K22[41] not only have high catalytic activity for aliphatic nitriles but also for other nitriles.

However, the relationship between the substrate types of nitrilases and their source strains is not fixed. As nitrilases are further explored, it is evident that most enzymes can hydrolyze two or more types of nitriles, displaying a complex substrate spectrum.[42] Consequently, nitrilases from the same source may demonstrate varying activities induced by different substrates, underscoring that the reaction pathway of nitrile compounds predominantly hinges on the stereoscopic and electronic properties of the substrate. It is worth noting that the substrate preference for specific nitrilase can be altered by modifying enzyme molecules.

2.2. Structure and Catalytic Mechanism. The catalytic activity and selectivity of nitrilases largely depend on the inherent spatial and electronic properties of each substrate. Grasping the recognition mechanism between enzymes and substrates is a crucial step in understanding how enzymes acquire new functions during evolution and is essential for targeted efforts to alter substrate specificity.

The first crystal structure with true nitrilase activity was resolved by Raczynska et al. on the *Pyrococcus abyssi* GE5-derived nitrilase[43] (PDB: 3IVZ). Based on the study of Brenner et al.,[44] which revealed the typical multimeric $\alpha-\beta-\beta-\alpha$ sandwich protein architecture of nitrilases as well as a conserved catalytic triad of Glu-Lys-Cys in the active center,[45] it was shown in detail for the first time how nitrilase catalyzes the hydrolysis reaction of nitrile (Figure 2A). With the deeper understanding of the function of nitrilases,

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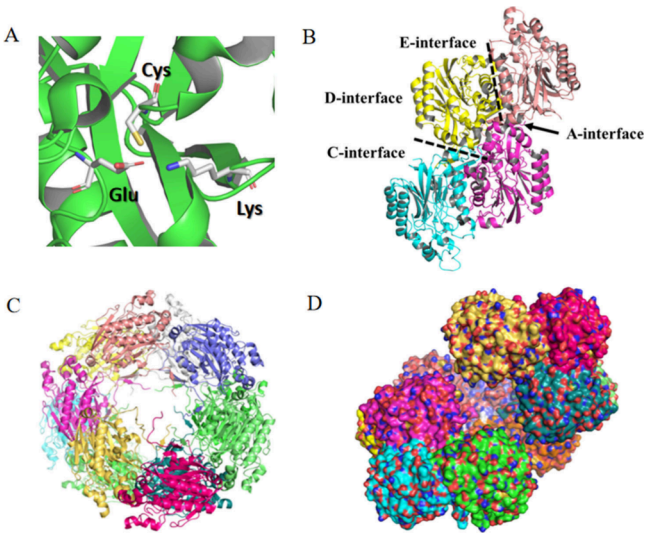

Figure 2. Three-dimension model of nitrilase from *Pseudomonas fluorescens* EBC191 (PDB: 6ZBY). (A) The catalytic triad of the nitrilase superfamily. (B) Interfaces of the nitrilase monomers in the supramolecular spirals or helices. (C, D) The crystal structure is presented in the form of carton and surface, respectively.

subsequent researchers have analyzed the crystal structures of more nitrilases and their superfamily members. For example, the resolution of the crystal structure of NIT6808 (PDB: 3WUY) from *Synechocystis* sp. PCC6803[46] and nitrilase from *Pseudomonas fluorescens* EBC191[47−49] (PDB: 6ZBY) provided richer structural information for the polymer structure, multiple interface types (such as A-interface, C-interface), and substrate specificity of nitrilases[50,51] (Figure 2B). Further studies have found that the multimeric structure of nitrilases can form not only short spiral or long filament,[52] but also various multimeric configurations, such as circular and C-shaped, which leads to diversity in stability, specificity and stereoselectivity (Figure 2C and D).

The development of crystal structure research on nitrilases has enabled researchers to gain a deeper understanding of their structure−function relationships, and the details of the catalytic mechanism have been gradually clarified. The glutamic acid in the catalytic triad is an acidic amino acid and acts as an alkaline catalyst. Cysteine, as a nucleophilic catalyst, features sulfhydryl group that can engage in nucleophilic reactions with the carbon atoms of the nitrile substrates, along with protonation of nitrogen, forming a tetrahedral structure known as the thiomidate intermediate. This intermediate first binds to a molecule of water, releasing ammonia, and then reacts with a second water molecule to form the corresponding carboxylic acid and enzyme. While lysine plays a role in stabilizing the intermediate complex during the catalytic process. The specific catalytic mechanism is shown in Figure 3.

Since the discovery of nitrilase, it has garnered growing interest among industrial biocatalysis researchers for its critical role in the organic synthesis of carboxylic acids from nitriles. The key enzymes for nitrile conversion include nitrile hydratase (NHase) and nitrilase, both directly using nitrile as a substrate but diverging into two distinct pathways of nitrile catabolism. Nitrile hydratase,[53] centered around sulfur atoms and cysteine sulfonic acid residues, initially hydrates nitrile substrates to form amides, which are then hydrolyzed to carboxylic acids by amidase. It is precisely because of the formation of covalent intermediates that nitrile can be catalyzed by nitrilase in one step to directly produce the corresponding carboxylic acid and ammonia with no amide formed in the process.

2.3. Recent Development of Application. The discovery of nitrilase offers a more favorable alternative for the derivation and conversion of toxic nitrile compounds. Nitriles are ubiquitous in nature, with many biological systems such as bacteria, fungi, and various plants and animals producing metabolites like indole-3-acetonitrile, phenylpropanonitrile, and ricinine. Common organic reagents like acetonitrile, important monomers for synthesizing polymers (fiber, rubber, resin) such as acrylonitrile, and herbicidal pesticides such as iodobenzonitrile all contain cyano functional groups.[54] These compounds, when used as raw materials, can be converted into nontoxic, high-value acids and amides through a series of reactions, which have been widely used in many fields such as medicine, agriculture, food, chemical industry[55] and environmental remediation.

As efficient, specific and green catalytic tools, nitrilases show great potential for biocatalytic synthesis of pharmaceutical intermediates. Global leading suppliers in the pharmaceutical, healthcare and life science sectors, such as Lonza and BASF, have been commercially producing nicotinic acid, nicotinamide and *(R)*-(−)-mandelic acid using nitrilase and nitrile hydratase since the last century.[56] They have also established facilities with capacities exceeding 1,000 tons per year and remain the core global players in nicotinamide supply to this day. Besides, nitrilases are increasingly favored in the preparation of drugs such as pyridine carboxylic acid, mandelic acid derivatives, mononitrile monoacids, and amino acids for medical purposes (Table 1), providing more innovative solutions for the pharmaceutical industry.[14,57]

In agricultural applications, nitrilases have been widely studied for the synthesis of highly effective pesticide precursors. For example, low-toxicity and efficient herbicides such as *L*-phosphinothricin can be synthesized through nitrilase-mediated selective reactions.[58] Meanwhile, nitrilases provide a molecular basis for enhancing crop growth and coping with climatic stress by regulating phytohormone metabolism and morphogenetic processes, and offer the potential for the development of chemical regulators (e.g., indoleacetic acid and its derivatives, Heatin, etc.) that can optimize plant growth.[59,60] In addition, nitrilases can hydrolyze cyanogenic glycosides formed in plant metabolism, converting them into low-toxicity compounds, effectively reducing the content of toxic substances in feed and improving feed safety.[61]

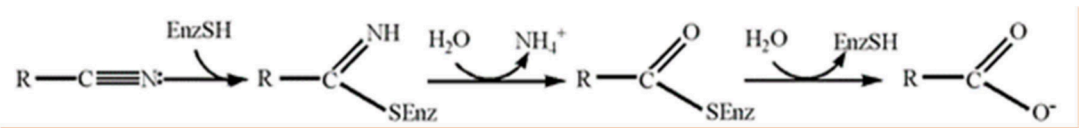

Figure 3. Catalytic mechanism of nitrile hydrolysis by nitrilase.

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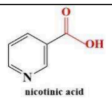
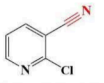
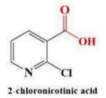
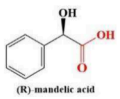
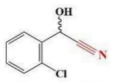
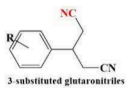
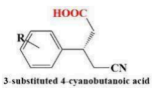
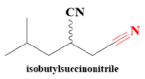
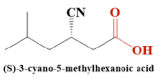
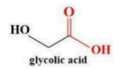
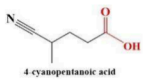
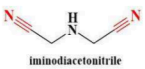
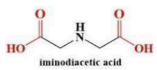
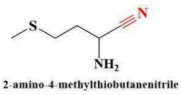
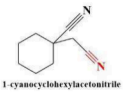
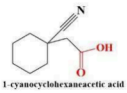
Table 1. Main Applications of Nitrilase in Drug Precursors and Fine Chemicals

Origin	Substrate	Product	Application	Reference
Rhodococcus rhodochrous J1 *Pseudomonas putida* CGMCC3830	 3-cyanopyridine	 nicotinic acid	preparation of isoniazid, nicoxamide and niacin inositol ester	22,68-70
Nocardia globerula NHB-2 *Pseudomonas putida* CGMCC3830	 4-cyanopyridine	 isonicotinic acid	synthesis of anti-tuberculosis drugs	71,72
Rhodococcus zopfii *Paraburkholderia graminis*	 2-chloronicotinonitrile	 2-chloronicotinic acid	synthesis of mirtazapine, and nevirapine precursors	73-75
S. wittichii RW1	 N-phenylglycinonitrile	 D-phenylglycine	synthesis of ampicillin and other β-lactam antibiotics	27
Alcaligenes sp. MTCC 10675 *Alcaligenes faecalis* ZJUTB10	 mandelonitrile	 (R)-mandelic acid	semi-synthetic penicillin and cephalosporin	76-78
Alcaligenes faecalis JM3 *Labrenzia aggregate* DSM 13394	 o-chloromandelonitrile	 (R)-o-chloromandelic acid	precursor to the synthesis of clopidogrel	5,79,80
Synechocystis sp. PCC6803 *Herbaspirillum* sp. GW103 *Bradyrhizobium japonicum* USDA 110	 3-substituted glutaronitriles	 3-substituted 4-cyanobutanoic acid	synthesis of chiral β-substituted γ-amino acids	81,82
Arabidopsis thaliana NIT1 *Brassica rapa*	 isobutylsuccinonitrile	 (S)-3-cyano-5-methylhexanoic acid	synthesis of optically active pregabalin	17,83,84
Alcaligenes sp. ECU0401	 glycolonitrile	 glycolic acid	synthesize medical materials and suppress tumors	85
Acidovorax facilis 72W	 2-methylglutaronitrile	 4-cyanopentanoic acid	synthesis of 1,5-dimethyl-2-piperidone	86,87
Arthrobacter aurescens CYC705 *Acidovorax facilis*	 iminodiacetonitrile	 iminodiacetic acid	production of glyphosate pesticides, chelators and surfactants	88,89
Acidovorax facilis	 2-amino-4-methylthiobutanenitrile	 methionine	nutritional supplement and adjuvant treatment	90
Acidovorax facilis ZJB09122	 1-cyanocyclohexylacetonitrile	 1-cyanocyclohexaneacetic acid	synthesis of gabapentin	91,92

In the food industry, nitrilases are being developed for green production of spices and food additives. Food additives such as cinnamic acid, nicotinic acid, and pantothenic acid can be obtained by nitrilase catalysis, making the production process cleaner and avoiding the use of high temperature, high pressure, and organic solvents in traditional chemical catalysis.[15,22] For example, the nitrilase BjNIT3397 can effectively hydrolyze 3-aminopropionitrile with a substrate concentration of up to 3 M to synthesize β-alanine.[62] As a functional food additive, β-alanine can enhance athletic performance, relieve fatigue, and provide antioxidant and antiaging benefits by increasing the content of carnosine in muscles.[63] It is widely used in the fields of sports nutrition and health food. In the future, nitrilases may also be investigated for optimizing the flavor of fermented foods by breaking down specific nitrile compounds to enhance the sensory attributes of the food.[61]

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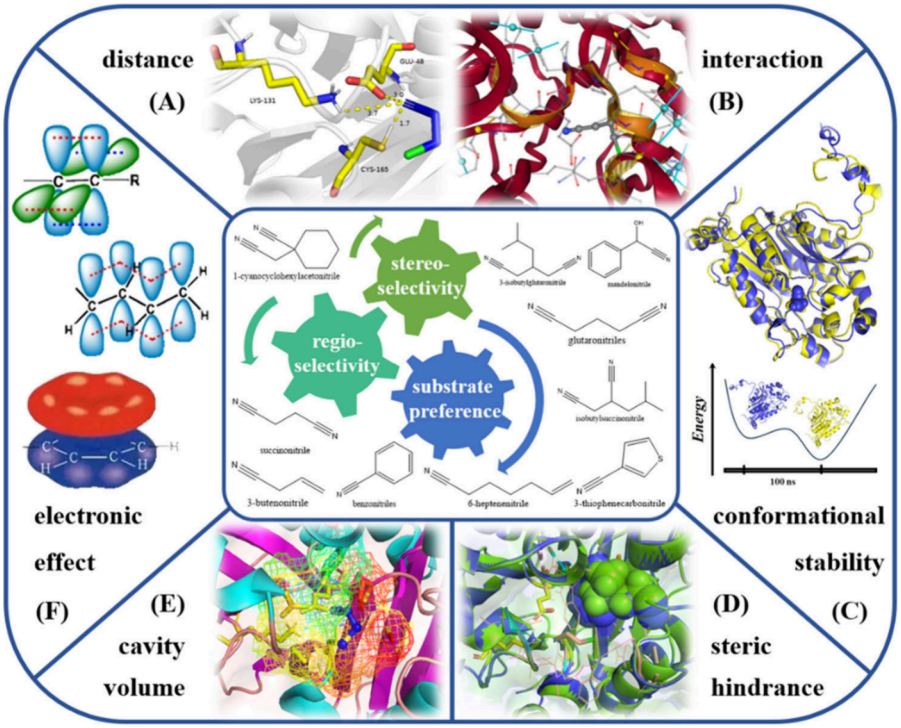

Figure 4. Possible factors affecting substrate specificity of nitrilase. (A) The distance between the cysteine residue sulfhydryl group and the substrate cyanide group. (B) Interaction between enzymes and substrates. (C) The conformational regulation and stability of proteins. (D) The steric hindrance effect of substrates and residues. (E) The volume of substrate−enzyme binding cavity. (F) The electronic effects of substrate molecules.

In addition, nitrilases have demonstrated excellent biodegradability for environmental remediation. Industrial and agricultural activities have inevitably elevated cyanide levels in the soil, wastewater, and air.[64] A heat-resistant nitrilase-producing strain coated *Bacillus* sp. UG-5B were reported to be effectively utilized to biodegrade mixtures of *ortho*-, *meta*-, and *para*-tolunitrile at various concentrations. In a laboratory-scale column bioreactor, the total degradation rate of nitriles reached over 90% after a two-step process lasting 9 h.[65] Similar studies can all provide an efficient green solution to reduce the environmental hazards of toxic nitrile wastes and important support for the realization of sustainable development.[66,67]

3. PROTEIN ENGINEERING STRATEGIES TO IMPROVE SUBSTRATE SPECIFICITY OF NITRILASE

Enhancing enzyme functionality through engineering and modification is crucial for overcoming the limitations in the industrial use of nitrilases. Integrating genetic engineering, enzyme engineering, protein modification, and process engineering can significantly broaden the specificity and range of applications for specific substrate types.[93] Current modifications to the substrate specificity of nitrilases primarily focus on substrate preference, regioselectivity, and stereoselectivity. The synthesis of chiral compounds, broadening of analogous substrates, and modifying the type of enzymatic reactions involved are critically important in fields like pharmaceuticals and chemical engineering.[94] A thorough understanding of the specific factors and mechanisms behind protein engineering modification of nitrilases will greatly aid in the precise regulation of their substrate specificity (Figure 4).

3.1. Substrate Preference.

Nitrilases are capable of catalyzing a diverse array of nitrile compounds, exhibiting substrate promiscuity, which implies their broad application range to some extent. However, in order to truly customize organic acids and amide compounds with high added value, the selectivity and catalytic activity of nitrilase toward natural substrates are insufficient to meet the needs of modern industrial production. Utilizing innovative protein engineering techniques to modify nitrilases and enhance their selectivity for specific types of reactions, especially to improve the preference of this multifunctional biocatalyst for a specific substrate, has proved to be an effective strategy.

Nit6803 is a promiscuous nitrilase with similar enzymatic activity to aliphatic and aromatic nitrile substrates derived from *Synechocystis* sp. Zhang et al.[46] found that the aromatic amino acid at position 146 in the nitrilase is absolutely required for its substrate preference and stability of substrate binding pockets. Meanwhile, the spatial relationship between the sulfhydryl group of the cysteine residue and the cyanide group of the substrate determines the catalytic activity of nitrilase toward different nitrile substrates (Figure 4A). Wang et al.[16] successfully developed an upgraded Ala-Leu-Phe scanning-(ALF-scanning)strategy that replaces the original amino acids in the active pocket with representative amino acids of different side chain sizes to reshape the active pocket, resulting in a synergistic combinatorial mutant V198L/W170G with strong aromatic nitrile preference and high catalytic activity. The dramatic improvement in the properties of this enzyme is mainly due to the introduction of stronger substrate-residue π-alkyl interaction in the active pocket and the acquisition of a substrate cavity with a significantly increased volume, making it easier for aromatic nitrile substrates to pass through the depth of the active cavity (Figure 4B and E). On the contrary,

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aliphatic nitrile substrates used for catalytic reactions are more difficult to be captured in the active pocket, thus showing a tendency to move away from the active center. This substrate preference mechanism has also been confirmed in the other three sources of nitrilases (LsNit, RsNit, SmNit).

This design effectively overcomes the problem of drastic changes in the geometric shape of the active pocket caused by alanine scanning, and also optimizes the efficiency of substrate entry into the catalytic center while maintaining the hydrophobic microenvironment of the active pocket. However, the strategy also has its limitations, especially in cases where the enzyme activity pocket structure is complex, which may require a combination of more computational simulations and experimental validation. In addition, the application of ALF-scanning strategy requires in-depth knowledge of the structure and substrate binding characteristics of the enzyme. So, its extensive application may need to be combined with other protein engineering methods, such as directed evolution, to jointly promote the performance of enzymes.

Another study successfully modified the 142 amino acid residue of nitrilase from *Rhodococcus rhodochrous* ATCC 33278 to change its substrate specificity,[95] and such simple substitutions may be effective in multiple systems. For example, mutants substituted with aromatic amino acids retained their activity toward aliphatic and aromatic nitriles; mutants substituted with nonpolar aliphatic amino acids favored aromatic nitrile substrates and showed a decrease in activity with increasing amino acid side-chain size; mutants containing charged amino acid substitutions were inactive toward any nitrile. The structure−function correlation strategy allowed us to understand in more detail the role of the electron acceptor played by the aromatic ring at the key active site, enabling electron transfer between the enzyme and substrate through a conjugated system (Figure 4F). The team then went on to discover that the positively charged amino acid at position 129 of the enzyme is essential for the activity of aromatic nitriles.[96] In contrast to the wild-type enzyme, the lysine- or histidine-substituted mutants were active only for the *meta*-substituted benzonitriles. The lack of activity of the mutants toward *para*-substituted benzonitriles may be attributed to steric hindrance between the *para*-substituent and the side chains (Figure 4D). However, the substrate specificity of an enzyme is not only determined by a few key residues, but also affected by the overall three-dimensional structure, so there may still be some uncertainty about the effect of each variant. Therefore, in the process of enzyme engineering, in addition to focusing on the effects of specific mutations, the potential impact of these mutations on other biological functions of the enzyme need to be considered comprehensively.

Furthermore, based on the sequence similarity of plant nitrilases, Woodward et al.[52] identified and characterized two nitrilases derived from *Capsella rubella*. The two enzymes differ in only 51 sites on the amino acid sequence, but show different preferences for 6-heptenenitrile and 3-butenonitrile. In order to locate the key residues, authors used the helix imprinting effect to mix these two nitrilases with different helix twisting properties. Combined with the binary search technique, it was determined that residue exchange at the site H80/F80 on the chimera resulted in an almost complete switch in substrate preference ratio. The study found that helix twist determines the size of the substrate-binding site, with larger helical twist angles (tighter helices) being suitable for smaller substrates and smaller angles for larger substrates. Even when the amino acid sequence of the enzyme is kept constant, changing the twist can alter substrate specificity. This is the first time that the substrate specificity of nitrilase has changed substantially in a predictable manner. The modulation of helical structure breaks through the limitation of the traditional modification based on the binding site residues and can effectively regulate the overall geometry of the substrate-binding site, which is applicable to enzymes that are highly homologous but have different substrate specificities. However, the method is limited in its applicability. Not all nitrilases are able to form mixed helices, which hinders the application of the helix imprinting strategy. It also requires a good understanding of the quaternary structure of enzymes, and the experimental design and validation process are relatively complicated.

In summary, the substrate preference and catalytic properties of nitrilases can be efficiently regulated by precise amino acid mutagenesis, helical structure modulation and other protein engineering tools. These strategies not only extend the application scope of enzymes, but also provide powerful technical support for industrialized biocatalysis. However, the complexity of different enzyme systems requires us to comprehensively consider the structural features of enzymes, substrate properties and their performances in specific reactions when applying these methods to optimize and tailor the enzyme functions.

3.2. Regioselectivity. The regioselectivity of nitrilases enables them to selectively hydrolyze a single cyano group in dinitriles to produce the corresponding monocyanocarboxylic acid without altering other readily hydrolyzable groups, which compensates for the drawbacks of high energy consumption and pollution associated with conventional chemical methods. The mechanism behind the regioselective hydrolysis catalyzed by nitrilases has been revealed, identifying that the carbon chain length and the type or position of substituents of the substrate are key factors affecting enzyme selectivity.[97] Both of these factors determine the enzyme-catalyzed reaction pathways by altering the steric hindrance and electronic effects of the substrate molecules (Figure 4D and F).

Specifically, substrates with shorter carbon chains tend to be converted to a single product, cyanocarboxylic acid (CCBA), while the production of dicarboxylic acid (DCBA) increases with increasing carbon chain length, resulting in a final hydrolysis product that may be a mixture of CCBAs and DCBAs[98] (Figure 5). When the carbon chain length of the substrate reaches a certain level (e.g., C8 or C10), DCBAs appear as a single product. For aromatic, heterocyclic and aliphatic dinitriles, the position of the substituent group of the substrate significantly affects the hydrolysis selectivity of the enzyme, and in general, weak hindrance of the substrate to the enzyme prompts the product to be a DCBA, while strong hindrance preferentially generates a CCBA.

Based on the mechanism, rational molecular modification through the structural information on the enzyme can not only enhance the regioselectivity of nitrilases, but also improve their activity, stability and substrate tolerance. This provides theoretical support for the application of nitrilase in industrialization. An *Acidovorax facilis* ZJB09122 derived nitrilase that mediates the production of 1-cyanocyclohexane-acetic acid exhibits good regioselectivity and hydrolysis activity toward aliphatic dinitriles. The improved nitrilase Mut-F168 V was obtained by mutating its Phe at position 168 into Val,[99] which could convert 1-cyanocyclohexylacetonitrile at low cost

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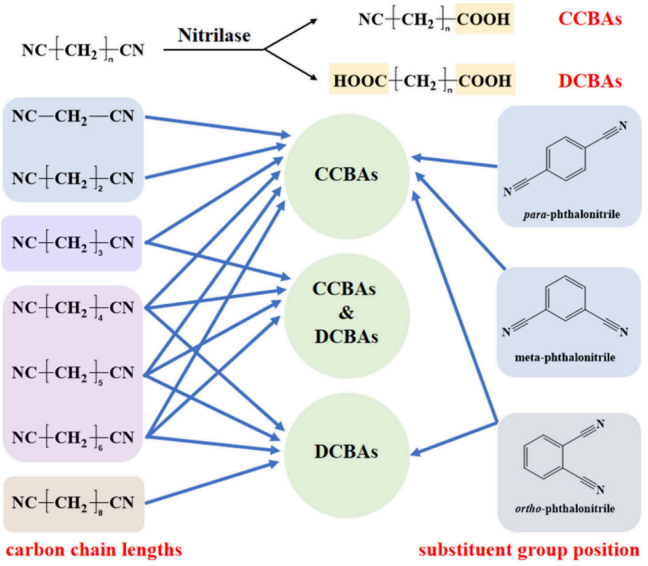

Figure 5. Products of dinitrile compounds with different characteristics hydrolyzed by nitrilases.

and almost 100% catalytic conversion to meet the needs of scale-up production. By studying the interaction between the mutations and substrate, it was shown that the distances of hydrogen bonds between the catalytic triad and the substrate were minimized to have a stable energy conformation and relax the conformation from close contacts[100] (Figure 4C). In recent years, the research group has further utilized various computer design algorithms and adopted a semirational design method based on sequence analysis to optimize the enzyme. The role of multiple key residues and the universality of mutation sites were studied through mutation/reverse mutation of homologous nitrilases.[101]

In addition to this, Chen et al.[102] systematically investigated the regioselective conversion of succinonitrile by two nitrilases bll6402 and blr3397 from *Bradyrhizobium japonicum* USDA110 through analyzing the structural model and substrate binding sites of the enzymes. They used MEGA and GENEDOC for multiple alignment based on the differences and similarities between amino acid sequences, and found that 163Ala in bll6402NIT and the corresponding 172Trp in blr3397NIT are very close to the key catalytic residue Cys, which may directly affect the size of substrate channel and the morphology of active center, switching their respective regioselectivity toward succinonitrile (Figure 4E). This hypothesis was verified in conjunction with site-directed saturated mutagenesis. Researches have shown that in the dinitrile-catalyzed reaction, the two cyano groups should be catalyzed sequentially.[103] It is the mutation of these key residues that alters the size of the active pocket and inhibits or facilitates the turnover of the intermediate 3-cyanopropanoic acid, thereby enhancing the specificity of the single conversion pathway of the substrate.

These studies combining molecular docking techniques to optimize the spatial conformation and physicochemical properties of the active pockets to achieve pathway-specific regioselectivity provide new ideas for the design of the regioselectivity of nitrilases, and lay the foundation for efficient conversion of nitriles with different chain lengths or substituents at the same time. However, there are still relatively few explorations in this direction. One of the reasons may be that the catalytic mechanism of nitrilases is inherently complex, and the size, shape, and electronic properties of the dinitriles may have a large impact on the catalytic performance of the enzyme. Engineering for selectivity to specific regions is therefore more challenging. With the development of computational simulations, structural biology techniques, and high-throughput screening methods, such strategies are gradually being proposed and optimized. In the future, functional modular enzymes can be developed by integrating multiple nitrilases with complementary substrate selectivities and designing synergistic catalytic pathways to adapt to the needs of stepwise transformation of complex substrates for a wider range of applications.

3.3. Stereoselectivity. Chiral pharmaceuticals represent a frontier within the pharmaceutical industry and are a primary focus of current international new drug research. At present, most of the drugs used are composed of chiral molecules, often exhibiting distinct biological activities, thus commanding a vast market potential and significant economic value.[104] Enzymatic catalysis allows the conversion of racemic nitrile compounds not only into optically active carboxylic acids and amides but also to attain substantial enantiomeric excess (*ee*) values. Significant advancements in asymmetric catalytic transformations facilitated by nitrilase provide an effective and reliable solution to this challenge,[105] with high reactivity and stereoselectivity consistently sought after objectives.

Combining traditional random mutations with site-directed mutations has become an important means to achieve stereoselective regulation of enzymes. In particular, achieving stereoselective flipping without loss of enzyme activity remains a challenge.[106] Therefore, many studies have adopted innovative mutagenesis strategies aimed at improving the enantioselectivity of nitrilases for specific substrates. Sun et al.[107] constructed a random mutagenesis library by error-prone PCR and identified four key sites (M113, R128, A136, and I168). These sites significantly affect the hydrolysis rates of *S*- and *R*-mandelonitrile by enzymes, which in turn determines the enantiomer preferences of nitrilases. They are not involved in the formation of the PpL19 helical structure, but directly or indirectly affect the interaction between enzymes and substrates (Figure 4B). Notably, certain substitutions of methionine at site 113 can reverse the enantioselectivity of PpL19 from *S*-selectivity to *R*-selectivity. And two enantiocomplementary nitrilase mutants, PpL19-LH and PpL19-GYY, were constructed by combining beneficial mutations that exhibited high *S*- and *R*-selectivity for mandelonitrile, respectively. These findings not only highlight the importance of key residues in the catalytic process, but also provide valuable insights into the stereoselective regulation of other enzymes.

In another study, Zhang et al.[17] obtained a chimeric nitrilase BaNit with high activity and enantioselectivity for isobutylsuccinonitrile (IBSN) through a single fragment swapping of two parent enzymes, AaNit and BrNit, based on structure−function information. With the help of this method, the evolutionary paths can be traced by reverse mutagenesis, and the functional residues with critical properties can be rapidly targeted. On this basis, a desired robust nitrilase, BaNIT/L223Q/H263D/Q279E, was further developed using mutation site-based reverse saturation mutagenesis. The enzyme simultaneously exhibited a 5.4-fold enhancement of whole-cell catalytic activity, a 3.3-fold increase in enzyme solubility, and achieved an enantiomeric ratio (*E*) better than 300. This study validated

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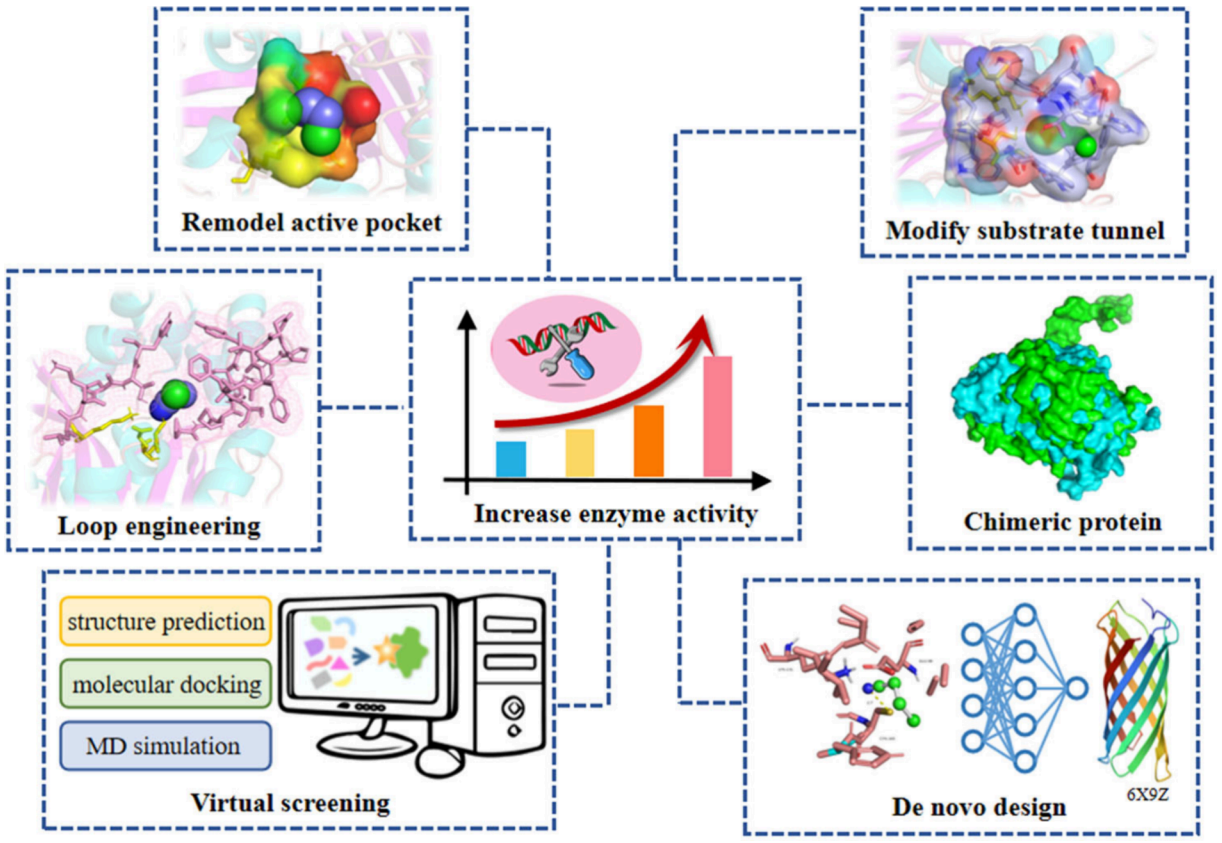

Figure 6. Multiple engineering strategies based on computer-aided enzyme modification.

the effect of asymmetric interaction between protein dimers on catalytic properties, where structural changes directly affect the conformation of high-level spiral structures, leaving the binding pocket in a highly reactive conformation. Residue 227, on the other hand, acts as a "gatekeeper" when the original cave shape at the entrance of the active pocket is replaced by a hydrophobic cleft one, which facilitates the interaction between the substrate and the active site and thus exhibits high enantioselectivity (Figure 4B and C). This was again demonstrated in the extended experiment on the chimeric enzyme BaNit.[108]

In the early years, a foreign team also obtained a higher enantioselectivity for the formation of *(R)*-mandelic acid by constructing chimeric enzymes.[109] For the nitrilase derived from *Pseudomonas fluorescens* EBC191, it has been found in several studies that the alanine residue[109] (Ala165) or cysteine residue[110] (Cys163) directly adjacent to the catalytic active cysteine residue can change its stereoselectivity and amide production (Figure 4D). It is evident that this method can rapidly locate key functional sites in the absence of precise crystal structure information and is versatile for the development of various industrial enzymes. However, the fragment exchange strategy relies strongly on the high sequence homology of parent enzymes, which may limit its application scope.

Similarly, for the selective hydrolysis of prochiral dinitrile compounds, Yu et al. used the nitrilase from *Synechocystis* sp. PCC6803 (SsNIT) as a model and proposed a "mirror-image strategy".[111,112] By analyzing the symmetry of the substrate binding pocket and identifying and exchanging two key amino acid residues W170 and V198 responsible for stereo-recognition, the enzyme successfully achieved the reversal of (*S*)-selectivity (90% *ee*) to (*R*)-selectivity (47% ee) for the substrate 3-isobutylglutaronitrile. Subsequent precise tuning of the binding pocket further yielded a mutant E8 with significantly enhanced activity and stereoselectivity (>99% *ee*, *R*).[113] Molecular dynamics simulation revealed that these mutations effectively enhanced the selectivity for the (*R*)-configuration by altering the substrate binding energy and the stability of catalytic conformation (Figure 4C). In addition, mutant E8 exhibited similar stereoselective reversal toward a range of 3-substituted glutaronitrile substrates, providing a generalizable strategy for the stereoselective modification of other nitrilases and related enzymes.

With the development of computational tools, calculation methods such as the classical binding free energy molecular mechanics/Poisson−Boltzmann surface area (MM/PBSA) have been widely used in enzyme design and optimization. Chen et al.[114] efficiently identified two hotspots of Trp57 and Val134 by the method and significantly enhanced the selectivity of nitrilase for the (*S*)-enantiomers through saturated and combinational mutagenesis (>99.9% *ee*, E > 300). These variants not only efficiently catalyzed the formation of (*S*)-3-cyano-5-methylhexanoic acid, but also completely avoided the generation of byproducts. The strategy reduces the workload of blind mutation screening while revealing the mechanism of improved stereoselectivity through molecular docking and dynamic simulation, namely adjusting the distance of substrate binding site and forming a stable hydrogen-bond network (Figure 4B). Although this method relies on high-quality structural information on the enzyme and the experimental validation process is still complicated, its remarkable screening efficiency and excellent modification effect provide a powerful tool for the development of

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Table 2. Representative Enzyme Substrate Specificity Modification Strategies

Improvement	Strategy	Enzyme	Method	Ref
substrate preference	optimize substrate channel room	4-decarboxylase	alanine scanning	122
	alternative active site confinement	squalene−hopene cyclase	tunnel simulation	123
	hydrophilicity-based engineering of the active pocket	*D*-amino acid oxidase	saturation mutagenesis	124
	virtual saturation mutagenesis	rhizopus oryzae lipase	Rosetta's Cartesian_ddg protocol	125
	semirational binding adaptation	aminotransferase	binding energy analysis	126
	semirationally designed substrate binding	cytochrome P450 PikC	noncanonical amino acid mutagenesis	127
	steric hindrance modification-based bidirectional pocket reshaping	leucine dehydrogenase	site-directed mutagenesis	128
	computational enzyme redesign	ammonia lyases	Rosetta Enzyme Design	129
	structure-guided engineering	rhamnosyltransferase	first-shell residue mutations	130
	structure-guided rational design	CYP154C2	mutation based on residual side chain length	131
	structure-guided rational engineering	MHETase	bioinformatics analysis	132
	directional-path modification	cutinase	bind groove remodeling and positive charge introduction	133
	stepwise loop insertion	phosphotriesterase-like lactonase	saturation mutagenesis libraries with the NNK randomization codon degeneracy.	134
	engineering hydrogen peroxide tunnels	cytochrome P450 monooxygenases	mutation based on residual side chain polarity	135
	loop engineering	CYP116B3	simultaneous saturation mutagenesis	136
	install a substrate recognition domain	catechol *O*-methyltransferase	Rosetta interface design of multidomain proteins	137
	de novo enzyme design	luciferases	deep-learning-based "family-wide hallucination"	138
	de novo enzyme design	lysozymes, CM, and MDH	a language model ProGen	139
	machine learning-aided engineering	PET hydrolase	3D self-supervised convolutional neural network MutCompute	140
	machine learning-aided engineering	malic enzyme	phylogenetic analysis with logistic regression	141
regioselectivity and stereoselectivity	engineering in reaction specificity and pocket space	P450s	repetitive construction of sequence chimeras	142
	flip the substrate	halohydrin dehalogenase	molecular docking	143
	scaffold sampling	CYP109B4	iterative saturation mutagenesis	144
	rational cavity design	imine reductase	hydrogen bond reorganization and cavity expansion	145
	expand the cation cage	squalene-hopene cyclase	homologous mutation	146
	structure-based redesign of the substrate cavity	indole monooxygenase	computational molecular mechanics force field	147
	adaptation strategies for substrate conformational regulation	*L*-threonine aldolases	site-directed and combinatorial mutagenesis	148
	terminal modification	7α-hydroxysteroid dehydrogenase	His-tags location and terminal truncation	149
	loop engineering	cumene dioxygenase	active-site loop variations	150
	gene duplication and fusion	4-oxalocrotonate tautomerase	directed evolution	151

stereoselective enzymes and lay an important foundation for the green industrial production of high-value chiral compounds.

These studies indicate that stereoselective regulation of enzymes is not dependent on a single strategy. By combining multiple mutation strategies, such as the combination of error-prone PCR and site-directed mutagenesis, fragment exchange and chimeric enzyme design, as well as semirational design based on molecular mechanics calculations, not only the performance of nitrilase has been effectively improved, but also important lessons have been provided for the optimization of other enzymes. With the further development of experimental validation and computational simulation, it is expected to identify key functional sites more accurately in the future, thus promoting the efficient modification of more enzyme systems.

4. CONCLUSION AND PERSPECTIVES

From the initial directed evolution to rational and semirational design, an increasing number of protein engineering strategies have relied on computer-aided modifications to provide effective pathways for improving the substrate specificity of nitrilases. In general, molecular modification of key regions such as active pockets, substrate channels, and loop regions of enzymes has emerged as research hotspots in the field of protein engineering (Figure 6). The ability of nitrilases to recognize and transform different substrates has been largely improved by structure-guided site-directed mutagenesis, gene splicing and fusion, as well as total gene synthesis technology. In addition, the advent of virtual screening technology has opened up vast potential avenues for enhancing the performance and functionality of nitrilases. Here, commonly used online servers such as PDBbind, FoldX, CASTp, Caver, PROTEINS PLUS, DeepSite, Molsoft, and fpocket, offer a more convenient and streamlined approach for comprehensive protein analysis. Simultaneously, the convergence of AlphaFold and molecular dynamics simulation has elevated the study of protein interactions and mechanisms to new heights, which relieving researchers from complex and burdensome tasks and

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unnecessary high costs, thereby significantly enhancing research efficiency and accuracy.

However, the engineering of nitrilases faces a series of challenges, including the complexity of the entire enzyme structure, the trade-off between catalytic efficiency and stability, and the prediction of synergistic effects of multiple mutation sites, resulting in the substrate-specific modification of nitrilases is still in the exploratory stage. Although the preceding text discusses numerous encouraging research advances in enhancing the substrate specificity of nitrilases, design algorithms have not penetrated deeply enough, and there is a need for further development on how to efficiently identify and modify key amino acids, as well as establish a new method for bidirectional precise regulation of substrate selectivity in nitrile hydrolysis reaction.[115] Exploring the research cases on various other enzymes can provide us with important inspiration, help us identify applicable strategies and establish new modification methods, further enhancing the potential of nitrilases in industrial applications. To this end, the following table lists representative substrate-specific modification strategies that have been devised using advanced computer-aided design techniques in recent years, demonstrating the current important research progress in the field of protein engineering (Table 2). These methods primarily rely on the homologous protein sequence alignment, existing information regarding protein three-dimensional structures or functional domains. By predicting and evaluating changes in structure, free energy, substrate binding energy, and other pertinent aspects of mutants, we can facilitate the modification of enzymes. However, to enhance the evolutionary potential of enzyme molecules, it is imperative to harness artificial intelligence for data collection, utilize classical machine learning and deep learning to aid enzyme modification, and incorporate more sophisticated technological approaches to break through the technical bottleneck of low efficiency inherent in traditional protein engineering and promote various neural network models to rapidly update and iterate in a more accurate and efficient direction.[116−120]

In the future, we should pay more attention to combining machine learning, molecular dynamics simulation and deep mutation scanning with other emerging technologies to further analyze the key regulatory factors and dynamic behaviors between nitrilases and substrates, in order to improve the quality of existing biocatalysts. Meanwhile, we will combine the synthetic biology tools to achieve the multifunctional and customized modification of nitrilases, and explore the integration of nitrilases with other enzymes in cascade reactions to realize the stepwise transformation of complex substrates, so as to further expand the prospects of their applications in green industrial synthesis. This will not only contribute to a deeper understanding of the structure−function relationship of enzymes, but also provide a generalized strategy for the specific regulation of other key enzyme species. It can be believed that with the continuous exploration in the field of biocatalysis, digital computational design will certainly become the mainstream trend of future research, and the substrate specificity engineering of nitrilases is poised to embrace broader opportunities for innovation.[121]

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Notes

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