·医药中间体生物合成·

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萜类化合物微生物合成中酶工程的研究进展与展望

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Amna Bibi, 苏立秋, 戴宗杰, 王钦宏. 萜类化合物微生物合成中酶工程的研究进展与展望[J]. 生物工程学报, 2024, 40(8): 2473-2488.
Amna Bibi, SU Liqiu, DAI Zongjie, WANG Qinhong. Enzyme engineering in microbial biosynthesis of terpenoids: progress and perspectives[J]. Chinese Journal of Biotechnology, 2024, 40(8): 2473-2488.

摘 要：萜类化合物以其结构与功能的多样性而闻名，在食品、化妆品、洗涤用品中广泛应用。微生物合成是萜类化合物绿色可持续生产的方式之一，近年来备受关注。然而，参与萜类物质合成

资助项目：国家自然科学基金(32161133019, 32301228)；ANSO青年人才奖学金
This work was supported by the National Natural Science Foundation of China (32161133019, 32301228) and the ANSO Scholarship for Young Talents.
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Received: 2024-02-28; Accepted: 2024-05-08

的天然酶存在催化活性低、特异性差、稳定性不足等问题，限制了萜类化合物的微生物生产效率。酶工程是酶蛋白结构及功能改造优化的重要手段。近年来研究人员通过使用不同的酶工程策略，使萜类合成相关酶的活性、选择性、稳定性得到了明显提升，为萜类化合物的可持续生产提供有力支持。本文综述了近年来微生物合成萜类物质关键酶改造的工程策略，包括提高酶活性、稳定性、改变特异性以及多酶协同促进传质等；同时展望了酶工程技术在萜类微生物合成中面临的挑战及未来发展方向。

关键词：萜类化合物；微生物合成；酶工程；酶改造；萜类合酶

Enzyme engineering in microbial biosynthesis of terpenoids: progress and perspectives

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Abstract: Terpenoids, known for their structural and functional diversity, are highly valued, especially in food, cosmetics, and cleaning products. Microbial biosynthesis has emerged as a sustainable and environmentally friendly approach for the production of terpenoids. However, the natural enzymes involved in the synthesis of terpenoids have problems such as low activity, poor specificity, and insufficient stability, which limit the biosynthesis efficiency. Enzyme engineering plays a pivotal role in the microbial synthesis of terpenoids. By modifying the structures and functions of key enzymes, researchers have significantly improved the catalytic activity, specificity, and stability of enzymes related to terpenoid synthesis, providing strong support for the sustainable production of terpenoids. This article reviews the strategies for the modification of key enzymes in microbial synthesis of terpenoids, including improving enzyme activity and stability, changing specificity, and promoting mass transfer through multi-enzyme collaboration. Additionally, this article looks forward to the challenges and development directions of enzyme engineering in the microbial synthesis of terpenoids.

Keywords: terpenoids; microbial synthesis; enzyme engineering; enzyme modification; terpene synthases

Terpenoids are highly diverse natural compounds, with over 80 000 distinct structures. They can be found in a broad range of organisms, including plants, fungi, bacteria, marine animals, insects, and archaea[1]. Although certain terpenes are largely responsible for protective toxins, others also have pleasant aromas. As a result, they have attracted a lot of interests in the food, drug, and cosmetic industries[2]. Terpenoids can be classified into distinct groups based on the number of isoprene (C5) units they possess. These groups include hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesterterpenoids (C25),

triterpenoids (C30), tetraterpenoids (C40), and polyterpenoids (C>40). Terpenoids can also be synthesized chemically, but these processes are energy intensive, costly and produce a lot of organic waste that is also unfriendly to the environment. The production of terpenoids using microbial hosts is considered as a safe, cost-competitive and scalable approach[3]. Biosynthesis of terpenoids employs dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) as building blocks, which are five-carbon isomers. These building blocks are produced through either the mevalonate (MVA) pathway or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in biological systems[4]. Prenyl diphosphate synthases then condense these building blocks to produce prenyl chains of varying sizes, with geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), serving as the substrates of terpene synthases (Figure 1).

Many efforts have been made in recent years to improve the production of terpenoid in microbes, such as *Saccharomyces cerevisiae* and *Escherichia coli*[5-6]. However, poor productivity (low titer, low yield, and low efficiency) and lack of stability of microorganisms during fermentation, is currently posing a challenge to establish strains

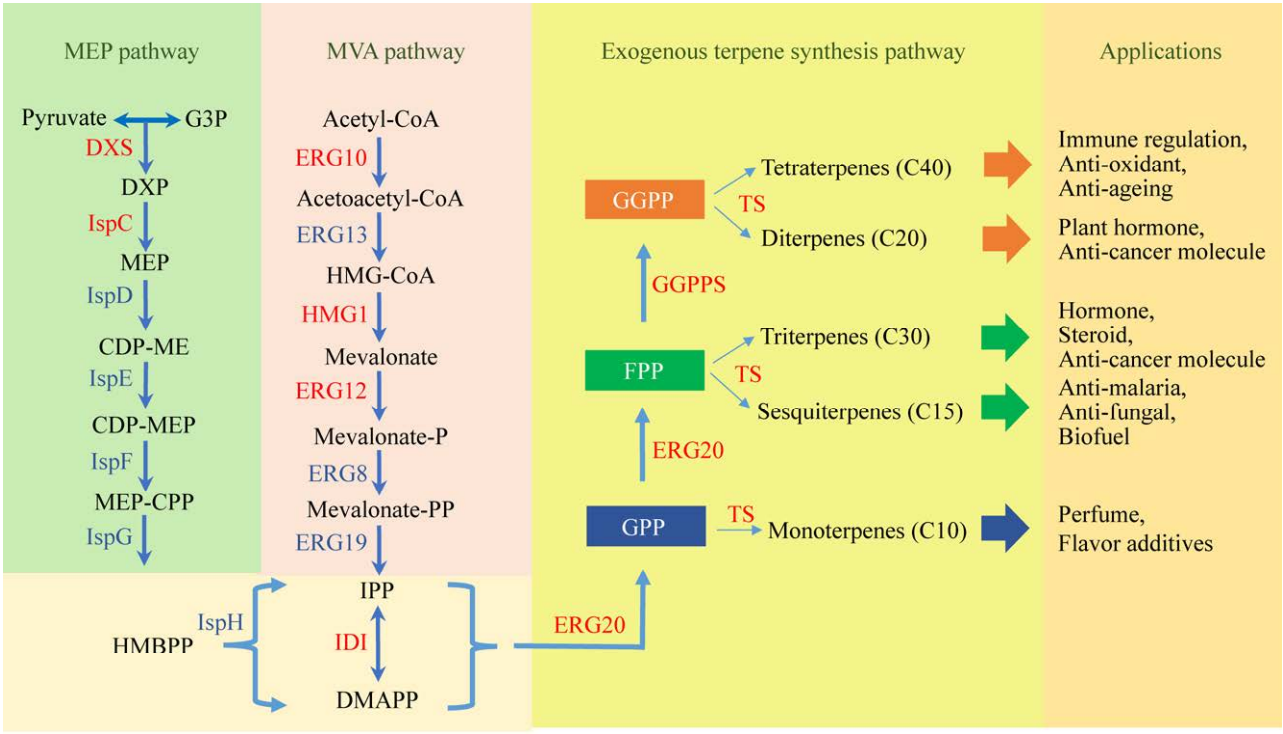

图 1　萜类微生物合成途径

Figure 1　Pathways for microbial synthesis of terpenoids. Highlighting key steps of upstream precursor supply by mevalonate (MVA) and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways, downstream terpene synthesis by exogenous terpenoid synthetic pathway, and engineered enzymes used for monoterpenes, sesquiterpenes, and diterpenes production discussed in this review are shown in red. G3P: Glyceraldehyde-3-phosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; CDP-ME: 4-diphosphocytidyl-2C-methyl-D-erythritol; CDP-MEP: 4-diphosphocytidyl-2C-methyl-D-erythritol phosphate; MEP-CPP: 2C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP: 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate; IPP: Isopentenyl pyrophosphate; DMAPP: Dimethylallyl pyrophosphate; GPP: Geranyl disphosphate; FPP: Farnesyl diphosphate; GGPP: Geranylgeranyl diphosphate.

for commercial production of terpenoids[7]. Competition between the heterologous terpenoid biosynthetic route and inherent metabolic processes of the host strain is a significant barrier to high-level terpenoid synthesis. Accumulation of hydrophobic terpenoids may contribute to the metabolic stress and damage the cells, causing cytotoxicity. A further major concern is the ineffective catalytic capability of terpenoid-related enzymes, which reduces the productivity of target products and necessitates an expensive purification procedure[8]. Therefore, in the context of heterologous microbial system, enzyme modification is crucial for refining enzyme activity beyond natural capabilities. In this study, we will shed some lights on several terpenoids biosynthesis related enzymes and highlight the significance of enzyme engineering in the yield of terpenoids *via* microbial systems.

1 Enzyme engineering strategies for terpenoids production

Enzyme engineering plays a crucial role in enhancing enzyme activity, stability, and broadening the availability of terpenoid biosynthetic products[9]. Among the key methods that have emerged are directed evolution, rational design, and semi-rational modification. Directed evolution, agnostic to enzyme structure and catalytic mechanisms, utilizes mutagenesis techniques such as error-prone PCR, DNA shuffling, and site-directed mutagenesis to create a diverse mutant library. Subsequently, selection pressures are applied to identify mutants with desired characteristics. Its advantage lies in its ability to transcend limitations in protein structure-function comprehension. However, it can be resource-intensive and requires efficient high-throughput screening methodologies[10-11]. In contrast, rational design heavily relies on knowledge of protein sequences, structures, and catalytic mechanisms. It involves meticulous modifications, leveraging computational tools such as molecular docking, dynamics simulations, and quantum mechanics. Rational design's primary benefit is its efficiency in narrowing down the mutant pool and targeting specific objectives. Nevertheless, its success is often constrained by incomplete knowledge of protein structures and the complexities of predicting mutational interactions[12]. Semi-rational design serves as a bridge between directed evolution and rational design. It integrates rational design principles within a directed evolution framework, confining random mutations to specific amino acid residues. This approach ensures a smaller mutant library, thereby increasing the likelihood of obtaining favorable mutants. However, it still requires a certain level of understanding of protein function and structure[10] (Figure 2). Each protein engineering strategy has unique strengths and weaknesses. The choice of strategy hinges on the specific objectives, the extant knowledge about the protein, and the available resources.

In addition to the main strategies stated above, protein engineering involves various other strategies to modify and optimize proteins for specific purposes, including protein fusion and chemical modification. Protein fusion combines different proteins to create hybrids with new or improved properties. Chemical modification alters amino acid residues to achieve the desired changes[13]. These enzyme engineering techniques have been particularly beneficial in promoting the microbial biosynthesis of terpenoids. They enable the optimization of enzyme stability, substrate specificity, product specificity, catalytic efficiency, and other desired characteristics. By employing these strategies, researchers can enhance the production and yield of terpenoids.

1.1 Enhancing catalytic efficiency

1.1.1 Modifications of active center

For the overall catalytic outcome, the amino acid residues of active center, are of great value. They play a vital role in shaping the modulation and variations of active sites as well. One crucial enzyme in the terpenoids biosynthesis pathway is mevalonate kinase (MK). To optimize MK activity, a study employed a directed evolution strategy in

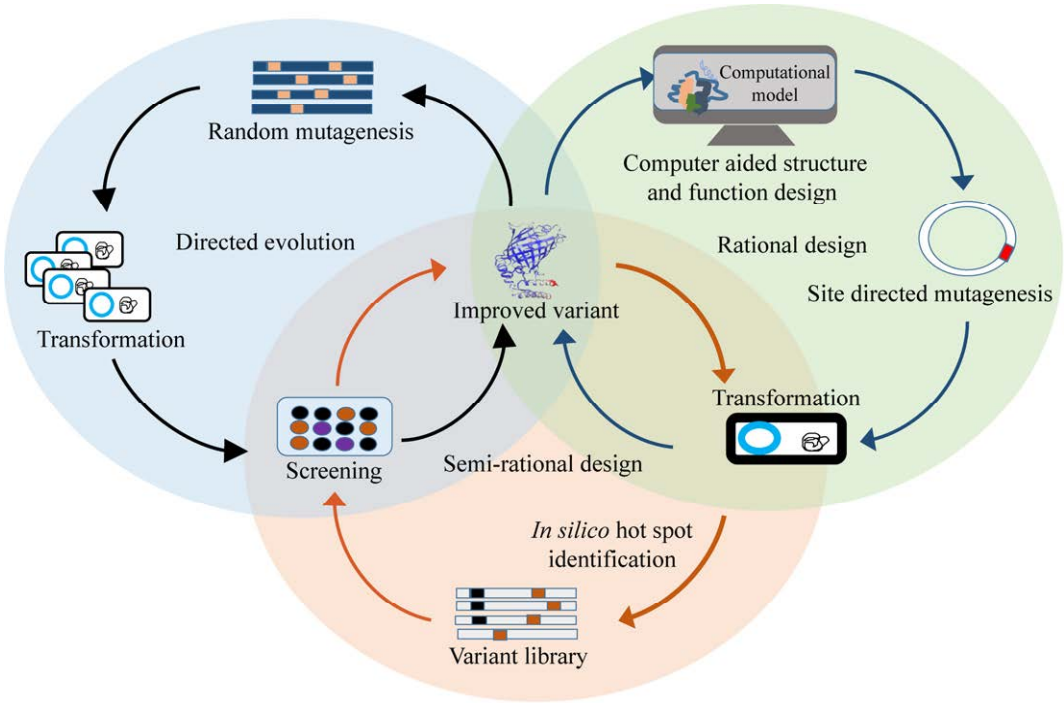

图 2　蛋白质工程策略

Figure 2　General strategies for protein engineering. Protein engineering primarily relies on directed evolution, rational engineering, and semi-rational design to effectively construct the desired enzyme variants.

E. coli, and three specific mutations (V13D, S148I, and V301E) which are located in the active center were identified[14]. Compared to the wild-type MK, the purified mutant MK exhibited a decrease of 74% in the value of K_m and an improvement of 26% in the value of k_{cat}, indicating improved substrate affinity and enhanced catalytic efficiency of the mutant enzyme[14]. These improvements led to a remarkable 2.4-fold increase in lycopene production when using the mutant MK compared to the wild-type MK (Figure 3A)[14].

In addition to MK, isopentyl diphosphate isomerase (IDI) is also the key enzyme in the terpenoids biosynthesis pathway. According to the structure of IDI, L141, Y195, and W256 are near the active pocket, forming hydrogen bonds with the neighboring β-phosphates of IPP[15]. Notably, F195 and C256, which are nonpolar amino acids, were positioned in proximity to the hydrophobic group of IPP, indirectly expanding the hydrophobic range of the active pocket[15]. The mutated IDI enzyme (Y195F and W256C) exhibited a 10% decrease in K_m and the k_{cat} was 28% improved compared to the original IDI[15]. Consequently, the enzyme activity of IDI mutation L141H-Y195F-W256C was remarkably enhanced by 2.53-fold[15]. This increase in enzyme activity corresponded to a substantial boost (2.10-fold) in lycopene production[15]. These findings highlight the significance of the identified mutations within the active pocket, leading to improved enzyme activity and ultimately improve the production of terpenoids.

1.1.2　Changing the preference of cofactors

Cofactors remain vital elements for the activity of terpenoid synthases. By manipulating the cofactors involved in enzymatic reactions, researchers have successfully modulated the catalytic efficiency of terpenoid synthases, ultimately leading to increased terpene yields.

For instance, in a study focused on the production of artemisinin, a valuable antimalarial

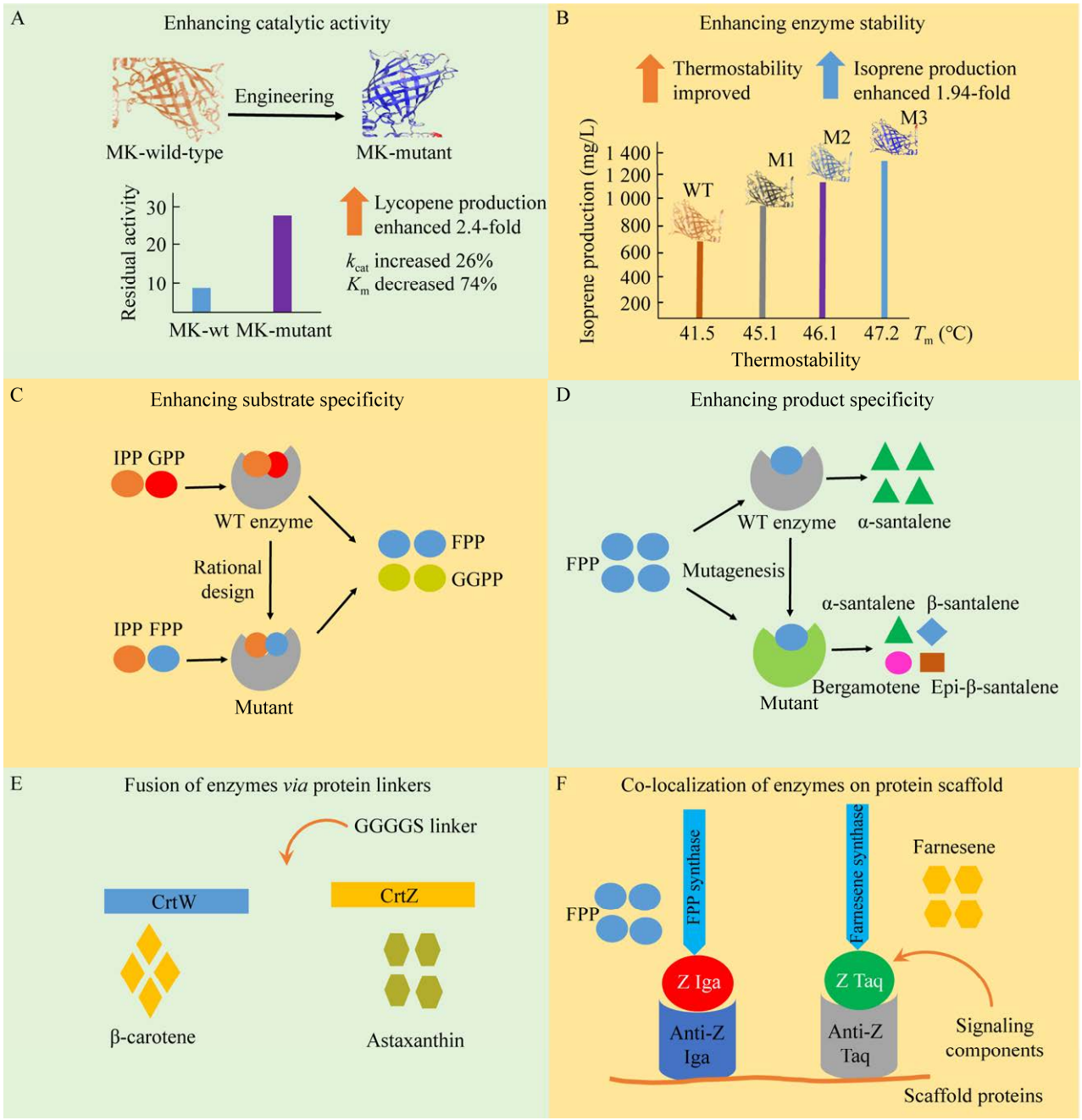

图 3　酶工程改造策略　提高催化活性(A)，提高酶稳定性(B)，提高底物(C)及产物(D)特异性，蛋白连接器促进酶融合(E)，蛋白脚手架促进酶共定位(F)

Figure 3　Various enzyme engineering strategies employed to enhance catalytic activity (A), stability (B), substrate and product specificity (C and D), enzyme fusion (E) and colocalization (F). CrtW: 4,4′ β-carotene oxygenase; CrtZ: 3,3′ β-carotene hydroxylase.

compound, cofactor changing was employed to improve the activity of amorpha-4,11-diene synthase (ADS)[16]. Through rational enzyme modification, the NADPH-binding sites of ADS were modified, resulting in an increased preference for NADH as the cofactor[16]. This alteration led to

a 3-fold enhancement in artemisinic acid production, ultimately contributing to the improved overall yield of artemisinin[16]. Similarly, in another investigation, boosting the biosynthesis of taxadiene, a precursor of the anticancer drug paclitaxel, cofactor engineering was utilized[17]. By introducing mutations in the taxadiene synthase (TS) enzyme, the researchers successfully altered its cofactor preference from NADPH to NADH. This modification resulted in a 2.4-fold increase in taxadiene production, thus facilitating the subsequent bioconversion steps towards paclitaxel production[17].

Some sesquiterpene synthases have been shown to utilize Mg^{2+} as a cofactor, while conifer monoterpene synthases, like pinene synthase, exhibit a preference for Mn^{2+}[18]. However, the cytosolic concentration of Mn^{2+} in *E. coli* is relatively low (around 10 μmol/L), whereas the concentration of Mg^{2+} is much higher (10−20 mmol/L). This difference creates a limitation for pinene production. In an interesting investigation, scientists conducted a single round of mutagenesis and screening on pinene synthase[18]. Surprisingly, the resulting variant of the enzyme demonstrated a shift in its metal dependency from Mn^{2+} to Mg^{2+}. This alteration enabled a remarkable increase in pinene productivity, with approximately 140 mg/L achieved in *E. coli* cultures[18]. These examples highlight the potential of cofactor preference changes in terpenoid synthases to enhance terpene production.

1.1.3 Enhancing solubility

In synthetic metabolic pathways, achieving optimal function and efficiency of enzymes is crucial for successful implementation. When it comes to expressing non-native proteins, such as enzymes derived from plants involved in terpenoid synthesis, in microbial systems, the *in vivo* properties of these enzymes, such as solubility and stability, often face challenges[19]. To overcome these challenges, researchers commonly use codon optimization and promoter engineering to enhance protein expression levels. When expressing membrane bound proteins in *E. coli*, most often a high amount of inclusion bodies are formed. This is due to the fact that *E. coli* lacks inner organelles and thus the native protein cannot be properly incorporated into membranes. Consequently, the hydrophobic regions are displayed and agglomerate to inclusion bodies[9]. To further improve the *in vivo* properties of enzymes, truncation of the membrane anchor region or replacement with solubilizing leader peptides from highly expressed heterologous P450s has been explored. These approaches have been shown to enhance the catalytic performance of enzymes and overcome issues related to low solubility and expression levels[9].

One successful example of enhancing the expression and function of enzymes is demonstrated by the replacement of the N-terminal membrane anchor region of CYP76AH4 from *Rosmarinus officinalis*, to improve soluble expression of heterologous protein CYP76AH4 by replacing this region with the leader sequence MAKKTSSKGK, researchers were able to achieve functional expression of CYP76AH4 in the cytosol of *E. coli*[20]. This modification enabled CYP76AH4 to catalyze the oxidation of abietatriene, resulting in the production of the diterpenoid ferruginol[20]. Another example illustrates the enhancement of 8-cadinene hydroxylase (CAH) expression by modifying the N-terminal membrane anchor. Researchers replaced the predicted transmembrane sequences of the native CAH with N-terminal sequences from CAH derived from other species, leading to a significant 5-fold improvement in the activity of 8-cadinene hydroxylase[21]. Overall, by employing strategies such as modifying of N-terminal membrane anchor sequences and adding leading peptides to target enzymes, researchers can overcome challenges related to enzyme expression levels and solubility.

1.2 Enhancing enzyme specificity

1.2.1 Substrate specificity

Proteins can acquire novel functions and specificities in response to various environmental changes through molecular evolution by changing substrate range and catalytic activity. Erg20 facilitates the biochemical reaction that transforms

IPP and DMAPP into FPP, which is a key precursor molecule in the biosynthesis of various terpenoids. Based on the structure of FPP synthase (*Gallus gallus* FPS1), the researchers utilized a homology modeling approach to construct a model of Erg20[22]. From this model, a specific set of residues was identified that form the active site cavity, namely Y95, F96, L97, Q168, and Y201[22]. These residues were selected due to their potential to create additional space within the cavity, which is necessary to accommodate FPP as a co-substrate for GGPP production, by introducing mutations to these residues, either individually or in combination, it would be possible to modify the active site cavity and enable it to accommodate FPP (Figure 3C)[22]. The resulting Erg20 variant with GGPP synthetic activity, co-expressed with 8-hydroxy copalyl diphosphate synthase and cultured in shake flasks, enhanced sclareol production levels by approximately 70-fold[23].

In another study, bulky residues in the middle of the catalytic site were removed and added to the prenyl diphosphate synthases through an enzyme-substrate docking technique to utilize longer and shorter substrates[24]. From results the engineering of prenyltransferases have been shown to accept unfamiliar substrates affording a series of novel terpenoids[24]. Besides this, a novel terpenes C11 containing 11-carbons was synthesized using a GPP methyltransferase, through which monoterpene precursor was methylated to give 2-methyl-GPP (2meGPP), and consumption efficacy of 2meGPP through various monoterpene synthases increased to 25%, showed the use of synthetic substrates and enhanced the chemical space of terpenes[24]. In an experimental set, ratio of C11 terpenes further increased in yeast by substituting tyrosine instead of phenylalanine at the 571st position in SfCinS1, results show about 6-fold enhancement in the affinity for 2meGPP and total catalytic efficiency remained the same[24]. These findings underscore the potential of harnessing enzymatic engineering to generate novel terpenoids.

1.2.2 Product specificity

Enzymes possess the ability to selectively catalyze specific chemical reactions and produce a particular product, which is referred to as product specificity. Enzymes exhibit varying degrees of product specificity, with some enzymes are highly specific and catalyzing only a single reaction, while others may catalyze multiple related reactions. Product specificity of enzymes is determined by the precise arrangement of their active sites, which allows them to bind and interact with specific substrates and facilitate the formation of specific products[25]. From *Abies grandis*, an important example is γ-humulene synthase using FPP as a substrate and produces more than fifty types of sesquiterpenes[26]. Researchers used the crystal structure of 5-epi-aristolochene synthase as a model to check the plasticity of γ-humulene synthase by identifying 19 different active-site residues based on homology structure[26]. Results showed by application of saturation mutagenesis to specific residues, four residues significantly affected catalytic product specificity: W315, M447, S484, and Y566, located at the active site[26]. Mutations to these residues shifted the relative selectivity (the amount of one product relative to another product) by 100- to 1 000-fold[26]. These results suggest that plasticity residues could significantly drive molecular evolution, and most of the substitutions in plasticity residues additively affect protein functions.

By modifying product specificity, the chemical space of terpenoids can be greatly improved. It is not only reflected in the modification of terpenoid cyclase, but also has the same effect on other terpenoid enzymes. Through targeted mutations, researchers successfully modified the active site of *Pinus taeda* abietadiene oxidase. These mutations involved hydrophobic residues of varying sizes, which influenced the type of products produced by the enzyme. Remarkably, these modifications altered the overall pocket volume without affecting its chemical properties. As a result, novel compounds including 18-hydroxy miltiradiene, 19-hydroxy miltiradiene, and 19-hydroxy manool were discovered. Notably, these compounds had not been previously reported in any other P450 enzyme[27].

In addition, terpenoid synthase product specific modification strategy can be applied to control the ratios of components in plant essential oils (PEOs) during biotechnological production (Figure 3D). The focus of the study was on analyzing the catalytic reaction pathways of two santalene synthases, SaSSy and SanSyn, utilizing advanced multiscale simulations. During the investigation, they identified a critical residue, F441, within the SanSyn enzyme that influences the conformational dynamics of intermediates[28]. This residue specifically facilitates direct deprotonation through the involvement of the general base T298, resulting in the predominant production of α-santalene[28]. To broaden the range of synthesized products, they introduced a mutation at this residue, generating a new mutant enzyme named SanSyn F441V[28]. This mutant enzyme exhibited the ability to produce both α- and β-santalenes and showed a favorable product profile, with 57.2% α-santalene, 28.6% β-santalene, 6.7% epi-β-santalene, and 7.6% exo-α-bergamotene[28]. These component ratios successfully achieved a desired blend of primary essential oil (PEO) constituents, aligning well with the ISO 3518:2002 standard[28]. The successful manipulation of the enzyme through enzyme modification highlights a remarkable approach in constructing biotechnological platforms for PEOs that possess desirable product ratios. The above analysis revealed that the majority of the introduced mutations have a significant role in determining the catalytic outcome of the enzymes.

1.3 Enhancing enzyme stability

Enzyme stability is a complex concept that refers to the capacity of enzymes to retain their three-dimensional structures, which are crucial for catalysis, in storage and operational processes[29]. This stability is influenced by a multitude of interactions, including hydrophobic bonds, hydrogen bonds, salt bridges, protein surface charge, disulfide bonds, and metal ions. Given the complexity of these interactions, enhancing enzyme stability presents a significant challenge, particularly due to the partial understanding of the structure-function relationship, which complicates the identification of suitable mutation targets.

However, advancements in enzyme engineering have led to the development of innovative strategies for enzyme stabilization[30]. Enzyme engineering techniques have successfully been employed to enhance the thermostability of isoprene synthase from *Ipomoea batatas* (IspSib), an enzyme previously identified as a limiting factor for isoprene production in engineered *E. coli*. To achieve this, scientists created a unique tripartite protein folding system called lac′-IspSib-′lac, this system linked the stability of IspSib to antibiotic ampicillin resistance, facilitating high-throughput screening of variants[31]. Through two rounds of random mutation and site-saturation mutation, three variants were identified: IspSib N397V-A476V, IspSib N397V-A476T, and IspSib N397V-A476C. These variants exhibited improved thermostability, which was confirmed through *in vitro* tests. The melting temperatures of N397V-A476V, N397V-A476T, and N397V-A476C were (45.1±0.9) ℃, (46.1±0.7) ℃, and (47.2±0.3) ℃, respectively. All of these values exceeded the melting temperature of the wild-type IspSib (41.5±0.4) ℃. Notably, the use of the IspSib N397V-A476T variant resulted in a significant increase in isoprene production during shake-flask fermentation. Isoprene production was boosted by 1.94-fold, reaching a level of 1.3 g/L[31] (Figure 3B). From another investigation, it has shown that Gly and Pro are significantly less mutable, compared to other amino acids in *E. coli* central metabolic enzymes. Gly and Pro mediated mutation of γ-humulene synthase increased the folding capacity of mutant variants at elevated temperatures. This increased the sesquiterpene production from 3.3-fold (at 20 ℃) to 220-fold (at 37 ℃), supporting the stability of enzymes in a heterologous system[32].

1.4 Enhancing mass transfer of multiple enzymes

Enzyme co-localization is a straightforward approach of consecutive coordination of enzymes to further enhance catalytic efficiencies[33]. When heterologous enzymes become unable to collaborate

with native enzymes as a result microbial metabolite production is reduced, this in turn, can result in the degradation, diffusion, or utilization of intermediates by competitive pathways, leading to a reduction in overall production and the release of toxic and reactive metabolites that pose a threat to the survival of the host cell[34]. To prevent the accumulation of toxic metabolites, co-localized enzymes are coordinated in a proximity, ensuring higher catalytic rates of sequential pathway enzymes[33]. The method is similar to natural enzymatic systems, in order to have enhanced metabolic products, their metabolic flux is increased by protein tunnel through direct substrate channeling[35]. In the process of terpene synthesis, the coordinated action of multiple enzymes is another key factor in improving the overall catalytic efficiency and product quality. Through enzyme engineering techniques, we can fuse enzymes or co-expressed in subcellular organelles to achieve more efficient substrate delivery and product accumulation. Substrate channeling is improved by bringing active sites closer through enzyme fusion, a straightforward approach (Figure 3E).

Production of α- and β-pinene can be improved by fusing and co-expressing enzymes. However, the stability and construction of the resulting proteins are dependent on the order of the domains. Comparing the fusion of GPPS to pinene synthase at the C and N-terminus, it was observed that fusion at the C-terminus had an unfavorable effect on pinene production. When pinene synthase and GPPS were fused in the correct order, pinene production in *E. coli* was enhanced by approximately 32 mg/L[36]. Therefore, it is essential to consider the order of enzyme fusion when attempting to improve terpene production[36]. Respectively, the production of β-phellandrene was significantly enhanced by a specific strategy involving fusion of β-phellandrene synthase with the β-subunit of phycocyanin, which is encoded by the endogenous *cpcB* gene, in *Synechocystis transformants*. The resulting improvement was remarkable, with a 3.2 mg/g of total dry cell weight that is almost 100-fold increase in β-phellandrene production[37]. In a similar research, the overexpression of chloramphenicol (*cmR*) and kanamycin (*nptI*) resistance genes in fusion with GPPS is investigated in *Synechocystis*[38]. Improved expression level of GPPS through fusion constructs, showing that both heterologous (such as *nptI* and *cmR*) or homologous (like *cpcB*) genes having high expressions are considered as leader sequence and play a crucial role in the enhanced expression of required gene, which is vital for expressing isoprenoid pathway genes. From subsequent studies, it has been revealed that in biotechnology for terpenoid production, enzyme fusion is considered one of the most promising approaches.

Moreover, besides fusion proteins colocalization can be further promoted by another alternative technique includes attachment of synthetic scaffolds to enzymes in a sequential and programmable order (Figure 3F). In synthetic scaffolds, desired enzymes are connected in a modular manner to interactive domains. It is found in studies that the intermediate pathway flux controls the production enzyme to scaffold ratio is found as an important factor[39]. Several scaffolds were produced synthetically with a diverse number of repeated metazoan signaling proteins, such as the PSD95/DlgA/Zo-1 (PDZ) domain, SH3 domain, and GTPase binding domain. It is suggested that through this mevalonate titer is improved up to 77-fold compared to synthetic scaffold, as the intermediate processing is higher due to enzyme colocalization[40]. The farnesene production was boosted up to 135% by enzyme scaffolding in fed-batch cultivations, showing enhanced overall farnesene compared with non-scaffolded pathway[41]. Activity loss and undesirable conformational changes may occur in scaffolded enzyme assemblies due to the dissimilar properties of individual enzyme components[41]. This drawback was overcome by examining interactions between short peptide tags comprising of the 18 amino acids RIAD peptide and 44 amino acids RIDD peptide, which originated from a kinase-anchoring protein, AMP-dependent protein kinase[42]. It is found that due to robust binding ability of RIDD and RIAD peptides at a

2:1 stoichiometry, without scaffold assemblies of modular enzymes. The interaction of both peptides was used and fused with geranylgeranyl diphosphate synthase (CrtE) and IDI to yield carotenoids in *S. cerevisiae* and *E. coli*. In *E. coli*, fusion of IDI with CrtE indicated an increase of 5.7-fold increase (276.3 mg/L) in the over-all carotenoid production[42]. Likewise, in *S. cerevisiae*, lycopene yield increases by up to 58% as a result of the IDI-CrtE complex formation, which leads to ultimate titer of 2.3 g/L in fed-batch fermentation[42]. The optimized strategies of genetic manipulation and novel enzyme fusions demonstrate the potential for improving the production of valuable compounds and also highlight the importance of exploring alternative techniques for enhancing metabolic pathways and generate desirable products.

2 Conclusion and prospects

This study has highlighted the various enzyme engineering approaches that have been used to enhance the production of terpenoids. These approaches include improving catalytic activity, varying the selectivity of substrates or products, enhancing stability, and enhancing mass transfer of multiple enzymes. Examples of enzyme engineering strategies are presented in Table 1−4, with detailed information on enzymes involved in the MVA pathway, MEP pathway, and exogenous terpenoid synthesis pathway and their respective genetic modifications, resulting in changed enzyme function and product yield. Enzyme engineering strategies hold immense potential for the microbial biosynthesis of terpenoids, and significant advancements have been made in recent years through directed evolution and rational design approaches. However, it still has the problems of labor-intensive directed evolution and the low accuracy of rational design. Below, we provide an outlook on the application of advanced enzyme engineering technology in terpene synthesis.

表1 提高萜类生物合成催化活性的酶工程策略

Table 1 Examples of enzyme engineering to enhance catalytic activities

Enzyme	Substrate	Product	Mutations	Biological effects	References
Hydroxymethyl glutaryl-CoA synthase (HMGS)	Acetoacetyl-CoA	HMG-CoA	(C159A)	Production of mevalonate enhanced by 25-fold	[43]
Mevalonate kinase (MK)	Mevalonate	Mevalonate phosphate	(V13D-S148I-V301E)	Production of lycopene enhanced by 2.4-fold	[14]
Isopentenyl disphosphate isomerase (IDI)	IPP	DMAPP	(L141H-Y195F-W256C)	Catalytic activity enhanced by 2.53-fold	[15, 44]
3-hydroxyl-3-methylglutaryl-CoA reductase (HMGR)	HMG-CoA	Mevalonate	(P200A-G206A-T239P-G319A-G352A-G417A-P428G-K474G-G495A)	Production of mevalonate enhanced by 3-fold	[32]
GGPP synthase (GGPPS)	GPP	GGPP	(P96C)	Production of sclareol enhanced by 70-fold	[23]
Deoxyxylulose 5-phosphate synthase (PtDXS)	G3P	DXP	(A147G-A352G)	Enzyme activity improved by 10.9-fold	[45]
8-cadinene hydroxylase (P450-CYP76AH4)	Cadinene	8-hydroxy cadinene	Substitution of lysine and serine residues at N-terminal region	Production of 8-hydroxycadinene enhanced by 5-fold	[21]

IPP: Isopentenyl pyrophosphate; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; GPP: Geranyl disphosphate; G3P: Glyceraldehyde-3-phosphate; DMAPP: Dimethylallyl pyrophosphate; GGPP: Geranylgeranyl diphosphate; DXP: 1-deoxy-D-xylulose 5-phosphate.

表2 改变萜类生物合成特异性的酶工程策略

Table 2 Examples of enzyme engineering to enhance substrate and product specificity

Enzyme	Substrate	Product	Mutations	Biological effects	References
1,8-cineole synthase (SfCinS1)	2meGPP	C11 terpenes	(N388S-I451A)	Enhanced substrate specificity; Production of C11 terpene enhanced by 6-fold	[24]
Santalene synthase (SanSyn)	FPP	Alpha, beta and epi-beta santalene	(F441V)	Mutant SanSyn F441V possesses the ability to produce both α- β- and Epi-β-santalenes	[6]
γ-humulene synthase	FPP	Bisabolene	(A336V-M447H-M562T)	Enhanced product promiscuisity; Production improved up to 1 000-fold	[26]
Lycopene cyclase	GGPP	β-carotene	(Y27R)	Enhanced product selectivity; production of β-carotene was significantly enhanced, by 1 441-fold	[46]
Pentalenene synthase	FPP	Pentalenene, β-chamigrene, β-elemene, sativene	(T182A)	Enhanced product diversity; with pentalene the sativene, β-chamigrene, thujopsene-I3 and β-elemene also produced	[47]

2meGPP: 2-methyl-geranyl disphosphate; FPP: Farnesyl diphosphate.

表3 提高萜类生物合成酶稳定性的酶工程策略

Table 3 Examples of enzyme engineering to enhance stability

Enzyme	Substrate	Product	Mutations	Biological effects	References
Isoperene synthase	DMAPP	Isoprene	(N397V-A476T)	Thermostability optimized and production of isoprene enhanced by 1.94-fold	[31]
IDI	IPP	DMAPP	(L141H-Y195F-W256C)	Enhanced enzyme stability, the half-lives at 25–50 ℃ were 2.6-6 times longer.	[15]
τ-muurolol synthase	FPP	τ-muurolol	C terminus truncation	Remains active at up to 78 ℃	[19]

表4 提高萜类生物合成多酶传质的酶工程策略

Table 4 Examples of enzyme engineering to enhance mass transfer of multiple enzymes

Co-localization strategy	Substrate	Product	Improved production	References
Fusion of CrtZ and CrtW using three different sizes of flexible linkers of glycine, serine and proline	β-carotene	Astaxanthin	Production of astaxanthin improved by 1.4-fold	[48]
Co-localization and spatial organization of NES and FPPS	FPP	Nerolidol	Production of nerolidol enhanced by 50-fold	[49]
Fusion of *ispA* and α-farnesene synthase using two repeats of the GGGGS linker	FPP	α-farnesene	Production of α-farnesene enhanced by 1.5-fold	[50]
Fusion of Erg20, Bts1 and SmCPS, SmKSL using GGGS linker	IPP, DMAPP, GGPP	Diterpenoids	Production of miltiradiene enhanced by 4.4-fold	[51]

Directed evolution has been discussed as an important technique when specific properties and structural information related to catalytic activities of enzymes are unknown[40]. However, the process of generating gene libraries with effective genetic diversification can be time-consuming, taking days or even weeks. In this regard, *in vivo* mutagenesis provides a promising alternative for achieving genetic diversity. A notable approach gaining attention is the continuous directed evolution of proteins *in vivo*, which includes techniques such as phage-assisted continuous evolution, directed evolution based on DNA polymerase mutations, and retroelement-based *in vivo* mutagenesis[52]. Efficient screening methods play a crucial role in the successful implementation of directed evolution technology. Recently in the field of synthetic biology has witnessed significant advancements, leading to the development of various biosensing components

that are utilized in biosensor-based screening systems. These biosensors have the ability to convert changes in ligand concentrations into easily detectable output signals through transcriptional or RNA regulation. This enables the coupling of ligand concentration with fluorescence signals or cell growth, facilitating high-throughput screening capabilities. By employing biosensor based screening systems, researchers can effectively identify and select improved biocatalysts for further optimization through directed evolution[53]. Isoprene is a fundamental structural component found in terpenoids, which are a diverse class of natural compounds. It has made significant progress in this field by developing a high-throughput screening method using a whole-cell biosensor for the detection of isoprene synthase[54]. This approach provides a solid foundation for the future development of sensors that can selectively respond to terpenoids[54].

One approach to streamline experimental screening and reduce library size is through "semi-rational" prescreening. In recent years, various computational tools have been developed to identify target residues that are more likely to yield positive mutations, resulting in higher-quality libraries. These include sequence-based methods, structure-based methods, molecular dynamics (MD), and machine learning. Sequence-based methods, such as FOLDEF and PoPMuSiC 2.1, analyze protein stability, while SIFT, PANTHER, and PROVEAN assess the functional impact of amino acid substitutions. Structure-based methods, on the other hand, utilize tools like CAVER 3.0 to analyze protein structure channels, CASTp, LPC, and CSU software for protein interface analysis, and Q-SiteFinder, SITEHOUND-web, LigPlot+, and HotSpot Wizard for ligand binding site analysis and mutation hotspot prediction. Molecular dynamics (MD) simulation is a powerful technique that captures detailed information about the position and velocity of every atom in a system, providing insights to information which is unobtainable through experiments alone. MD simulation-assisted directed evolution methods have been successfully employed in the design of terpene synthases with different product specificities[55]. With the rapid development of data science, machine learning is becoming increasingly widespread in enzyme engineering. Its main purpose is to construct a sequence-function mapping through measured data and guide experiments to explore the relevance more effectively. However, to further enhance the precision and reliability of these predictions, the integration of quantum mechanics/molecular mechanics (QM/MM) techniques has become increasingly pertinent. QM/MM offers a unique blend of computational approaches, combining the accuracy of quantum mechanical calculations with the computational efficiency of molecular mechanical methods. This hybrid approach allows for a more detailed and accurate understanding of the intermolecular interactions at play, thereby leading to more reliable predictions of mutational outcomes. By incorporating QM/MM into our arsenal of computational tools, we can expect to achieve even higher-quality mutation libraries, with mutations that are not just positive but also optimized for specific desired outcomes[56]. It is believed that with the continuous development of computational tools, the directed evolution of terpene bioproduct-related enzymes will become faster and more efficient[52].

To avoid long screening process, rational design utilizing computational methods has been considered as another potential approach. Such as *de novo* design will become increasingly important in enzyme engineering, it is based on the physical and chemical principles of protein folding, and various structures can be designed from scratch under the guidance of computational molecular simulation without relying on existing protein structures as templates[57]. Efficient employment of these strategies, based on the structure and chemical principles of protein folding, leads to optimal terpenoids production. In the future, the identification and construction of novel enzymes involved in terpenoid biosynthesis will be facilitated by modern techniques in computational biology.

Recently, the development of self-driving laboratories has revolutionized enzyme engineering. The self-driving autonomous machines for protein landscape exploration (SAMPLE) is an advanced system that revolutionizes enzyme engineering by automating and expediting the process. It seamlessly integrates automated learning, decision-making, protein design, and experimentation to efficiently explore fitness landscapes and discover optimized proteins. This cutting-edge technology serves as a promising gateway for future research endeavors in the field[58].

With the continuous development of enzyme engineering technology, its application in terpene synthesis will become more extensive and in-depth. In the future, we are expected to use enzyme engineering technology to achieve the development of more efficient, selective, and stable terpene synthases. This will provide more bioactive substances and solutions for the fields of biomedicine, agriculture, and chemicals. At the same time, through the in-depth study of interdisciplinary research such as synthetic biology and metabolic engineering, we will be able to better understand and optimize the terpene synthesis pathways of microorganisms, achieving more precise and efficient production of terpene compounds.

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