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Review

Recent Advances in Multiple Strategies for the Synthesis of Terpenes by Engineered Yeast

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Abstract: Terpenes are an important class of natural secondary metabolites with a wide range of applications in food, pharmaceuticals, and biofuels. Currently, the traditional production methods of terpenes almost depend on plant extraction and chemical conversion. The plant extraction method consumes a lot of natural resources and makes it difficult to separate the target compound from the extractives, while the chemical conversion method has a complex synthesis route and leads to severe environmental pollution. Compared to plant extraction and chemical conversion methods, the microbial synthesis method has the advantages of preferable sustainability, low production cost and environmental friendliness, and is a potential way to achieve efficient terpenes production in the future. Yeast is a conventional platform for bio-chemical production and is also engineered to synthesize terpenes due to their abundant intracellular acetyl-CoA, high metabolic flux of the MVA pathway, high local concentrations of substrates and enzymes, and fewer by-products. At present, a variety of terpenes including α-farnesene, squalene, limonene, β-carotene have been successfully synthesized by the engineered yeast via the application of multiple strategies. This work summarized the progress of research on these strategies conducted in the synthesis of terpenes from several aspects, including the adaptive screening and expression of terpene synthases, the regulation of synthesis pathways, and the application of intracellular compartmentalized expression strategy. The perspectives and challenges were also discussed, from which it was hoped that some useful views for future research on the synthesis of terpenes in yeast would be provided.

Keywords: yeast; terpenes; metabolic engineering; rate-limiting steps; central carbon flux; intracellular compartmentalized expression

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1. Introduction

Terpenes are a class of natural compounds composed of isoprene as structural units, mainly including monoterpenes, sesquiterpenes, diterpenes, and triterpenes (Figure 1) [1]. Many terpene compounds have wide applications in food and pharmaceutical fields. For example, lycopene, β-carotene, and other colorful carotenoids have broad applications in the food industry [2]; squalene and patchoulene are important vaccine adjuvant and drug precursors in the pharmaceutical industry [3]. In addition, some terpenes have the potential to be a feedstock for advanced biofuels due to the high density and high combustion heat, such as β-caryophyllene, sabiene, α-pinene, and so on [4,5]. Therefore, terpenes have high commercial value (Table 1) and have attracted a great deal of attention in recent years.

Table 1. The potential applications and market values of partial terpenes.

Terpenes	Applications	Market Value	Current Production by Microbes (g/L)	Reference
Squalene	Pharmaceuticals, health products, and vaccine additives	241.9 million USD	21.2	[6–9]
Limonene	Food additives, pharmaceuticals, cosmetics, biomaterials, and biofuels	1.9 billion USD	2.23	[10–15]
Farnesene	Food additives, pharmaceuticals, cosmetics, biofuels	315.8 million USD	130	[16–20]
β-carotene	Food additives, health products, pharmaceutical, and cosmetics	300 billion USD	39.5	[21–24]
Astaxanthin	Health products cosmetics, and pharmaceuticals	663.89 million USD	1.18	[25–27]

The traditional production methods of terpenes mainly relied on plant extraction and chemical catalysis. However, plant extraction consumed large amounts of natural resources due to the low contents of terpenes in plants, and some similar terpenes in the extracts made the separation process more complex, which severely limited the extraction efficiency [28]. Although chemical catalysis was a conventional method for chemical production, it was difficult to synthesize some terpenes with complex structures. Moreover, severe climate change and resource scarcity have also exposed the shortcomings of chemical methods [29]. The development of an alternative method has been the focus in the production of terpenes.

The microbial conversion method has the advantages of environmental friendliness, high renewability, and low production cost, which has been successfully applied to produce a variety of biochemicals and was thus a potential way to achieve efficient terpenes' production [30]. Currently, the microbial synthesis of terpenes mainly depended on the 2-methyl-D-erythritol-4-phosphate (MEP pathway) and the mevalonate pathway (MVA pathway), while the most common hosts for terpenes' biosynthesis were *Escherichia coli* and yeast. *E. coli* had the advantages of rapid growth and easy genetic manipulation, however, its natural MEP pathway was inefficient in the synthesis of terpenes [31]. In addition, *E. coli* had low tolerance to the toxicity of most terpenes, which also impeded the accumulation of products [32]. Compared with *E. coli*, yeast was a preferable platform for terpene production due to the excellent tolerance to complex environments, the complete organelles and protein expression system, fewer branched pathways, and byproducts [33]. Furthermore, the endogenous MVA pathway had high activity, which laid a good foundation for the production of terpenes in yeast. At present, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, and *Pichia pastoris* have been engineered to synthesize various terpenes. For instance, *S. cerevisiae*, as the commonly used host, has been used to efficiently produce lycopene and squalene [9,34]. *Y. lipolytica* as unconventional oil-producing yeasts have high acetyl-CoA flux and have been engineered to synthesize α-pinene and bisabolene [35,36]. *P. pastoris* could achieve high-density fermentation using cheap fatty acids and methanol as carbon sources, which was successfully modified to produce α-farnesene, β-carotene and lycopene [37].

With the development of metabolic engineering and synthetic biology, yeast has gradually developed as the main platform for terpene synthesis. Nevertheless, the titers of most terpenes were still too low to meet the industrial production requirements. In this review, we summarized the recent research progress on the strategies conducted in the synthesis of terpenes in yeast. The problems in the research were detail analyzed and perspectives were also proposed, which were aimed at providing some available ways to promote the industrial production of terpenes in yeast.

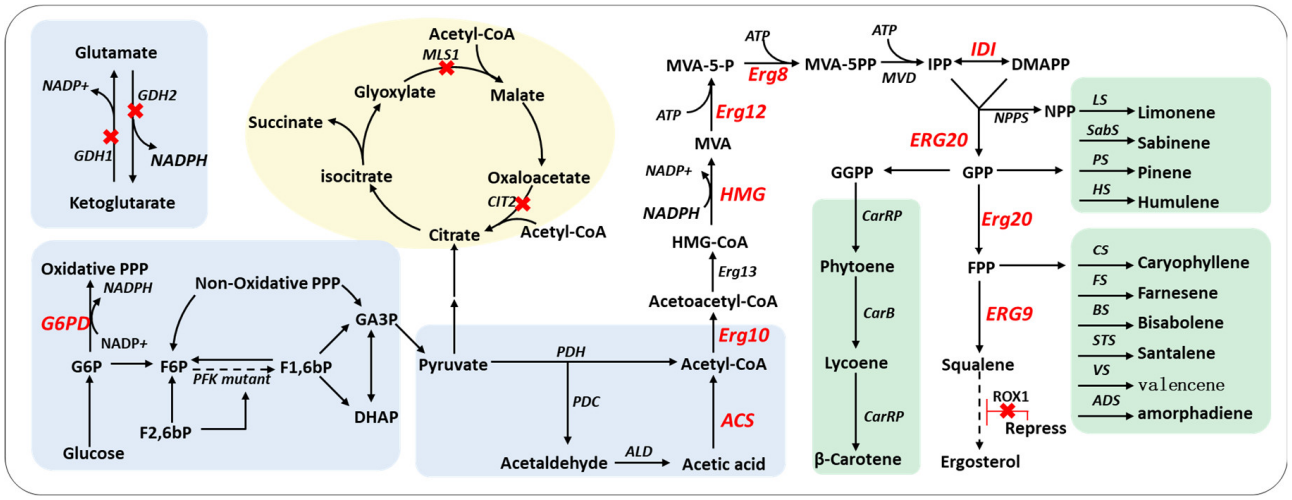

Figure 1. The biosynthesis and regulation pathways of terpenes. LS, limonene synthase; SabS, sabinene synthase; PS, pinene synthase; HS, humulene synthase; CS: caryophyllene synthase; FS, farnesene synthase; BS, bisabolene synthase; STS: santalene synthase; VS, valencene synthase; ADS, amorphadlene synthase; CarRP, bifunctional enzymes phytoene synthase/lycopene cyclase; CarB, phytoene dehydrogenase; ACS, acetyl-CoA synthase; ERG10, acetyl-CoA acetyltransferase; HMG: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; ERG12, mevalonate kinase; ERG13, hydroxymethylglutaryl CoA synthase; ERG8, phosphomevalonate kinase; MVD, mevalonate diphosphate decarboxylase; IDI, isopentenyl diphosphate isomerase; ERG20, farnesyl diphosphate synthase; ERG9, squalene synthase; NPPS, neryl diphosphate synthase; ALD, acetaldehyde dehydrogenase; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; MLS1, malate synthase; CIT2, citrate synthase; GDH1, glutamate dehydrogenase 1; GDH2, glutamate dehydrogenase 2; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; NPP, neryl diphosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; MVA-5PP, Mevalonate diphosphate; MVA-5-P, mevalonate-5-phosphate; MVA, mevalonate; HMG-CoA, hydroxymethylglutaryl-CoA; F6P, fructose-6-phosphate; F2,6bP, fructose-2,6-bisphosphate; F1,6bP, fructose-1,6-bisphosphate; GA3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate.

2. The Adaptive Screening and Expression of Terpene Synthases in Yeast

The endogenous MVA pathway provided a large number of precursors for terpene synthesis, and the exogenous introduction of terpene synthases was thus the key to successful construction of terpene biosynthesis pathways in yeast. However, the low expression of terpene synthases have been identified as one of the major limitations in the synthesis of terpenes due to the lack of terpene synthase modification mechanisms [38]. Therefore, the adaptive screening and expression of terpene synthases was considered an essential strategy to promote the synthesis of terpenes in yeast.

2.1. The Screening of Terpene Synthases from Different Origins

Terpenes, as important secondary metabolites, are widely present in various species, and plenty of synthases with the same function have been identified. The introduction of terpene synthases from different origins has usually led to differential results. The adaptive screening strategy was thus proposed to compare their effects in yeast. According to the report, more than 140 kinds of α-farnesene synthases have been identified. In order to improve the production of α-farnesene in the engineered *P. pastoris*, Liu et al. selected the appropriate α-farnesene synthase by constructing the phylogenetic tree. Coupled with the comparison of α-farnesene production, the synthase from *Malus domestica* showed the highest catalytic efficiency [37]. Keasling et al. constructed the bisabolene synthesis pathway in *S. cerevisiae* and the effects of five kinds of synthases were compared. It was indicated that the bisabolene synthase from *Abies grandis* showed the best performance and led to the titer of bisabolene up to 78 mg/L, which was about 2 ~ 20 times higher than the

results by expressing the synthase from other origins [39]. There was endogenous squalene synthase in yeast, but some works demonstrated that the heterologous squalene synthase from human and *Thermosynechococcus elongatus* had higher catalytic efficiency [40]. On this basis, Xun et al. analyzed the catalytic–kinetic efficiency of squalene synthase from plants, animals, fungus, and algae. The synthase from *Botryococcus braunii* was selected with obvious advantage in terms of catalytic–kinetic efficiency and 270 mg/L squalene was obtained by the engineered *S. cerevisiae.* [41]. These works indicated that an appropriate synthase directly affected the efficiency of terpene synthesis. The possible reasons were that: (1) the terpene synthases from various origins differ greatly in amino acid sequences and genetic relationships; (2) the cell compatibility limits the optimal performance of heterologous enzymes. Therefore, the adaptive screening of the synthases provided a feasible strategy to enhance the productivity of terpenes in yeast.

However, some studies have also identified a problem when applying the adaptive screening strategy. Namely, some synthases exhibited catalytic variation and produced multiple products when they were overexpressed in yeast. For instance, when the sabinene synthase was expressed in microbes, a variety of similar monoterpene byproducts could be generated, including α-pinene, myrcene, and 3-carene [42,43]. In addition, the limonene synthase was identified in nine different plants. However, when they were introduced into microbes, only the synthases from *Citrus limon* and *Mentha spicata* had relatively high product selectivity [44]. The adaptive screening was helpful to select the synthase with high activity from different origins but could not change the catalytic properties. With the development of protein engineering, more strategies have been developed to solve the issues, such as the resolution of protein structures, the artificial design of amino acid sequences, and the directed evolution of the proteins, and so on.

2.2. The Enhancement of Terpene Synthases' Expression in Yeast

The low expression of terpene synthases is a key factor limiting terpene production in yeast. It is a common strategy to enhance the expression of the synthases via increasing the number of the copies. It was shown that several monoterpenes including limonene, phellandrene, and carene were synthesized from neryl diphosphate (NPP) rather than geranyl diphosphate (GPP) (Figure 1) [45]. The expression levels of neryl diphosphate synthase (NPPS) and monoterpene synthases directly impacted the yield of target products. Cheng et al. investigated the effects of increasing the number of NPPS and LS copies in limonene synthesis, respectively. It was found that the yield of limonene was proportional to the copies' number of the enzymes. Meanwhile, the enhancement of LS expression was more beneficial to improve the yield of limonene, which resulted in a two-fold increase in yield compared to increasing the number of NPPS copies [46]. On this basis, Zhang et al. introduced 2–6 copies of NPPS-LS module in yeast by homologous recombination, which also led to a significant improvement of limonene production by the engineered *S. cerevisiae* [15]. Liu et al. constructed the β-carotene synthesis pathway in *Y. lipolytica*. The content of the precursor geranylgeranyl diphosphate (GGPP) was over accumulated when the GGPP synthase from *Archaeoglobus fulgidus* was introduced, which meant that the low-expressed bifunctional enzymes phytoene synthase/lycopene cyclase (CarRP) the conversion of GGPP was limited. After increasing the number of CarRP copies, the yield of β-carotene was increased by 62% [47]. These works demonstrate that increasing the number of terpene synthase copies was an effective strategy to improve the efficiency of product synthesis. However, it also had the concern of the issue that increasing the number of gene copies would make the plasmid construction process more cumbersome and would also add more burdens to the cells [48]. Therefore, the optimum number of gene copies should be optimized by taking into account cell growth and product synthesis.

Aside from increasing the genes copies, the application of strong promoters was also an available way to enhance the expression of enzymes. It has been reported that a strong promoter could increase the gene transcription levels by more than six-fold [49]. Makalena et al. conducted the optimization of promoter strength, and it was indicated that the

expression of GGS1, phytoene dehydrogenase (CarB), and CarRP controlled by the strong promoter TEF1P led to β-carotene production, increasing about 6.3-fold [50]. However, there were not many high-intensity promoters that could be applied in yeast, and the establishment of a more abundant promoter library would be one of the research priorities for future work.

2.3. The Artificial Design of Amino Acid Sequences Based on the Computer-Aided Analysis

Partial nature terpene synthases have signal peptides at the N-terminal end which help the initial translation product target to the cytoplasm and then the signal peptides are hydrolyzed off the protein. These truncated terpene synthases are a "pseudo-mature" form with more preferable solubility and efficient catalytic activity [51,52]. However, due to the lack of cytoplasm targeting and hydrolysis modification mechanisms in yeast, the heterogenous expression of complete terpene synthases possibly resulted in the low expression level and poor enzyme activity [33]. Some works thus proposed to artificially design the amino acid sequences based on computer-aided analysis to improve the expression efficiency of the terpene synthases in yeast. Jia et al. analyzed the sequence of sabinene synthase (SabS) using the PSIPRED Workbench (http://bioinf.cs.ucl.ac.uk/psipred/ accessed on 15 December 2020) and designed three kinds of truncated SabS. After overexpression, the truncated SabS in *Y. lipolytica*, the t34SabS with truncated protein-binding sites led to the highest sabinene yield [53]. Similarly, limonene synthase (LS) consists of two domains: a C-terminal domain exhibiting a class I terpene cyclase fold and an N-terminal transit domain [54]. Cao et al. attempted to engineer *Y. lipolytica* to synthesize limonene via overexpressing LS from *Agastache rugosa*, but no product was detected. After analyzing the sequence of LS, a truncated tLS was designed, which was co-overexpressed with the neryl diphosphate synthase and led to the yield of limonene to 0.006 mg/g dry cell weight [55]. Chen et al. analyzed the sequence of pinene synthase from *Pinus taeda* (PtPS) using the PSIPRED platform and designed the truncated tPtPS with N-terminus truncation from 2A to 51P, which improved the production of α-pinene about 2.23-fold in the engineered *S. cerevisiae* [56]. Identically, Liu et al. overexpressed the truncated tPtPS into *Y. lipolytica* and also successfully obtained a high α-pinene accumulation [35]. These works demonstrated that the artificial design of amino acid sequences based on computer-aided analysis was an effective strategy to improve the activity of terpene synthases. In further works, more effective strategies can be developed on the basis of computer-aided sequences design and combinationally applied to improve the expression of heterogenous enzymes in yeast.

Although the expression efficiency of terpene synthases was improved by the screening and expression optimization strategy and the artificial design of amino acid sequences strategy, the low activity of heterologous synthase was still a limitation in the synthesis of terpenes in yeast. The issue might be caused by several factors: (1) the expression mechanism of heterologous synthase was not completely understood in yeast; (2) some synthases might need to be activated with cofactors; (3) some synthases could only function in the specific cellular compartments. Hence, more efforts should be made to understand the expression and activation mechanism of heterologous synthase in yeast, and more effective strategies could be applied to improve the activity including the co-expression of a molecular chaperone, remodeling of the active sites of the enzymes, localized expression the enzymes and so on.

3. The Strategies for the Regulation of the Synthesis Pathway

3.1. The Regulation of the Rate-Limiting Steps in the Pathway

Terpene biosynthesis involves multiple reactions that are highly regulated at the genetic and metabolic levels. Regulation of the rate-limiting steps in the MVA pathway is essential to increase its metabolic flux. One of the key rate-limiting enzymes in the MVA pathway is 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG). It has been proven that the overexpression of HMG could significantly promote the availability of the precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) and was essential

in most metabolic engineering efforts for terpene synthesis in yeast [57]. In the biosynthesis of bisabolene, ten genes in the MVA pathway were overexpressed to compare their contribution in the production of bisabolene by the engineered *Y. lipolytica*, and the overexpression of endogenous HMG was found to reveal the best results [36]. Similarly, Matthäus et al. reported a 6.9-fold increase in lycopene production after the overexpression of endogenous HMG in *Y. lipolytica* [58]. There were two HMG isozymes in *S. cerevisiae*, namely HMG1 and HMG2, while HMG1 contributed to at least 83% of the enzyme activity in wild-type strains [59]. According to the reports, the activity of HMG1 was impacted by the feedback regulation and cross-regulation of the MVA pathway due to the special SSD structural domain which could lead to protein degradation in *S. cerevisiae* [60]. The truncated SSD structural domain was found to avoid the post-transcriptional degradation of HMG1, which was beneficial to improving its catalytic activity [61,62]. Therefore, the overexpression of the truncated tHMG1 was widely applied to increase the flux of the MVA pathway and improve terpene production. Ro et al. introduced the amorphadiene synthesis pathway into *S. cerevisiae* and the overexpression of endogenous tHMG resulted in an approximately 5-fold increase in production [63]. Analogously, in the synthesis of carotenoids, the expression of tHMG1 increased total carotenoid levels by 7-fold, and a significant color variation was observed in the engineered *S. cerevisiae* compared to the control cells [64]. As the overexpression of the truncated tHMG from *S. cerevisiae* could significantly promote the synthesis of terpenes, some studies proposed to explore its universality in *Y. lipolytica*. However, compared with the tHMG1 from *S. cerevisiae*, the endogenous HMG was more effective in the improvement of limonene and squalene biosynthesis by the engineered *Y. lipolytica* [55]. This might be due to the fact that the two yeasts were genetically distant and had different mechanisms of enzyme expression. In addition, co-overexpression of HMG1 with other enzymes in the MVA pathway could further promote terpene production. For instance, the production of squalene was increased 10-fold via co-expressing HMG, farnesyl diphosphate synthase (ERG20), and squalene synthase (ERG9) in *S. cerevisiae* [65]. In the synthesis of lycopene, the co-expression of HMG1 with geranylgeranyl diphosphate synthase (GGS1) showed a more positive effect than overexpressing the enzymes separately, which resulted in a 10.8-fold increase of lycopene production by the engineered *Y. lipolytica* [58]. This indicated that the combinational regulation of the rate-limiting step and the synthesis pathway was a more desirable strategy in the promotion of terpene synthesis.

Besides HMG, the isopentenyl diphosphate isomerase (IDI) is also a critical enzyme in the synthesis of terpenes, which catalyzes the interconversion of precursors IPP and DMAPP and regulates the steady state of the DMAPP/IPP ratio [66,67]. In 1993, Ladeveze et al. first demonstrated that the activity of IDI was associated with the accumulation of ergosterol in yeast [68]. Afterward, the overexpression of IDI became a common strategy for upregulating the synthesis of terpenes. Cao et al. introduced IDI from *E. coli* into *S. cerevisiae* and the poor downstream metabolic pathway was effectively improved [69]. Ignea et al. integrated the overexpressed IDI into the genome of *S. cerevisiae* which led to the production of cineole increasing about 24-fold, compared to the original strain [70]. Similarly, Liu et al. engineered *Y. lipolytica* to produce β-carotene and the yield was increased by 6.5-fold after co-overexpression of IDI, geranylgeranyl diphosphatesynthase (GPS), and mevalonate kinase (ERG12) [47]. These works indicated that regulation of IDI expression was also a feasible strategy to enhance terpenes synthesis in engineered yeast.

Acetyl-CoA acetyltransferase (ERG10) is responsible for the first step of the MVA pathway, catalyzing the condensation of two molecules of acetyl-CoA to form acetylacetyl-CoA, which directly impacts the flux of the MVA pathway [71]. Therefore, ERG10 is commonly overexpressed to promote the flow of acetyl-CoA to the synthesis of terpenes. Monireh et al. overexpressed endogenous ERG10 in *Y. lipolytica* and promoted the production of amorphadiene [72]. Kwak et al. demonstrated a synergistic effect of ERG10 and tHMG on squalene production by the engineered *S. cerevisiae*, and their co-expression led to squalene production increasing by 44% and 130% using glucose and xylose as carbon sources, respectively [73]. Similar results were also obtained in the synthesis of bisabolol,

in which a 67% increase of the production was achieved when ERG10 and tHMG were co-overexpressed in *S. cerevisiae* [74]. These works demonstrated that the flux of the MVA pathway could be effectively broadened via the enhancement of EGR10. Coupled with the combinational regulation of the other rate-limiting steps, the synthesis of terpenes could be substantially promoted.

The overexpression of rate-limiting enzymes is the most commonly used strategy in metabolic engineering, which can simply and effectively increase the throughput of the pathway and thus improve terpene production. However, there is usually more than one rate-limiting enzyme in cell growth and product synthesis pathways. The simultaneous regulation of multi-enzyme expression not only burdens the metabolism of the strain, but also causes poor fitness of the metabolic nodes in the pathway, which would lead to the accumulation of intermediate metabolites. Therefore, some more effective strategies should be applied to regulate the expression of multiple enzymes, such as modifying the active sites of enzymes based on the computer-aided analysis to block the catalytic limitations, establishing an orthogonal system for growth and products synthesis, sequentially expressing the enzymes by the control of biosensors, and so on.

3.2. The Regulation of Cofactor NADPH Supply

NADPH is a universal electron carrier in yeast, which is involved in cellular electron transfer reactions and drives biosynthetic pathways [28]. It also plays an important role in the biosynthesis and derivatization of terpenes. The expression of the MVA pathway leads to a large depletion of the cellular NADPH pool, thereby disrupting intracellular redox homeostasis. The regulation of cofactor NADPH supply is beneficial for the maintenance of intracellular redox status and facilitates the biosynthesis of terpenes. The synthesis and regeneration of NADPH depends on the activity of NADH kinase and NADP-dependent dehydrogenase. NADH kinase phosphorylates NADH to form NADPH, while the dehydrogenase reduces $NADP^+$ to form NADPH [28]. There were a series of cytosolic NADPH regeneration pathways in yeast functioned by NADP-dependent dehydrogenase, including glucose-6-phosphate dehydrogenase (ZWF), 6-phosphogluconate dehydrogenase (GND), sorbitol dehydrogenase (MnDH1 and MnDH2), malic enzyme (MAE1), cytosolic isocitrate dehydrogenase (IDP), succinate semialdehyde dehydrogenase (UGA2), and glutamate dehydrogenase (GDH) [75]. Liu et al. investigated the effects of these NADP-dependent dehydrogenases to enhance the synthesis of squalene in *Y. lipolytica* and MnDH2 was identified as the most appropriate NADPH regeneration pathway, which led to the production of squalene increasing by 11% [76]. The pentose phosphate pathway was the main route for the formation of NADPH, while ZWF was the key target for increasing the carbon flux flowing into the pathway. The mitochondrial NADH kinase (Pos5P) could phosphorylate NADH to form NADPH. When ZWF1 and POS5 were overexpressed in the carotenoid-producing *S. cerevisiae*, the total yield of lycopene and β-carotene was increased by 59.9% and 81.4%, respectively [77]. The mutation of phosphofructokinase (PFK) usually led to the accumulation of glucose-6-phosphate, a precursor substance of the pentose phosphate oxidation pathway. Suryang et al. overexpressed the mutated PFK and ZWF1 in *S. cerevisiae*, and the production of amorphadiene was 3.7-fold higher than that of the control strain [78].Glutamate dehydrogenase (GDH) was an essential enzyme catalyzing the oxidative deamination of glutamate to α-ketoglutarate. There were three types of GDH in yeast, namely GDH1, GDH2, and GDH3, and GDH1 was reported to catalyze the reaction depending on NADPH as the cofactor [79]. Asadollahi et al. proposed deleting GDH1 in *S. cerevisiae* to supply more NADPH for the synthesis of sesquiterpene, which finally led to an 85% increase in production [80]. Moreover, it was found that the overexpression of GDH2 would lead to the efficient conversion of glutamate to α-ketoglutarate, but the over-accumulated α-ketoglutarate would activate the activity of GDH1 [81]. Zhang et al. deleted GDH2 in *S. cerevisiae*, and the yield of limonene was increased by 30.33% [15]. In another work, it was reported that the overexpression of GDH2 could increase the consumption of NADH, thereby altering the NADH/NADPH balance and favoring NADPH

availability [82]. Scalcinati et al. proved that the production of α-sandalene was negatively impacted by the deletion of GDH1 but was improved approximately 6-fold via the overexpression of GDH2 in the engineered *S. cerevisiae* [83]. It might be suggested that GDH had a complex regulatory mechanism, and three types of GDH showed variant effects on the synthesis of different products in yeast.

The regulation of cofactor NADPH supply has already been a well-applied metabolic engineering strategy to overcome the limitations imposed by cellular redox limitation. But excessive regulation of NADPH regeneration pathways could also lead to an imbalance in the distribution of energy in cells growth and products synthesis. The establishment of a regulatory strategy for the autonomous regeneration and distribution of NADPH was the focus of research in the effective synthesis of terpenes in yeast.

3.3. The Enhancement of the Central Carbon Flux

The complex metabolic network contains multiple metabolite synthesis pathways, and the active metabolic branches waste central carbon flow and lead to a significant accumulation of by-products. Downregulation of the metabolic branches helps to reduce the loss of central carbon flux, thus enhancing the synthesis of target products.

In the MVA pathway, GPP was the direct precursor for monoterpene synthesis, which was generated from the condensation of IPP and DMAPP (Figure 1). ERG20 was reported to have a dual catalytic function in yeast, which could catalyze the formation of GPP and the conversion of GPP to farnesyl diphosphate (FPP). However, the sequential reaction was detrimental to the synthesis of monoterpenes due to the lack of precursor GPP [84]. The complete deletion of ERG20 was lethal because the mutant was unable to synthesize sterol for cell growth. Codruta et al. proposed weakening the activity of ERG20 to inhibit the consumption of GPP, and ERG20 was subsequently mutated. The inactivated form ERG20^{ww} showed a poor ability to convert GPP to FPP, which was able to significantly increase the synthesis of sabinene without an obvious effect on cell growth [85]. Similarly, in the production of non-endogenous sesquiterpenoids and carotenoids products using FFP as precursor, the carbon flux also needed to be transferred to the target synthesis pathway, rather than the sterol production. Downregulation of ERG9 was usually applied to enhance the carbon flux. Kildegaard et al. truncated the natural promoter of ERG9 from *Y. lipolytica* to screen the promoter mutant with different strengths. When a weak mutant was used to replace the natural promoter of ERG9, the production of β-carotene was increased by 2.5-fold [86]. However, it was also found that the downregulation of ERG9 led to the over accumulation of FPP, which led to the generation of farnesol via rapid dephosphorylation. The deletion of dephosphorylases LPP1 and DPP1 was proven to be effective in reducing central carbon flow to farnesol [82,87]. Therefore, despite various kinds of terpenes generated from the MVA pathway, the downregulation of the branches was an available way to enhance carbon flux to the synthesis of target products.

Acetyl-CoA is the metabolic link between cell growth and multiple metabolic pathways, including the TCA pathway, the lipid synthesis pathway, the polyketide synthesis pathway, and the terpenes synthesis pathway. Acetyl-CoA is the carbon skeleton of mevalonate and its intracellular content determines the efficiency of terpene synthesis [88]. In yeast, pyruvate could be converted to acetyl-CoA through the pyruvate dehydrogenase bypass functioned by pyruvate decarboxylase (PDC), cytoplasmic acetaldehyde dehydrogenase (ALD), and acetyl-CoA synthase (ACS). ACS catalyzed the conversion of the intermediate metabolite acetate into acetyl-CoA with two molecules of ATP consumed, which was considered to be the limiting step in the pyruvate dehydrogenase bypass. Multiple studies have been performed to manipulate the acetyl-CoA pool by overexpressing the ACS [89,90]. It was shown that the overexpression of ACS could increase the levels of intracellular acetyl-CoA about 2–5 fold in *S. cerevisiae*, which was hence considered an available way to enhance the production of terpenes [91]. Shiba et al. significantly increased the production of amorphadiene by regulating the pyruvate dehydrogenase bypass, demonstrating that regulation of the pyruvate dehydrogenase bypass was effective for the

production of terpenes in yeast [90]. Jim et al. found that the titer of squalene was increased by 56.6% after enhancing the expression of endogenous ACS in *S. cerevisiae* [9]. Lian et al. further compared the effects of ACS from different origins, and it was indicated that the ACS variant $SeACS^{L641P}$ from *Salmonella enterica* was more efficient than the endogenous, which increased the production of limonene by 11.94% by the engineered *S. cerevisiae* [15]. Moreover, it was found that peroxisomal citrate synthase (CIT2) consumed acetyl-CoA to generate citrate, while cytoplasmic malate synthase (MLS1) catalyzed the conversion of acetyl-CoA to C4 organic acid, which caused a diversion of the central carbon flow. Chen et al. and Zhang et al. proposed to delete these two genes in engineered *S. cerevisiae* to disrupt the glyoxylate shunt, which led to the production of α-santalene and limonene, increasing more than 2.27 and 1.4 fold, respectively [15,88]. These works demonstrated that the enhancement of acetyl-CoA supply was a beneficial strategy to facilitate the synthesis of terpenes.

Although it was a feasible strategy to regulate the central carbon flux by weakening the competitive pathways and strengthening the precursor supply, the efficient distribution of central carbon flux in cell growth and product synthesis could not be achieved through the simple suppression or overexpression of the pathways. The complexity of the intracellular metabolic network required a more refined and dynamic regulation to maximize the benefits of carbon flux. With the development of modern synthetic biology, new regulatory approaches have been developed, including the construction of the logic circuit to control carbon flux, the application of biosensors to promote the dynamic expression of pathways, and the establishment of orthogonal metabolic systems to improve the utilization of the substrate. These strategies provided operational tools to achieve rational regulation of metabolic pathways and would further promote the biosynthesis of terpenes in yeast.

4. The Intracellular Compartmentalized Expression of Metabolic Pathways

There are a variety of organelles in yeast cells, including mitochondria, endoplasmic reticulum, lipid droplets, the Golgi apparatus, and peroxisomes, each providing a unique physicochemical environment for different metabolites, enzymes, and cofactors. Compared to cytoplasmic engineering, organelle engineering has more unique advantages, such as high local concentrations of substrates and enzymes, less interference of branch pathways, and fewer by-products, due to its highly specialized subcellular compartments. In recent years, an increasing number of studies have introduced terpene biosynthesis pathways into the organelles of yeast, while mitochondria, endoplasmic reticulum, liposomes, and peroxisomes were proven to be effective reaction chambers (Figure 2). Additionally, the dual regulation of cytoplasmic and organelle engineering has shown great potential to increase the yield and productivity of terpenes.

4.1. The Promotion of Terpene Biosynthesis via Mitochondria Engineering

As the multifunctional subcellular organelle in eukaryotic cells, mitochondria are the important site for the TCA cycle, oxidative phosphorylation, amino acid and lipid metabolism, and the synthesis of iron–sulfur clusters [92]. Mitochondria are rich in acetyl-CoA, ATP, and cofactors, which provide a compact compartment for terpene biosynthesis pathways. Terpenes are lipophilic compounds that are able to diffuse between the mitochondria and the cytoplasm [93]. Yuan et al. successfully demonstrated that the FPP synthesis pathway could be functionally expressed in mitochondria, which significantly promoted the accumulation of amorpha-4,11-diene [94]. Lv et al. compared the effects of cytoplasmic and mitochondrial engineering on the synthesis of isoprene in *S. cerevisiae*. It was found that the expression of the synthesis pathway in mitochondria led to a higher production of isoprene [95]. In order to avoid the depletion of GPP by the downstream pathway, Yee et al. targeted the biosynthetic pathway of geraniol to the mitochondria and obtained six times higher yields than the corresponding cytoplasmic engineered *S. cerevisiae* [96]. Farhi et al. constructed the valencene synthesis pathway in *S. cerevisiae*, and the yield of valencene was increased 8-fold via the combinational application of both mitochondrial and cytoplasmic

engineering strategies [97]. These works demonstrated that mitochondria were potential organelles for terpene production in yeast. It was suspected that it was not only owed to the abundant precursors and cofactors in mitochondria, but the mitochondrial membrane physically isolated the competing pathways and reduced the loss of carbon flux.

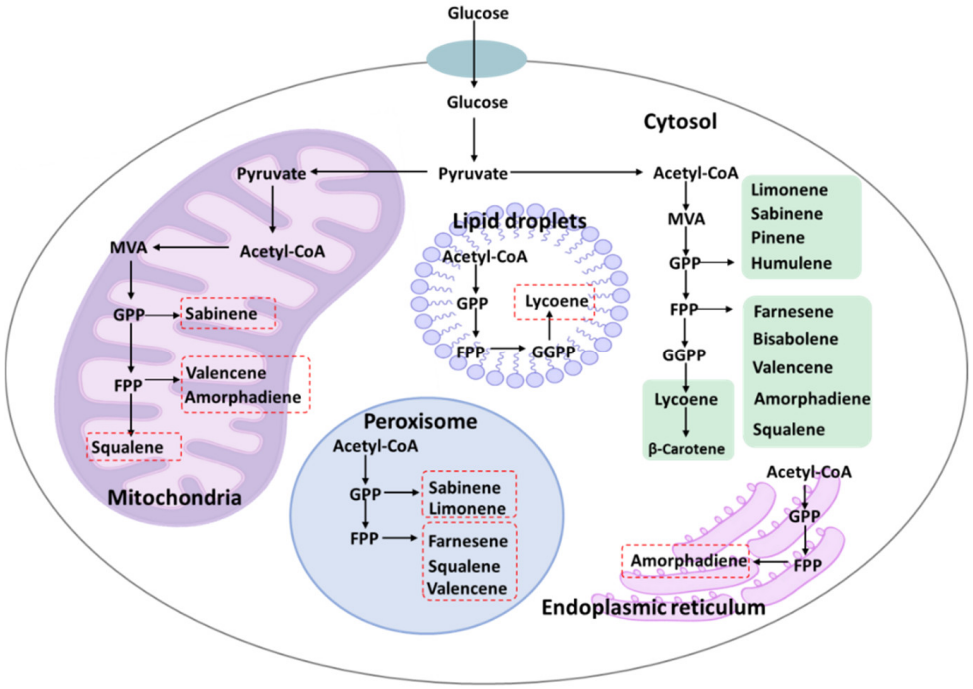

Figure 2. The introduction of terpene biosynthesis pathways into different organelles using the intracellular compartmentalized expression strategy. The red box represents the terpenes that can currently be synthesized in organelles. MVA, mevalonate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

However, it was also found that the introduction of the MVA pathway into the mitochondria slowed cell growth and significantly reduced biomass, which might be caused by the accumulation of the toxic intermediate metabolites in the MVA pathway. In the previous study, IPP/DMAPP were proven to be toxic intermediates for cells. When the exogenous MVA pathway was introduced into *E. coli* or *Bacillus subtilis*, the significant cytotoxic effects on cell growth were observed and the inhibition was diminished after IPP/DMAPP depletion [80]. In addition, some other phosphorylated metabolites of the MVA pathway were more toxic to mitochondria, including mevalonate 5-P and mevalonate 5-PP, because they could not cross the mitochondrial membrane directly and could only be excreted out of mitochondria after conversion to IPP/DMAPP [9]. To address this issue, Zhu et al. proposed to overexpress the mevalonate diphosphate decarboxylase (ERG19) localized in the mitochondria so as to reduce the toxicity of the intermediates by promoting the conversion of mevalonate 5-PP to IPP/DMAPP. Coupled with the further regulation of other rate-limiting steps, squalene synthesis and cell growth were significantly improved [9]. Although lowering the concentration of toxic metabolites in mitochondria was beneficial for cell growth and terpene potency to some extent, the inhibition could not be completely eliminated and the functional inhibition of mitochondria by phosphorylated metabolites, as well as the restoration of cell growth, needed to be further investigated.

4.2. The Promotion of Terpene Biosynthesis via Peroxisome Engineering

Peroxisomes are the major organelles involved in cellular detoxification and play a crucial role in cellular lipid metabolism, and are the only site for fatty acid β-oxidation and generate large amounts of acetyl-CoA [98]. Meanwhile, the peroxisomal NADP-dependent isocitrate dehydrogenase isoenzyme can provide a reductive redox state in the organelle,

thus meeting the NADPH demand of some pathways. In addition, peroxisomes have a monolayer membrane structure, which allows the powerful transport of large amounts of compounds [99]. Moreover, peroxisomes are not essential for cell viability under most culture conditions, and their numbers and size can be modified in various ways almost without any negative effects [100,101]. Due to these features, some studies have explored the feasibility of using peroxisomes as subcellular compartments for terpene production in yeast. Guo et al. demonstrated that the peroxisomes were lipophilic organelles and subsequently introduced the synthesis pathway of squalene into the peroxisomes of *S. cerevisiae*. The high yield of squalene demonstrated that the peroxisomes were appropriate subcellular compartments for the biosynthesis and storage of lipophilic terpenes [102]. Similarly, Dusséaux et al. overexpressed the complete MVA pathway in peroxisomes to establish a GPP synthesis microfactory and efficiently promoted the production of monoterpenes by the engineered *S. cerevisiae*, which further indicated the potential of peroxisomes as subcellular compartments for the synthesis of terpenes in yeast [103]. On this basis, Guo et al. introduced the entire α-humulene biosynthetic pathway into the peroxisomes using the peroxisome-targeting signal in *Y. lipolytica*, and the final yield of α-humulene was 50-fold higher than that of the control strain [104]. These studies suggested that peroxisomes were promising organelles for the effective synthesis of terpenes.

Nevertheless, some issues should be considered to further promote efficiency in peroxisome engineering. Firstly, the content of acetyl-CoA in peroxisomes was lower than that it in mitochondria, which might limit the flux of the MVA pathway and impact the synthesis of terpenes. Secondly, a large amount of NADH was also generated in the degradation of fatty acid in peroxisomes, while NADPH was the major cofactor to drive the MVA pathway, which might exacerbate the contradiction between NADPH/NADH supply and demand. Therefore, improvement of acetyl-CoA and the conversion between NADH and NADPH is needed as a focus in peroxisome engineering to release the restriction and promote the synthesis of terpenes.

4.3. The Development of Other Organelles for Terpene Biosynthesis

Similar to peroxisomes, lipid droplets are also lipophilic organelles consisting of triacylglycerols (TAG) and sterol esters, which are also regarded as promising organelles for the synthesis of lipophilic compounds. It has been reported that bottlenecks in the biosynthesis of esters could be alleviated after transferring the intracellular localization of the enzyme from the mitochondria to lipid droplets in yeast [105]. Ma et al. regulated the TAG metabolism pathway to increase the intracellular accumulation of lipophilic compounds in *S. cerevisiae*. It was indicated that the production of lipophilic lycopene could be effectively promoted through enhancing liposome synthesis in the engineered *S. cerevisiae* [106]. This provided a new strategy for the exploration of lipid droplets as subcellular compartments for terpenes production.

The endoplasmic reticulum (ER) is a dynamic organelle responsible for the synthesis and modification of proteins, and is especially an essential site for the heterologous expression of partial enzymes, such as P450 enzymes. The membrane size of the ER is a key determinant of protein folding efficiency, and the tremendous expansion of the membrane was beneficial for the heterologous expression of enzymes, which could enhance the metabolic capacity of target synthesis pathway [107]. In addition, partial enzymes in the MVA and ergosterol biosynthesis pathways resided in the ER, which meant that ER was also a promising subcellular organelle for terpene synthesis. Arendt et al. proposed to expand ER via the disruption of the phosphatidic acid phosphatase in *S. cerevisiae*, which significantly stimulated the production of triterpenoid and triterpene saponin [108]. However, there has been little work about the promotion of terpene production via ER engineering so far, and its potential application needed to be further developed.

5. Conclusions and Perspectives

Facing the challenges of social development and human health, the production of terpenes has become a key focus of present research, due to diverse functions and promising applications. With the development of synthetic biology and metabolic engineering, the biosynthesis of terpenes based on a yeast platform has become a research hotspot. This has been the common strategy conducted in the engineering of yeast to promote terpene bio-production, including the adaptive screening and expression of terpene synthases, the regulation of the synthesis pathway, and the application of the intracellular compartmentalized expression of metabolic pathways. These strategies have made significant contributions to improving the expression and activity of terpene synthase, enhancing the central carbon flux in the target pathway, and increasing the supply of precursor acetyl-CoA and cofactors. However, there are still some issues that need to be addressed.

Firstly, the heterologous expression mechanism of terpene synthases was not clear in yeast and the lack of appropriate post-translational modifications led to poor enzyme activities and low catalytic efficiency. The development of bioinformatics and protein engineering provided feasible ways to resolve the expression mechanism of terpene synthases and would lay a theoretical basis for the regulation of the expression process. Moreover, some current strategies could be investigated to enhance the activity of synthases, such as screening molecular chaperones to help the post-translational modifications, remodeling enzyme active regions to improve catalytic efficiency, establishing a promoter library, and improving the enzymes' expression using strong promoters.

Secondly, the phosphorylated intermediate metabolites in the MVA pathway were toxic to cells. It inhibited the normal growth of cells and also limited the application of the intracellular compartmentalized expression strategy. The exploitation of more genetic manipulation tools in synthetic biology, would be potential routes to relieve the inhibition via exploring new synthesis pathways with few toxic metabolites synthesized, screening new enzymes to accelerate the conversion of phosphorylated intermediates, constructing logical circuit, and orthogonal metabolic system to reduce the growth inhibition.

Finally, the metabolic network has complexity, and the simple overexpression or deletion of multiple enzymes possibly led to metabolic burden to cells, as well as the accumulation of intermediate metabolites, which negatively impacted the carbon conversion rate. The establishment of biosensors for intermediate metabolites and target products could be an available way to solve the problem, which could promote the dynamic distribution of carbon flux in cell growth and product synthesis pathways according to the status and demand of cells in different stages. It was believed that these problems would be broken through with the development and application of various metabolic engineering strategies, and new progress would be achieved in the biosynthesis of terpenes by yeast.

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