Advances in biotechnological production of santalenes and santalols

Wen-long Zha, Jia-chen Zi, a,b,*

a Biotechnological Institute of Chinese Materia Medica, Jinan University, Guangzhou 510632, China
b College of Pharmacy, Jinan University, Guangzhou 510632, China

A B S T R A C T

Sandalwood essential oil has been widely used not only as natural medicines but also in perfumery and food industries, with sesquiterpenoids as its major components including (Z)-α-santalol and (Z)-β-santalol and so on. The mature heartwoods of Santalum album, Santalum austrocaledonicum and Santalum spicatum are the major plant resources for extracting sandalwood essential oil, which have been overexploited. Synthetic biology approaches have been successfully applied to produce natural products on large scale. In this review, we summarize biosynthetic enzymes of santalenes and santalols, including various santalene synthases (STSs) and cytochrome P450 monoxygenases (CYPs), and then highlight the advances of biotechnological production of santalenes and santalols in heterologous hosts, especially metabolic engineering strategies for constructing santalene- and santalol-producing Saccharomyces cerevisiae.

© 2020 Tianjin Press of Chinese Herbal Medicines. Published by ELSEVIER B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Contents

1. Introduction .................................................................................................................. 90
2. Biosynthesis of santalenes and santalols ........................................................................ 91
   2.1. Santalene synthase .................................................................................................. 91
   2.2. Cytochrome P450 monoxygenases (CYPs) involved in santalol biosynthesis .......... 92
3. Metabolic engineering strategies for production of santalenes and santalols in S. cerevisiae ................................................................. 92
   3.1. Restriction of branch pathways ............................................................................. 94
   3.2. Improvement of acetyl-CoA supply ..................................................................... 94
   3.3. Tuning of NADPH supply .................................................................................. 94
   3.4. Linking santalol biosynthesis to GAL regulatory system ....................................... 94
   3.5. Optimization of fermentation ............................................................................ 94
4. Production of santalolenes in other heterologous hosts ............................................. 95
5. Conclusion ................................................................................................................... 95
6. Declaration of Competing Interest ............................................................................ 95
7. Acknowledgments ........................................................................................................ 95
8. References .................................................................................................................... 95

* Corresponding author.
E-mail address: jiachen_zi@163.com (J.-c. Zi).

https://doi.org/10.1016/j.chmed.2020.11.002
1674-6384/© 2020 Tianjin Press of Chinese Herbal Medicines. Published by ELSEVIER B.V.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Dey, 2013; Saneja, Kaushik, Kaushik, Kumar, & Kumar, 2009), and anticancer activity (Bommareddy, Hora, Cornish, & Dwivedi, 2007; Bommareddy, Rule, VanWert, Santha, & Dwivedi, 2012; Kim et al., 2006; Lee, Bohmann, Reeves, Levenson, & Risinger, 2015; Saraswati, Kumar, & Alhaider, 2013; Zhang et al., 2010). Moreover, it is also used in food, cosmetic and perfume industries.

Sandalwood essential oil is mainly obtained from the heartwoods of mature sandalwood trees (Santalum album, Santalum austrocaledonicum and Santalum spicatum) by steam distillation (Jones, Plummer, & Barbour, 2007). Due to the harsh growth environment and long growth period of Santalum trees, sandalwood supply cannot meet the growing market demands of sandalwood oil, and overexploitation has seriously threatened the sandalwood resources. To date, sandalwood essential oil has become one of the most precious essential oils in the world (Subasinghe, Gamage, & Hettiarachchi, 2013).

Recently, synthetic biology has made great progresses in large-scale production of natural isoprenoids. In a masterwork, the biosynthetic pathway of artemisinic acid (the key precursor of artemisinin) was reconstructed and elaborately tuned in Saccharomyces cerevisiae, and consequently an artemisinic acid yield of 25 g/L was achieved (Paddon et al., 2013). Moreover, the microbial platforms for high-yield production of ginsenosides (Dai et al., 2013, 2014; Hu et al., 2019; Yan et al., 2014), taxadiene (Engels, Dahm, & Jennewein, 2008) and hydrocortisone (Chen et al., 2020) were also reported.

Recently, the biosynthetic pathway of the santalenes (referring to α-santalene, β-santalene, epi-β-santalene and exo-α-bergamotene herein) and santalols (referring to α-santalol, β-santalol, epi-β-santalol, exo-α-bergamotol herein) has been revealed (Fig. 1). And some successful efforts were made to produce santalenes or santalols in various heterologous hosts (Chen, Daviet, Schalk, Siewers, & Nielsen, 2013; Jia et al., 2019; Scalcinati et al., 2012a, 2012b; Tippmann, Scalcinati, Siewers, & Nielsen, 2016; Yin & Wong, 2019; Zha et al., 2020; Zhan, Zhang, Chen, & Simonsen, 2014) (Table 1). This review summarizes the knowledge on santalene and santalol biosynthesis and recent advances on their biotechnological production, especially the metabolic engineering approaches in S. cerevisiae cell factories.

2. Biosynthesis of santalenes and santalols

2.1. Santalene synthase

Like all the terpenoids, santalenes and santalols are synthesized from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which are generated via the mevalonate (MVA) pathway.

![Fig. 1. Biosynthetic pathway of santalenes and santalols in planta. IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; eFPS: (E,E)-farnesyl diphosphate synthase; (E,E)-FPP: (E,E)-farnesyl diphosphate; STS: santalene/bergamotene synthase; CYP: cytochrome P450 monoxygenase; CPR: cytochrome P450 reductase.](image-url)
in plant cytosol. Farnesyl diphasphate synthase (FPP) catalyzes the condensation of one molecule of DMAPP and two molecules of IPP to yield farnesyl diphasphate (FPP). From *Santalum* species and *Cinnamomum camphora*, multiple isoenzymes of santalene/bergamotene synthase (STS) were characterized. All of these enzymes cyclize (*E, E*)-FPP were used to yield santalenes (including *α*-santalene, *β*-santalene, *epi*-*β*-santalene and *exo-* and *α*-bergamotene) (Beekwilder, van Houwelingen, Bosch, Lentzen, Melillo, & Wisselink, 2020; Jones et al., 2011; Rani, Ravikumar, Reddy, & Kush, 2013; Srivastava et al., 2015). Unlike these typical product-promiscuous STSs, SanSyn from *Clusia laurina* produces *α*-santalene as well as a trace of *exo-* and *α*-bergamotene using (*E, E*)-FPP as the substrate (Schalk, 2011). In 2009, a novel santalene biosynthetic pathway was found in the wild tomato *Solanum habrochaites* (Sallaud et al., 2009). Therein, it was reported that a (*Z,Z*)-FPP synthase (*2FPS*) is responsible for generation of (*Z,Z*)-FPP from DMAPP and IPP, and SBS cyclizes (*Z,Z*)-FPP to afford *α*-santalene, *epi*-*β*-santalene, *endo-* and *exo-*bergamotene and (*end*o-*endo*)-bergamotene (*Table 2*) (Matsuba et al., 2013; Sallaud et al., 2009). Intriguingly, SaSSy from *S. album* was found to be capable of not only cyclizing (*E,E*)-FPP to yield *α*-santalene, *β*-santalene, *epi*-*β*-santalene and *exo-* and *α*-bergamotene but also converting (*E,E*)-FPP into *α*-santalene, *β*-santalene, *epi*-*β*-santalene, *endo-* and *exo-* and *Z,Z*-farnesene (*Table 2*) (Jones et al., 2011).

### 2.2. Cytochrome P450 monooxygenases (CYPs) involved in santalol biosynthesis

Santalols are synthesized from oxidation of the corresponding santalenes under catalysis of CYPs (*Fig. 1, Table 3*). In the previous studies, ten CYPs have been functionally characterized from *S. album* which hydroxylate C–12 of santalenes, yielding *β*-santalol, *exo-* and *α*-bergamotol and *epi-* and *β*-santalol (*Table 3*). Among them, CYP76F41, CYP76F42 and CYP76F39v1 produce both *Z* and *E*-stereoisomers of *α*-santalol, *β*-santalol, *exo-* and *α*-bergamotol and *epi-* and *β*-santalol (Diaz-Chavez et al., 2013). CYP76F39v2 yields seven products including (*Z*)- and (*E*)-santalol, (*Z*)- and (*E*)-bergamotol and (*E*)- and (*Z*)- and (*E*)- and (*Z*)- and (*E*)- and (*Z*)-bergamotol and (*E*)- and (*Z*)- and (*E*)- and (*Z*)- and (*E*)-bergamotol when co-expressed with *SaSSy* in *S. cerevisiae* and in vitro enzymatic assays (Diaz-Chavez et al., 2013). Unlike the above five CYPs, CYP76F37v1, CYP76F37v2, CYP76F38v1 and CYP76F38v2 only generate *E*-stereoisomers of *α*-santalol, *β*-santalol and *exo-* and *α*-bergamotol when co-expressed with *SaSSy* in *S. cerevisiae* and in vitro enzymatic assays (Diaz-Chavez et al., 2013). While CYP736A167 was found to selectively produce all the *Z*-type products (Celedon et al., 2016). Identification and characterization of STSs and CYPs laid a foundation for production of santalenes and santalols by biotechnological approaches.

### 3. Metabolic engineering strategies for production of santalenes and santalols in *S. cerevisiae*

#### 3.1. Reconstruction of santalenes and santalols biosynthetic pathway and optimization of DMAPP, IPP and FPP synthesis in *S. cerevisiae*

*S. cerevisiae* is one of the most used microbial hosts for natural product production due to its fast growth rate, high tolerance against harsh industrial conditions and multiple organelles which provide various compartments and environments for enzyme expression and catalysis (Bian, Deng, & Liu, 2017; Lian, Mishra, & Zhao, 2018). Moreover, intensive researches on *S. cerevisiae* metabolic engineering have brought about numerous and ease genetic engineering technologies (Heavner & Price, 2015; Liang, Ning, & Zhao, 2013).

### Table 1

| Host            | Carbon sources | Products         | Titer          | References                        |
|-----------------|----------------|------------------|----------------|-----------------------------------|
| *S. cerevisiae* | Glucose        | *α*-Santalane    | 91.96 mg/L     | Scalcinati et al., 2012a          |
| *S. cerevisiae* | Glucose        | *α*-Santalane    | 0.036 Cmol (g biomass)^−1^ h^−1^ | Scalcinati et al., 2012b          |
| *S. cerevisiae* | Glucose        | *α*-Santalane    | 8.29 mg/L      | Chen et al., 2013                 |
| *P. patens*     | N/A            | *α*-Santalane    | 0.04/0.04 mg/g d.w. | Zhan et al., 2014                |
| *S. cerevisiae* | Glucose        | *α*-Santalane    | 163 mg/L       | Tippmann et al., 2016             |
| *N. tabacum*    | N/A            | Santalene/bergamotene | 1.98±0.35 μg/g f.w. | Yin & Wong, 2019               |
| *Y. lipolytica* | Glucose        | *α*-Santalane    | 27.92 mg/L     | Jia et al., 2019                  |
| *S. cerevisiae* | Glucose and galactose | Santalenes/santalols | 0.3/1.3 g/L   | Zha et al., 2020                 |

### Table 2

| Genes      | Species              | GenBank ID  | Substrates | Products                                      | References                        |
|------------|----------------------|------------|------------|-----------------------------------------------|-----------------------------------|
| SaSSy      | *S. album*           | HQ343276   | (*Z,Z*)-FPP | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Jones et al., 2011 |
|           |                      |            | (*E,E*)-FPP | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *endo-* and *α*-bergamotene | Jones et al., 2011 |
|           |                      |            |            | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Schalk, 2011                      |
| SavSSy     | *S. austrocaledonicum* | HQ343277   | (*E,E*)-FPP | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Jones et al., 2011 |
|           |                      |            |            | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Jones et al., 2011 |
|           |                      |            |            | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Beekwilder et al., 2020          |
| SppSSy     | *S. spicatum*        | HQ343278   | (*E,E*)-FPP | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Schalk, 2011                      |
| CiSSy      | *C. camphora*        | LQ880194   | (*E,E*)-FPP | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Schalk, 2011                      |
| SanSyn     | *C. laurina*         | HQ452480   | (*E,E*)-FPP | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Schalk, 2011                      |
| SBS        | *S. habrochaites*    | FJ194970   | (*Z,Z*)-FPP | *α*-Santalene, *β*-santalene, *epi*-*β*-santalene, *exo-* and *α*-bergamotene | Sallaud et al., 2009             |
S. cerevisiae synthesizes IPP and DMAPP through the MVA pathway (Fig. 2). In this pathway, ERG10 (acetoacetyl-CoA thiolase) catalyzes the condensation of two molecules of acetyl-CoA to generate one molecule of acetoacetyl-CoA which is converted into 3-hydroxy-3-methyl-gluraryl-CoA (HMG-CoA) by ERG13 (HMG-CoA synthase). Subsequently, HMG-CoA is reduced by HMG1 or HMG2 (HMG-CoA reductase) to produce the core intermediate mevalonic acid, from which IPP is yielded through a series of conversions successively catalyzed by ERG12 (mevalonate-5-kinase), ERG8 (phosphomevalonate kinase) and ERG19 (mevalonate pyrophosphate decarboxylase). Finally, the reversible conversion between IPP and DMAPP is achieved under the catalysis of IDI1. FPP is then synthesized from DMAPP and IPP by catalysis of ERG20 (FPP synthase). As mentioned above, the biosynthetic pathway of santalenes and santalols can be reconstructed by introducing exogenous STSs, CYPs and cytochrome P450 reductases (CPRs) in S. cerevisiae.

In order to increase the supply of IPP and DMAPP, much effort has been made to optimize MVA pathway in S. cerevisiae. Reduction of HMG-CoA is the major rate-limiting step in MVA pathway. Both HMG1 and HMG2 contain an anchoring transmembrane domain and a catalytic domain, and overexpression of the truncated HMG1 (tHMG1, the catalytic domain of HMG1) has been reported to be an efficient strategy for enhancement of terpenoid production in S. cerevisiae (Dai et al., 2014; Donald, Hampton, & Fritz, 1997; Huang et al., 2019). The transcription factor UPC2 plays

### Table 3

| Genes       | GenBank ID | Substrates                  | Products                                    | References     |
|-------------|------------|------------------------------|---------------------------------------------|----------------|
| CYP76F37v1  | KC533717   | α-Santalene, β-santalene     | (E)-α-Santalol, (E)-exo-α-bergamotol, (E)-β-santalol | Diaz-Chavez et al., 2013 |
| CYP76F37v2  | KC698966   | exo-α-bergamotene           | (E)-β-santalol                              |                |
| CYP76F38v1  | KC533715   |                             |                                             |                |
| CYP76F38v2  | KC533718   |                             |                                             |                |
| CYP76F39v1  | KC533716   |                             |                                             |                |
| CYP76F41    | KC698969   | (Z)-α-Santalol, (E)-α-santalol, (Z)-β-santalol, (E)-β-santalol |                |
| CYP76F42    | KC698965   |                             | (Z)-β-santalol, (E)-β-santalol, (Z)-epi-β-santalol, (E)-epi-β-santalol |               |
| CYP76F40    | KC698968   | (Z)-α-Santalol, (E)-α-santalol, (Z)-β-santalol, (E)-β-santalol, (Z)-epi-β-santalol, (E)-epi-β-santalol, (Z)-exo-α-bergamotol, (E)-exo-α-bergamotol |               |
| CYP736A167  | KU169302   | (Z)-α-Santalol, (Z)-β-santalol, (Z)-epi-β-santalol, (Z)-exo-α-bergamotol |                |
| CYP736A167  |           | (Z)-exo-α-bergamotol        |                                             | Celedon et al., 2016 |

**Fig. 2.** Reconstruction of biosynthetic pathway of santalenes and santalols in S. cerevisiae. Red and blue arrows represent the catalytic steps by native enzymes and exogenous enzymes, respectively. Dash arrow: the step that is depressed. HMG-CoA, 3-hydroxy-3-methyl-gluraryl-CoA; ERG10, acetoacetyl-CoA thiolase; ERG13, HMG-CoA synthase; tHMG1, a truncated HMG-CoA reductase; ERG12, mevalonate-5-kinase; ERG8, phosphomevalonate kinase; ERG19, mevalonate pyrophosphate decarboxylase; ERG20, (E,E)-FPP synthase; ERG9, squalene synthase; SaSSy, S. album santalene/bergamotene synthase; CYP736A167, S. album cytochrome P450 monoxygenase; SaCPR2, S. album NADPH-cytochrome P450 reductase.
a key role in activating expression of the gene members of the MVA pathway, and overexpression of its mutant UPC2-1 can increase the efficiency of the MVA pathway (Ro et al., 2006; Yan et al., 2014). Hence, tHMG1, UPC2-1 and ERG20 are often overexpressed to improve synthesis of FPP in santalene- and santalol-producing \textit{S. cerevisiae} (Scalcinati et al., 2012a, 2012b; Tippmann et al., 2016; Zha et al., 2020).

3.2. Restriction of branch pathways

FPP, the direct precursor of sesquiterpenes, is largely consumed for synthesis of squalene (the common precursor of triterpenes) in \textit{S. cerevisiae} (Daum, Lees, Bard, & Dickson, 1998) (Fig. 2). ERG9, the \textit{S. cerevisiae} squalene synthase, is responsible for condensation of two molecules of FPP to yield one molecule of squalene which is oxidized by ERG1 (squalene epoxidase) into 2,3-oxidosqualene. Then, through a series of cyclization and modification reactions, 2,3-oxidosqualene is converted into ergosterol (Bard et al., 1996; Gachotte et al., 1999; Zweytick, Hrastnik, Kohlwein, & Daum, 2000). Due to the substantial demand for sterols in \textit{S. cerevisiae}, biosynthesis of ergosterol is very active and consumes most of FPP (Keesler, Laster, & Parks, 1992; Kennedy, Barbach, & Bard, 1999) in order to redirect FPP flux towards sesquiterpenes, the biosynthetic pathway of ergosterol can be depressed by replacing the endogenous promotor ERG9 with a weak promoter. \textit{MET3} and \textit{CTR3} promoters have been used to depress \textit{ERG9} expression in the patchouli- and artemisinic acid-producing strains (Asadollahi et al., 2008; Paddon et al., 2013; Ro et al., 2006). Later, a glucose-induced promotor HXT1 was found to be more efficient in inhibition of \textit{ERG9} expression in construction of squalene-producing \textit{S. cerevisiae}, resulting in a 3.4-fold increase of squalene titer compared with that of the control strain (Ozcan & Johnston, 1999; Scalcinati et al., 2012a, 2012b). In our recent research, 5.9- and 7.1-fold higher levels of squalene and santalol titers were achieved in the same way to depress \textit{ERG9} expression (Zha et al., 2020).

The other branch pathway from FPP to farnesol involves LPP1 and DPP1, both of which encode lipid phosphate phosphatases (Toke et al., 1998a, 1998b). Knockout of DPP1 resulted in a significant improvement of santalene titer and inhibition of farnesol generation (Scalcinati et al., 2012a, 2012b).

3.3. Improvement of acetyl-CoA supply

Acetyl-CoA is a central metabolite in the entire metabolism network. In \textit{S. cerevisiae} cells, ALD (acetaldehyde dehydrogenase) (Meaden et al., 1997; Saint-Prix, Bönquist, & Dequin, 2004), ACS (acetyl-CoA synthetase) (De Virgilio et al., 1992) and ADH (alcohol dehydrogenase) (Hazelwood, Daran, vanMaris, Pronk, & Dickinson, 2008) play key roles in acetyl-CoA synthesis/regeneration. After decarboxylation of pyruvic acid to acetaldehyde under catalysis of pyruvate decarboxylase, ALD and ACS successively catalyze acetaldehyde dehydrogenation and ligation of acetic acid and CoA to yield acetyl-CoA. And ADH can catalyze the reversible conversion between acetaldehyde and ethanol (Hazelwood et al., 2008). Since ethanol production is a dominant metabolic process in \textit{S. cerevisiae} due to Crabtree effect (Vemuri, Eiteman, McEwen, Olsson, & Nielsen, 2007), it has been reported that overexpression of ADH2, ALD6, and a codon-optimized \textit{S. enterica} ACS L641P mutate enhanced not only acetyl-CoA synthesis from pyruvate but also its regeneration from ethanol (Shiba, Paradise, Kirby, Ro, & Keasling, 2007; Starai, Gardner, & Escalante-Semerena, 2005).

Moreover, acetyl-CoA is massively consumed in peroxisome and cytosol through glyoxylate cycle in which CIT2 (peroxisomal citrate synthase) and MLS1 (cytosolic malate) respectively catalyze synthesis of citrate and malate from acetyl-CoA (Chen, Siewers, & Nielsen, 2012). Knockout of these two enzymes led to a four-fold increase in \(\alpha\)-santalene production compared with that of the starting \textit{S. cerevisiae} strain (Chen et al., 2013).

3.4. Tuning of NADPH supply

NADPH is an important enzymatic cofactor participating in redox reactions. Both \textit{HMGI} and CYPs need NADPH to function, and hence enhancement of NADPH concentration benefits terpenoid production in \textit{S. cerevisiae}. Some successful strategies have been adopted to improve NADPH supply in \textit{S. cerevisiae}, including overexpression of mBDH1 (2,3-butanediol dehydrogenase mutant) (Celtion, Goelzer, Camarasa, Fromion, & Dequin, 2012; Ehsani, Fernández, Biosca, & Dequin, 2009; Li & Zhang, 2015), ZFW1 (glucose 6-phosphate dehydrogenase) (Kwon et al., 2006), and POS5 (mitochondrial NADH kinase) (Paramasivan & Mutteri, 2017). Scalcinati and the co-workers genetically ablated GDH1 (NADP-dependent glutamate dehydrogenase) and overexpressed GDH2 (NAD-dependent glutamate dehydrogenase) to decrease NADPH consumption by the ammonium assimilation pathway, which led to a significant increase of \(\alpha\)-santalene yield in the engineered \textit{S. cerevisiae} (Scalcinati et al., 2012b).

3.5. Linking santalol biosynthesis to GAL regulatory system

GAL promoters are broadly used for tuning enzyme expression in construction of cell factories of natural products since the expression of the target enzymes under control of GAL promoters can be easily controlled by tuning galactose content in culture of \textit{S. cerevisiae} (Paddon et al., 2013; Ro et al., 2006). In our recent research, we linked biosynthetic pathway of santalol to GAL regulatory system by expressing key biosynthetic enzymes (i.e. \textit{HMGI}, \textit{STS} and \textit{CYP736A167}) and \textit{UPC2-1} under control of the GAL promoters. Meanwhile, GAL4 (the transcriptional activator of GAL genes) (Stagoj, Comino, & Kemel, 2006; Wang et al., 2017) was overexpressed for strengthening the inducible effect of galactose on the target gene expression, and PGM2 (phosphoglyceromutase) (García-Sánchez, Hahn-Hagerdal, & Gorwa-Grauslund, 2010) was also overexpressed to increase galactose uptake. Consequently, the santalol titer exceeded 1 g/L in the final engineered strain (Zha et al., 2020).

3.6. Optimization of fermentation

Fermentation process can significantly affect the yield of the target products in their microbial hosts (Lenihan, Tsuruta, Diola, Renninger, & Regentin, 2008; van Hoek, de Hulster, van Dijken, & Pronk, 2000). Various fermentation methods, such as fed-batch fermentation and double-phase fermentation, have been broadly used in microbial production of natural products. An in situ product removal chemostat cultivation process was utilized in fermentation of \(\alpha\)-santalene-producing \textit{S. cerevisiae}, and an \(\alpha\)-santalene yield of 0.036 Cmmol / (g biomass) \(j\) was achieved by optimization of the dilution rate (Scalcinati et al., 2012a, 2012b). In another study, an RQ-controlled exponential feed strategy was used, resulting in an \(\alpha\)-santalene yield of 163 mg/L (Tippmann et al., 2016). In our research, a fed-batch fermentation was employed, and after carefully tuning the ratio of galactose and glucose in batch-phase medium and feeding phase medium, the yields of santalenes and santalols reached two folds of those in flask-based fermentation (Zha et al., 2020).
4. Production of santalenes in other heterologous hosts

The other heterologous hosts have been used to produce santalenes (Table 1), as well, including Physcomitrella patens, Nicotiana tabacum and Yarrowia lipolytica (Jia et al., 2019; Yin & Wong, 2019; Zhan et al., 2014). Optimization of MVA pathway was also performed in P. patens, N. tabacum and Y. lipolytica. For plant hosts (P. patens and N. tabacum), targeting STS into chloroplasts proved efficient in enhancement of santalene yields. In Y. lipolytica, modulation of carbon source concentration can increase α-santalene yield. Compared with S. cerevisiae, Y. lipolytica can use a broader range of carbon sources, particularly raw materials (e.g. molasses) (Ledesma-Amaro & Nicaud, 2016), enabling the potential of developing low-cost processes for microbial production of santalenes and santalols. Nevertheless, construction of high-yield Y. lipolytica platforms is still challenging due to its limited genetic tools. For plant hosts, their application is hampered by substantial difficulties in genetic editing and slow growth rate, compared with microbial hosts.

5. Conclusion

Synthetic biology techniques possess the advantages, such as with low cost and environmentally benefits, and are widely used in production of plant-derived natural products (Lian et al., 2018). In spite of distinct hosts for santalene and santalol production, S. cerevisiae has been most used. And many strategies have been utilized to increase santalene and santalol yields. Especially, in our study, linking the biosynthetic pathway of santalols to GAL regulatory system resulted in a total santalene/santalol yield of 1.6 g/L (Zha et al., 2020). Recent advances in metabolic engineering enable some potential strategies for further increasing santalene and santalol yields:

(1) Discovery or engineering of more matched cytochrome P450 reductases (CPRs) for CYP736A167

Since CYPs need association of CPRs to function, different combinations of CYPs and CPRs likely have significant impacts on oxidation efficiency (Zhang et al., 2019; Zhu et al., 2018). Accordingly, identification or engineering of more matched CPR partners of CYP736A167 may dramatically increase santalol yield. Besides, fusion of CYP and CPR proteins is also a potential method to improve oxidation efficiency (Zhao et al., 2016).

(2) Further optimization of NADPH supply

As described above, improvement of NADPH supply is an effective strategy to increase santalene and santalol production. Up to now, only knockout of GDH1 and overexpression of GDH2 were used to enhance NADPH supply in the santalene—producing S. cerevisiae (Scalcinati et al., 2012b). It has been reported that other enzymes also have impacts on NADPH supply, such as mBDH1, ZWF1 and POS5 (Celton et al., 2012; Kwon et al., 2006; Paramasivan & Muttoni, 2017). Overexpression of these enzymes may further enhance santalene and santalol yields.

(3) Compartmentalizing the entire biosynthetic pathway of santalols to endoplasmic reticulum (ER) and ER engineering

Santalene synthesis locates at cytosol in engineered yeast, but santalols are synthesized on ER membranes because CYP736A167 expresses on ER membranes. This separate distribution may reduce santalol yields due to santalene translocation and diffusion. This might be removed by compartmentalizing MVA pathway and STS to ER membranes by simply tethering an ER routing tag to each relevant enzyme (Thodey, Galanie, & Smolke, 2014). Furthermore, regulation of ER size and morphology by knockout of PAH1 (phosphatidic acid phosphatase) and overexpression of INO2 (a phospholipid biosynthesis transcription factor) were reported to be effective in improving the ability of synthesizing ER-associated proteins (Arendt et al., 2017; Kim et al., 2019), thereby potentially providing a larger reaction space for santalol synthesis.

Declaration of Competing Interest

The authors declare no conflict of interests.

Acknowledgments

This work was supported by National Natural Science Foundation of China (No. 81673530), Natural Science Foundation of Qinghai province (No. 2018-ZJ-907), Qinghai Provincial Key Laboratory of Phytochemistry for Tibetan Plateau (2017-ZJ-19Y) and Guangdong Provincial Key R&D Programme (2020B111120002).

References

Arendt, P., Miettinen, K., Pollier, J., De Rycke, R., Gallaertw, N., & Goossens, A. (2017). An endoplasmic reticulum-engineered yeast platform for overproduction of triterpenoids. Metabolic Engineering, 40, 165–175.
Asadollahi, M. A., Maury, J., Møller, K., Nielsen, K. F., Schalk, M., Clark, A., & Nielsen, J. (2008). Production of plant sesquiterpenes in Saccharomyces cerevisiae: Effect of ERG9 repression on sesquiterpene biosynthesis. Biotechnology and Bioengineering, 99(3), 666–677.
Baldovini, N., Delasalle, C., & Joulain, D. (2011). Phytochemistry of the heartwood from fragrant Santalum species: A review. Flavour and Fragrance Journal, 26(1), 17–29.
Bard, M., Bruner, D. A., Persson, C. A., Lees, N. D., Biermann, B., Frye, L. E., ... Barbuch, R. (1996). Cloning and characterization of ERG25, the Saccharomyces cerevisiae gene encoding C4-sterol methyl oxidase. Proceedings of the National Academy of Sciences, 93(1), 186–190.
Beekwilder, M. J., van Houwelingen, A. M. M. L., Bosch, J. H., Lenton, G. F., Melillo, E., & Wisselink, H. W. (2020). Santalene synthase. U.S. Patent, US0010822 A1.
Benencia, F., & Courrèges, M. C. (1999). Antiviral activity of sandalwood oil against Herpes simplex viruses-1 and-2. Phytomedicine, 6(2), 119–123.
Bian, G., Deng, Z., & Liu, T. (2017). Strategies for terpenoid overproduction and new terpenoid discovery. Current Opinion in Biotechnology, 48, 234–241.
Bommedredev, A., Rule, B., VanWirt, A. L., Santia, S., & Dwiwedi, C. (2012). Santalol, a derivative of sandalwood oil, induces apoptosis in human prostate cancer cells by causing caspase-3 activation. Phytomedicine, 19(8–9), 804–811.
Bommedredev, A., Hora, J., Cornish, B., & Dwiwedi, C. (2007). Chemoprevention by z-santalol on UVB radiation-induced skin tumor development in mice. Anticancer Research, 27(4B), 2185–2188.
Burdam, G. A., & Carabin, I. G. (2008). Safety assessment of sandalwood oil (Santalum album L); Food and Chemical Toxicology, 46(2), 421–432.
Celedon, J. M., Chiang, A., Yuen, M. M., Diaz-Chavez, M. L., Madliao, L. L., Finnegan, P. M., ... Bohlmann, J. (2016). Heartwood-specific transcriptome and metabolite signatures of tropical sandalwood (Santalum album) reveal the final step of (Z)-santalol fragrance biosynthesis. The Plant Journal, 86(4), 289–299.
Celton, M., Goelzer, A., Camarasa, C., Fromion, V., & Dequin, S. (2012). A constraint-based model analysis of the metabolic consequences of increased NADPH oxidation in Saccharomyces cerevisiae. Metabolic Engineering, 14(4), 366–379.
Chen, J., Fan, F., Qi, G., Tang, J., Xi, Y., Bi, C., ... Zhang, X. (2020). Identification of Absidia orichoides steroid 11b-hydroxylation system and its application in engineering Saccharomyces cerevisiae for one-step biotransformation to produce hydrocortisone. Metabolic Engineering, 57, 31–42.
Chen, Y., Daviet, L., Schalk, M., Sievers, V., & Nielsen, J. (2013). Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metabolic Engineering, 15, 48–54.
Chen, Y., Sievers, V., & Nielsen, J. (2012). Profiling of Cysolic and Peroxiominal acetyl-CoA Metabolism in Saccharomyces cerevisiae. PLoS ONE, 7(8), e42475.
Dai, Z., Liu, Y., Zhang, X., Shi, M., Wang, B., Wang, D., ... Zhang, X. (2013). Molecular biology of ginsenosides in baker’s yeast. Scientific Reports, 4, 3698.
Daum, G., Lees, N. D., Bard, M., & Dickson, R. (1998). Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae. Yeast, 14(16), 1471–1510.
De Virgilio, C., Bürckert, N., Barth, C., Neuhaus, J. M., Boller, T., & Wiemken, A. (1992). Cloning and disruption of a gene required for growth on acetate but not on ethanol: The acetyl-coenzyme A synthetase gene of Saccharomyces cerevisiae. Yeast, 8(12), 1043–1051.
Diaz-Chavez, M. L., Moniodis, J., Madiallo, L. L., Jancsik, S., Keeling, C. I., Barbour, E. L., ... Bohlmann, J. (2013). Biosynthesis of Sandalwood Oil: Santalum album CYP76F cytochromes P450 produce santalols and bergamotol. Plos ONE, 8(9).

Donald, K. A. G., Hampton, R. Y., & Fritz, I. B. (1997). Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthase in Saccharomyces cerevisiae. Applied and Environmental Microbiology, 63, 3341–3344.

Ehsani, M., Fernández, M. R., Biosca, J. A., & Dequin, S. (2009). Reversal of squalene synthase in yeast as a first step towards Taxol (Paclitaxel) production. Metabolic Engineering, 11(2), 201–206.

Gachotte, D., Sen, S. E., Eckstein, J., Barbach, R., Krieger, M., Ray, B. D., & Bard, M. (1999). Coenzyme specificity of 2,3-butanedioyl dehydrogenase from Saccharomyces cerevisiae and in vivo functional analysis. Biotechnology and Bioengineering, 64(2), 381–389.

Engels, B., Dahm, P., & Jennewein, S. (2008). Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metabolic Engineering, 10(3), 201–206.

Kennedy, M. A., Barbuch, R., & Bard, M. (1999). Transcriptional Regulation of the Flavivirus Flavivirus Genome. Journal of Virology, 73(1), 157–164.

Koch, C., Reichling, J., Schneele, J., & Schnitzler, P. (2008). Inhibitory effect of santonin from Indian sandalwood on the central nervous system in mice. Phytomedicine, 25(2), 201–206.

Kwon, D. H., Kim, M. D., Lee, T. H., Oh, Y. J., Ryu, Y. W., & Seo, J. H. (2006). Elevation of a-santalol and b-santalol from sandalwood on the central nervous system in mice. Phytomedicine, 2(2), 119–126.

Li, J., & Zhang, Y. (2015). Modulating betulinic acid production in Saccharomyces cerevisiae by managing the intracellular supplies of the co-factor NADPH and oxygen. Journal of Bioscience and Bioengineering, 119(1), 77–81.

Mohankumar, A., Shanmugam, G., Kalaichelvi, D., Levenson, C., Nivitha, S., Thirumurugan, G., & Sundararaman, K. (2008). East Indian sandalwood (Santalum album L.) oil confers neuroprotection and geroprotection in Caenorhabditis elegans via activating SKN-1/Nrf2 signaling pathway. RSC. Advances, 8(59), 33753–33774.

Oktawa, H., Ueda, R., Matsutomo, K., Kawanishi, K., & Kato, A. (1995). Effect of a-santalol and b-santalol from sandalwood on the central nervous system in mice. Phytomedicine, 2(2), 119–126.

Ozcan, S., & Johnston, M. (1995). Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. Molecular and Cellular Biology, 15(4), 1564–1572.
*cerevisiae* DPP1 gene encoding diacylglycerol pyrophosphate phosphatase. *Journal of Biological Chemistry*, 273(6), 3278–3284.

Toke, D. A., Bennett, W. L., Oshiro, J., Wu, W. I., Voelker, D. R., & Carman, G. M. (1998a). Isolation and characterization of the *Saccharomyces cerevisiae* LPP1 gene encoding a Mg2+-independent phosphatidate phosphatase. *Journal of Biological Chemistry*, 273(23), 14331–14338.

van Hoek, P., de Hulster, E., van Dijken, J. P., & Pronk, J. T. (2000). fermentative capacity in high-cell-density fed-batch cultures of baker’s yeast. *Biotechnology and Bioengineering*, 68(3), 517–523.

Vermuri, G. N., Eiteman, M. A., McEwen, J. E., Olsson, L., & Nielsen, J. (2007). Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*, 104(7), 2402–2407.

Wang, F., Lv, X., Xie, W., Zhou, P., Zhu, Y., Yao, Z., ... Yu, H. (2017). Combining Gal4p-mediated expression enhancement and directed evolution of isoprene synthase to improve isoprene production in *Saccharomyces cerevisiae*. *Metabolic Engineering*, 39, 257–266.

Yan, X., Fan, Y., Wei, W., Wang, P., Liu, Q., Wei, Y., ... Zhou, Z. (2014). Production of bioactive ginsenoside compound K in metabolically engineered yeast. *Cell Research*, 24(6), 770–773.

Yin, J. L., & Wong, W. S. (2019). Production of santalenes and bergamotene in *Nicotiana tabacum* plants. *PLoS ONE*, 14(1), e0203249.

Zha, W., An, T., Li, T., Zhu, J., Gao, K., Sun, Z., ... Zi, J. (2020). Reconstruction of the biosynthetic pathway of santalols under control of the GAL regulatory system in yeast. *ACS Synthetic Biology*, 9(2), 449–456.

Zhan, X., Zhang, Y. H., Chen, D. F., & Simonsen, H. T. (2014). Metabolic engineering of the moss Physcomitrella patens to produce the sesquiterpenoids patchoulol and α/β-santalene. *Frontiers in Plant Science*, 5, 636.

Zhang, R., Zhang, Y., Wang, Y., Yao, M., Zhang, J., Liu, H., ... Yuan, Y. (2019). Pregnenolone overproduction in *Yarrowia lipolytica* by integrative components pairing of the cytochrome P450ccc system. *ACS Synthetic Biology*, 8(12), 2666–2678.

Zhang, X., Chen, W., Guillermo, R., Chandrasekher, G., Kaushik, R. S., Young, A., ... Dwiwedi, C. (2010). Alpha-santalol, a chemopreventive agent against skin cancer, causes G2/M cell cycle arrest in both p53-mutated human epidermoid carcinoma A431 cells and p53 wild-type human melanoma UACC-62 cells. *BMC Research Notes*, 3(1), 220.

Zhao, F., Bai, P., Liu, T., Li, D., Zhang, X., Lu, W., & Yuan, Y. (2016). Optimization of a cytochrome P450 oxidation system for enhancing protopanaxadiol production in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, 113(8), 1787–1795.

Zhu, M., Wang, C., Sun, W., Zhou, A., Wang, Y., Zhang, G., & Li, C. (2018). Boosting 11-oxo-α-amyrin and glycyrrhetinic acid synthesis in *Saccharomyces cerevisiae* via pairing novel oxidation and reduction system from legume plants. *Metabolic Engineering*, 45, 43–50.

Zweytick, D., Hrastnik, C., Kohlwein, S. D., & Daum, G. (2000). Biochemical characterization and subcellular localization of the sterol C-24(28) reductase, erg4p, from the yeast *Saccharomyces cerevisiae*. *FEBS Letters*, 479(1), 83–87.