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Review

Advances in biotechnological production of santalenes and santalols

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ABSTRACT

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 monooxygenases (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*.

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Contents

1. Introduction

The core components of sandalwood essential oil are sesquiterpene olefins and sesquiterpene alcohols, mainly including (Z)-α-santalol, (Z)-β-santalol, (Z)-epi-β-santalol, (Z)-exo-α-bergamotol, α-santalene, β-santalene, exo-α-bergamotene and epi-β-santalene (Baldovini, Delasalle, & Joulain, 2011; Burdock & Carabin, 2008; Howes, Simmonds, & Kite, 2004). Sandalwood essential oil possesses various medicinal properties, such as neuroprotection (Mohankumar et al., 2018; Okugawa, Ueda, Matsumoto, Kawanishi, & Kato, 1995), antiviral (Benencia & Courrèges, 1999; Koch, Reichling, Schneele, & Schnitzler, 2008; Paulpandi et al., 2012), antimicrobial (Jirovetz et al., 2006), antioxidant (Misra &

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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 heart-woods 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
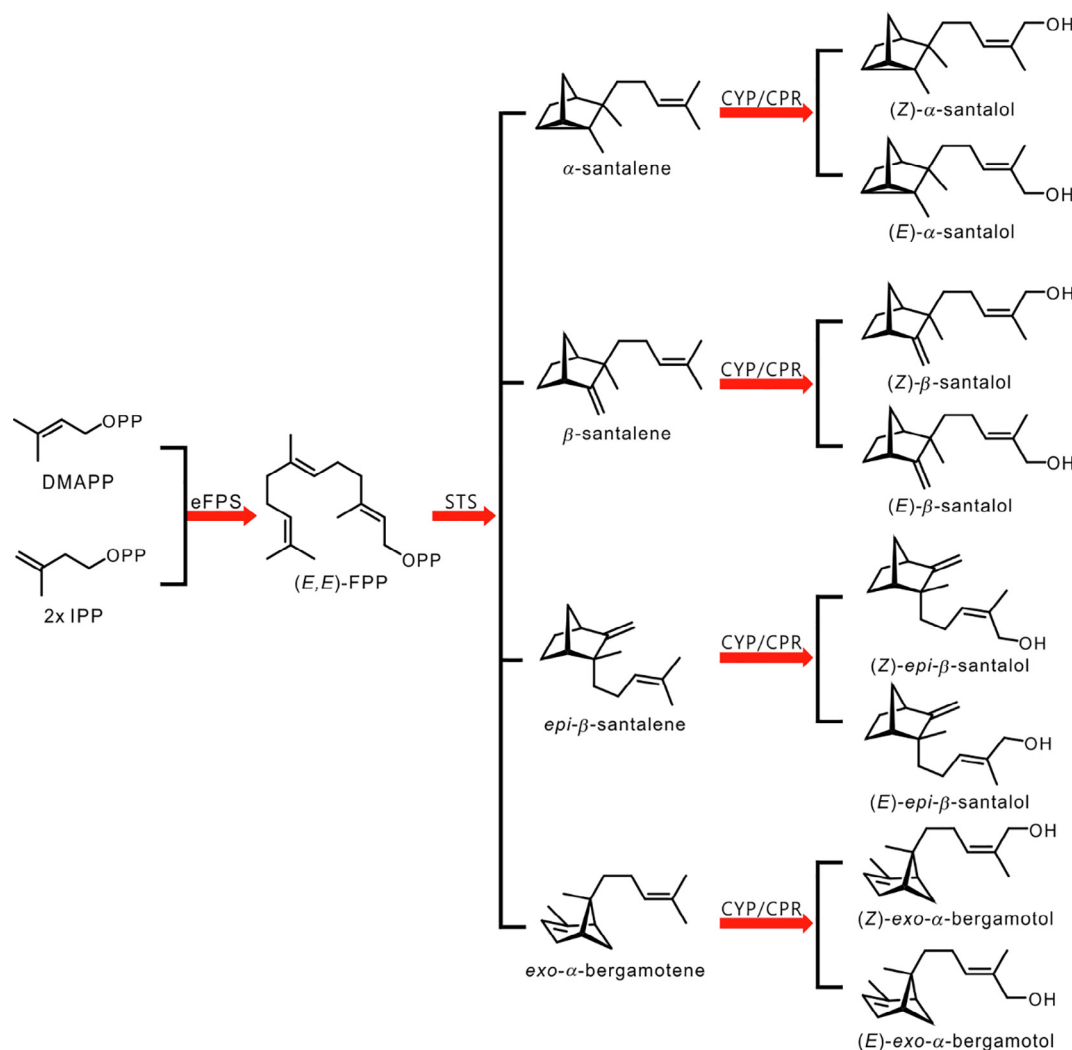
Fig. 1. Biosynthetic pathway of santalenes and santalols in planta. IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; eFPS: (E,E)-farnesyl diphosphate synthase; (E,E)-FPP: (E,E)-farnesyl diphosphate; STS: santalene/bergamotene synthase; CYP: cytochrome P450 monooxygenase; CPR: cytochrome P450 reductase.

Table 1
Biotechnological production of santalenes and santalols.

Host	Carbon sources	Products	Titer	References
S. cerevisiae	Glucose	α-Santalene	91.96 mg/L	Scalcinati et al., 2012a
S. cerevisiae	Glucose	α-Santalene	0.036 Cmmol (g biomass)$^{-1}$h^{-1}.	Scalcinati et al., 2012b
S. cerevisiae	Glucose	α-Santalene	8.29 mg/L	Chen et al., 2013
P. patens	N/A	α-Santalenes	0.04/0.04 mg/g d.w.	Zhan et al., 2014
S. cerevisiae	Glucose	α-Santalene	163 mg/L	Tippmann et al., 2016
N. tabacum	N/A	Santalenes/ bergamotene	1.98/0.35 μg/g f.w.	Yin & Wong, 2019
Y. lipolytica	Glucose	α-Santalene	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

in plant cytosol. Farnesyl diphosphate synthase (FPS) catalyzes the condensation of one molecule of DMAPP and two molecules of IPP to yield farnesyl diphosphate (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-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 *Clausena lansium* produces α-santalene as well as a trace of *exo-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 (zFPS) 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-α*-bergamotene, *exo-α*-bergamotene and *endo-β*-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-α*-bergamotene but also converting (*Z,Z*)-FPP into α-santalene, β-santalene, *epi-β*-santalene, *endo-α*-bergamotene and (*Z*)-β-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 α-santalol,

β-santalol, *exo-α*-bergamotol and *epi-β*-santalol (Table 3). Among them, CYP76F41, CYP76F42 and CYP76F39v1 produce both *Z*- and *E*-stereoisomers of α-santalol, β-santalol, *exo-α*-bergamotol and *epi-β*-santalol (Diaz-Chavez et al., 2013). CYP76F39v2 yields seven products including (*Z*)-α-santalol, (*E*)-α-santalol, (*Z*)-β-santalol, (*E*)-β-santalol, (*E*)-*epi-β*-santalol, (*Z*)-*exo-α*-bergamotol and (*E*)-*exo-α*-bergamotol, and CYP76F40 only produces (*Z*)-α-santalol, (*E*)-β-santalol and (*E*)-*exo-α*-bergamotol (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-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 2
STSs from various plant species.

Genes	Species	GenBank ID	Substrates	Products	References
SaSSy	*S. album*	HQ343276	(*Z, Z*)-FPP	α-Santalene, β-santalene, *epi-β*-santalene, *endo-α*-bergamotene, (*Z*)-β-farnesene	Jones et al., 2011
			(*E, E*)-FPP	α-Santalene, β-santalene, *epi-β*-santalene, *exo-α*-bergamotene	
SauSSy	*S. austrocaledonicum*	HQ343277	(*E, E*)-FPP	α-Santalene,	Jones et al., 2011
SspiSSy	*S. spicatum*	HQ343278		β-santalene,	Jones et al., 2011
CiCaSSy	*C. camphora*	LQ880194		*epi-β*-santalene, *exo-α*-bergamotene	Beekwilder et al., 2020
SanSyn	*C. lansium*	HQ452480	(*E, E*)-FPP	α-Santalene, *exo-α*-bergamotene	Schalk, 2011
SBS	*S. habrochaites*	FJ194970	(*Z, Z*)-FPP	α-Santalene, *epi-β*-santalene, *endo-α*-bergamotene, *exo-α*-bergamotene, *endo-β*-bergamotene	Sallaud et al., 2009

Table 3
CYPs for santalols biosynthesis.

Genes	GenBank ID	Substrates	Products	References
CYP76F37v1	KC533717	α-Santalene, β-santalene, *epi*-	(E)-α-Santalol,	Diaz-Chavez et al., 2013
CYP76F37v2	KC698966	β-santalene,	(E)-*exo*-α-bergamotol,	
CYP76F38v1	KC533715	*exo*-α-bergamotene	(E)-β-santalol	
CYP76F38v2	KC533718			
CYP76F39v1	KC533716		(Z)-α-Santalol, (E)-α-santalol, (Z)-β-santalol, (E)-β-santalol, (Z)-*epi*-β-santalol,	
CYP76F41	KC698969		(E)-*epi*-β-santalol, (Z)-*exo*-α-bergamotol, (E)-*exo*-α-bergamotol	
CYP76F42	KC698965			
CYP76F39v2	KC698967		(Z)-α-Santalol, (E)-α-santalol, (Z)-β-santalol, (E)-β-santalol, (E)-*epi*-β-santalol, (Z)-*exo*-α-bergamotol, (E)-*exo*-α-bergamotol	
CYP76F40	KC698968		(Z)-β-Santalol, (E)-β-santalol, (E)-*exo*-α-bergamotol	
CYP736A167	KU169302		(Z)-α-Santalol, (Z)-β-santalol, (Z)-*epi*-β-santalol, (Z)-*exo*-α-bergamotol	Celedon et al., 2016

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-glutaryl-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
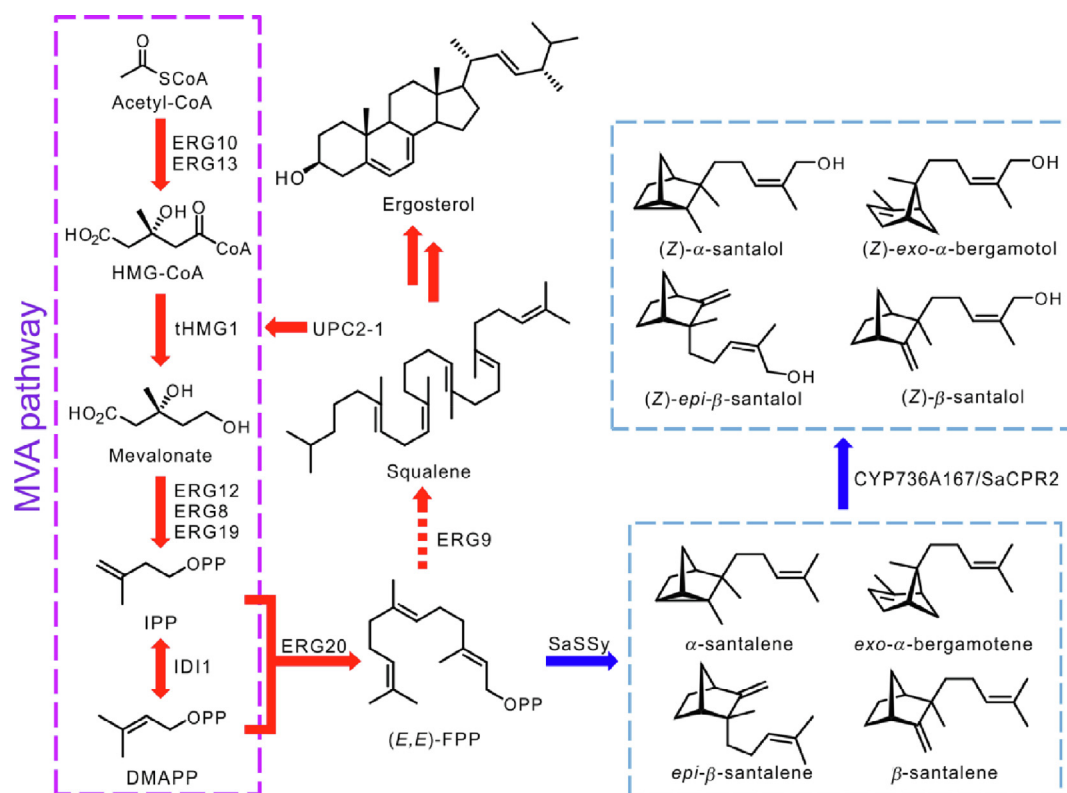
Fig. 2. Reconstruction of biosynthetic pathway of santalenes and santalols in *S. cerevisiae*. Red and blue arrows represent the catalytic steps by native enzymes and exogenous enzymes, respectively. Dash arrow: the step that is depressed. HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; ERG10, acetoacetyl-CoA 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 monooxygenase; 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 *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 *S. cerevisiae* (Daum, Lees, Bard, & Dickson, 1998) (Fig. 2). ERG9, the *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 *S. cerevisiae* cells, biosynthesis of ergosterol is very active and consumes most of FPP (Keesler, Laster, & Parks, 1992; Kennedy, Barbuch, & 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 promotor. *MET3* and *CTR3* promoters have been used to depress *ERG9* expression in the patchoulol- and artemisinic acid-producing strains (Asadollahi et al., 2008; Paddon et al., 2013; Ro et al., 2006). Later, a glucose-induced promoter *HXT1* was found to be more efficient in inhibition of ERG9 expression in construction of santalene-producing *S. cerevisiae*, resulting in a 3.4-fold increase of santalene titer compared with that of the control strain (Ozcan & Johnston, 1995; Scalcinati et al., 2012a, 2012b). In our recent research, 5.9- and 7.1-fold higher levels of santalene and santalol titers were achieved in the same way to depress 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 *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 *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 *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 α-santalene production compared with that of the starting *S. cerevisiae* strain (Chen et al., 2013).

3.4. Tuning of NADPH supply

NADPH is an important enzymatic cofactor participating in redox reactions. Both tHMG1 and CYPs need NADPH to function, and hence enhancement of NADPH concentration benefits terpenoid production in *S. cerevisiae*. Some successful strategies have been adopted to improve NADPH supply in *S. cerevisiae*, including overexpression of mBDH1 (2,3-butanediol dehydrogenase mutant) (Celton, Goelzer, Camarasa, Fromion, & Dequin, 2012; Ehsani, Fernández, Biosca, & Dequin, 2009; Li & Zhang, 2015), ZWF1 (glucose 6-phosphate dehydrogenase) (Kwon et al., 2006), and POS5 (mitochondrial NADH kinase) (Paramasivan & Mutturi, 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 α-santalene yield in the engineered *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 *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. tHMG1, STS and CYP736A167) and UPC2-1 under control of the GAL promoters. Meanwhile, GAL4 (the transcriptional activator of GAL genes) (Stagoj, Comino, & Komel, 2006; Wang et al., 2017) was overexpressed for strengthening the inducible effect of galactose on the target gene expression, and PGM2 (phosphoglucomutase) (Garcia Sanchez, 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 α-santalene-producing *S. cerevisiae*, and an α-santalene yield of 0.036 Cmmol / (g biomass) /h 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 a α-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 & Mutturi, 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 rel-

evant 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

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