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Authors

Liu, Chun-Li

Xue, Kai

Yang, Yankun

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Metabolic engineering strategies for sesquiterpene production in microorganism

Chun-Li Liu[1, 2, 3, 4], Kai Xue[1, 3, 4], Yankun Yang[1, 3, 4], Xiuxia Liu[1, 3, 4], Ye Li[1, 3, 4], Taek Soon Lee[5, 6, *], Zhonghu Bai[1, 3, 4, *], Tian-Wei Tan[2, *]

[1] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi, 214122, China;

[2] College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, P. R. China;

[3] The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University;

[4] Jiangsu Provincial Research Center for Bioactive Product Processing Technology, Jiangnan University;

[5] Joint BioEnergy Institute, 5885 Hollis Street, Emeryville, CA 94608, USA;

[6] Biological Systems & Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA;

*Corresponding authors:

Dr. Tianwei Tan, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing Chaoyang District North Sanhuan Road no. 15, Beijing 100029. P. R. China; Phone: +86-10-6441-6691, Fax: +86-10-6471-5443, E-mail: twtan@mail.buct.edu.cn;

Dr. Zhonghu Bai, Jiangnan University, Jiangsu Province Wuxi City Binhu District Lihu Road no. 1800, Wuxi 214122. P. R. China; Phone: +86-510-8532-9306, Fax: +86-510-8532-9306, E-mail: baizhonghu@jiangnan.edu.cn; Dr. Taek Soon Lee, Joint BioEnergy Institute, 5885

Hollis St. 4th floor, Emeryville, CA 94608, USA; Phone: +1-510-495-2470, Fax: +1-510-495-2629, E-mail: tslee@lbl.gov.

Abstract

Sesquiterpenes are a large variety of terpene natural products, widely existing in plants, fungi, marine organisms, insects, and microbes. The value-added sesquiterpenes are extensively used in industries as food, drugs, fragrances and fuels. With increase of the market demands and the price of sesquiterpenes, biosynthesis of sesquiterpenes by microbial fermentation methods from renewable feedstocks acquires increasing attention. The synthetic biology provides robust tools of sesquiterpene production in microorganisms. This review presents a summary of sesquiterpene biosynthesis and metabolic engineering strategies on the host and pathway engineering for sesquiterpene production. The diversity, native producers, and the synthases of sesquiterpenes are presented. Advances in synthetic biology provide new strategies on creation of the desired hosts for sesquiterpene production. Especially, metabolic engineering strategies for production of sesquiterpenes such as amorphadiene, farnesene, bisabolene, and caryophyllene are emphasized in *Escherichia coli*, *Saccharomyces cerevisiae*, and some other microorganisms. Future perspectives on strain and process improvements for sesquiterpene production are also discussed.

Keywords

Sesquiterpene biosynthesis, sesquiterpene synthase, metabolic engineering strategies, MEP pathway, MVA pathway.

1. Introduction

Terpenes are a large and diverse class of organic compounds, with basic C5 isoprene-unit hydrocarbons. According to the number of C5 isoprene-unit, terpenes are classified as hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri- (C30) and tetra- (C40) terpenes (Bohlmann and Croteau, 1999, Chen et al. , 2011). Sesquiterpene, the largest subgroup of terpenes, has several thousands of representatives and more than 300 different skeletons (Breitmaier, 2008, Connolly and Hill, 1991, Sacchettini and Poulter, 1997). Sesquiterpenes mostly consist of hydrocarbons possessing a multitude of different carbon skeletons and some unsaturated bonds, and their structures present acyclic, monocyclic, bicyclic, and tricyclic compounds (Kramer and Abraham, 2011). They present more structures after oxidation or rearrangement based on C15 skeleton. Sesquiterpenes are mostly isolated from a variety of natural sources, including plant, fungi, microorganisms, and insect (Sacchettini and Poulter, 1997). In plants and microorganisms, sesquiterpenes are important components of volatile oil, existing in form of alkene, alcohol, ketone, and lactone, emitted respectively for spreading fungal spores, protecting themselves from enemies and communicating with other organisms (Rohlfs and Churchill, 2011).

In these natural sources, sesquiterpene synthases are responsible for compound biosynthesis from the common intermediate substrate farnesyl diphosphate (FPP) condensed from isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) provided by mevalonate (MVA)-dependent pathway discovered in 1950s and 2-C-methyl-D-erythrito1-4-phosphate (MEP) pathway disclosed in 1993 (Rohmer, et al. , 1993, Schwender et al. , 1996). The MVA pathway exists in some prokaryotes and almost all eukaryotes including high plants. Many, but not all eubacteria (Rohmer, 1999, Rohmer et al. , 1993, Trutko et al. , 2005), and cyanobacteria (Proteau, 1999) make exclusive use of the MEP pathway. The MVA pathway, located in cytosol, is suggested to be responsible for synthesis of sesquiterpenes (C15),

terpenoid aldehydes (C15 and C25) and steroids/triterpenoids (C30). While all MEP-derived isoprenoids are generally considered as typical and exclusive plastidial hemi- (C5), mono- (C10), diterpenes (C20) and carotenoids (C40) (Bouvier et al. , 2005, Opitz et al. , 2014, Pazouki and Niinemets, 2016). Although the MVA pathway and the MEP pathway operate in two different subcellular compartments (cytosol and plastid, respectively), metabolite exchanges or cross-regulation known as crosstalk occur between them (Hemmerlin et al. , 2003).

Because many sesquiterpenes have complex molecular structures, inherent bioactivity, and aroma, they could be applicated as functional precursors of fragrances, medicines, and fuels (Enserink, 2005, Little et al. , 1950, Nour et al. , 2009, Peralta-Yahya et al. , 2011). Although sesquiterpenes and their derivatives display a broad range of application from medicineto fuels, their production is usually at low levels in native producers (Nour et al. , 2009). The small quantities and poor purities limit the scale-up of sesquiterpene production through natural extraction or chemical synthesis. Given the great market demands and limitations of high production of sesquiterpenes, metabolic engineering strategies are attempted to produce them, such as amorphadiene (Redding-Johanson et al. , 2011, Ro et al. , 2006), farnesene (Meadows et al. , 2016, Su et al. , 2015, Yang et al. , 2016c), bisabolene (Kirby et al. , 2014b), and caryophyllene (Yang et al. , 2016a). Synthetic biology provides an alternative approach to produce plant natural product sesquiterpenes in sufficient quantities using microbial fermentation (Ro et al. , 2006), which is enabled to be an economically feasible choice by recent advances.

Even though there have been examples of sesquiterpene biosynthesis (Block et al. , 2019, Kramer and Abraham, 2011) and analytical techniques for them (Merfort, 2002), it is meaningful to summarize and update the advances in sesquiterpene biosynthesis and metabolic engineering strategies. Here, we review the main aspects regarding the applications of

sesquiterpenes, natural sesquiterpene biosynthesis and metabolic engineering strategies on sesquiterpene production in engineered microorganisms. First, we briefly characterize diversities and applications of sesquiterpenes. Next, we systematically discuss the natural producers of the compounds and terpene synthases (TPSs). Then, we summarize the strategies on metabolic regulation of isoprenoid pathways as well as other relating engineering networks for increasing sesquiterpene production. Finally, we illustrate the great potentials and challenges of microbial production of sesquiterpenes using metabolic engineering strategies.

2. Sesquiterpenes are widely-existing biosynthetic compounds

The spread applications of sesquiterpenes are systematically summarized here shown in Figure 1. Many sesquiterpenes have been extracted from plants by mankind for different purposes - as fragrances and flavors in cosmetic or food industries, pharmaceutical agents, insecticides and fuels (Agger et al. , 2008). For the natural fragrance of sesquiterpenes and their derivations, they could be used as blends in cosmetic.

Various plants are popular traditional Chinese medicines to prevent or treat different diseases for centuries. One of the effective constituents of the pharmacopoeia is sesquiterpene derivatives, and the bioactivities make them to exhibit useful medicinal properties. Sesquiterpene lactones make a huge group of bio-active constituents isolated from plant families. They have been reported for various potential effects of anti-microbial activities, antioxidant and anticancer potentials, served as antifeedants (Knight, 1995, Mori and Matsushima, 1995, Mullin et al. , 1991), antimigraine, anti-inflammatory (Pfaffenrath et al. , 2002, Tassorelli et al. , 2005), antitumor, and antiulcer (Cho et al. , 2000) for treatment of stomach-ache and skin infection (Heinrich et al. , 1998). From sesquiterpenes and their oxides, considerable interests are gained, such as well-known amorphadiene-derived artemisinin for antimalarial activity (Ecksteinludwig et al. , 2003), xanthatin for antitumor activity (Nibret et al. , 2011), parthenolide for curing migraine (Kwok et al. , 2001), and thapsigargin for treating

prostate cancer (Drew et al. , 2013). Furanoeudesma-1,3-diene and curzarene are responsible for the analgesic effects of myrrh by interacting with brain opioid receptors (Massoud et al. , 2010). β-2-Himachalen-6-ol, a novel sesquiterpene alcohol unique to Lebanese wild carrot, was demonstrated with potent anti-cancer activity (Taleb et al. , 2016). *Trans*-caryophyllene is a bicyclic sesquiterpene produced by plants in response to herbivore damage (Köllner et al. , 2008). Valerenadiene and valerenic acid have been suggested as the active ingredients responsible for sedative effect in *Valeriana officinalis* (*V. officinalis*) (Pyle et al. , 2012). Some plants synthesize mixture of sesquiterpenes which are believed to be ecologically more effective than single compound, such as the case of attraction of pollinators or parasites of insects (Tholl, 2006). Additionally, some sesquiterpenes are also ingredients of pheromones and juvenile hormones or their precursors (Izquierdo-Bueno et al. , 2018, Lamers, 2003).

Sesquiterpenes exist in many natural foods, such as fruits, drinks, and oil. They are comprehensively investigated in essential oil producing plants, such as peppermint, conifers, and citrus fruits (Croteau, 2001, Croteau et al. , 2005, Voo et al. , 2012). Sesquiterpenes accumulate in specialized tissues such as glandular trichomes, oil ducts or secretory cavities (Lange and Turner, 2013). Over 60 sesquiterpenes have been identified in whole grapes or skins (Petronilho et al. , 2014); α-Farnesene was found on natural coating of apples (FE and KE, 1966); (+)-valencene is an aroma compound of citrus fruits, used in flavor foods and drinks (Frohwitter et al. , 2014). Through BLAST analysis, 19 TPSs and 13 sesquiterpenes were identified in unripe fruits of black pepper (*Piper nigrum*), including β-caryophyllene, δ-elemene, α-copaene, cubebene, α-humulene, δ-cadinol, γ-cadinene and δ-cadinene (Jin et al. , 2018). Insects are repelled by subjecting clove oil and patchouli oil, and the functional ingredients are sesquiterpenes and their oxides: seychellene, α-guaiene, α-bulnesene, β- and iso-caryophyllene, β-caryophyllene oxide and ketone, α-humulene, β-patchoulene, α-patchoulene, β-elemene, (+)-longipinene, (−)-isolongifolene, (+)-longifolene, and so on

(Zhang et al. , 2011). Therefore, sesquiterpenes may have potential interests as preservative in food. Many sesquiterpenes, β-elemene, γ-elemene, α-caryophyllene, germacrene D and δ-cadinene, exist in fruit of *Zanthoxylum schinifolium*, a kind of flavourful spice and Chinese medicine (Wang et al. , 2011b). α-Humulene and α-caryophyllene are contained in the essential oil of *Humulus lupulus*, an inevitable ingredient for beer brewery (Connolly and Hill, 1991). Interestingly, potent odorants in wine are from some mixed terpenoids, of which the formation mechanism encompasses enzymatic and non-enzymatic steps (Schwab and Wust, 2015).

In addition, due to high energy density, comparable cold properties and similar carbon length with cetane, sesquiterpenes reveal great interests as next-generation jet fuel substitutes (Harrison and Harvey, 2017). Especially, the ring and branching portion of sesquiterpenes could increase the gravimetric density and the volumetric energy density of fuel. Some sesquiterpenes have been proposed as jet fuel precursors due to their low hygroscopicity and high energy density. Yoshikuni and coworkers introduced TPS mutants into *E. coli* for terpene production, obtaining eight different terpenes (α-farnesene, sabinene, γ-humulene, sibirene, longifolene, α-longipinene and β-bisabolene), most of which have potential as biofuels (Yoshikuni et al. , 2006). The biosynthesized bisabolene was chemically hydrogenated to generate bisabolane, which was identified as an alternative to D2 diesel by comparing its fuel properties with those of diesel fuels (Peralta-Yahya et al. , 2011). Sesquiterpene thujopsene, α-cedrene and β-cedrene in cedarwood oil, were hydrogenated to generate fuel blends with 12% higher volumetric net heat of combustion than conventional jet fuel (Harrison and Harvey, 2017). Sesquiterpene epi-isozizaene, pentalenene and α-isocomene are even proven to have excellent fuel properties by heat of combustion calculation (Liu et al. , 2018).

Figure 1

3. Sesquiterpene biosynthesis

3.1 Sesquiterpene producers

In nature, sesquiterpenes widely exist in plants, fungi, marine organisms, and insects (Figure 2) (Connolly and Hill, 1991, Finefield et al. , 2012). Most of sesquiterpenes are essential for the life of their producers, such as steroid hormones in mammals, carotenoids and chlorophylls in plants, and ubiquinone or menaquinone in bacteria (Sacchettini and Poulter, 1997, Thomas, 1995). In plants, sesquiterpenes and monoterpenes are important constituents of essential oils. As summarized in Figure 2, they are components of volatile oil in form of alcohol, ketone, lactone or glycoside in Asteraceae, Umbelliferae, Euphorbiaceae, Wedaceae, Leguminous, Cucurbitaceae and Ranunculaceae (Connolly and Hill, 1991). Volatile oil from plants is usually consistent of sesquiterpenes: in oil of *Xanthium strumarium*, germacrene D, guaia-4, -6-diene, β-elemene, β-caryophyllene and β-copaene were detected (Li et al. , 2016a). Sesquiterpenes are modulated by enzymes or other enzymes being induced by environmental stress factors. For example, UV-radiation (Back et al. , 1998), infection (Poole and Llewellyn, 2005) and herbivore attack (Yuan et al. , 2008) have been identified to enhance volatile sesquiterpene production. It was reported that H_2O_2 could induce sesquiterpene production by increasing expression of gene *AsTPS10*, *AsTPS16* and *AsTPS19* responsible for sesquiterpene biosynthesis in wounded *Aquilaria sinesis* (Lv et al. , 2019). *Solidago canadensis* majorly produces germacrene D, accounting for more than 90% of the total compounds (Niwa et al. , 2008, Schmidt et al. , 1998, Weyerstahl et al. , 1993). Asteraceae family plant *Parthenium hysterophorus* produces guaia-4,6-diene, whose chemical structure was previously confirmed by nuclear magnetic resonance (NMR) studies (Bohlmann et al. , 1977). Sesquiterpene 7-epizingiberene produced and stored in glandular trichomes is toxic and repellent properties (Bleeker et al. , 2012). Through gas-chromatographic-mass spectrometric (GC-MS) analysis of dynamic gas from the native scent of *Solanum tuberosum L.* leaves, the following sesquiterpenes have been identified: *trans*- and *cis*-caryophyllenes, α-ylangene, α-copaene, β-bourbonene, β-elemene, Z-β-farnesene, aromadendrene, α-humulene, α-elemene, β-bisabolene,

γ-cadinene and δ-cadinene (Khalilov et al. , 1999). In flowers of *Cananga odorata var. fruticose*, bulk of the 49 VOCs were identified as sesquiterpenes: β-caryophyllene, farnesene, γ-muurolene, humulene, β-germacrene D, cedrene, δ-cadinene, α-patchoulene, α-bergamotene, β-ylangene, β-copaene, and β-cubebene (Jin et al. , 2015).

Fungi is another rich resource of terpenes, mostly existing in the format of VOCs (Figure 2). Two producers *Ascomycota* and *Basidiomycota* usually produce sesquiterpenes in late growth phase. Many species of the phylum *Ascomycota* have been reported to produce volatile sesquiterpenes (Kramer and Abraham, 2011). Caryophyllene was emitted from *Phialophora fastigata*, and α-curcumene was biosynthesized by *Penicillium commune* and *Paecilomyces variotii* (Sunesson et al. , 1995). Six TPSs and fifteen sesquiterpenes were respectively characterized and identified in basidiomycete *Coprinus cinereus* (Agger et al. , 2009). Genus *Penicillium*, belonging to phylum *Ascomycota*, is a kind of rich sesquiterpene producer: germacrene A and germacrene B were respectively produced by *P. cyclopium* and *P. expansum*, and β-elemene and β-guaiene were synthesized by *P. clavigerum* (Larsen, 1998). *P. roqueforti* could emit multiply sesquiterpenes β-patchoulene, β-elemene, β-elemene-isomer, diepi-a-cedrene, β-gurjunene, β-patchoulene-isomer, aristolochene, valencene, a-selinene, β-himachalene, α-chamigrene, β-bisabolene, and α-panasinsene (Jeleń, 2002). The fungal phylum Basidiomycota produces multiply sesquiterpenes via transforming and rearranging humulene (Abraham, 2001).

Many terpenes from plants and fungi have been described, but relatively fewer microbial sesquiterpenes and TPSs have been functionally summarized. As we summarized here, prokaryote producers of sesquiterpene are mainly identified belonging to Actinomyces, marine bacteria and Cyanobacteria (Figure 2). *Streptomyces* is a rich source of bioactive secondary metabolites, such as antiparasitic avermectins and sesquiterpene-derived antibiotic pentalenolactone (Chou et al. , 2010, Omura et al. , 2001, Quaderer et al. , 2006).

Sesquiterpenoid antibiotic pentalenolactone is a commonly occurring metabolite, isolated from more than 30 species of *Streptomyces* (Koe et al. , 1957, Takahashi et al. , 1983). Bioinformatics analysis of *Streptomyces* and *Kitasatospora* genomes identified 20 terpenes, including sesquiterpene β-elemene, α-muurolene, cadinadiene, epi-isozizaene, germacrene A and pentalenene (Yamada et al. , 2015a, Yamada et al. , 2015b). Many marine bacteria are found to produce many sesquiterpenes with great medical values. Four sesquiterpenes—euplotin A, euplotin B, euplotin C and their biogenetic precursor preuplotin—have been isolated from a eukaryotic unicellular marine ciliate *Euplotes crassus* (Dini et al. , 1993). Sesquiterpenes exclusively produced by marine bacteria ciliate protozoon *E. crassus* have the bioactivity of inhibiting growth and killing protozoa *L. major* and *L. infantum* in vitro (Savoia et al. , 2004).

In addition, insects were reported to synthesize sesquiterpenes (Finefield et al., 2012, Naya et al. , 1978). Some investigation suggests that insects possess a specific set of C-C cyclases for sesquiterpenoids. Volatile oil from the secretion of a scale insect *Ceroplastes ceriferus*, is consisting of numbers of sesquiterpenes: germacrene-D, β-bourbonene, α-copaene, β-bourbonene and β-elemene (Naya et al. , 1978). The presence of some cedrene-type sesquiterpenoids was reported in scale insect *Laeeifer lacea Kerr* (Singh et al. , 1974).

Figure 2

3.2 Sesquiterpene synthases

In nature, terpene synthases and their carbocationic reaction mechanisms are responsible for huge varieties of terpene structures and biosynthesis, and they have garnered attentions of chemists and biologists for many years. Starting from farnesyl diphosphate (FPP), sesquiterpene skeleton is cyclized by different TPSs (Benedict et al. , 2001, Cane, 1990). From analysis of TPSs, DDXXD motifs were found to serve as a crucial site for divalent action and substrate binding (Marrero et al. , 1992, Prosser et al. , 2004). Many TPSs, such as farnesene

248 synthase from *C. junos*, δ-selinene synthase and λ-humulene synthase, were discovered through
249 searching the motifs (Little and Croteau, 2002, Maruyama et al. , 2001).
250 Large amount of natural TPSs are extracted from plants. A TPS forming carbocyclic
251 skeleton of δ-guaiene was isolated from Aquilaria cells (Kumeta and Ito, 2010). α-Farnesene
252 synthase cDNA from peel tissue of apple fruit was cloned and characterized in *E. coli* (Pechous
253 and Whitaker, 2004). Germacrene A synthase from *Lactuca sativa L.* was expressed in
254 engineered *E. coli*, and β-elemene was extracted by thermally rearrangement (Bennett et al. ,
255 2002, Kraker et al. , 1998). The caryophyllene synthase was characterized from *Artemisia*
256 *annua* (Cai et al. , 2002) and *Mikania micrantha* (Wang et al. , 2009). Multiple TPSs are often
257 expressed in different organs of one organism, and several sesquiterpenes are synthesized
258 through one TPS catalysis. Therefore, several sesquiterpenes are often produced by one
259 organism or one TPS catalysis of substrate FPP. For example, β-humulene synthase and δ-
260 selinene synthase are expressed in *Abies grandis*, each catalyzing at least 52 and 36
261 sesquiterpenes, respectively (Steele et al. , 1998). Chamomile, with great pharmaceutical
262 values and economic essential oil, was excavated five TPSs, four of which MrTPS1, MrTPS3,
263 MrTPS4 and MrTPS5 were exclusively expressed in above-ground organs and respectively
264 catalyzed (-)-(E)-β-caryophyllene, (+)-germacrene A, (E)-β-ocimene and (-)-germacrene D.
265 Enzyme MrTPS2, mainly expressed in roots, catalyzed several Asteraceae-specific
266 sesquiterpenes: (-)-(E)-β-caryophyllene, α-humulene, silphinene, modeph-2-ene, β-isocomene
267 and the major product α-isocomene (Irmisch et al. , 2012). Based on transcriptome sequencing
268 of *Santalum album*, five TPSs were characterized, including two sesquisabinene synthases
269 (SaSQS1, SaSQS2), bisabolene synthase (SaBS), santalene synthase (SaSS) and farnesyl
270 diphosphate synthase (SaFDS) (Srivastava et al. , 2015). In grand fir, δ-selinene synthase and
271 λ-humulene synthase each enzyme synthesizes three major products respectively, and a third
272 TPS catalyze to form only (E)-α-bisabolene (Bohlmann et al. , 1998, Steele et al. , 1998). Six

TPSs from *C. cinereus* were described: Cop1 and Cop2 each synthesizes germacrene A as their major product; Cop3 was identified as an α-muurolene synthase but producing β-elemene, δ-muurolene, germacrene D, and δ-cadinene; Cop4 synthesizes δ-cadinene as its major product; Cop5 has not been characterized any compounds; Cop6 catalyzes specific α-cuprenene (Agger et al. , 2009). Two TPSs were identified to synthesize a-farnesene and germacrene D from kiwifruit flowers (Nieuwenhuizen et al. , 2009). In root of *V. officinalis*, two TPSs VoTPS1 and VoTPS2 were identified to produce germacrene C/germacrene D and valerenadiene, respectively (Pyle et al. , 2012). In flowers of grapevine *Vitis vinifera L.*, 69 putative TPSs were discovered through probe cross-hybridizing, and numbers of sesquiterpenes were identified in heterologous yeast and the plant flowers (Smit et al. , 2019).

Recent years, the availability of genome sequences led to the discoveries of some TPSs from actinomycetes and cyanobacteria (Agger et al. , 2008, Cane and Watt, 2003, Giglio et al. , 2008, Gust et al. , 2003, Komatsu et al. , 2008, Lin et al. , 2006a, Zhao et al. , 2008). Based on bioinformatics analysis, a biosynthetic protein (AncCL1) was reconstructed and identified to catalyze major product epi-zizaene and minor product epi-isozizaene (Guzzetti et al. , 2016). In *Streptomyces coelicolor*, epi-isozizaene synthase (EIZS) catalyzes cyclization of FPP to a novel tricyclic epi-isozizaene (Lin et al. , 2006b). Pentalenene synthase (PentS) from *Streptomyces* UC5319 catalyzes to pentalenene (Cane et al. , 1994), and a mixture of pentalenene and caryophyllene is synthesized by site-directed mutagenesis PentS N219D (Seemann et al. , 2002). Similarly, the types or the product distribution of sesquiterpenes could be changed when mutating TPSs, such as MrTPS2 (Irmisch et al. , 2012), bisabolene synthase (*BIS*) (Kirby et al. , 2014a) and amorphadiene synthase (*ADS*) (Li et al. , 2016b).

Figure 3

4. Metabolic engineering strategies on sesquiterpene production in microorganisms

To explore the potential interest of sesquiterpenes, fermentation-based production process is used to produce diverse sesquiterpenes through engineering metabolic pathways to efficiently drive conversion of the renewable resources. Some industrial model microorganisms are metabolically engineered from isoprenoid pathways, TPSs and other relative metabolic networks for sesquiterpene production. Herein, we describe the general strategies on host and pathway engineering for sesquiterpene production in engineered microorganisms.

4.1. Metabolic engineering strategies on sesquiterpene production in *E. coli*

4.1.1. Metabolic regulation on MEP pathway for sesquiterpene production in *E. coli*

E. coli employing the MEP pathway to accumulate FPP is a widely used host for sesquiterpene production. Metabolic engineering and strategies for sesquiterpene production in *E. coli* are summarized in Table 1 and Figure 4. Via the *E. coli* native MEP pathway, sesquiterpene production is generally considerable low when only over-expressing TPSs. For example, *E. coli* BL21 (DE3) harboring only gene farnesene synthase produced 0.325 mg/L α-farnesene (Zhu et al. , 2014). When codon-optimized gene *coEIZS*, *coPentS* and *coMrTPS2* were simply over-expressed in *E. coli* DH1, the titers of tricycle sesquiterpene epi-isozizaene, pentalenene and α-isocomene were 0.54 mg/L, 0.19 mg/L and 0.01 mg/L, respectively (Liu et al. , 2018). In *E. coli* BL21 (DE3), over-expressing wildtype *valerenadiene synthase* (*wvds*) gene rendered 12 μg/L valerenadiene (Nybo et al. , 2017).

For metabolic regulation on the MEP pathway, over-expression of rate-limiting genes and TPSs is the most popular strategy to increase sesquiterpene production. Upon co-expressing gene *Dxs*, *isopentenyl-diphosphate isomerase* (*Idi*) and *ispA* in SOE4 operon, amorphadiene titer increased 3.6-fold comparing to that achieved by only *ADS* gene expression strain (Martin et al. , 2003). Medium optimization and over-expression of gene *Dxs* from *Streptomyces avermitilis*, *Idi* from *Bacillus subtilis*, *ispDF* and *ADS* increased amorphadiene titer to 331.70

mg/L, 15.5-fold of the initial titer in *E. coli* MG1655 (Wang et al. , 2015). The aim of over-expressing and deleting genes is to enrich the vital MEP pathway precursors. To enrich precursor phosphoenolpyruvate and pyruvate, carbohydrate phosphotransferase system (PTS) genes were deleted, resulting in amorphadiene titer increased to 182 mg/L in OPT2 medium (Zhang et al. , 2013, Zhang et al. , 2015). To enrich the vital precursor DXP, a novel route of pentose phosphate to it annotated as putative xylose reductase gene *yajO* and *ribB* mutant was screened. Through fusing *ribB* mutant to *Dxr* gene and over-expressing *ispA*, *ispDF* and *Idi* gene, bisabolene titer increased above 4-fold, about 8.8 mg/g DCW (dry cell weight) (Kirby et al. , 2015). Engineering the MEP pathway by cross-lapping in vitro assembly (CLIVA) method and expressing iron sulfur (Fe–S) cluster operon increased amorphadiene titer to ~300 mg/L (Zou et al. , 2013). Additionally, to increase sesquiterpene production, efflux transporter engineering was carried out: Over-expressing the screened outer membrane protein tolC, together with ABC family transporters (macAB) or MFS family transporters (emrAB or emrKY) increased amorphadiene titer by more than 3-fold, up to 180 mg/L (Zhang et al. , 2016); Over-expressing two copies of *TolC* and *AcrB* improved amorphadiene yield 118% with 404.83 mg/L (Wang et al. , 2013). For valerenadiene titer, besides over-expressing gene *Dxs*, *Idi*, FPP synthase (*FFPS*) and *cvds*, supplying glycerol into fermentation medium increased it to 11.0 mg/L (Nybo et al. , 2017).

4.1.2. Metabolic regulation on MVA pathway in *E. coli*

The low sesquiterpene production may be resulted from the insufficient FPP supplementary via native MEP pathway which is tightly controlled by endogenous regulation (Phulara et al. , 2016). The heterogenous MVA pathway was introduced to *E. coli* for dramatically improving flux to FPP and terpene production (Martin, Pitera, 2003). In *E. coli* DYM1, the synthetic *ADS* gene from *A. annua* and MVA pathway from *S. cerevisiae* were expressed, achieving approximately 112.2 mg/L of amorphadiene (see Table 1) from LB + 0.8%

glycerol culture (Martin, Pitera, 2003). Afterwards, employing a two-phase partitioning bioreactor with dodecane led this engineered strain to produce 0.5 g/L amorphadiene (Newman et al. , 2006). Although introduction of the MVA pathway dramatically increased amorphadiene production, bottlenecks limiting the yield still exist. To investigate them, balancing the heterologous MVA pathway flux was proposed. All the actions of supplementing MVA into medium, increasing copies of mevalonate kinase gene (*mvk*) or plasmid with *ADS* gene and global sensitivity analysis with an equation model for the MVA pathway using kinetic parameters identified the rate-limiting genes *HMGR*, *mvk* and *ADS* (Anthony et al. , 2009, Pitera et al. , 2007, Weaver et al. , 2015). Gene titration studies and metabolomics using liquid chromatography–mass spectrometry (LC-MS) excavated a bottleneck - insufficient HMGR leading to HMG-CoA accumulation, being toxic to cells by inhibiting fatty acid biosynthesis and generalizing membrane stress (Kizer et al. , 2008, Pitera et al. , 2007). Sequentially, three strategies were carried out: upregulating *tHMGR* gene, over-expressing *HMGS* and *HMGR* gene from *Staphylococcus aureus* (Tsuruta et al. , 2009), and cofactor engineering using NADH-dependent *HMGR* variants from *Delftia acidovorans* (Ma et al. , 2011). Then, a commercially relevant amorphadiene titer of 27.4 g/L was achieved through a superior fed-batch fermentation process of restricted glucose and nitrogen (Tsuruta et al. , 2009). By supplying more ATP, polyphosphate kinase (PPK)-catalyzed synthesis increased amorphadiene titer 2.5-fold (Shimane et al. , 2012). To characterize strain variation in engineered *E. coli* for increasing the productivity, a multi-omics-based workflow was developed (Brunk et al. , 2016). Especially, targeted proteomics helped to identify *mvk* and *phosphomevalonate kinase* (*pmk*) as potential bottleneck genes in amorphadiene production. Based on this, codon-optimizing *mvk* and *pmk* gene, and replacing P_{lac} with a stronger P_{trc} were conducted, improving amorphadiene titer 3-fold (>500 mg/L) (Redding-Johanson, Batth, 2011). Assisted by metabolomics and targeted proteomics, metabolic pathway optimization with RBS

variants and combinatorial gene assembly reduced toxic intermediate accumulation and increased amorphadiene production about 5-fold (Nowroozi et al. , 2014). Accumulation of toxic intermediates (HMG-CoA, IPP/DMAPP and FPP) of the MVA pathway resulted in cell growth issues. Through toxicity stress and whole-genome transcriptional analysis identifying FPP-responding promoter P_{rstA} and P_{gadE}, dynamic control of the MVA pathway was established, and it doubled amorphadiene titer to 1.4 g/L (Dahl et al. , 2013). Afterwards, this dynamic control strategy was used to produce other sesquiterpenes: dynamic control of the MVA pathway expression and comparative proteome analysis made engineered *E. coli* BW25113 to produce 722.46 mg/L zeaxanthin (Shen et al. , 2016); Together promoter engineering with this dynamic control or inducible control of the MVA pathway, 727.9 mg/L epi-isozizaene, 780.3 mg/L pentalenene and 77.5 mg/L α-isocomene were produced when gene *coEIZS*, *coPentS* and *coMrTPS2* were over-expressed in *E. coli* DH1, respectively (Liu et al. , 2018).

α-Farnesene and β-farnesene are isomers, differing by the location of one double-bond. The commercially available farnesene used as perfume and fuel is a mixture of the isomers. Expressing a codon-optimized farnesene synthase gene made *E. coli* to produce 1.2 mg/L α-farnesene. Augmenting the metabolic flux for FPP synthesis through expressing the MVA pathway and over-expressing *Dxs*, *Idi*, and *ispA* gene conferred a 48-fold improvement. Together with protein fusion of FPPS and farnesene synthase, 380.0 mg/L of α-farnesene was produced (Wang et al. , 2011a). Through engineering the heterologous MVA pathway, 8.74 g/L β-farnesene was produced in a lab bioreactor (You et al. , 2017). Although up-regulating bottleneck genes and down-regulating competitive pathway improved sesquiterpene production, the ratios of enzymes in the reconstructed pathway are hardly precisely evaluated. Therefore, targeted engineering analysis was employed: the MVA pathway was reconstructed *in vitro* to produce farnesene using purified enzymes, and the molar ratio of

AtoB:ERG13:tHMG1:ERG12:ERG8:MVD1:Idi:ispA:AFS was slightly adjusted to 1:10:2:5:5:2:5:2:2. Based on this information, a rationally designed strain was constructed *in vivo* for a high titer of farnesene, achieving 1.1 g/L after 96 h post induction in *E. coli* BL21(DE3) (Zhu, Zhong, 2014). The *in vitro* biosynthesis of sesquiterpenes starting from acetic acid using the MVA pathway was also investigated: a ten-step cascade plus added cofactor regeneration systems led conversion of farnesyl pyrophosphate >40% and conversion of sesquiterpenes >90% (Dirkmann et al. , 2018).

Bisabolene was identified as a biosynthetic precursor of a novel alternative to D2 diesel fuel. *E. coli* expressing the MVA pathway from *S. cerevisiae* and plant-derived bisabolene synthase produced 388 mg/L bisabolene. Codon-optimization of *tHMGR*, *HMGS*, *mvk* and *pmk* genes, and insertion of a P_{trc} in front of *mvk* gene increased bisabolene titer to 912 mg/L (Peralta-Yahya et al. , 2011). Later, this *E. coli* variant was re-engineered to produce 363 μg/L cedrol under shaking flask culture conditions for 72 h (Luo et al. , 2019). To use the industrial biomass pretreated by ionic liquid (IL) for biofuel production, *E. coli* containing plasmid with bisabolene pathway genes was engineered to be resist tolerant to the IL with an inner membrane transporter regulated by an IL-inducible repressor (Ruegg et al. , 2014). Sequentially, principal component analysis of proteomics (PCAP) was proposed to increase bisabolene titer to 1,150 mg/L in strain CB with an extra copy of *BIS* (Alonso-Gutierrez et al. , 2015). These above MVA pathway genes and sucrose utilization genes were also integrated into chromosome to address issues associated with *E. coli* as an industrial platform for isoprenoids and growth ability on sucrose. Although a CRISPR-Cas9 system for replacing promoter sequences increased the bisabolene titer 5-fold, low titer of ~435 μg/L was finally resulted probably due to lower enzymes levels from chromosomally integrated pathway than the strains using plasmids to express pathway proteins (Alonso-Gutierrez et al. , 2018). Similar with the dynamic control, quorum sensing (QS) system controlling gene expression in response to

population density, was applicated to establish an inducer-free system of bisabolene production. The LuxI/R system from *Vibrio fischeri* was employed to construct sensor and response plasmids and variants, of which a QS chromosome-integrated strain produced 1.1 g/L bisabolene without inducers added, increasing 44% comparing to the previous inducible system (Kim et al. , 2017). In addition, simulation including of molecular simulation to study the membrane permeability of terpenoids (Vermaas et al. , 2018), CFD and kinetic-based modeling to optimize the sparger design of a large-scale photobioreactor (Ali et al. , 2019) were also employed to improve bisabolene production.

β-Caryophyllene is a bio-based product with economic potentials and a promising precursor candidate for petroleum-based products. Glucose-6-phosphate dehydrogenase (*G6PH*) for increasing NADPH, geranyl diphosphate synthase (*GPPS2*) and β-caryophyllene synthase (*QHS1*) genes, were co-overexpressed with the MVA pathway in engineered *E. coli* YJM59, producing 1,520 mg/L β-caryophyllene in fed-batch fermentation (Yang et al. , 2016a). To convert acetic acid to β-caryophyllene, genes *QHS1*, *GPPS2*, acetyl-CoA synthase (*ACS*) and acetoacetyl-CoA synthase (*AACS*) were co-expressed along with the heterologous MVA pathway in *E. coli* YJM67 and produced up to 1.05 g/L of β-caryophyllene accumulated after 72 h (Yang and Nie, 2016). By expressing the MVA pathway and gene *QHS1* in *E. coli*, 406 mg/L terpene mix enriched in 100 mg/L caryophyllene was produced (Wu et al. , 2018).

For α-humulene production, the MVA pathway genes from *Streptomyces* (from *HMGS* to *Idi2*) and yeast (*Idi1*), and rat gene acetoacetate-CoA ligase (*Aacl*) and α-humulene synthase of shampoo ginger were co-expressed, and the resultant *E. coli* strain produced 958 mg/L α-humulene when supplemented with a lithium acetoacetate (LAA), a 13.6-fold increase compared with strain expressing only humulene synthase (Harada et al. , 2009). These resulted from that exogenous LAA is efficiently converted into acetoacetyl-CoA by mammalian *Aacl* and used as a dominant precursor for isoprenoid biosynthesis. Strategy of a probable

mechanism of adaptive evolution was used to engineer *tHMGR* and terpene synthases (λ-humulene synthase, bisabolene synthase, sibirene synthase, longifolene synthase, and a-longipinene synthase) in *E. coli*, and thereby eliminate the bottleneck from inefficient enzymes, increasing the productivity by ~1,000 fold (Yoshikuni et al. , 2008). Protoilludene was successfully produced by overexpressing a hybrid exogenous MVA pathway, endogenous ispA, and protoilludene synthase of *Omphalotus olearius*, and was improved to 1199 mg/L by altering promoters and copy numbers (Yang et al. , 2016b). Additionally, 62.0 mg/L valerenadiene was produced via expressing the MVA pathway and *cvds* gene respectively promoted by P_{trc} and P_{T7} (Nybo et al. , 2017). Co-expression of condon-optimized longifolene synthase from *Picea abies*, *FPPS*, and the MVA pathway genes in *E. coli* BL21 (DE3) rendered 382 mg/L longifolene in a 5-L bioreactor (Cao et al. , 2019).

Figure 4

Table 1

4.2. Metabolic engineering strategies for sesquiterpene production in *S. cerevisiae*

S. cerevisiae harboring the MVA pathway is one of the most favorite microorganisms for industrial production of isoprenoids. The low productivity limits commodity scale production of sesquiterpene. The metabolic engineering strategies, synthetic biology technologies and advanced tools (summarized in Figure 5 and Table 2) have exhibited potential to convert *S. cerevisiae* to a high sesquiterpene-producing host.

In *S. cerevisiae*, up-regulating the upstream MVA pathway for increasing a precursor FPP synthesis is the most common strategy to increase sesquiterpene production. Over-expressing *tHMGR* gene increased amorphadiene titer approximately 5-fold, and down-regulating *ERG9* encoding squalene synthase using a methionine repressible promoter (P_{MET3}) increased it an additional 2-fold by increasing FPP pool (Paradise et al. , 2008, Ro et al. , 2006). In *S. cerevisiae*, a global transcription factor *UPC2* was mutated to regulate biosynthesis of sterols,

an additional copy of *tHMGR* was integrated into chromosome, and *ERG20* encoding FPP synthase was over-expressed, generating FPP-biosynthesis platform EPY224 which produced 153 mg/L amorphadiene (Ro et al. , 2006). Over-expressing heterologous ADS, replacing promoter by P_{MET3} for repressing ERG9 gene and enzyme fusion made amorphadiene titer to 25.02 mg/L in *S. cerevisiae* MTCC 3157 (Baadhe et al. , 2013). P_{CTR3} repressed by cheap $CuSO_4$ was slight better to suppress *ERG9* expression to reduce FPP flux to squalene than P_{MET3} repressed by methionine (Paddon et al. , 2013, Peng et al. , 2017). To achieve high sesquiterpene, fermentation process needs to be optimized via such as response surface methodology (Baadhe et al. , 2014) and feeding various carbon sources. Feedback-controlled ethanol pulse feed has several significant advantages over restricted glucose feed. This strategy is successfully applied to amorphadiene with titer of 41 g/L and artemisinic acid production in *S. cerevisiae* CEN.PK2 Y293 (Paddon et al. , 2013, Westfall et al. , 2012). With development of genomics and relevant technologies, comprehensive metabolic models were developed. Model prediction via network models has been applied to sesquiterpene production: OptGene (Burgard et al. , 2003, Patil et al. , 2005), as an extension modeling framework of OptKnock, was subjected to minimization of metabolic adjustment (MOMA) method to identify *GDH1* coding NADPH-dependent glutamate dehydrogenase as the best knockout target. Then, through over-expressing *tHMG1* and replacing NADH-dependent *GDH2* with *GDH1* gene, sesquiterpene titer increased to 30.1 mg/L from 11.3 mg/L (Asadollahi et al. , 2009). Flux balance analysis (FBA) and MOMA methods were used for predicting knockout targets, resulting in 54. 55 mg/L amorphadiene in the highest strain *hxk2D:: his3::ADS* (Sun et al. , 2014). Assisted by 3D model of ADS protein, mutability landscape guided enzyme engineering was performed to improve the catalytic activity, increasing catalytic efficiency ~ 4-fold in mutant H448A and k_{cat} by 5-fold in mutant T399S/H448A (Abdallah et al. , 2018).

Based on FPP-biosynthesis platform EPY224, many studies were carried out: through replacing *ADS* gene with *BIS* gene, more than 900 mg/L bisabolene was produced (Peralta-Yahya et al. , 2011). Then, this EPY224-derived strain was applied to evolving BIS, enriching a mixed population and removing little bisabolene producing strains in presence of selective pressure nonionic surfactants Tween 20 (T20), for the protection of bisabolene against the disruptive action of T20 (Kirby et al. , 2014a). Recently, *S. cerevisiae* EPY224 was re-engineered to produce 344 mg/L pentalenene by replacing gene *BIS* with gene *coPentS* (Liu et al. , 2018). In terpene production, screening color distribution of strains harboring carotenoid-producing pathway is a tool of screening the targeted mutants (Furubayashi et al. , 2014, Wadhwa and Bachhawat, 2019, Yuan and Ching, 2014) and identifying gene deletions contributing to improving isoprenoid production (Ozaydin et al. , 2013). Combination of the deletions and MVA pathway modifications increased bisabolene titer to 5.2 g/L in a fermentation process (Ozaydin et al. , 2013).

In yeast, *FS* gene from *Malus domestica* led to higher α-farnesene titer (4 mg/L) than plant-origins of *C. junos* and *A. annua*. Gene *FS* and *tHMG1* were over-expressed in a high-copy plasmid in *S. cerevisiae* CEN.PK113-5D, which produced 170 mg/L farnesene in fed-batch fermentations with a respiratory quotient (RQ)-controlled exponential feeding (Tippmann et al. , 2016). After *FS* gene was replaced by santalene synthase from *Clausena lansium*, this engineered *S. cerevisiae* produced 163 mg/L santalene under RQ-controlled exponential feeding (Tippmann et al. , 2016). Before this, dynamic control of gene expression including using P_{HXT1} promoter to control *ERG9* expression, overexpression of gene *tHMGR* and deletion of gene *LPP1* and *DPP1* led strain to produce 92 mg/L α-santalene by diverting the carbon flux FPP towards α-santalene (Scalcinati et al. , 2012a). In yeast, protein fusion strategy could also make multi-enzyme complexes for increasing metabolic flux and compound production. Comparing with that observed in a non-fused control, over-expression of Tya-

fused (Tya: Ty1 retrotransposon element) enzymes tHmg1, ispA and FS increased farnesene production 4-fold, yielding 930 mg/L after 7 days in *S. cerevisiae* ATCC200589 (Han et al. , 2018). Specifically, scaffold of FFPS and FS protein through recognizing affibodies to their anti-idiotypic partners, the farnesene yield on glucose was improved by 135% (Tippmann et al. , 2017a). Sequentially, acetoacetyl-CoA synthase (*nphT7*) identified to catalyze the irreversible condensation of malonyl-CoA and acetyl-CoA to acetoacetyl-CoA from *Streptomyces sp.* CL190, was expressed to increase flux, however, it was detrimental for growth and farnesene production (Tippmann et al. , 2017b). Many important insights into microbial metabolism have been achieved using metabolic nodes to disturb metabolic flux and efficiency. Pantothenate (vitamin B5) was utilized as a "metabolic switch" for tuning synthesis rates of molecules relying on CoA intermediates β-farnesene (Sandoval et al. , 2014). To overcome the metabolic inefficiencies including a low ATP/oxygen ratio and challenges of commodity-scale production of anabolic molecules in *S. cerevisiae*, gene aldehyde dehydrogenase acylating (*ADA*), xylulose-5-phosphate specific phosphoketolase (*xPK*), phosphotransacetylase (*PTA*) and NADH-*HMGR* were over-expressed, and DL-glycerol-3-phosphatase (*Rhr2*) was deleted. They rewired the central carbon metabolism, reduced ATP requirement and carbon loss, and improved pathway redox balance of cytosolic acetyl-CoA biosynthesis, leading to farnesene titer exceeded 130 g/L using ~25% less glucose and ~75% less O_2 than previous strains in a fed-batch fermentation (Meadows et al. , 2016, Yu and Chang, 2016). Additionally, evolutionary engineering discovered that truncated tTcb3p protein could increase tolerance of replacement jet fuels blend AMJ-700t (10% cymene, 50% limonene, 40% farnesene) in *S. cerevisiae* (Brennan et al. , 2015).

Compared with *S. cerevisiae* BY4741, industrial *S. cerevisiae* EG60 showed more advantages on cineole and sesquiterpene production levels (Ignea et al. , 2011). In *S. cerevisiae* EG60, through over-expressing gene *IDI1*, *HMG2* (K6R) variant, *ERG20* and *SF* homologue

P330 from *Salvia pomifera*, and deleting a single copy of *ERG9* gene, the titers of *trans*-β-caryophyllene, α-cubebene, α-copaene and δ-cadinene were respectively ~10 mg/L, ~7 mg/L, ~12 mg/L and ~38 mg/L (Ignea et al. , 2011). This strain was further engineered to produce 125 mg/L caryophyllene through replacing *Salvia fruticosa* caryophyllene synthase Sf126 with *SF*, as well as deleting the ubiquitin ligases ubc7 and ssm4/doa10 and endoplasmic reticulum associated degradation resident protein pho86 (Ignea et al. , 2012). In *S. cerevisiae*, over-expressing gene *tHMG1* and down-regulating gene *ERG9* are two widely-used strategies, and the effect on sesquiterpene biosynthesis was specially studied (Asadollahi et al. , 2008, Asadollahi et al. , 2010). *S. cerevisiae* YZL141 was metabolically engineered to be a sesquiterpene production platform by over-expressing gene *tHMG1*, *ERG20* and *FgJ03939*. Based on this platform, sesquiterpene cyclase and skeletons of fusariumdiene and fusagramineol from *Fusarium graminearum* were characterized, identifying 8 compounds - nerolidol, (−)-α-acorenol, (E)-β-farnesene, (+)-α-bisabolol, (−)-acoradiene, fusariumdiene, epi-fusagramineol and fusagramineol (Bian et al. , 2018). Mutation of *ERG9* gene and uptake of exogenous ergosterol (sue) made *S. cerevisiae* WAT11 to produce about 90 mg/L 5-epi-aristolochene, 90 mg/L premnaspirodiene and 20 mg/L valencene when respectively introducing gene *TEAS* (tobacco epi-aristolochene synthase), *HPS* (*Hyoscyamus muticus* premnaspirodiene synthase) and *CVS* (citrus valencene synthase) (Takahashi et al. , 2007). Thereafter, applying this strategy in *S. cerevisiae* BY4741, co-expressing *HPS* gene with an amino-terminal truncated *tHMGR* gene made it to produce 170.23 mg/L premnaspirodiene (Zhuang and Chappell, 2015). Protein fusion engineering is an efficient strategy in sesquiterpene production both in *E. coli* and yeast. Besides farnesene, it was also applied to germacrene A production increasing 6-fold to 190.7 mg/L by over-expressing *tHMGR* gene and fusing FPPS and germacrene A synthase (Hu et al. , 2017), and hirsutene and Δ6-protoilludene production by fusion of hirsutene synthase (*HS*) from *Stereum hirsutum* to an

HMGS domain (Flynn and Schmidt-Dannert, 2018). Optimizing FPP branch point, modulating MVA pathway, modifying ammonium assimilation pathway and enhancing the transcriptional activator activity as well as a specifically developed continuous fermentation process were combined to produce 0.036 mg/g DCW of α-santalene (Scalcinati et al. , 2012b). Engineering protein degradation of FPPS protein is an effective regulatory strategy to regulate monoterpene and sesquiterpene production in *S. cerevisiae* (Peng et al. , 2018).

Figure 5

Table 2

4.3. Metabolic engineering strategies for sesquiterpene production in other microorganisms

Besides *E. coli* and *S. cerevisiae*, other microorganisms are also engineered to produce sesquiterpenes, such as *Corynebacterium glutamicum*, *B. Subtilis*, *Lactococcus lactis*, cyanobacterium and some eucaryons. The metabolic engineering and strategies for sesquiterpene production in these microorganisms are summarized in Table 3. In *C. glutamicum*, due to the low provision of FPP, heterologous expression of *CVS* gene from *Citrus sinensis* could not enable (+)-valencene production. Upon deleting two endogenous prenyltransferase genes and expressing either gene *ispA* from *E. coli* or gene *ERG20* from *S. cerevisiae*, (+)-valencene production was observed. Employing *CVS* gene from *Nootka cypress* further improved (+)-valencene titer to 2.41 mg/L, increasing 10-fold comparing to *VS* gene from *C. sinensis* (Frohwitter et al. , 2014).

B. subtilis and *Lactococcus lactis* are two Gram-positive prokaryotes reported to be engineered for sesquiterpene production. *B. subtilis* is a promising microbial host for chemical production purpose because of its fast growth rate and generally regarded as safe status. In *B. subtilis*, protein translation engineering was conducted by fusing the highly positive charged six-arginine tag to *ADS* to increase protein expression. Coupled with expressing gene *Dxs* and *Idi* under P_{xylA} and systematic media optimization, ~20 mg/L amorphadiene was produced in

596 shake flask scale (Zhou et al. , 2013). *L. lactis*, harboring MVA pathway, is also an attractive
597 host for characterizing sesquiterpene production: TPS *VMPSTS* from *Vanda Mimi Palmer* was
598 characterized multiple sesquiterpenes with the dominating germacrene D (Song et al. , 2012).
599 Comparing with the β-sesquiphellandrene titer when only expressing β-sesquiphellandrene
600 synthase, co-expression of *mvk* and *mvaA* gene doubled the amount to 15.5 mg/L (Song et al. ,
601 2014). An industrial bacterium *Rhodobacter sphaeroides* engineered by co-expressing MVA
602 operon from *Paracoccus zeaxanthinifaciens* and *CVS* from *Callitropsis nootkatensis* produced
603 352 mg/L valencene in shaker flasks, 14-fold higher than that of only *CVS* expressing in *S.*
604 *cerevisiae* (Beekwilder et al. , 2014).
605 Cyanobacterium, as a photosynthetic prokaryote, was engineered to convert CO_2 and H_2O
606 directly into high-valued biofuel sesquiterpenes (Lin and Pakrasi, 2019). Filamentous
607 cyanobacterium *Anabaena sp.* PCC 7120 produced a farnesene photosynthetic productivity of
608 69.1 $\mu g \cdot L^{-1} \cdot OD^{-1} \cdot d^{-1}$ through its endogenous MEP pathway and *coFS* gene using CO_2,
609 mineralized water and light (Halfmann et al. , 2014). Unicellular cyanobacterium
610 *Synechococcus elongatus* PCC 7942 produced 4.6 mg/L farnesene or 19.8 mg/L amorphadiene
611 when over-expressing MEP pathway gene *Dxs*, *Idi* and *ispA*, with *FS* gene from apple or *ADS*
612 gene from *A. annua* with a dodecane overlay in 7 days (Choi et al. , 2016, Lee et al. , 2017).
613 With *QHS1* gene from *A. annua* integrated into cyanobacterium *Synechocystis sp.* PCC6803
614 genome, ~ 4 mg $gDCW^{-1}$ β-caryophyllene was measured by a methanol: chloroform extraction
615 method (Reinsvold et al. , 2011). *Synechococcus sp.* PCC 7002 engineered by expressing *A.*
616 *grandis BIS* gene and deleting *glgC* gene produced more than 0.6 mg/L bisabolene with a
617 dodecane overlay (Davies et al. , 2014). Recently, through genetic engineering, serial enzyme
618 loading and artificial micro RNA-based repression of competing pathways, green microalga
619 *Chlamydomonas reinhardtii* produced 10.3 mg bisabolene$\cdot g^{-1}$ CDW, yielding up to 11.0 mg/L
620 by mixotrophic cultivation in 7 days (Wichmann et al. , 2018).

For eukaryote microorganism, *Yarrowia lipolytica*, *Rhodosporidium toruloides*, *Streptomyces venezuelae* and *Xanthophyllomyces dendrorhous* are reported to produce sesquiterpenes for their simple industrial scale-up operations. *Y. lipolytica* Po1h, with *tHMG1*, *Idi*, *ERG20* and *coFS* gene integrated into the genome, produced 57.08 mg/L of *α*-farnesene, a 20.8-fold increase over the initial titer in shake flasks with YPD medium. Bioreactor scale-up in PM medium led *α*-farnesene concentration up to 259.98 mg/L (Yang et al. , 2016c). The carotenogenic yeast *R. toruloides* was reported to utilize lignocellulose hydrolysate to produce bisabolene and amorphadiene. *R. toruloides* showed superior growth in corn stover hydrolysates prepared by two pretreatment methods: biocompatible IL choline α-ketoglutarate resulting in 261 mg/L bisabolene at bench scale; and an alkaline resulting in 680 mg/L bisabolene in a high-gravity fed-batch bioreactor (Yaegashi et al. , 2017). In *S. venezuelae*, 10.52 mg/L bisabolene was produced after deleting FPP and GPP consumption gene *SVEN_0269* and *SVEN_7111*, and inserting P_{SV}-coAgBis and P_{ermEp}-FPPS1 at the aatP chromosomal site (Phelan et al. , 2015). Comparing α-cuprenene production in *E. coli*, *S. cerevisiae* and *X. dendrorhous* expressing *Cop6* gene from *C. cinereus*, α-cuprenene titer in *X. dendrorhous* reached to 80 mg/L, far higher than the other mutants (Melillo et al. , 2013).

Table 3

5. Conclusions and perspective

Biosynthesizing sesquiterpene by engineered microorganisms is a worthy-studied theme for their widely promising applications and values. More and more sesquiterpenes and their synthases are discovered from all kinds of organisms. Although great values contained in sesquiterpenes, minute amount of sesquiterpenes are biosynthesized by native producers. Benefit from the development of metabolic engineering and synthetic biology, microorganisms have become tractable cell factories for sustainable biomanufacturing of the high-value sesquiterpenes. Recent advances in cells and process engineering have made fermentation to

be an attractive route for sesquiterpene production from renewable, low-cost biomass feedstocks.

Sesquiterpene production depends on metabolic strategies, and metabolic strategies also need biotechnology innovation. Recent progress in synthetic biology of terpenoid synthases has opened new avenues of research by providing diversities of strategies. Biotechnological applications made possible by recent molecular advances including the engineering of industrial microbiology and regulation of two isoprenoid pathways to increase the desirable sesquiterpenes. The designing, engineering and optimizing processes are served to establish a high efficiency fermentation-based process and stable sesquiterpene production hosts. Although there have been successful applications of sesquiterpenes, like amorphadiene (Westfall et al. , 2012) and farnesene (Harvey et al. , 2014), more studies and challenges need to be performed and overcame to expand its transformational efficiency, stability and recovery. For example, the use of solvents reduced sesquiterpene evaporation during fermentation and improved product recovery, but it resulted in higher costs, due to the additional equipment cost for solvent-product separation (Cuesta et al. , 2019). Further understanding of the enzymatic mechanism and optimal expression of isoprenoid pathway enzymes will be necessitated. Challenges on making bio-based sesquiterpene more competitive in applications come from aspects of searching high-activity enzymes, supplying energy and cofactor, and balancing metabolic flux, as well as efficient recovery and purification of sesquiterpenes from fermentation broth. Furthermore, issues concerning microbial biosynthesis for achieving higher production help to develop approaches of identifying regulatory effects in metabolic pathways for redirecting carbon fluxes to terpenoid pathways.

Ethical Statement

This article does not contain any studies with human participants performed by any of the authors.

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Competing interests

The authors declare that they have no competing interests.

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Table 1. Metabolic engineering of *E. coli* for sesquiterpene biosynthesis.

Sesquiterpene	Strain	Description	Titer	Reference
Amorphadiene	*E. coli* DYM1	Over-expressing *ADS* gene from *A. annua* and MVA pathway genes from *S. cerevisiae*, with *mvk*, *pmk*, *mvaD*, *idi* and *ispA* gene under control of P_{LAC} (plasmid pMBIS); *atoB*, *HMGS* and *tHMGR* gene under control of P_{LAC} (plasmid MevT)	112.2 mg/L	(Martin et al. , 2003)
	E. coli K12 MG1655 ΔrecA, ΔendA	Containing plasmid P_{BAD}-Dxs-Idi-ispDF and P_{ACM}-ADS, over-expressing multidrug-resistant membrane efflux transporter protein TolC and ABC family transporters (macAB) or MFS family transporters (emrAB or emrKY)	~180 mg/L	(Zhang et al. , 2016)
	E. coli K12 MG1655	Containing plasmid P_{BAD}-Dxs-Idi-ispDF and P_{ACM}-ADS, deleting PTS genes, using optimized OPT2 medium	182 mg/L	(Zhang et al. , 2013)
	E. coli MG1655 DE3	ΔpstHIcrr::FRT, containing plasmid pETA-TM2-galP-glk and pACM-P_{T7}-dxs-P_{T7}-idi-ADS-ispA	201 mg/L	(Zhang et al. , 2015)
	E. coli DP10	Containing plasmid pMBIS, supplementing 20 mM MVA to medium	~290 mg /L	(Pitera et al. , 2007)
	E. coli DH1	Plasmid pAM4: lacUV5 promoters for codon-optimized MevT operon and MBIS operon, Cm^R, Plasmid pAM94: pTrc99A derivative containing *ADS* and codon-optimized *mvk*, Ap^R; fermentation for 75 h.	293 mg/L/OD_{600}	(Anthony et al. , 2009)
	E. coli Bl21-Gold DE3	Over-expressing *Dxs*, *Idi*, *ispA* and *ADS* gene	~300 mg/L	(Yuan and Ching, 2014)
	E. coli BL21 (DE3)	Co-expressing *Dxs2* from *S. avermitilis* and *Idi* from *B. subtilis*	331.7 mg/L	(Wang et al. , 2015)
	E. coli BL21 (DE3)	Harboring pET21c-ADS, P_{T7}-tolC-tolC-acrB operon	404.8 mg/L	(Wang et al. , 2013)
	E. coli W3110 (ATCC 27325)	Containing plasmid pMBIS and MevT, employing a two-phase partitioning bioreactor with a dodecane organic phase	500 mg/L	(Newman et al. , 2006)
	E. coli DH1	Codon-optimizing *mvk* and *pmk* gene, and replacing P_{lac} with a stronger P_{trc}	>500 mg/L	(Redding-Johanson et al. , 2011)

	E. coli DH1	Bearing *D. acidovorans HMGR* by increasing intracellular NADH level using a NADP-dependent formate dehydrogenase (*fdh1*) from *Candida boidinii*, along with formate supplementation after 48 h of fermentation.	700 mg/L	(Ma et al. , 2011)
	E. coli DH1	Dynamic control the MVA pathway expression using the identified FPP-responding P_{rstA} and P_{gadE}	1400 mg/L	(Dahl et al. , 2013)
	E. coli DH1	Metabolic pathway optimization using rbs variants and combinatorial gene assembly, pTrc-RBS.ADS-MBIS, using 3% glycerol	3550 mg/L	(Nowroozi et al. , 2014)
	E. coli DH1	Heterologous MVA pathway with *HMGS* and *HMGR* from *S. aureus*, using a fermentation process of restricted glucose and nitrogen with NaOH pH control	29.7 g/L	(Tsuruta et al. , 2009)
α-Farnesene	*E. coli* DH5a	Codon-optimizing *FS* gene, augmenting metabolic flux for FPP synthesis by introducing MVA pathway and over-expressing *Dxs*, *Idi* and *ispA* gene, fusing *FPPS* and *FS*	380.0 mg/L	(Wang et al. , 2011a)
	E. coli BL21 (DE3)	Developing a reconstituted in vitro farnesene biosynthetic system, rationally designing strain F4 with P_{lac} for *ERG12*, *ERG8*, *MVD1* and *Idi* gene with pBBR1MCS ori, and P_{T7} for *AFS*, *ispA* and *Idi* gene with pBBR322 ori	1100 mg/L	(Zhu et al. , 2014)
	E. coli BL21 (DE3)	Expressing heterologous MVA pathway, and high-expressing *Idi* and *ispA* gene in a lab bioreactor.	8740 mg/L	(You et al. , 2017)
Bisabolene	*E. coli* BL21 (DE3)	Harboring plasmid pBbA1k-AgBIS-ispA-ispDF-idi, fusing nDXP gene *ribB* to *Dxr* gene,	8.8 mg/g DCW	(Kirby et al. , 2015)
	E. coli DH1	F-, λ-, endA1, recA1, gyrA96, thi-1, glnV44, relA1, hsdR17(rK- mK+), DSMBT7, rbsR::P_{T7}-BS-idi-ispA, poxB::P_{T7}-MevT	~435 μg/L	(Alonso-Gutierrez et al. , 2018)
	E. coli DH1	Screening BIS enzyme, codon-optimizing *HMGS*, *tHMGR*, *mvk* and *pmk* gene, and inserting a strong P_{trc} before *mvk* gene (pJBEI-2999)	> 900 mg/L	(Peralta-Yahya et al. , 2011)
	E. coli DH1	Using quorum sensing LuxI/R system from *Vibrio fischeri* to construct sensor plasmid carrying *luxI*-*luxR* genes, and response plasmid carrying bisabolene producing pathway genes under control of P_{luxI}	1100 g/L	(Kim et al. , 2017)

	E. coli DH1	Harboring plasmid pBbA5c-CL1-T61-CL2-T21-AgBISþpTrc-AgBIS	1150 mg/L	(Alonso-Gutierrez et al. , 2015)
β-Caryophyllene	*E. coli* DH1	Expressing MVA pathway and *QHS1* gene	100 mg/L	(Wu et al. , 2018)
	E. coli BL21 (DE3)	Co-expressing *ACS*, *QHS1*, *GPPS2*, *AACS* gene and heterologous MVA pathway	1050 mg/L	(Yang and Nie, 2016)
	E. coli BL21 (DE3)	Over-expressing MVA pathway and *GPPS*, *G6PH*, *QHS1* genes, fed-batch fermentation for 60 h	1520 mg/L	(Yang et al. , 2016a)
α-Humulene	*E. coli* BL21 (DE3)	Expressing *Streptomyces* MVA pathway genes from *HMGS* to *Idi2*, yeast *Idi1* and rat *Aacl* gene, α-humulene synthase gene of shampoo ginger, supplying with LAA	958 mg/L	(Harada et al. , 2009)
5-Epi-aristolochene	*E. coli* XL1-Blue	P_{lac}-crtM-crtN, *TEAS* from *Nicotiana tabacum*, using the screened TEASmut7 Q481R by scoring their substrate consumption based on color loss of cell harboring carotenoid pathways	160 mg/L	(Furubayashi et al. , 2014)
Protoilludene	*E. coli* DH5α	Over-expressing a exogenous MVA pathway, endogenous ispA, and protoilludene synthase of *Omphalotus olearius*	1199 mg/L	(Yang et al. , 2016b)
Valerenadiene	*E. coli* BL21 (DE3)	Codon-optimizing gene *cvds*, over-expressing *Dxs*, *Idi* and *FPPS*, optimizing fermentation medium with glycerol as supplementary	11 mg/L	(Nybo et al. , 2017)
Longifolene	*E. coli* BL21 (DE3)	pTrcLower: pTrcHis2B harboring *S. cerevisiae erg19*, *erg8*, *erg12* and *idi1*; pA-mvaESispALFS: pACYCDuet-1 harboring *E. faecalis mvaE*, *mvaS*, *E. coli ispA* and *P. abies* longifolene synthase	382 mg/L	(Cao et al. , 2019)

1289 **Table 2. Metabolic engineering of *S. cerevisiae* for sesquiterpene biosynthesis.**

Sesquiterpene	Strain	Description	Titer	Reference
Amorphadiene	*S. cerevisiae* WAT11	Using flux balance analysis (FBA) and MOMA methods for predicting knockout targets, strain MATa (leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, *hxk2D*::*his3::ADS*	54.6 mg/L	(Sun et al. , 2014)
	S. cerevisiae EPY224	Upregulating *HMGS*, *mvk* and *pmk* gene by upc2-1 expression; over-expressing a truncated *tHMGR, ispA* and *ADS* gene; *erg9*: P_{met3}-ERG9	153 mg/L	(Ro et al. , 2006)
	S. cerevisiae CEN.PK2 Y293	Genotype: MATa, erg9Δ::kan^r P_{MET3}-ERG9, leu2-3,112::HIS P_{GAL1}-MVD1, P_{GAL10}-ERG8, his3Δ1::HIS, P_{GAL1}-ERG12, P_{GAL10}-ERG10, ade1Δ::P_{GAL1}-tHMG1, P_{GAL10}-IDI1 ADE1, ura3-52::P_{GAL1}-tHMG1, P_{GAL10}-ERG13, URA3trp1 -289::P_{GAL1}-tHMG1, P_{GAL10}-ERG20 TRP1; gal80Δ::nat^r; plasmid pAM426: P_{GAL1}-tHMG1. Fermentation with unrestricted ethanol pulse feed with lower oxygen uptake rate (OUR)	41 g/L	(Westfall et al. , 2012)
Bisabolene	*S. cerevisiae* EPY224	EPY224 platform through replacing *ADS* gene with *BIS* gene	900 mg/L	(Peralta-Yahya et al. , 2011)
	S. cerevisiae EPY224	EPY224 platform through replacing *ADS* gene with *BIS* gene, enriching a mixed population and removing little or no bisabolene producing strains using selective pressure nonionic surfactants (T20)	375 mg/L	(Kirby et al., 2014a)
	S. cerevisiae CEN.PK2-1C	Genotype: MATa ura3-52 trp1-289 leu2-3,112 his3D 1 MAL2-8C SUC2, YPRCd15::KANMX-PGAL1-C.O.BisSyn~ERG20/PGAL10-tHMG, gal80::HIS5, ypl064w::hph yjl064w::Ca.URA3, harboring pRS-AgBIS	5.2 g/L	(Ozaydin et al. , 2013)
β-Farnesene	*S. cerevisiae* CEN.PK113-5D	Genotype: MATa, MAL2−8c, SUC2, ura3−52, lpp1Δ::loxP, dpp1Δ::loxP, PERG9Δ::loxP-P_{HXT1}, P_{PGK1}-Z_{IgA}-ERG20, P_{TEF1}-Z_{Taq}-FarnSyn-Cj, P_{TEF1}-anti-Z_{Taq}-anti-Z_{IgA} with $(SSSSG)_4$ linker	0.813 mg/g	(Tippmann et al. , 2017a)
	S. cerevisiae CEN.PK113-5D	Genotype: MATa, MAL2-8^c, SUC2, ura3-52, lpp1Δ::loxP, dpp1Δ::loxP, P_{ERG9}Δ::loxP-P_{HXT1}, gdh1Δ::loxP, P_{TEF1}-ERG20, P_{PGK1}-GDH2, over-expressing *FS* and *tHMG1* gene in a 2-μm plasmid with respiratory quotient-controlled exponential feeding	170 mg/L	(Tippmann et al. , 2016)

	S. cerevisiae ATCC200589	Over-expressing structural component Ty1 retrotransposon element Tya-fused enzyme tHmg1, ispA and FS in a two-phase partitioning fed-batch fermentation for 7 days	930 mg/L	(Han et al. , 2018)
	S. cerevisiae CEN.PK2-1C	Genotype: MATα, erg9Δ::P_{GAL4}(OC)-GAL4_syn_P_{MET3}-ERG9, trp1-289:: P_{GAL1}-tHMG1_P_{GAL10}-ERG20_T_{RP1}, leu2-3,112::HISMX_P_{GAL1}-MVD1_ P_{GAL10}-ERG8, ade1Δ::P_{GAL1}-tHMGR_P_{GAL10}-IDI1_ADE1, his3Δ1::HISMX_ P_{GAL1}-ERG12_P_{GAL10}-ERG10_HIS3, ura3-52:: P_{GAL1}-tHMG1_P_{GAL10}-ERG13 _URA3, gal80Δ::syn_P_{GAL1}-ERG12, erg9Δ::P_{GAL4}(OC)-GAL4_syn_P_{MET3}-ERG9, hxt3Δ::syn_P_{TDH3}-ERG10-T_{AHP1}_P_{YPD1}-Bj.HMGs-T_{CCW12}_P_{TUB2}-Ca.THL-T_{HXT3}, dit1Δ::syn_P_{GAL1}-Aa.FS_A-T_{CYC1}, ndt80Δ:: P_{GAL1}-Aa.FS_A-T_{CYC1}_P_{HXT3}-ACS2-T_{PGK1}_P_{GAL7}-Zm.PDC-T_{TDH3}, hoΔ::syn, gal2Δ::syn, adh5Δ:: P_{GAL1}-Aa.FS_B-T_{ADH1}_P_{GAL10}-Aa.FS_A-T_{CYC1}_LEU2_P_{GAL1}-Aa.FS_B -T_{ADH1}_P_{GAL10}Aa.FS_A-T_{CYC1}; ald4Δ::P_{GAL1}-Dz.eutE-T_{PGK1}_syn_P_{GAL1}-Dz.eutE-T_{PGK1}, ald6Δ::syn, rhr2Δ::syn_P_{TDH3}-Lm.PK-T_{TDH3}, erg13Δ::syn_ P_{HXT1}-GAL4*, ygr250cΔ::syn_P_{ACT3}-PDR3, bdh1Δ::P_{TDH3}-Lm.PK-T_{TDH3}_syn _P_{ACT1}-Ck.PTA-T_{PGK1}, adh1Δ::P_{FBA1}-ACS2_syn_P_{TDH3}-Sp.HMGr-T_{HMG1}, P_{TDH3}-ERG13*_P_{GAL10}-Sp.HMGr-T_{HMG1}; 200,000-L industrial bioreactors.	130 g/L	(Meadows et al. , 2016)
α-Santalene	*S. cerevisiae* CEN.PK113-5D	Genotype: MATa MAL2-8c SUC2 ura3-52 lpp1Δ::loxP dpp1Δ::loxP P_{ERG9}Δ::loxP-P_{HXT1}, plasmid pICK01 containing a copy of tHMG1 and santalene synthase	92 mg/L	(Scalcinati et al. , 2012a)
	S. cerevisiae CEN.PK113-5D	Genotype: MATa, MAL2-8^c, SUC2, ura3-52, lpp1Δ::loxP, dpp1Δ::loxP, P_{ERG9}Δ::loxP-P_{HXT1}, gdh1Δ::loxP, P_{TEF1}-ERG20, P_{PGK1}-GDH2, over-expressing santalene synthase and *tHMG1* gene in a 2-μm plasmid with respiratory quotient-controlled exponential feeding	163 mg/L	(Tippmann et al. , 2016)
Caryophyllene	*S. cerevisiae* EG60	Genotype: Mat a/α, P_{Gal1}-HMG2-(K6R) x2, P_{Gal1}-HA-ERG20, ura3, trp1, his3, leu2, Δerg9:: HIS5, pYESmyc/P330, 2μ, URA3, P_{GAL1}-myc-P330	~ 10 mg/L	(Ignea et al. , 2011)
	S. cerevisiae EG60	Genotype: Mat a/α, PGal1-HMG2(K6R):: HOX2, ura3, trp1, his3, PTDH3-HMG2(K6R)X2-::leu2 ERG9/erg9, UBC7/ubc7, SSM4/ssm4, PHO86/pho86	125 mg/L	(Ignea et al. , 2012)
α-Cubebene	*S. cerevisiae* EG60	Genotype: Mat a/α, P_{Gal1}-HMG2-(K6R) x2, P_{Gal1}-HA-ERG20, ura3, trp1, his3, leu2, Δerg9:: HIS5, pYESmyc/P330, 2μ, URA3, P_{GAL1}-myc-P330	~ 7 mg/L	(Ignea et al. , 2011)

α-Copaene	*S. cerevisiae* EG60	Genotype: Mat a/α, P_{Gal1}-HMG2-(K6R) x2, P_{Gal1}-HA-ERG20, ura3, trp1, his3, leu2, Δerg9:: HIS5, pYESmyc/P330, 2μ, URA3, P_{GAL1}-myc-P330	~12 mg/L	(Ignea et al. , 2011)
α-Cadinene	*S. cerevisiae* EG60	Genotype: Mat a/α, P_{Gal1}-HMG2-(K6R) x2, P_{Gal1}-HA-ERG20, ura3, trp1, his3, leu2, Δerg9:: HIS5, pYESmyc/P330, 2μ, URA3, P_{GAL1}-myc-P330	~38 mg/L	(Ignea et al. , 2011)
Pentalenene	*S. cerevisiae* EPY	Upregulating *HMGS*, *MK* and *PMK* gene by upc2-1 expression; over-expressing *tHMGR, ispA* and *PentS* gene; *erg9*: P_{met3}-ERG9	344 mg/L	(Liu et al. , 2018)
5-Epi-aristolochene	*S. cerevisiae* WAT11	Mutating gene *ERG9*, uptake of exogenous ergosterol (sue), introducing gene *TEAS* in plasmid pESC-HIS3-GPD-TEAS	90 mg/L	(Takahashi et al. , 2007)
Premnaspirodiene	*S. cerevisiae* WAT11	Mutating gene *ERG9*, uptake of exogenous ergosterol (sue), introducing gene *HPS* in plasmid pESC-HIS3-GPD-HPS	90 mg/L	(Takahashi et al. , 2007)
	S. cerevisiae BY4741	Expressing *HPS* gene with an amino-terminal truncated in plasmid pESC-HIS3-GPD-HPS, catalytic form of tHMGR gene, uptake of exogenous ergosterol (sue)	170.23 mg/L	(Zhuang and Chappell, 2015)
germacrene A	*S. cerevisiae* SCIGS22a	MATa MAL2-8c SUC2 ura3-52 lpp1Δ::loxP dpp1Δ::loxP PERG9Δ::loxP-P_{HXT1} gdh1Δ::loxP P_{TEF1}-ERG20, P_{GK1}-GDH2 P_{TEF1}-tHMG1, P_{TEF1}-ERG20-GGGGS-$LTC2_{opt}$ aaaaaaaaaa P_{PGK1}-tHMG1	190.7 mg/L	(Hu et al. , 2017)
Valencene	*S. cerevisiae* YIP-00-03 (CEN.PK113-5D)	MATa MAL2-8c SUC2 ura3-52, erg9::P_{MET3}-ERG9, pESC-URA 2μ URA3 P_{GAL1}- GFTpSD	2 mg/L	(Asadollahi et al. , 2008)
	S. cerevisiae WAT11	Mutating *ERG9* gene, uptake of exogenous ergosterol (sue), introducing *CVS* gene	20 mg/L	(Takahashi et al. , 2007)

1292 **Table 3. Metabolic engineering of other organisms for sesquiterpene biosynthesis.**

Sesquiterpene	Strain	Description	Titer	Reference
(+)-Valencene	*C. glutamicum* ATCC 13032	Deleting two endogenous prenyltransferase genes, expressing heterologous gene *ispA* from *E. coli* or gene *ERG20* from *S. cerevisiae*	2.41 mg/L	(Frohwitter et al. , 2014)
Amorphadiene	*B. subtilis* 1A1 (BGSC)	Expressing gene *ADS* under P_{grac}, gene *Dxs* and *Idi* under P_{xylA}, protein translation engineering fused six-arginine tag to N terminus of gene *ADS*, optimizing media by adding pyruvate and dipotassium phosphate	~20 mg/L	(Zhou et al. , 2013)
β-Sesquiphellandrene	*L. lactis* NZ9000	Introducing β-sesquiphellandrene synthase, co-expressing *mvk* and *mvaA* gene	15.5 mg/L	(Song et al. , 2014)
Valencene	*R. sphaeroides*	Co-expressing MVA operon from *Paracoccus zeaxanthinifaciens* and *CVS* from *Callitropsis nootkatensis* on plasmid (pBBR-MEV) in shaker flasks	352 mg/L	(Beekwilder et al. , 2014)
Farnesene	*Anabaena sp.* PCC 7120	Expressing a *coFS* gene, through its endogenous MEP pathway using CO_2, mineralized water, and light	69.1μg·L^{-1}·O D^{-1}·d^{-1}	(Halfmann et al. , 2014)
	S. elongatus PCC 7942	*NSI::Bb1s-dxs-idi-ispA, NSII::Bb1k-AFS* direct conversion of CO_2, with a dodecane overlay in 7 days	4.6 mg/L	(Lee et al. , 2017)
Amorphadiene	*S. elongatus PCC 7942*	*NSI::Bb1 s-dxs-idi-ispA NSII::Bb2 k-ADS*, direct conversion of CO_2, with a dodecane overlay in 7 days	19.8 mg/L	(Choi et al. , 2016)
Caryophyllene	*Synechocystis sp.* PCC6803	Inserting *QHS1* gene from *A. annua* via double homologous recombination into genome, converting CO_2 and H_2O, measured by a methanol: choloroform extraction method	~ 4 mg/g DCW	(Reinsvold et al. , 2011)
Bisabolene	*Synechococcus sp.* PCC 7002	Expressing heterologous *A. grandis BIS* gene, deleting *glgC* gene and applying a dodecane overlay	>0.6 mg L^{-1}	(Davies et al. , 2014)
	C. reinhardtii	Through genetic engineering, serial enzyme loading and artificial micro RNA-based repression of competing pathways	11.0 mg/L	(Wichmann et al. , 2018)
Farnesene	*Y. lipolytica* Po1h	Integrating *tHMG1*, *Idi*, *ERG20* and *coFS* gene into genome in a bioreactor scale-up with PM medium	259.98 mg/L	(Yang et al. , 2016c)

Amorphadiene	*R. toruloides* IFO0880	Mating type A2, P_{GAPDH}-BIS-TNOS, in corn stover hydrolysates prepared by a biocompatible ionic liquid (IL) choline α-ketoglutarate pretreatment	36 mg/L	(Yaegashi et al. , 2017)
Bisabolene	*R. toruloides* IFO0880	Mating type A2, plasmid P_{GAPDH}-BIS-TNOS, with corn stover hydrolysates prepared by an alkaline pretreatment, in high-gravity fed-batch bioreactor	680 mg/L	(Yaegashi et al. , 2017)
Bisabolene	*S. venezuelae* ATCC 10712	Deleting gene *ΔSVEN_0269* and *ΔSVEN_7111*, inserting P_{SV}-coAgBis and P_{ermEp}-FPPS1 at the aatP chromosomal site	10.52 mg/L	(Phelan et al. , 2015)
α-cuprenene	*X. dendrorhous*	Integrating gene *Cop6* in the rDNA from *C. cinereus*	80 mg/L	(Melillo et al. , 2013)

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