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Research progress on improving Santalene production by metabolic engineering of Saccharomyces cerevisiae

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Abstract. Santalene, as a major sesquiterpene component of sandalwood oil, possesses broad pharmacological activities and considerable commercial value. However, traditional plant-extraction methods are inefficient, costly and unsustainable. Metabolic engineering of Saccharomyces cerevisiae to achieve biological synthesis of santalene has emerged as a highly promising alternative. This review summarizes the key recent advances in the field, including reconstruction and optimization of the Mevalonate (MVA) pathway to enhance precursor supply; redirecting metabolic flux toward santalene by suppressing competing branch pathways such as Squalene Synthase (ERG9) and Cathepsin C (DPP1), achieving 3.4- and 5.9-fold increases; optimization of cofactor regeneration systems [e.g., Nicotinamide Adenine Dinucleotide Phosphate Reduced (NADPH) and Acetyl Coenzyme A (Acetyl-CoA)]; and fermentation-process improvements that raised α-santalene titers to 163 $mg \cdot L^{-1}$. These systemic modifications have led to significant yield improvements. Finally, we discuss future directions for combining systems biology and synthetic biology tools to further optimize cell-factory performance and advance industrial-scale production.

Keywords: sandalwood essential oil, santalene, biosynthesis, Saccharomyces cerevisiae, metabolic engineering

1. Introduction

Complex mixes of several volatile, low-molecular-weight, frequently aromatic chemicals make up plant essential oils. They are oily substances that typically have strong odors [1]. Different plant parts, including leaves, flowers, seeds, bark, heartwood, fruit, and roots, contain essential oils, which can be extracted and distillated. These oils consist mainly of volatile compounds, among which terpenes are prominent. At present, steam distillation is widely used for commercial extraction of essential oils [2]. It has been proven that several essential oils have therapeutic qualities beyond their conventional applications, including antibacterial, antiviral, anti-inflammatory and anticancer activities [3]. In addition, plant-derived essential oils—for example, sandalwood oil—are important aroma ingredients in a variety of commercial applications, particularly in the cosmetics and fragrance sectors..

Traditional approaches rely on direct plant extraction; due to low yields, high production costs, and substantial time and land requirements, these methods are particularly problematic for sandalwood oil [4]. Sandalwood trees grow in harsh environments and have long maturation periods, so supply cannot satisfy the rising market demand for sandalwood oil; overharvesting poses a severe threat to sandalwood resources. One of the most valuable essential oils in the world today is sandalwood oil [5]. On the other hand, fermentation-based production of plant natural products from renewable feedstocks is made possible by microbial biosynthesis, providing a sustainable and affordable manufacturing method with significant benefits for the economy and environment.

Sandalwood essential oil is primarily obtained by steam distillation of the heartwood of mature sandalwood trees [6]. Its core components are sesquiterpene hydrocarbons and sesquiterpene alcohols, most notably α-santalene, β-santalene, epi-β-santalene and exo-α-bergamotene among the sesquiterpenes [7]. The high contents of santalols and santalene in sandalwood oil underlie its diverse pharmacological and medicinal activities. Preclinical and clinical studies have shown that sandalwood extracts have antibacterial and anticancer qualities in addition to anti-inflammatory, antioxidant, antihyperglycemic, and antihyperlipidemic activities [1, 4].

Recently, the biosynthetic pathways for santalene and santalol have been elucidated. This review focuses primarily on current knowledge of santalene biosynthesis and the latest biotechnological advances in its production, with particular emphasis on metabolic engineering strategies applied to S. cerevisiae cell factories.

2. Biosynthetic pathway of Santalene

Although terpenoids display vast structural diversity, they all originate from two universal C5 building blocks: Isopentenyl Diphosphate (IPP) and Dimethylallyl Diphosphate (DMAPP). From these precursors, larger prenyl diphosphates such as Geranyl Diphosphate (GPP), farnesyl diphosphate (FPP), and Geranylgeranyl Diphosphate (GGPP) are formed sequentially [8-10]. These intermediates are most commonly synthesized via the Mevalonate (MVA) pathway, which is native to the cytoplasm of plants and other eukaryotes, and has now been successfully introduced into yeast [11] and bacteria [12]. The MVA pathway produces IPP and DMAPP (Figure 1).

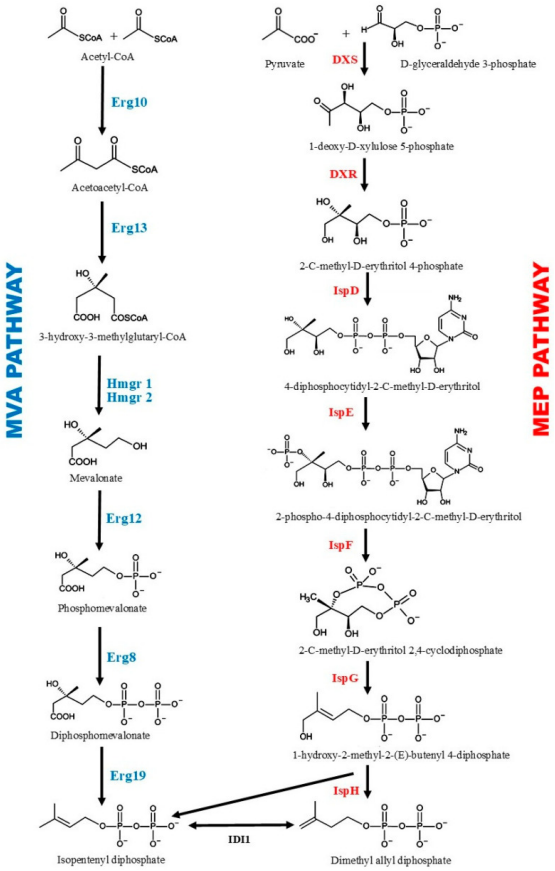

Figure 1. MVA pathway of isoprenoid biosynthesis (left) [4]

FPP is the primary substrate needed for the synthesis of santalene and the universal precursor of all sesquiterpenes. FPP is produced by a series of prenyl transfer reactions. First, a DMAPP carbocation is electrophilically alkylated with one IPP unit, and then it is deprotonated to produce GPP. FPP is created when another IPP unit is added to GPP, and Farnesyl Diphosphate Synthase (FPS) catalyzes these prenylation processes [13]. Sandalwood and other plant species have been found to contain several isoenzymes of santalene/bergamotene Synthase (STS) [14]. In sandalwood species, the conversion of (E,E)-Farnesyl Pyrophosphate [(E,E)-FPP] into a combination of sesquiterpenes is mostly dependent on STS, primarily α-santalene, β-santalene, epi-β-santalene, and exo-α-bergamotene [15].

3. Metabolic engineering strategies for improving Santalene production in Saccharomyces cerevisiae

Saccharomyces cerevisiae has been one of the most widely used microorganisms for the synthesis of valuable biomolecules for centuries. Being a eukaryotic, unicellular microbe that is a member of the fungus kingdom, it is among the most thoroughly studied eukaryotes and provides fundamental insights into eukaryotic cell biology [16]. Due to its nonpathogenic nature, the organism S. cerevisiae is regarded as Generally Recognized as Safe (GRAS). The yeast is well known for its versatility in using carbon sources and for its simplicity of changing sterol pathways by introducing foreign genes, making it a significant asset in biotechnology that presents several prospects for innovation and improvement, such as Terpene Synthases (TPS), and for its adaptability to harsh conditions [17]. These features make S. cerevisiae an indispensable platform for biotechnological production of terpenoids.

3.1. Reconstruction of the Santalene biosynthetic pathway in S. cerevisiae and optimization of DMAPP, IPP, and FPP synthesis

In S. cerevisiae, IPP and DMAPP are produced via the MVA pathway (Figure 2). In this pathway, ERG10 catalyzes the condensation of two acetyl-CoA molecules to produce acetoacetyl-CoA, which is then transformed into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by ERG13 (HMG-CoA synthase). HMG-CoA is subsequently reduced by HMG1 or HMG2 (HMG-CoA reductases) to produce the key intermediate mevalonate. Through sequential catalysis by ERG8 (phosphomevalonate kinase), ERG12 (mevalonate-5-kinase), and ERG19 (mevalonate diphosphate decarboxylase), IPP is generated. IDI1 catalyzes the reversible interconversion between IPP and DMAPP. Then FPP is synthesized from DMAPP and IPP via ERG20 [18].

Much work has been done to enhance the MVA route in S. cerevisiae in order to boost the supply of IPP and DMAPP. The primary rate-limiting step in the MVA pathway is the decrease of HMG-CoA. Huang et al. [19] demonstrated that a shortened form of HMG1 (tHMG1, which only contains the catalytic domain) can be overexpressed to increase terpenoid synthesis in yeast. Both HMG1 and HMG2 have a transmembrane anchor domain and a catalytic domain.

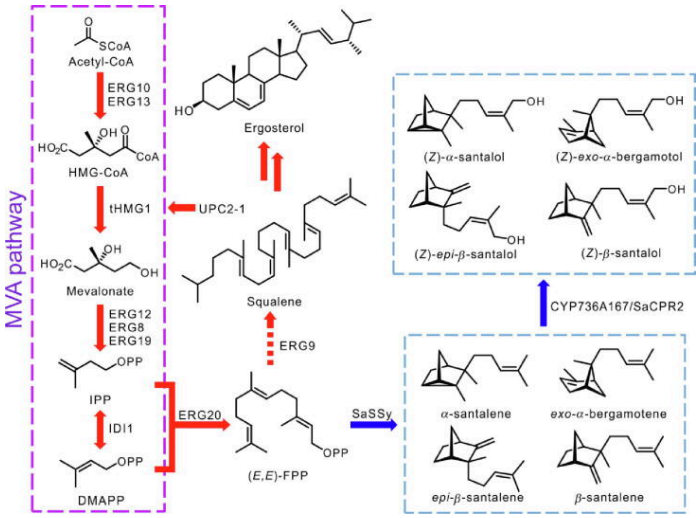

Figure 2. Biosynthetic pathway of α-santalene and α-santalol in S. cerevisiae [18]

Red and blue arrows indicate steps catalyzed by native and exogenous enzymes, respectively.

3.2. Limiting competing branch pathways

Due to the high cellular demand for sterols, ergosterol biosynthesis in yeast is highly active, consuming the majority of FPP [20]. To redirect FPP flux toward sesquiterpene production, ergosterol biosynthesis can be attenuated by replacing the native ERG9 promoter with a weaker promoter. Asadollahi et al. [21] reported that In strains producing patchoulol and artemisinic acid, ERG9 expression has been suppressed using MET3 and CTR3 promoters. Later, Scalcinati et al. [22, 23] found that the glucose-inducible PHXT1 promoter is primarily activated at high glucose levels in S. cerevisiae. Thus, downregulation of ERG9 is expected to increase the yield of target products while reducing by-product formation. In fact, this method dramatically reduced ERG9 expression, which led to a 3.4-fold rise in santalene titer as compared to control strains. More recently, Zha et al. [24] reported a 5.9-fold increase in santalene titer through ERG9 suppression.

Furthermore, two phosphatase genes, LPP1 and DPP1, which encode lipid phosphate phosphatases, are involved in the farnesol biosynthesis pathway, which branches from FPP [25]. Downregulation of DPP1 markedly improved santalene synthesis in yeast while inhibiting farnesol formation [22, 23]. This strategy is commonly applied for high-yield production of other terpenoids, such as patchoulol and trans-nerolidol [26].

3.3. Adjustment of NADPH supply

NADPH is an essential cofactor for redox reactions. For cytochrome P450 enzymes (CYPs) and tHMG1 to perform their biological tasks, NADPH is necessary.; therefore, increasing intracellular NADPH levels can enhance terpenoid biosynthesis in S. cerevisiae [27]. Overexpression of mBDH1 (a mutant 2,3-butanediol dehydrogenase) is one of several effective tactics that

have been used to increase the supply of NADPH [28], ZWF1 (glucose-6-phosphate dehydrogenase) [29], and POS5 (mitochondrial NADH kinase) [30].

Moreover, increasing the synthesis of the NAD-dependent glutamate dehydrogenase GDH2 results in a distinct pathway for ammonium consumption, which causes the modified yeast to have a different cofactor balance and a higher NADH flux.

To reduce NADPH consumption during ammonium absorption, Scalcinati et al. [22] carried out genetic changes by overexpressing GDH2 and deleting GDH1.

3.4. Enhancement of Acetyl-CoA supply

In S. cerevisiae, Aldehyde Dehydrogenase (ALD), Acetyl-CoA Synthetase (ACS), and Alcohol Dehydrogenase (ADH) play crucial roles in acetyl-CoA synthesis and regeneration. Pyruvate is converted to acetaldehyde by pyruvate decarboxylase, which then combines with acetate and CoA to form acetyl-CoA. Acetaldehyde is reversibly converted to ethanol by ALD and ACS working in tandem [31]. Genes encoding acetyl-CoA synthetase, for example, ACS1 and ACS2, can improve acetyl-CoA physiology in yeast. Chen et al. [32] reported that this enhanced tolerance to elevated ethanol levels, activated key genes in the MVA pathway for terpenoid synthesis, and raised intracellular acetyl CoA and ATP levels. Furthermore, Ding et al. [33] found that overexpression of ACS2 enhanced acetate tolerance, indicating that ACS2-mediated uptake during fermentation promotes acetate detoxification. Overexpression of ACS2 therefore improves the efficiency of converting acetate into acetyl-CoA.

In addition, Chen et al. [34, 35] found that acetyl-CoA is substantially consumed via the glyoxylate cycle in peroxisomes and the cytosol, with peroxisomal citrate synthase (CIT2) and cytosolic malate synthase (MLS1) catalyzing the conversion of acetyl-CoA to citrate and malate, respectively. Knockout of these two enzymes increased α-santalene titers fourfold compared with the parental yeast strain.

3.5. Fermentation optimization

The final yield of target compounds in engineered microbial hosts is significantly influenced by fermentation, a crucial step in product formation. Numerous techniques, including two-phase fermentation and fed-batch fermentation, have been extensively used for the microbial generation of natural compounds. Scalcinati et al. [22, 23] used in situ product removal in S. cerevisiae producing α-santalene and optimized dilution rates to achieve a production rate of 0.036 Cmmol/(g biomass)/h. In another study, Tippmann et al. [36] employed an RQ-controlled exponential feeding strategy, achieving an α-santalene titer of 163 mg/L. Process parameter optimization (for instance, agitation and mixing, oxygen supply and aeration, fermentation time, and timing of harvest) and medium optimization (such as selecting carbon and nitrogen sources, controlling pH and temperature, and adding growth agents and cofactors) are examples of fermentation optimization techniques.

4. Conclusion

Metabolic engineering of S. cerevisiae for santalene production has emerged as an effective alternative to traditional plant extraction. Strategies including MVA pathway optimization, suppression of competing branches (e.g., ERG9, DPP1), enhancement of NADPH and acetyl-CoA supply, and fermentation process control have significantly increased santalene yields. Systematic modifications not only improved precursor utilization efficiency but also reduced by-product formation, achieving highly directed metabolic flux. Future research should focus on integrating systems biology and synthetic biology tools to explore and optimize key enzyme activities, enhancing the stability and tolerance of cell factories. Additionally, development of low-cost media and efficient two-phase fermentation processes will help reduce downstream separation costs, promoting industrial-scale microbial production of santalene and better meeting sustainable demand in pharmaceutical, fragrance, and related industries.

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