

MINIREVIEW

Engineering yeast metabolism for production of terpenoids for use as perfume ingredients, pharmaceuticals and biofuels

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One sentence summary: Discuss advances, challenges and perspectives of engineering *Saccharomyces cerevisiae* as a cell factory to produce terpenoids.

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ABSTRACT

Terpenoids represent a large class of natural products with significant commercial applications. These chemicals are currently mainly obtained through extraction from plants and microbes or through chemical synthesis. However, these sources often face challenges of unsustainability and low productivity. In order to address these issues, *Escherichia coli* and yeast have been metabolic engineered to produce non-native terpenoids. With recent reports of engineering yeast metabolism to produce several terpenoids at high yields, it has become possible to establish commercial yeast production of terpenoids that find applications as perfume ingredients, pharmaceuticals and advanced biofuels. In this review, we describe the strategies to rewire the yeast pathway for terpenoid biosynthesis. Recent advances will be discussed together with challenges and perspectives of yeast as a cell factory to produce different terpenoids.

Keywords: terpenoids; terpenes; yeast; metabolic engineering

INTRODUCTION

Terpenoids are a large and diverse class of natural compounds (more than 55 000 structures), with significant commercial applications (Maimone and Baran 2007; Pateraki, Heskes and Hamberger 2015). The term terpenes are defined as biological originated hydrocarbons with carbon skeletons derived from isoprene units (2-methylbuta-1,3-diene; IUPAC 1997). Terpenoids, on the other hand, are complex natural products derived from terpenes that may contain oxygen in various func-

tional groups and their carbon skeletons may differ from the strict additive structures of isoprene units (IUPAC 1997).

In fact, the building blocks of terpenoids are not exactly isoprene units but their activated forms, the isopentenyl diphosphate (IPP) and the dimethylallyl diphosphate (DMAPP), which are derived from either the mevalonate pathway (MVA) or the methylerythritol phosphate pathway (MEP; Chang, Song and Liu 2013; Paramasivan and Mutturi 2017). In one isoprene unit, the methyl group with the double bond group is referred

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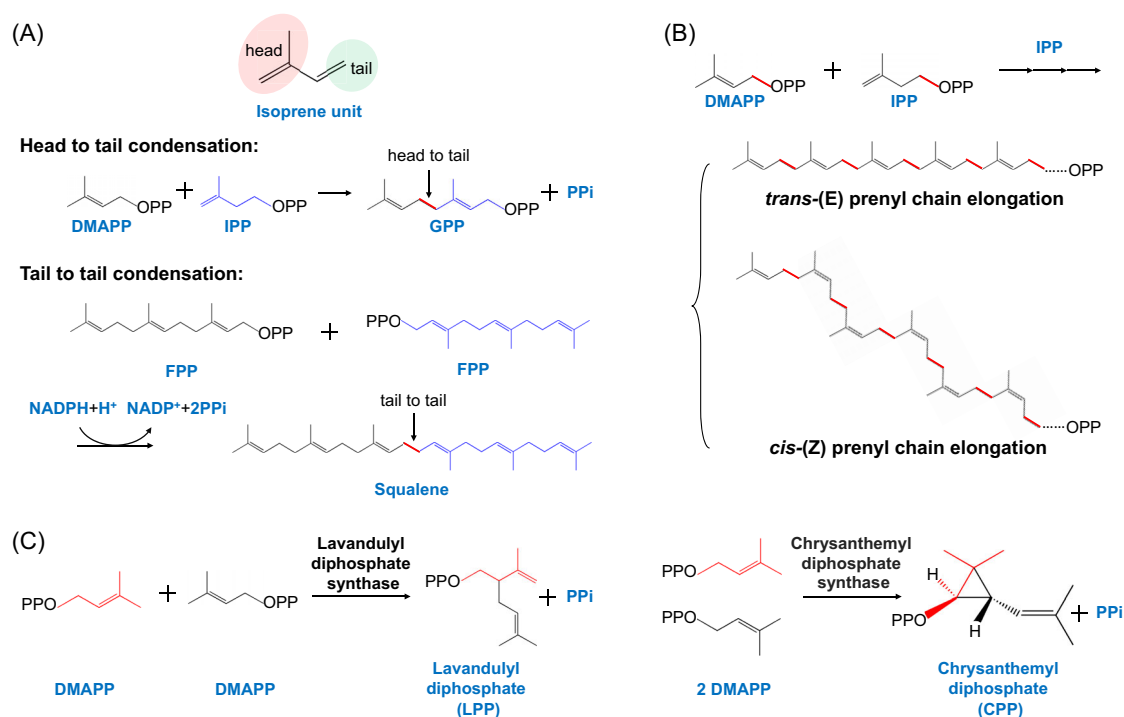


Figure 1. The condensation reaction of terpenoid biosynthesis. (A) In one isoprene unit, the methyl group with double bond group is referred as the head, while the other side as the tail. The condensation reaction of GPP biosynthesis is in a head-to-tail manner, while condensation reaction of squalene biosynthesis is in a tail-to-tail manner. (B) The terpenoid elongation can be *trans*- (E) or *cis*- (Z) prenyl chain elongation. (C) Irregular terpenoids can be biosynthesized by alternative condensation reactions, e.g. the biosynthesis of LPP and CPP.

as the head, and the other side as the tail (Fig. 1A; Ouellette and Rawn 2014). The regular linear terpenoids are synthesized in a head-to-tail manner: a DMAPP is the starting point and its allylic group is coupled to the double bond of an IPP to give a condensation compound, which still contains an allylic group at the tail and in turn can be elongated by another round of condensation with another IPP, and so on (Fig. 1A; Ruzicka 1953; Ouellette and Rawn 2014). In addition to head-to-tail condensation, these reactions may also be carried in an irregular tail-to-tail or head-to-head fashion. Triterpenes (e.g. squalene) and tetraterpenes (e.g. β -carotene and lycopene) tend to possess a tail-to-tail bond as condensation of tails of two pyrophosphate compounds (Fig. 1A; Zografos and Anagnostaki 2016), while head-to-head condensations are found in some other terpenoids, such as some terpenoids in petroleum (Moldovan and Seifert 1979). Additionally, the terpenoid condensation steps can be either *cis*- (or Z) or *trans*- (or E) prenyl chain elongation catalyzed by either *cis* or *trans* types of prenyltransferases (Fujihashi et al. 2001; Sallaud et al. 2009; Schillmiller et al. 2009). The geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), precursors of different classes of terpenoids, are catalyzed by *trans*-prenyltransferases, while the biosynthesis reactions of neryl diphosphate (NPP), peptidoglycan in prokaryotes, glycoprotein in eukaryotes and natural rubbers are through *cis*-prenyltransferases (Fig. 1B; Fujihashi et al. 2001; Schillmiller et al. 2009). In addition, irregular terpenoids can be biosynthesized by alternative condensation reactions, e.g. the biosynthesis reactions of lavandulyl diphosphate (LPP), a precursor of lavandulol (an important perfume ingredient) and chrysanthemyl diphosphate (CPP), a precursor of pyrethrins (an important natural insecticide), are catalyzed by lavandulyl diphosphate synthase and chrysanthemyl diphosphate synthase (Fig. 1C; Demissie et al. 2013).

In terpenoid biosynthesis, the carbon skeletons may be rearranged (e.g. by cyclization) or modified (e.g. loss or addition of carbon atoms). Thus, the carbon number may differ from the 5n pattern, but still terpenoids are typically classified based on the number of isoprene units from which they are biogenetically derived (isoprene rule): hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15), diterpenoids (C20), sesquiterpenoids (C25), triterpenoids (C30), tetraterpenoids (C40) and polyterpenoids (C > 40) (Fig. 2; Ruzicka 1953). Monoterpenoids (C10) are volatile compounds contained in plant essential oils (essence of odor and flavor), e.g. geraniol, linalool, menthol, limonene and camphor (Buchanan, Gruissem and Jones 2015) and some monoterpenoids are found in marine organisms, e.g. halmon from red algae *Portieria hornemannii* a potential antitumor agent (Fuller et al. 1992). Monoterpenoids are derived from GPP, NPP, LPP and CPP (Demissie et al. 2013). They are widely used as perfume ingredients, food flavors, pharmaceutical agents and insecticides (Buchanan, Gruissem and Jones 2015). Sesquiterpenoids (C15) constitute a large group of compounds derived from FPP and they can be used as perfume ingredients, e.g. nerolidol, farnesol and patchoulol (Chan et al. 2016; Liao et al. 2016; Hu et al. 2017), as insect repellents and insecticides, e.g. Nootkatone (Leonhardt and Berger 2015), as pharmaceuticals, e.g. the anti-malaria drug artemisinin and the antifungal agent capsidiol (Lozoya-Gloria 1999; Tu 2011), and as biofuels, e.g. farnesene and bisabolene (Peralta-Yahya et al. 2011). For example, artemisinin is isolated from *Artemisia annua* (A. annua) and has been identified to be the active component of an old Chinese medicine used to treat malaria and it has since then been used as a key drug to treat this disease (Tu 2011). Diterpenoids (C20) constitute a large group of compounds derived from GGPP, and they can be used in cosmetics and pharmaceuticals, e.g. sclareol as a precursor of Ambrox that is responsible

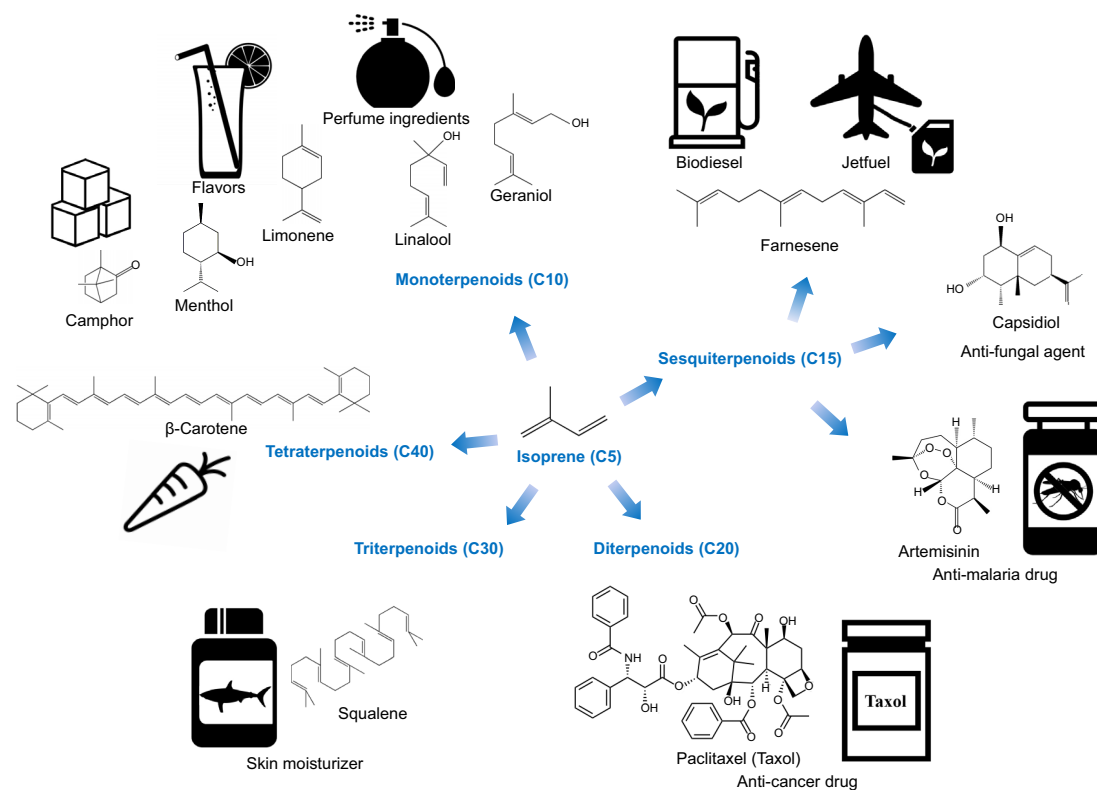


Figure 2. Terpenoid classifications and some examples of their commercial applications.

for the odor of ambergris and highly priced in perfume industry (Zerbe and Bohlmann 2015), carnosic acid as a preservative in the cosmetic and food industry (Ignea et al. 2016), and taxol as an anti-cancer drug (Engels, Dahm and Jennewein 2008). Triterpenoids (C30) constitute a large group of compounds derived from squalene or squalene 2,3-epoxide and they can be modified by multiple steps of cyclization and carbon skeleton rearrangements. For examples, squalene is used as skin moisturizer in cosmetics and immunologic adjuvant in vaccines (Reddy and Couvreur 2009; Bhilwade et al. 2010), cholesterol is the essential structural component of all animal cell membranes, and hydrocortisone is a major mammalian adrenal glucocorticoid and an important intermediate of steroidal drug (Szczepara et al. 2003). Tetraterpenoids (C40) are derived from phytoene, and often referred as carotenoids. For examples, β -carotene and lycopene that assist the light reactions of photosynthesis and can be used as food colorants (Mata-Gomez et al. 2014). Currently, terpenoids are produced predominantly in plants and their storage in complex mixtures makes it difficult and costly for the downstream extractions and purifications (Wu et al. 2006). Total chemical synthesis of many terpenoids is still costly and inefficient (Nicolaou et al. 1994); therefore, there are on-going demands for efficient biological production of terpenoids.

There are two pathways for terpenoid biosynthesis in plant cells, the MVA and the MEP, and plants balance the use of these two pathways to meet different terpenoid biosynthetic needs (Chang, Song and Liu 2013; Paramasivan and Mutturi 2017). Naturally, prokaryotes only possess the MEP pathway and fungi only use the MVA (Chang, Song and Liu 2013; Paramasivan and Mutturi 2017). *Saccharomyces cerevisiae* has a wide range of advantages in terms of large-scale production of terpenoids, including tolerance to low pH, osmotic stress, as well as resistance towards

phage infections. *Saccharomyces cerevisiae* also has status as generally regarded as safe, which facilitates approval of products produced by this cell factory. More importantly, *S. cerevisiae* endogenously synthesizes precursors of most terpenoids, including GPP, FPP, GGPP and squalene, and this allows easy modifications of the biosynthetic pathway (Fig. 3). Furthermore, with successful examples of engineering yeast for industrial scale terpenoid production, such as β -farnesene production with titers exceeding 130g/L in bioreactor (Meadows et al. 2016), yeast has been demonstrated as an excellent cell factory for terpenoid production. In this review, we will discuss general strategies to improve terpenoid production, as well as challenges and perspectives of yeast as a cell factory to produce terpenoids.

GENERAL STRATEGIES TO IMPROVE TERPENOID PRODUCTION IN YEAST

Terpenoid synthases and cytochromes P450-dependent monooxygenases

The diversity and complexity of terpenoids are generated primarily by terpenoid synthases (TSs; Boutanaev et al. 2015). TSs are defined as biosynthetic enzymes involved in producing terpenoid hydrocarbon backbones, including sequential isoprenyl elongation by prenyltransferases, such as *trans*-isoprenyl diphosphate synthases and squalene synthases, and subsequently cyclization or rearrangement through class I terpene synthases (TPSs) or class II terpene cyclases (Gao, Honzatko and Peters 2012). The class I TPSs are designated for catalyzing ionization of the allylic pyrophosphate ester bond, which is often followed by forming an intramolecular C=C double-bond, and then followed by cyclization, carbon rearrangements or forming a

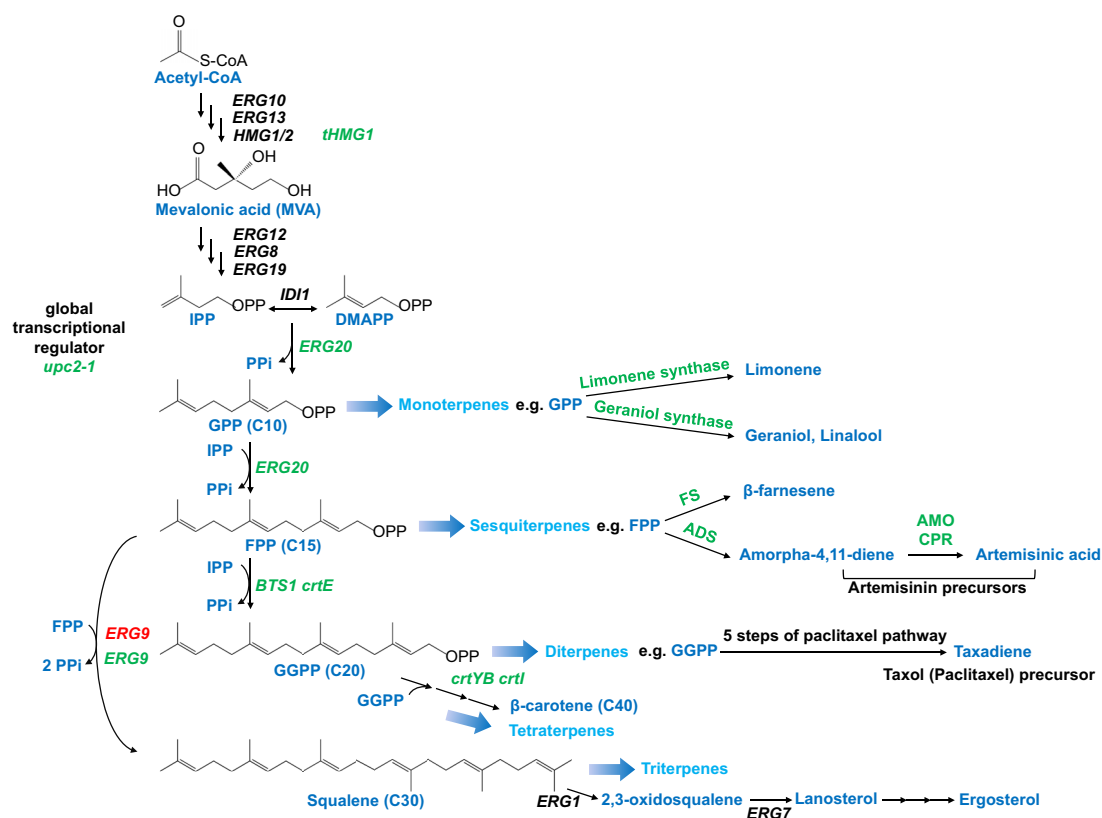


Figure 3. The biosynthesis of different classes of terpenes in *S. cerevisiae* using the mevalonate pathway. The green gene names indicate strategies of over-expression, while the red gene names indicate strategies of gene-repression. FS: farnesene synthase, ADS: amorpha-4,11-diene synthase, AMO: amorphadiene oxidase, CPR: cytochrome P450 reductase, crtE: GGPP synthase from *X. dendrorhous*, crtYB: bifunctional lycopene cyclase/phytoene synthase from *X. dendrorhous* and crtI: phytoene desaturase from *X. dendrorhous*.

hydroxyl group (Gao, Honzatko and Peters 2012). For examples, monoterpene synthases and sesquiterpene synthases are the class I TPSs. The class II terpene cyclases are characterized by distinct cyclization reactions by protonating to either a C=C double-bond or epoxide to form multi-cyclic structures (Gao, Honzatko and Peters 2012). Triterpene synthases are the class II cyclases, for example, oxido-squalene cyclases that can catalyze complex cyclization reactions from one to five rings. Some TPSs are bifunctional (both class I and II), e.g. abietadiene synthase, a diterpene synthase from *Abies grandis* that can catalyze an initial cyclization of GGPP to copalyl diphosphate, followed by the ionization of allylic group and subsequent cyclization reaction steps to form abietadiene (Gao, Honzatko and Peters 2012). There are a large number of TPSs in plants. For examples, there are 32 putative TPS genes in *Arabidopsis thaliana* and 69 in *Vitis vinifera* (Chen et al. 2011). TSs can be heterologously expressed in *S. cerevisiae*, and utilize yeast endogenous precursors (GPP, FPP, GGPP and squalene) to produce diverse terpenoids.

The hydrocarbon products from TPS can be further modified into terpenoids by the diverse family of cytochrome P450-dependent monooxygenases (P450s or CYPs; Urlacher and Girhard 2012). P450s catalyze a wide variety of oxidation reactions including introduction of atmospheric oxygen to sp³ hybridized C atoms, epoxidation of C=C double bonds, N-oxidation, deamination, dehalogenation and dealkylation, in addition, P450s also catalyze unusual rearrangement reactions, cleavage of C-C bonds and other reactions (Urlacher and Girhard 2012). P450s need redox partners for catalytic activity, such as NAD(P)H-dependent cytochrome P450 oxido-reductases (CPR

or POR), to provide reducing equivalents, NADH or NADPH (Urlacher and Girhard 2012). There are totally three P450s in *S. cerevisiae*, and they are Erg5 and Erg11 in ergosterol pathway and Dlt2 (putative cytochrome P450 involved in the synthesis of N,N-bisformyl dityrosine). There are 246 P450 genes and 26 pseudogenes in the genomes of *Arabidopsis thaliana* (Urlacher and Girhard 2012). *Saccharomyces cerevisiae* has been a successful host for expressing functionally active plant derived P450s (Paramasivan and Mutturi 2017). For example, by over-expression of CYP71AV1, CPR1 and CYB5 (encoding a cytochrome b₅ reductase) from *A. annua*, the MVA engineered *S. cerevisiae* can produce artemisinic acid at the titer of 25 g/L in bioreactor (Table 2; Paddon et al. 2013).

Metabolic engineering in mevalonate pathway

To increase the production of terpenoids in yeast, the most crucial step is to increase the precursor pool, which can be achieved by directing more flux into the MVA (Kampranis and Makris 2012). HMG-CoA reduction is reported as the rate-limiting step in sterol biosynthesis (Liscum et al. 1985), and is therefore often the target for over-expression to promote flux through the mevalonate pathway. There are two HMG-CoA reductases in yeast, Hmg1 and Hmg2, and the N-termini of both proteins anchor to the endoplasmic reticulum membrane (Liscum et al. 1985). Over-expression of N-terminal truncated version of HMG1 (tHMG1) resulted in the catalytic C-terminal region being located to the cytosol and dramatically increased squalene production (Polakowski, Stahl and Lang 1998). Hmg2 is the predominant

isoform under low oxygen conditions (Mantzouridou and Tsimidou 2010). The Hmg2 (K6R) variant is a stabilizing mutant of Hmg2 and over-expression of this mutant resulted in the squalene production increased by 20-fold comparing to the wild-type strain (Mantzouridou and Tsimidou 2010). A similar strategy has been used for increasing the activity of the transcription factor Upc2. Upc2 and Ecm22 have been identified as transcriptional activators of genes in mevalonate and ergosterol pathway (Vik and Rine 2001). Over-expressing *upc2-1*, a constitutively active point mutant of Upc2, resulted in a significant increase in the uptake of sterols and expressions of genes involved in the MVA comparing to the wild-type strain (Crowley et al. 1998; Ro et al. 2006). In addition, over-expression of *IDI1*, an IPP: DMAPP isomerase, also enhanced monoterpene production (Ignea et al. 2011). In some recent studies, most of the structural genes in the MVA (*ERG10*, *ERG13*, *tHMG1*, *ERG12*, *ERG8*, *IDI1* and *ERG20*) have been over-expressed to increase production of terpenoids (Westfall et al. 2012; Meadows et al. 2016). Furthermore, the precursor pool can also be increased by down-regulating the downstream genes or genes in competitive pathways. For example, *ERG9*, an essential gene encoding a squalene synthase, competes with sesquiterpenoid and diterpenoid synthase for the FPP pool. As *ERG9* is essential, it is important to have conditionally regulation of this gene to ensure that there can be maintained flux towards ergosterol at least in some parts of the fermentation process. By down-regulation of *ERG9* through replacing its endogenous promoter with the methionine promoter, P_{MET3} , which is repressed in the presence of methionine, the FPP pool is increased as well as the production of sesquiterpenes and diterpenes (Cherest, Nguyen and Surdin-Kerjan 1985; Ro et al. 2006; Westfall et al. 2012). However, the use of the methionine-regulated promoter to control expression of *ERG9* is sometimes problematic as yeast will consume methionine and hence relieve the repression of *ERG9*, and it is therefore advantageous to use the *HXT1* promoter as this allows for down-regulation of *ERG9* when the glucose concentration in the fermentation is low, which can be achieved either by growth on ethanol or by using a fed-batch fermentation (Scalcinati et al. 2012a).

Acetyl-CoA, energy cost and cofactors

Cytosolic acetyl-CoA and NADPH are the key precursor and cofactor for biosynthesis of multiple bioproducts, including terpenoids. Over-expression of structural genes in the MVA to increase terpenoid production may drain the cytosolic acetyl-CoA and NADPH pools and affect yeast cell growth, and this may in return affect the terpenoid production. Acetyl-CoA can be generated in different compartments in *S. cerevisiae*, such as mitochondria, peroxisome and nucleus. Acetyl-CoA is mainly generated by pyruvate dehydrogenase (PDH) in mitochondria; however, mitochondrial acetyl-CoA cannot be transported into the cytosol for fatty acid and terpenoid biosynthesis. Cytosolic acetyl-CoA is mainly synthesized via the PDH bypass pathway, which involves pyruvate decarboxylase (PDC), aldehyde dehydrogenases (ALD) and acetyl-CoA synthetase (*Acs1/2*; Fig. 4). Several studies were attempted to increase the cytosolic acetyl-CoA pool by manipulating the PDH-bypass and removing other competitive pathways (Shiba et al. 2007; Chen et al. 2013); however, the energy cost of the PDH-bypass is high: the reaction catalyzed by *Acs1/2* includes hydrolysis of 1 molecule of ATP to AMP and pyrophosphate, which is energetically equivalent to hydrolysis of 2 molecules of ATP to ADP. Other pathways with less energy cost for productions of cytosolic acetyl-CoA have therefore been tested. Meadows et al. established a synthetic metabolic

pathway to substitute the PDH-bypass and increase the cytosolic acetyl-CoA levels and lower ATP cost. They combined the acetaldehyde dehydrogenase (ADA) that converts acetaldehyde to acetyl-CoA, with xylulose-5-phosphate (X5P)-specific phosphoketolase (xPK) and phosphotransacetylase (PTA), which convert X5P to acetyl-CoA (Fig. 4 the green route; Meadows et al. 2016). To balance the NADPH and NADH, Meadows et al. (2016) used an NADH-consuming HMG-CoA reductase (NADH-HMGr). This combination pathway resulted in increased production of β -farnesene while requiring 75% less oxygen (Meadows et al. 2016). Another strategy is to express a cytosolic PDH complex to substitute PDH-bypass. Kozak et al. (2014) were able to express a cytosolic ATP-independent PDH complex and fully replace the PDH-bypass for cytosolic acetyl-CoA synthesis. Moreover, another strategy is to apply the ACL pathway. In mammalian cells, mitochondrial citrate is transported to the cytosol where it can be cleaved into acetyl-CoA and oxaloacetate by ATP citrate lyase (ACL), with hydrolysis of 1 molecule of ATP to ADP. Oxaloacetate can be converted to malate by malate dehydrogenase isoenzyme (MDH), and then to pyruvate by malic enzyme (ME; Fig. 4 the purple route). Zhou et al. were able to increase the production of free fatty acids and fatty acid-derived oleochemicals in *S. cerevisiae* by expressing cytosolic MDH3, ME and ACL with over-expressing yeast *CTP1*, the mitochondrial citrate transporter (Zhou et al. 2016). However, up till now, the methods of both cytosolic PDH complex substitution and ACL pathway have not been tested for terpenoid production.

The supply of NADPH can be increased by promoting pentose phosphate pathway or converting NADH to NADPH. For examples, glucose-6-phosphate dehydrogenase (*Zwf1*) catalyzes the rate-limiting step of pentose phosphate pathway and over-expression of *ZWF1* resulted in a 1.6-fold improvement of lycopene production and 1.2-fold improvement of β -carotene production (Zhao, Shi and Zhan 2015). Moreover, over-expression of *POS5*, which encodes a mitochondrial NADH kinase and converts NADH to NADPH, also increased the final yields of lycopene and β -carotene by 1.9 and 1.6-fold, respectively, respectively (Zhao, Shi and Zhan 2015).

PROGRESS OF TERPENOID SYNTHESIS IN YEAST

Monoterpenoids

Monoterpenoids are natural products predominantly produced in plants, with over 4000 structures and diverse commercial applications as fragrances, flavors, biofuels, pharmaceutical agents and insect repellents (Loza-Tavera 1999; Pateraki, Heskes and Hamberger 2015). Presently, monoterpenoids are produced through extraction from plants or by chemical synthesis. Although microbial production of monoterpenes has certain advantages, so far it is still far more costly than the conditional chemical route, particularly due to the high toxicity of many monoterpenes for microbes (Jongedijk et al. 2016).

Saccharomyces cerevisiae does not contain any endogenous monoterpene synthases (Jongedijk et al. 2016), but by transformation with monoterpene synthases originated from plants or bacteria and recruitment of the endogenous mevalonate pathway, *S. cerevisiae* can produce specific monoterpenes, as shown in Table 1. In *S. cerevisiae*, *Erg20*, the only FPP synthase, converts 1 molecule of DMAPP and 2 molecules of IPP to FPP, with low availability of GPP as the intermediate product (Jongedijk et al. 2016). GPP is the precursor for biosynthesis of most monoterpenoids,

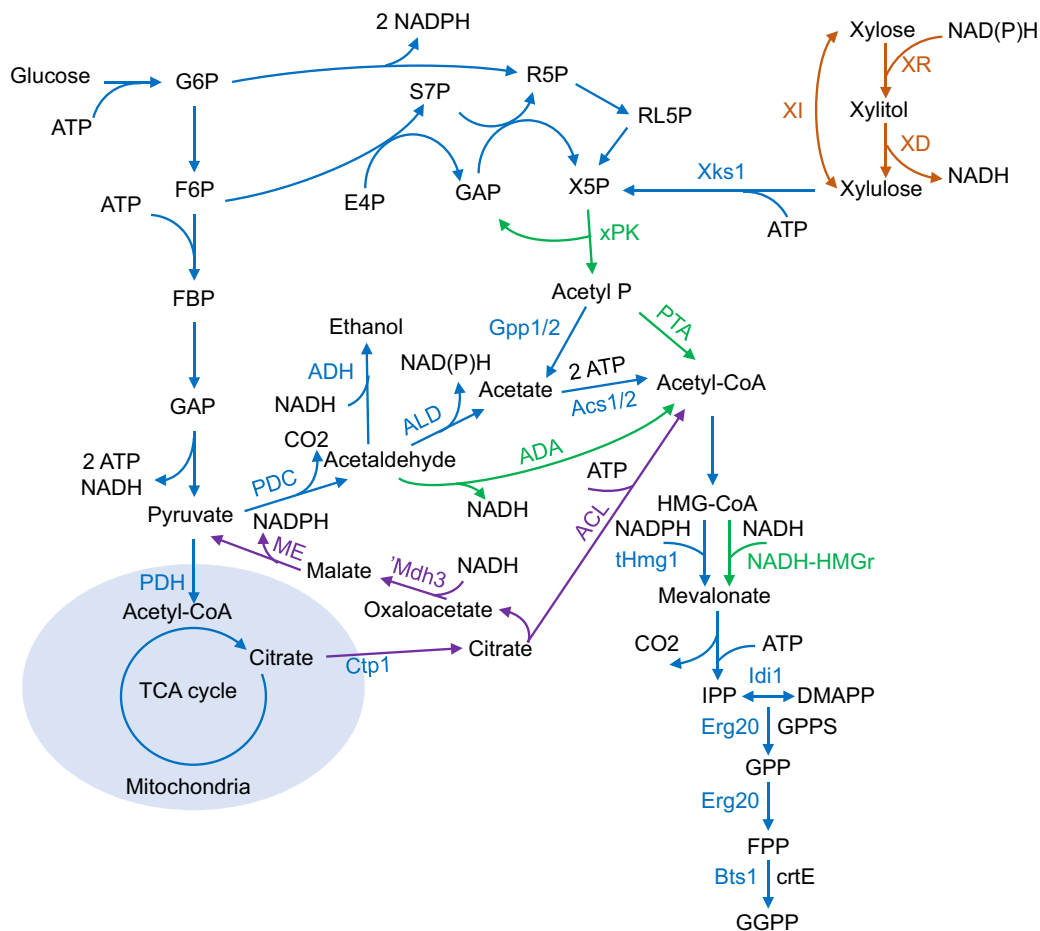


Figure 4. Manipulate metabolic pathways for optimizing biosynthesis of acetyl-CoA-derived terpene precursors and balancing cofactors in *S. cerevisiae*. The blue routes are the *S. cerevisiae* endogenous pathway, the green routes are the xPK-PTA-ADA pathway (Meadows et al. 2016), the purple routes are the ACL-based acetyl-CoA pathway, and the brown routes are xyllose pathway. PDH: pyruvate dehydrogenase complex, PDC: pyruvate decarboxylase isozymes, ADH: alcohol dehydrogenases, ACL: aldehyde dehydrogenases, xPK: xylulose-5-phosphate phosphoketolase, PTA: phosphotransacetylase, ADA: acetaldehyde dehydrogenase (acylating), NADH-HMG: NADH-specific HMG CoA reductase, ACL: ATP: citrate lyase, Ctp1: citrate transporter 1, 1^{mdh3}: cytoplasmic malate dehydrogenase isoenzyme 3, ME: malic enzyme, XR: Xyllose reductase, XD: Xylitol dehydrogenase, XI: Xylose isomerase, and GPPS: Geranyl pyrophosphate synthase.

and this is the rate-limiting step for monoterpenoid production in *S. cerevisiae*. To increase the GPP pool, one approach has been to mutate Erg20 at the position K197 to E, whereby further extension of GPP is blocked, resulting in an increased GPP production and reduced FPP production (Blanchard and Karst 1993; Oswald et al. 2007). Fischer et al. (2011) extended this approach by screening a library of Erg20 K197 mutations, and they found that the mutations of K197 to G, A, L, C, S, T, D and E improved the production of geraniol, linalool and citranellol by 10–20-folds with the expression of geraniol synthases from *Ocimum basilicum*. Besides Erg20 K197 mutants, Ignea et al. identified the double mutant of Erg20^{WW} (F96W and N127W) that also allowed for increasing GPP pool and the production of sabinene was increased by 10.4-fold (Ignea et al. 2014). Besides promoting the GPP pool, engineering of monoterpene synthases is important as well. There are several recent reports that prenyltransferase-terpene synthase fusion proteins that significantly increased monoterpene production, as fusion enzymes may promote the efficiency of sequential reactions (Table 1). Deng et al. (2016) used the fusion protein of Erg20^{K197E} and (S)-linalool synthase AaLS1 from *Actinidia arguta* and the production of (S)-Linalool was increased by 1.7-fold comparing to the strains with two independent enzymes. Ignea et al. (2014) applied the fusion protein of Erg20^{WW}

and *Salvia pomifera* sabinene synthase to the HMG2 (K6R) over-expression yeast and enabled a 340-fold increase in the production of sabinene. Zhao et al. (2017) had recently reported the highest production of geraniol so far at titer of 1.69 g/L in bioreactor, and their strategies included over-expressing a fusion protein of Erg20^{WW} and tVoGES (truncated geraniol synthase from *Valeriana officinalis*), over-expressing tHMG1, IDI1 and *upc2-1*, controlling ERG20 expression by using the HXT1 promoter, and deleting OYE2 or ATF1 to avoid geraniol conversion to other terpenoids. To improve monoterpene production, toxicity is another important aspect. To address this issue, adaptive evolution of yeast strains in monoterpene environments and process development of more actively removing monoterpenes from the fermentation broth, may be carried out to improve the production of monoterpenes in *S. cerevisiae* (Zhuang and Chappell 2015).

Sesquiterpenoids

Sesquiterpenoids are a large group of terpenoids with around 14 000 compounds identified so far (Pateraki, Heskes and Hamberger 2015). By transformation with sesquiterpenoid synthases originated from plant or bacteria, *S. cerevisiae* can

Table 1. Examples of engineering *S. cerevisiae* for monoterpene and monoterpene production.

Product	Approaches	Yield	Reference(s)
(-)-Limonene	• (-)-limonene synthase from <i>Perilla frutescens</i> • Erg20 K197G	0.49 mg/L Headspace trapping	Jongedijk et al. (2015)
Limonene	• Limonene synthase from <i>Citrus limon</i> • P_{Gal1}-tHMG1, P_{Gal1}-upc2-1	1.48 mg/L Dodecane organic phase	Behrendorff et al. (2013)
(S)-Linalool	• Over-expression of Erg20^{K197E} and (S)-linalool synthase from <i>Actinidia arguta</i> fusion protein • diploid	0.26 mg/L in bioreactor	Deng et al. (2016)
Geraniol	• Geraniol synthase from <i>Ocimum basilicum</i> • Erg20 K197G, A, L, C, S, T, D, and E	Around 5 mg/L	Fischer et al. (2011)
Sabinene	• 2μ plasmid of P_{TDH3}-ERG20 (F96W-N127W)- sabinene synthase (<i>Salvia pomifera</i>) fusion protein • diploid ERG9/erg9, ERG20/erg20, • P_{Gal1}-HMG2 (K6R), P_{TDH3}-HMG2 (K6R) $\times$ 2	17.5 mg/L	Ignea et al. (2014)
Geraniol	• 2μ plasmid of P_{TEF1}-tVoGES-(GGGS)-ERG20^{WW} fusion protein • 2μ plasmid of P_{TEF1}-tHMG1, P_{PGK1}-IDI1, P_{TEF1}-upc2.1 • P_{HXT1}-ERG20, oye2Δ	1.69 g/L with pure ethanol feeding in fed-batch fermentation	Zhao et al. (2017)

produce specific sesquiterpenoids. For example, amorphadiene, a key precursor for semi-synthesis of the anti-malarial drug artemisinin, can be produced in *S. cerevisiae* through FPP cyclization by amorphadiene synthase (ADS; Peralta-Yahya et al. 2011). Due to their physicochemical properties, many sesquiterpenes are suitable diesel and jet-fuel substitutes. With these wide applications, there is an increasing attention and significant advancements in terms of sesquiterpene production by yeast (as seen in Table 2; Ro et al. 2008; Albertsen et al. 2011; Peralta-Yahya et al. 2011; Scalcinati et al. 2012a; Meadows et al. 2016).

Several studies have successfully engineered *S. cerevisiae* for sesquiterpene production followed by manipulations of genes in the yeast MVA to increase flux towards sesquiterpenes. As described above, the established strategies are over-expression of tHMG1, over-expression of upc2-1, and down-regulation of ERG9 to increase the FPP pool (Peralta-Yahya et al. 2011). Combining these engineering strategies with over-expression of ERG20 and ADS by a high copy plasmid, Ro et al. (2008) were able to produce amorphadiene at titer up to 800 mg/L in shake flasks. Westfall et al. (2012) applied a similar strategy with the *S. cerevisiae* strain CEN.PK2 integrated additional copies of ERG10, 13, 12, 8 and IDI1 genes under control of galactose-inducible promoters, together with over-expression of upc2-1 and 3 copies of tHMG1, and deletion of GAL80, and reached the highest production of amorphadiene so far at titer of 40 g/L in bioreactor. As mentioned above, Meadows et al. (2016) rewired the yeast pathway (xPK-PTA-ADA) to increase cytosol acetyl-CoA and balanced the co-factors by expressing an NADH-HMGr and were able to produce β -farnesene at titer of 130 g/L in a 200 000-litre industrial bioreactor.

Diterpenoids

Diterpenoids (C20) constitute a large group of compounds derived from GGPP with over 12 500 structures (Pateraki, Heskes and Hamberger 2015). By expressing diterpene synthase, yeast can utilize GGPP to synthesize diterpenes. For example, taxadiene, a diterpene precursor to the anti-cancer drug taxol (paclitaxel), was successfully synthesized in *S. cerevisiae* by expressing the taxadiene synthase from *Taxus brevifolia* (Engels, Dahm and Jennewein 2008). However, compared to yeast production

of sesquiterpenes, including titers of 130 g/L of β -farnesene and 40g/L of amorphadiene in bioreactors, yeast production of diterpenoids is far less efficient. One important reason is that yeast Bts1, the only GGPP synthase, catalyzes the rate-limiting step in diterpene synthesis. The general strategies to increase the GGPP pool are to over-express Bts1 or a heterologous GGPP synthase, and down-regulate ERG9 expression to reduce competitive ergosterol pathway (Dai et al. 2012). Recent successful approaches for diterpene production in yeast are to apply the Bts1-ERG20 fusion protein, as fusion enzymes can increase the association of successively acting enzymes and allow intermediates for channeling towards the end-products. For example, militradiene, a diterpene precursor to tanshinones, a group of natural products in Chinese medicinal herb *Salvia miltiorrhiza*, was produced in MVA engineered *S. cerevisiae* through over-expression of fusion protein of diterpene synthases SmCPS and SmKSL with GGPP synthase from *Sulfolobus acidocaldarius* as well as an Erg20-Bts1 fusion protein, at titer of 488 mg/L in fed-batch bioreactor (Dai et al. 2012). Similarly, geranylgeraniol, a valuable diterpene alcohol for perfume ingredients and pharmaceutical products, was produced in diploid prototrophic strain of *S. cerevisiae* with multicopy integrations of HMG1, BTS1-DPP1 fusion genes and ERG20- BTS1 fusion genes, at titer of 3.31 g/L in fed-batch bioreactor (Tokuhito et al. 2009).

For synthesis of more complex diterpenoids, diverse modifying enzymes such as P450s need to be identified (Andersen-Ranberg et al. 2016; Guo et al. 2016). Ignea et al. (2015) have developed an interchangeable three module system, module 1 is prenyl diphosphate synthases for carbon skeleton elongation, module 2 is class I and class II TSs, and module 3 is P450s to further modify the products. They applied this platform to test a promiscuous library of cytochrome P450s for structurally distinct diterpenes and reported interchangeable production of a varieties of diterpenes including militradiene (26 mg/L), manoyloxide (35 mg/L), sclareol (87 mg/L), cis-abienol (3.9 mg/L), manool (96 mg/L) and Z-biformene (1.3 mg/L; Ignea et al. 2015). Then, they applied this platform to establish the biosynthetic pathway of the anti-oxidant carnosic acid in *S. cerevisiae* and identified that the combination activities of four P450s in *Lamiaceae* (CYP76AH24, CYP71BE52, CYP76AK6 and CYP76AK8) are responsible for all the oxidation events for synthesizing carnosic acid (Ignea et al. 2016).

Table 2. Examples of engineering *S. cerevisiae* for sesquiterpene and sesquiterpenoid production.

Product	Approaches	Yield	Reference(s)
β -Farnesene	• Over-expression of Farnesene synthase, ADA, xPK, PTA, NADH-HMGr • <i>adh1</i>Δ, <i>ald4</i>Δ, <i>ald6</i>Δ, <i>gpp1</i>Δ, <i>gal2</i>Δ, <i>bdh1</i>Δ • Over-express enzymes of the MVA pathway to Erg20 • Downregulation of ERG9 • Multiple rounds of mutagenesis	130 g/L Industrial bioreactor	Meadows et al. (2016)
Amorpha-4,11-diene	• Over-expression of ADS on a 2μ <i>leu2d</i> plasmid • Additional copies of ERG10, 13, 12, 8, and IDI1 genes integrated and under control of galactose-inducible promoters, 3 copies of tHMG1, over-expression of <i>upc2-1</i>, and <i>gal80</i>Δ • Downregulation of ERG9	>40 g/L Fermenter Restricted ethanol-pulse feeding and glucose feeding	Westfall et al. (2012)
Artemisinic acid	• Over-expression of ADS and CYP71AV1 on a 2μ plasmid • Over-expression of CPR1, CYB5, ALDH1 from <i>A. annua</i> (2 copies), ADH1 from <i>A. annua</i> (2 copies), HEM1, and CTT1 under control of galactose-inducible promoters • <i>gal80</i>Δ • Down-regulation of ERG9 by CTR3 promoter	25 g/L in bioreactor	Paddon et al. (2013)
Artemisinic acid	• Over-expression of ADS/(AMO or CYP71AV1)/CPR1 on a 2μ <i>leu2d</i> plasmid • Over-expression of tHMG1, ERG20, <i>upc2-1</i> • Downregulated ERG9 expression	250 mg/L in shake flask and 1 g/L in bioreactor.	Ro et al. (2008)
Bisabolene	• Over-expression of <i>upc2-1</i>, tHMG1, ERG20 and BIS (bisabolene synthase) • Downregulation of ERG9	>900 mg/L Dodecane Fermenter	Peralta-Yahya et al. (2011)
α -santalene	• Over-expression of tHMG1 • <i>lpp1</i>Δ, <i>dpp1</i>Δ • Downregulation of ERG9	92 mg/L Fermenter	Scalcinati et al. (2012a)
Patchoulol farnesol	• Over-expression of ERG20 and PatTps177 on a 2μ plasmid • Downregulation of ERG9	40.9 mg/L 42.1 mg/L Fermenter	Albertsen et al. (2011)

Triterpenoids

Triterpenoids are the largest compounds in known terpenoid family with over 23 000 structures, including plant growth regulators or animal hormones (Pateraki, Heskes and Hamberger 2015). For synthesis of triterpene precursor, two molecules of FPP are condensed into a squalene by squalene synthase, and then oxidized into 2,3-squalene epoxide by squalene epoxidase, consuming a cofactor of NADPH. In addition, 2,3-squalene epoxide is cyclized by various cyclases to produce a wide variety of structures with two to five rings, and these structures are further modified into diverse triterpenoids. For example, botryococene, a triterpene source for biodiesel and major oil component of the green algae *Botryococcus braunii*, was produced in *S. cerevisiae* by over-expression of SSL1-SSL3 (squalene synthase like genes in *Botryococcus braunii*) in the ERG9 knock-out strain at titer of 70 mg/L (Niehaus et al. 2011). In addition, heterologous expressions of P450-CPR combinations are critical for complex triterpenoid production in yeast (Fukushima et al. 2013). Hydrocortisone, a major mammalian adrenal glucocorticoid and an important intermediate of steroidal drug, was fully biosynthesized in engineered yeast by rerouting endogenous sterol pathway by expressing eight mammalian proteins (CYP11A1, adrenodoxin (ADX), mitochondrial ADX, ADX reductase (ADR), CYP11B1, 3 β -HSD, CYP17A1 and CYP21A1) with knocking-out of GCY1, YPR1 and ATF2 for reducing side reactions (Szczubara et al. 2003).

Tetraterpenoids (carotenoids)

Tetraterpenoids are derived from phytoene, which is condensation of two GGPP. Tetraterpenoids often referred to as carotenoids, including lycopene, β -carotene, γ -carotene, torulene and astaxanthin (Pateraki, Heskes and Hamberger 2015; Paramasivan and Mutturi 2017). Carotenoids are valuable molecules, not only because they can function as food supplements serving as vitamin A1 precursors, but also can serve as colorants with yellow to red colors. Because of extensive system of conjugated double bonds, carotenoids also have antioxidant properties, which promotes health by scavenging oxidative stress (Fiedor and Burda 2014; Mata-Gomez et al. 2014). Carotenoids are naturally produced by plants, algae, red yeast and phototrophic bacteria (Mata-Gomez et al. 2014). The production of carotenoids is still limited by low yields from plant extraction or biosynthesis, and it is therefore interesting to produce carotenoids using microorganisms (Mata-Gomez et al. 2014).

Although *S. cerevisiae* naturally does not produce carotenoids, it produces GGPP, the precursor of phytoene. With two additional carotenogenic enzymes, phytoene synthase (*crtYB*) and phytoene desaturase (*ctrl*) from a red yeast species *Xanthophyllomyces dendrorhous* (*X. dendrorhous*), *S. cerevisiae* can produce β -carotene (Verwaal et al. 2007). The conversion of FPP to GGPP by Bts1 is the rate limiting step in carotenoid production, and expression of an additional GGPP synthase encoded by *crtE* from *X. dendrorhous* resulted in increased production of β -carotene to

5.9 mg/g dry cell weight (DCW; Verwaal et al. 2007). Moreover, Chen et al. (2016) compared different combinations of crtE, phytoene synthase (crtB) and crtI from diverse species for lycopene production, and reported a final production at 55.56 mg/g DCW using fed-batch fermentation.

It is worth to mention that, one application of carotenoid production is to serve as a biosensor for screening yeast libraries for strains with high supply of FPP, GPP and GGPP. The visual sensor from the intensity of redness will be an indication of GGPP levels in yeast, which can be used for phenotypic screen of the yeast library to reveal new genes or mutations resulting in high GGPP pool (Ozaydin et al. 2013). By applying the same deletions or mutations from the results of screening into another specific terpenoid production strain, the production of specific terpenoid may be increased. For example, Ozaydin et al. (2013) introduced carotenoid biosynthetic enzymes into the yeast deletion collection to identify gene deletions that could improve carotenoid production and showed that the same deletions (*rox1*, *yil064w* and *ypl062w*) can improve the production of another strain for bisabolene by around 2-folds.

CHALLENGES AND OUTLOOK

Key lessons from the above-discussed studies are that engineering yeast metabolism for high-levels of terpenoid production requires three elements: (1) expression of an efficient pathway leading to the final product; (2) over-expression of key, or in some cases even all, upstream enzymes of the mevalonate pathway; and (3) rewiring yeast metabolism for efficient provision of cytosol acetyl-CoA and co-factors, in particular NADPH. Most work so far has focused on over-expressing enzymes of the mevalonate pathway, and several recent studies, including the complete rewiring of yeast metabolism as described by Meadows et al. (2016), have shown that increasing acetyl-CoA supply can significantly improve terpenoid production (Asadollahi et al. 2009; Chen, Siewers and Nielsen 2012; Scalcinati et al. 2012b). There are, however, many other ways to increase cytosol acetyl-CoA supply that have not yet been tested. One example is using the ACL pathway, which may provide additional acetyl-CoA for terpenoids production from citrate (Zhou et al. 2016). In choice of pathway it is, however, important to consider not only the rate, but also the overall yield that can be obtained with the specific pathway, and it may therefore be necessary to combine different pathways for provision of acetyl-CoA as illustrated in the study of Meadows et al. (2016), who combined the xPK-PTA pathway with a pathway for the conversion of acetaldehyde to acetyl-CoA.

If the product is used as a biofuel or commodity chemical it is important that costs are extremely low, and it may therefore be necessary to consider the use of cheap feedstocks, such as biomass hydrolysates. In these cases, it is necessary to engineer the yeast to utilize other carbon sources besides glucose, in particular xylose and arabinose, as this enables yeast to utilize agro-waste such as corn stalks. By integrating either xylose reductase and xylitol dehydrogenase or xylose isomerase, the yeast can ferment xylose (Fig. 4 the brown route), but it is generally necessary to optimize the uptake rate of xylose as these pathways are relatively inefficient. Such optimization may require adaptive laboratory evolution or extensive engineering of the central carbon metabolism.

A challenge with the production of terpenoids is that some of these molecules are toxic for yeast. This is particularly true for many monoterpenes, which generally have anti-microbial activity, and this might be the reason for the very low titers obtained

for these chemicals. Parveen et al. (2004) has performed genome-wide expression analysis of *S. cerevisiae* grown in the presence of 0.02% α -terpene, a monoterpene, and found more than 2-fold expression changes of 435 genes. Furthermore, de Bont and Poolman (1995) found that intra-membrane accumulation of cyclic sesquiterpenes results in cell toxicity by disrupting the membrane integrity. The best way to overcome these toxicity issues is to perform adaptive laboratory evolution where the cells are exposed to increasing concentration of the terpenoids of interest (Zhuang and Chappell 2015). Alternatively, one may continuously remove the product from the culture medium during fermentation, for example, using a two-phase system or by headspace removal. Most terpenoids have low water solubility and the product may therefore associate with gas bubbles and hereby be stripped from the bioreactor, even though their flash point is much higher than that of water. In order to reduce toxicity and stripping of the product from the bioreactor, *in situ* product removal (ISPR) is often applied to capture the product (Stark and von Stockar 2003). For this dodecane has shown to be well suited for capturing terpenoids, in particular sesquiterpenes, as it has good biocompatibility with *S. cerevisiae* (Tippmann et al. 2016). Using ISPR, it is hereby possible to constantly extract the product into an organic phase and reduce potential toxicity to the cells, and the product can subsequently easily be extracted from the organic phase that can then be reused.

In conclusion, recent developments have clearly demonstrated that it is possible to engineer *S. cerevisiae* for high-level production of terpenoids, and despite several challenges we are therefore confident that this yeast may serve as an excellent cell factory platform for wider bio-based production of terpenoids in the future.

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