



REVIEW ARTICLE



Progress in terpene synthesis strategies through engineering of *Saccharomyces cerevisiae*

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ABSTRACT

Terpenes are natural products with a remarkable diversity in their chemical structures and they hold a significant market share commercially owing to their distinct applications. These potential molecules are usually derived from terrestrial plants, marine and microbial sources. *In vitro* production of terpenes using plant tissue culture and plant metabolic engineering, although receiving some success, the complexity in downstream processing because of the interference of phenolics and product commercialization due to regulations that are significant concerns. Industrial workhorses' viz., *Escherichia coli* and *Saccharomyces cerevisiae* have become microorganisms to produce non-native terpenes in order to address critical issues such as demand-supply imbalance, sustainability and commercial viability. *S. cerevisiae* enjoys several advantages for synthesizing non-native terpenes with the most significant being the compatibility for expressing cytochrome P450 enzymes from plant origin. Moreover, achievement of high titers such as 40 g/l of amorphadiene, a sesquiterpene, boosts commercial interest and encourages the researchers to envisage both molecular and process strategies for developing yeast cell factories to produce these compounds. This review contains a brief consideration of existing strategies to engineer *S. cerevisiae* toward the synthesis of terpene molecules. Some of the common targets for synthesis of terpenes in *S. cerevisiae* are as follows: overexpression of *tHMG1*, *ERG20*, *upc2-1* in case of all classes of terpenes; repression of *ERG9* by replacement of the native promoter with a repressive methionine promoter in case of mono-, di- and sesquiterpenes; overexpression of *BTS1* in case of di- and tetraterpenes. Site-directed mutagenesis such as *Upc2p* (G888A) in case of all classes of terpenes, *ERG20p* (K197G) in case of monoterpenes, *HMG2p* (K6R) in case of mono-, di- and sesquiterpenes could be some generic targets. Efforts are made to consolidate various studies (including patents) on this subject to understand the similarities, to identify novel strategies and to contemplate potential possibilities to build a robust yeast cell factory for terpene or terpenoid production. Emphasis is not restricted to metabolic engineering strategies pertaining to sterol and mevalonate pathway, but also other holistic approaches for elsewhere exploitation in the *S. cerevisiae* genome are discussed. This review also focuses on process considerations and challenges during the mass production of these potential compounds from the engineered strain for commercial exploitation.

ARTICLE HISTORY

Received 5 May 2016
Revised 20 August 2016
Accepted 28 November 2016

KEYWORDS

Terpenes; terpenoids;
Saccharomyces cerevisiae;
metabolic engineering;
sterol pathway

Introduction

Natural products have their origin from diverse living organisms including terrestrial plants, microbes and marine species. There are more than 200,000 plant natural products identified so far [1]. Natural products could be either primary or secondary metabolites. The classes of secondary metabolites include polyketides (antibiotics), phenylpropanoids (flavonoids, stilbenes and lignin), alkaloids and terpenoids. Terpenes or terpenoids (oxygenated forms of terpenes) otherwise called as isoprenoids (as they are built from isoprene, C_5H_8 units) are the oldest known, structurally diverse and largest category of molecules with more than 55,000

structures elucidated so far [2]. Terpenes include both primary as well as secondary metabolites and forms the main constituent of essential oils. Terpenes could be used as flavors, fragrances, colorants and medicine in the food, cosmetics and pharmaceutical industries and have a surging demand in forthcoming years [3]. The Asian flavor and fragrance industry is estimated to reach \$9.6 billion and the global flavor and fragrance industry is estimated to reach a total market size of \$33.5 billion in 2019 [4].

Terpene biosynthesis involves two distinct pathways, produced by the mevalonate (MVA) pathway in yeast (Figure 1(A)) [5], whereas in prokaryotes like

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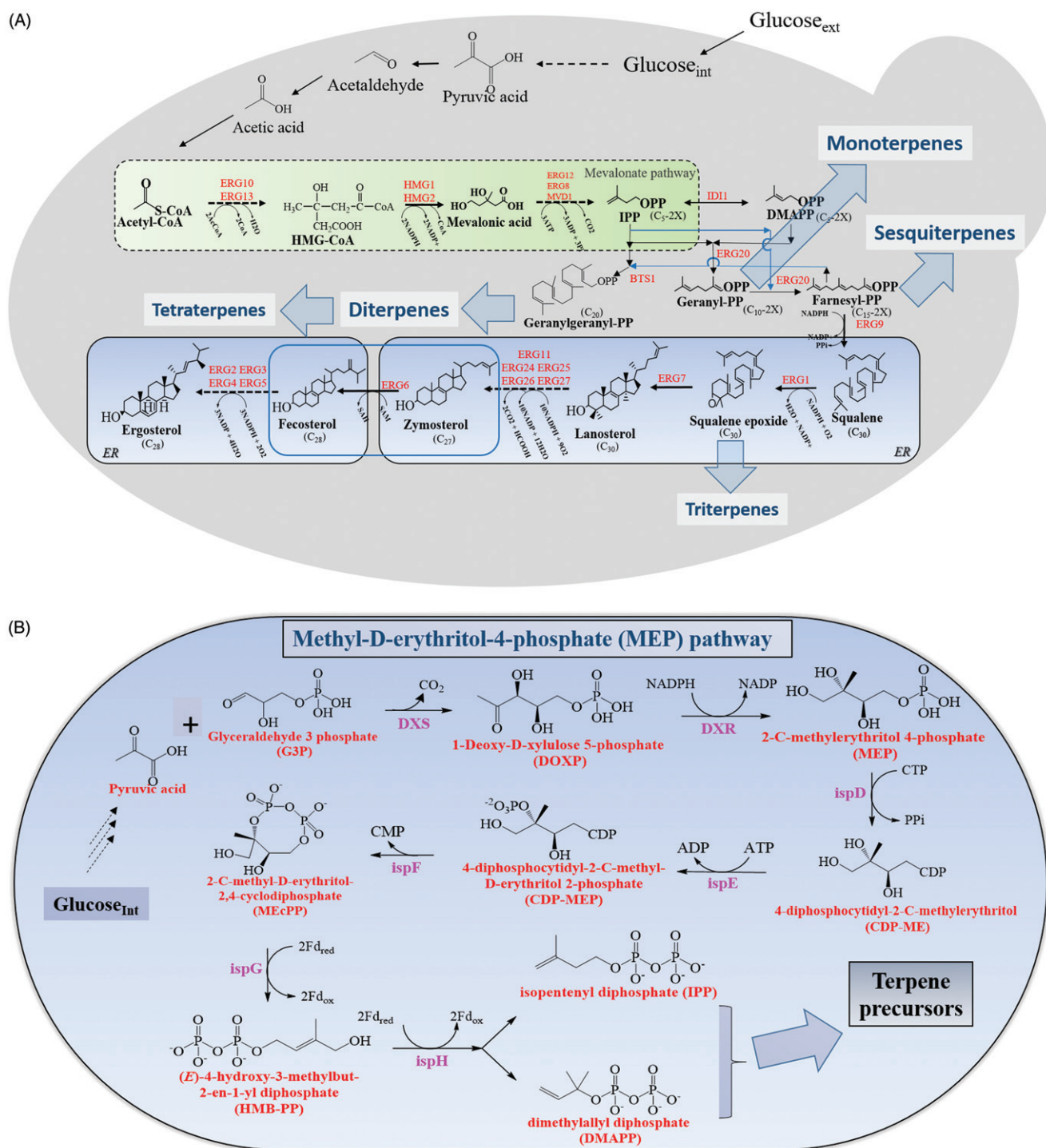


Figure 1. (A) Compartmentalized depiction of sterol pathway in *S. cerevisiae*. The solid and dotted arrows indicate single and multiple steps, respectively. The genes involved in individual or multiple reactions are shown above the arrow in red font. The box around fecosterol and zymosterol indicates the reaction occurring inside mitochondrion via translocation reactions (SAM, S-adenosyl-L-methionine; SAH, S-Adenosyl-L-homocysteine) [114]. The energy (ATP) and electron ((NADPH) balances are stoichiometrically correct. (B) The depiction of MEP pathway in *E. coli* (adapted from ecocyc.org). The solid and dotted arrows indicate single and multiple steps, respectively. DXS, DOXP synthase; DXR, DOXP reductase; ispD, MEP cytidyltransferase; ispE, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; ispF, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ispG, HMB-PP synthase; ispH, HMB-PP reductase.

Escherichia coli, these compounds are produced via the methyl-D-erythritol-4-phosphate (MEP) pathway (Figure 1(B)) [6]. A study shows in some organisms possess the both pathways. In such cases, MEP pathway is involved in primary metabolite formation whereas the MVA pathway in secondary metabolite formation [7]. Plants employ both pathways, while the MEP pathway is localized in plastids. Pyruvate and glyceraldehyde-3-phosphate, G3P act as precursors for the MEP pathway while two molecules of acetyl-CoA form the basis for MVA pathway. The three main processes of terpenoid production are plant cell culture, microbial fermentation and chemical synthesis. However, microbial fermentation is more desirable owing to its low cost of production and eco-friendly nature [8]. Since the publication of "Life with 6000 genes" [9] followed by release of the entire genome in the public domain, *Saccharomyces cerevisiae* has become an indispensable eukaryotic system and considerable knowledge of its physiology, genetics, biochemistry, gene manipulation techniques [10], cultivation strategies and computational methods has accumulated, thereby enhancing its scope for industrial exploitation. Synthetic biology efforts have led to the "cutting edge" development of the synthetic version of yeast cells with modification of the whole genome in a project called Sc.20 [11,12]. Though there are several advantages of using *S. cerevisiae* as a host organism, the pathways are compartmentalized in yeast which complicates the process as the intermediates have to translocate themselves from one compartment to another to carry out certain reactions in order to continue the pathway. Hence, the availability of precursors varies amongst the compartments. Unlike bacterial cells, *S. cerevisiae* cells do not have their genes organized as operons inside which make the genetic manipulation for strain improvement a more laborious process when compared to prokaryotes. Moreover, unlike plant cells, *S. cerevisiae* do not possess diverse class of Cytochrome P450 (CYP-CPR) enzymes which carry specific oxygenation reactions to produce structurally diverse terpenes. Hence, they needed to be heterologously expressed from plant origin. To some extent, these challenges have been addressed and are reported in subsequent sections in this review.

Because of the exclusive nature so far published in terpenoid production studies, the need for a comprehensive review article on *S. cerevisiae* considering the production of a diverse class of terpene molecules, the present review is relevant. Data "mining" was carried out with a special focus on the recent literature during in the last 5 years in order to consolidate information on this subject. This review covers the following topics: elucidation of the stoichiometric balance for terpene

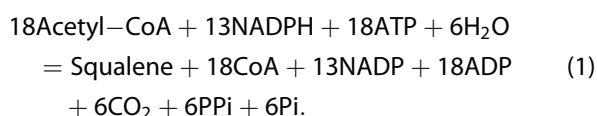
synthesis from the sterol pathway, description of different terpenes produced in yeast, genetic perturbations carried within and outside the sterol pathway to improve terpene production, application of *in silico* strategies, process considerations and finally, in conclusion, a consideration of future perspectives on this subject.

Sterol pathway and terpene theoretical yield calculations

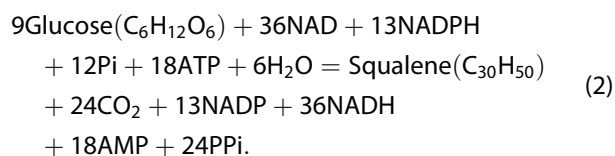
The MVA pathway extends into ergosterol (ERG) biosynthesis in *S. cerevisiae*, and the synthesis of ERG from acetyl-CoA is referred as the sterol pathway (Figure 1(A)). Figure 1(A) depicts the compartmentalized sterol pathway spanning the cytoplasm and endoplasmic reticulum of *S. cerevisiae*. The synthesis of ERG from acetyl-CoA involves 20 distinct metabolic steps with specialized structural genes which fuel these reactions as shown in Table S1. Following assumptions have been considered for framing the stoichiometric equations:

- Glucose to pyruvate formation does not include pentose-phosphate pathway.
- Pyruvate to acetyl-CoA is via pyruvate dehydrogenase (PDH)-bypass where NAD is selected as cofactor for reaction involving *ALD5*.

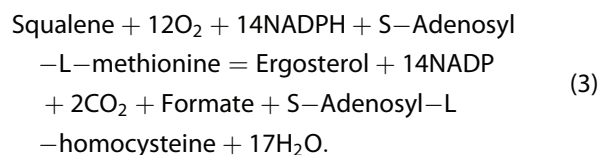
The overall stoichiometry for squalene production from acetyl-CoA summarized below indicates that 18 moles both acetyl-CoA and ATP are required in this energy-intensive pathway to produce a single mole of squalene:



From glucose, the overall stoichiometry for squalene production is as follows:

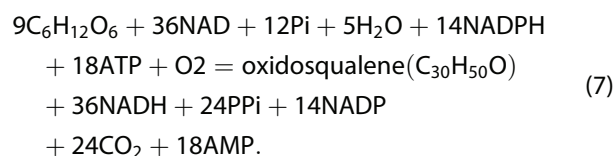
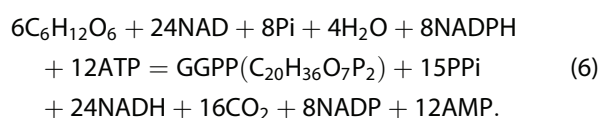
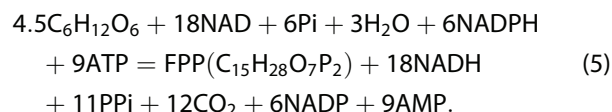
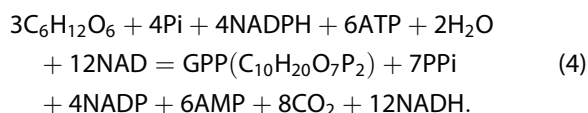


Further downstream reactions of squalene leading to ERG require oxygen and the stoichiometric balance is as follows:



Also, both Equations (1) and (3) demonstrate the high requirement of NADPH as a source of electrons. In Equation (1), the reactions carried by HMG-CoA reductases (Hmg1p, Hmg2p) and squalene synthase (Erg9p) are NADPH-dependent (Figure 1(A)). Whereas, in the lower half of sterol pathway represented by Equation (3), NADPH is utilized in most of the reactions including Erg11p and Erg5p, which belong to the cytochrome P450 family.

The stoichiometric closure for overproduction or heterologous production of a compound facilitates and sometimes becomes *a priori* for pathway engineering as it dictates and demonstrates the energy requirement that governs the efficiency of producing the required compound. Based on stoichiometric calculations [13], it was observed that the pathways where reducing equivalents are generated (NADH/NADPH) along with product formation are usually inefficient in terms of yield in comparison to those pathways which require supply of reducing equivalents for product formation. The individual stoichiometric balance for each of the terpene class precursors from glucose as the substrate is provided in separate equations below:



These Equations (4–7) indicate the requirement of NADPH and ATP for the synthesis of the precursor molecules with concomitant release of NADH. This NADH can be either utilized during other biosynthetic pathways or end up in a futile pathway as a sink. The yield for the different classes of terpene molecules were calculated according to the framework developed by Dugar and Stephanopoulos [13] and is tabulated in Table 1. It can be observed from this table, that the pathway yields (Y^P , $Y^{P,G}$ and $Y_{Cl}^{P,G}$) are lower than the maximum yields for all the terpenes due to the generation of excess reducing equivalents (NADH). This data also indicated that the actual yields will be either lower

Table 1. Estimation of possible yields for different terpenes produced in *S. cerevisiae* [13].

Terpene type	Compound/s	Formula	Yields (g terpene/g glucose) ^a			
			Y^E	Y^P	$Y^{P,G}$	$Y_{Cl}^{P,G}$
Mono-	Cineole, Geraniol	$C_{10}H_{18}O$	0.317	0.317	0.239	0.102
	Limonene, Sabinene	$C_{10}H_{16}$	0.324	0.324	0.236	0.101
Sesqui-	Amorphadiene, Bisabolene, Caryophyllene, Copaene, Cubebene, Epiaristolochene, Humulene, Cadinene, Premnaspirodiene, Valencene, α -santalene	$C_{15}H_{24}$	0.324	0.236	0.101	0.076
	Epicedrol, Farnesol	$C_{15}H_{26}O$	0.352	0.256	0.110	0.083
	Texadiene, Abietadiene, Casbene	$C_{20}H_{32}$	0.324	0.236	0.101	0.076
	Geranylgeraniol	$C_{20}H_{34}O$	0.345	0.251	0.108	0.081
	Scareol	$C_{20}H_{36}O_2$	0.367	0.267	0.114	0.086
	β -amyrin	$C_{30}H_{50}O$	0.338	0.246	0.111	0.079
	β -carotene, Lycopene	$C_{40}H_{56}$	0.331	0.232	0.099	0.075
Tri-						
Tetra-						

^a Y^E is maximum yield (γ_s/γ_p), Y^P is pathway yield (without cofactor imbalance), $Y^{P,G}$ is terpene yield with concomitant production of glycerol, which is assumed to be a sink for excess NADH (without cofactor imbalance), $Y_{Cl}^{P,G}$ is terpene yield with concomitant production of glycerol, a sink for NADH, with cofactor imbalance.

or close to the estimated yields. Varman et al. [14] built an empirical equation from various experimental studies for yield determination based on the type of gene manipulations carried out in *S. cerevisiae* for producing these compounds. Their methodology predicts the yield of a compound for a given set of genetic manipulations. Among these calculations, the yields of terpenes were in agreement with the yields compiled in Table 1. Thus, this kind of stoichiometric analysis would not only provide insight into the yield but also indicates pointers for genetic manipulation.

Genetic perturbations to improve terpene synthesis

The essence of metabolic engineering is to systematically improve the properties of cells by using analytical and computational methods to quantify fluxes and their control employing molecular tools and techniques [15–17]. Major strategies for global and pathway specific metabolic engineering, as described in [18] are:

- Increasing precursor and cofactor supply.
- Heterologous expression of non-native genes.
- Blocking or down regulating competing pathways.
- Overexpression of transcriptional regulators.
- Improving enzyme specificity.

The objective of this section is to present different specific strategies carried by several researchers in order to produce native and primarily non-native terpenes in

S. cerevisiae. The section is focused and classified based on the metabolic strategy rather than a class of terpenes. Furthermore, it is divided into perturbations carried inside the sterol pathway and more generic manipulations outside the sterol pathway to produce the target terpene. The consolidated information on the list of terpenes produced in *S. cerevisiae* along with details of metabolic strategies, titers generated and fold increment are provided in Table S2. This table is referred to in subsequent sections to discuss specific strategies implemented leading to robust strain development and the increase of the specific terpene upon perturbation.

Manipulations inside sterol pathway

Heterologous expression of terpene synthases

As discussed earlier, the sterol pathway leading to ERG formation involves intermittent compounds such as farnesyl pyrophosphate (FPP), geranyl pyrophosphate (GPP), geranylgeranyl pyrophosphate (GGPP) and squalene epoxide which can be diverted to specialized terpenes (Figure 2). This diversion of flux involves a class of enzymes known as terpene synthases that are not present in *S. cerevisiae* in contrast to plants and other organisms. The striking feature among all these synthases contains aspartate-rich motifs (DDXXDD) as conserved portions. Most of the heterologous genes for the functional expression of terpenes synthases are obtained from plant sources, or they are synthetically generated (from NCBI gene accession number) for expression in *S. cerevisiae*. S-Linalool synthase (LIS) was one among the earlier identified monoterpene synthase (MTS) and was isolated from stigmata of *Clarkia breweri*, a flowering plant [19]. Rico et al. [20] reported the successful expression of LIS cDNA in *S. cerevisiae* to produce 132 µg/l of linalool, a monoterpene used as a perfume ingredient. The studies on *C. breweri* were followed by isolation of terpene synthases from selected plant species and a list was compiled for both MTS and sesquiterpene synthases [21], which also includes gene bank accession numbers. Both monoterpenes and sesquiterpenes production in *S. cerevisiae* have been widely studied in comparison to others (Figure 2(A) and (B)). The diterpene synthases (DTS) list has also been compiled which provides details on its sources covering bacteria, fungi and plants [22]. Taxadiene synthase, a DTS from *Taxus brevifolia*, was successfully expressed in *S. cerevisiae* to produce taxadiene, a precursor for anti-cancer drug taxol [23]. Kirby et al. [24] investigated DTS produced in plants belonging to the *Euphorbiaceae* family for functional expression in *S. cerevisiae* to produce potential diterpenes such as neocembrene and casbene

(Figure 2(C)). The details on triterpene synthases (TTS), often referred to as oxido squalene cyclases, are comprehensively covered by [25]. Several TTS have been expressed in *S. cerevisiae*, the prominent being for the production of β-amyrin (Figure 2(D)). In the case of tetraterpenoids, the synthesis begins by condensation of two GGPP molecules to form phytoene (Figure 2(E)). Tetraterpenoids derived from phytoene, often referred to as carotenoids, includes lycopene, β-carotene, γ-carotene, torulene and astaxanthin. *S. cerevisiae* does not have the natural ability to produce these carotenoids. A red yeast species, *Xanthophyllomyces dendrorhous* has the capacity to produce carotenoids and is the source for isolation of genes for heterologous expression of carotenoids in *S. cerevisiae* [26] (Figure 2(E)).

Overexpression of structural genes in sterol pathway

One of the easiest and most widely applied strategy in metabolic engineering is to identify a rate-limiting reaction and overexpress the gene or genes that affect this reaction. However, it must be understood that the concept of a single rate-limiting step in isolation does not justify flux distribution for the entire pathway [27,28]. Hence, the term flux control coefficient has been adopted to suggest that some reactions possess a higher flux control coefficient relative to other reactions in a pathway. One such flux control reaction in the *S. cerevisiae* sterol pathway has been identified as HMG-CoA conversion to MVA which is catalyzed by Hmg1p or Hmg2p, products of *HMG1* and *HMG2*, respectively (Table S1) [29]. Further studies showed that these two isoforms contain a membrane binding domain (1–552 amino acids) which spans ER and a catalytic domain (553–1046 amino acids) [30]. Later, it was observed that overexpression of the only catalytic domain of *HMG1*, termed *tHMG1* (truncated *HMG1*), has improved squalene concentrations in *S. cerevisiae* [31]. This laid the foundation for overexpression of *tHMG1* in a large number of studies involving the production of terpenes in *S. cerevisiae* (Table S2). Hmg2p, an isoform of this reductase, is usually dominant during low oxygen conditions and hence has become the target for overexpression during anaerobic cultivation to produce squalene [32]. Products of *IDI1* and *ERG20* are key enzymes which lead to GPP and FPP formation that in turn could be diverted to monoterpene and sesquiterpene synthesis, respectively. In various studies, shown in Table S2, both these enzymes are overexpressed to produce the terpenes of interest in *S. cerevisiae*. A branch of the reaction from the main sterol pathway, catalyzed by Bts1p brings FPP and isopentenyl-PP (IPP), to synthesize GGPP, forms the precursor for diterpenes synthesis (Figure 2(B)). Hence, diterpene synthesis in *S. cerevisiae* is

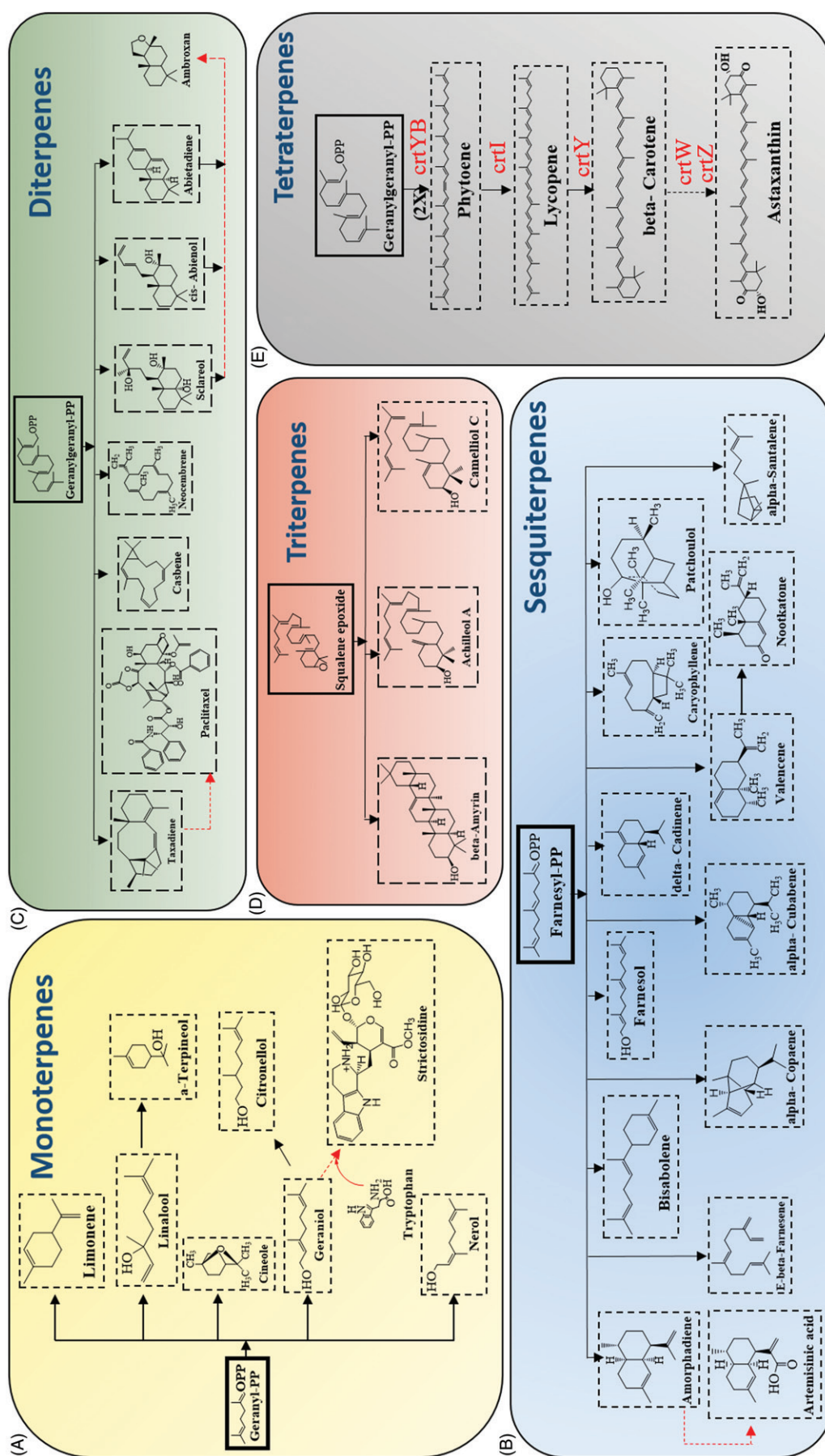


Figure 2. Biosynthetic routes to produce different class of terpenes from their corresponding precursor involving the heterologous expression of specific terpene synthases in *S. cerevisiae*. (A) Monoterpenes. (B) Sesquiterpenes. (C) Diterpenes. (D) Triterpenes. (E) Tetraterpenes.

sometimes facilitated by overexpression of *BTS1* [23,33] (Table S2). It is interesting to see in the studies by [34], they have overexpressed all the structural genes in the MVA pathway (*ERG10*, *tHMG1*, *ERG13*, *ERG12*, *ERG8*) and *ERG20* during synthesis of amorphaadiene (Table S2) and, as a result of this study, artemisinic acid production has been doubled whereas amorphaadiene production has been increased 10-fold to a titer value of ~40 g/l. During synthesis of acchileol A, a triterpene [35] overexpressed lanosterol synthase coded by the *ERG7* and different variants of the enzyme by introducing mutations in the *ERG7* gene. Essentially, overexpression of structural genes, prominently the ones upstream of squalene in the sterol pathway, has been targetted for producing terpenes in *S. cerevisiae*.

Knockout and knockdown

Deletion or down-regulation of a competing pathway has been proven as a successful strategy for developing an efficient cell factory [18]. Usually, this strategy is implemented by completely eliminating a gene involved in a competing pathway (referred as knockout) or lowering its expression levels by incorporating a weak promoter (referred as knockdown) and also sometimes enabling a mechanism to control the expression using a regulated or tunable promoter (referred as repression). The knockout strategy is preferred only if the target is a non-essential gene. *ERG6* is one of the non-essential genes that has been used as a target during squalene synthesis in *S. cerevisiae* [32]. Squalene synthase, coded by *ERG9*, is one of the key enzymes involved in sterol formation. Hence, *ERG9* has become the key target. Moreover, it is an essential gene. Knockdown and repression strategies for this gene have been widely studied and have proven successful. The *ERG9* gene was down-regulated by replacing its endogenous promoter with the tunable methionine promoter, P_{MET3} [24,34,36,37]. *ERG9* downregulation using the same strategy when combined with overexpression of *tHMG1* expression lead to a two-fold increase in amorphaadiene production [37]. Glucose-regulated promoters, P_{HXT1} and P_{HXT2} have also been used to exert tight control over the *ERG9* gene and has been successful in downregulation [38–40]. Comprehensive information on the level of expression using various promoters in *S. cerevisiae* can be obtained [41]. Single allele deletion of *ERG9* in a diploid strain was used as a strategy [42]. Homologous deletion of a gene in a diploid strain leads to a 50% reduction in its protein level in most of the cases [38].

Site-directed mutagenesis

Site-directed mutagenesis is an indispensable molecular tool for the direct evolution of enzymes.

Direct evolution leads to the enzyme characteristic enhancement such as their stability, solubility, substrate and cofactor specificity thereby leading to the development of tailor-made enzymes [43]. The crux of *in vitro* mutagenesis is to create a willful mutation at a specific position in the structural gene thereby generating a modified codon for translation. This approach has been an indispensable tool in protein engineering to enhance its functional properties and has been exploited to facilitate terpene production in *S. cerevisiae*. As *ERG20* fuels both FPP and GPP synthesis reactions, efforts have been made to manipulate this gene in order to change the ratios at which it catalyzes each of these condensation reactions. In one of the early studies, the AAG codon of *ERG20* gene was point mutagenized to GAG, thereby causing conversion of lysine (K) at the 197 position to glutamic acid (E) in the amino acid sequence of Erg20p [44]. This mutation has triggered production of prenyl alcohols (geraniol and linalool) which are otherwise not produced in *S. cerevisiae*. This study has inspired to probe into Erg20p conserved region, K197, in order to generate several point mutations and observe the response of monoterpene yields [45]. They have observed that K197G, A, L, C, S, T, R, D, E, N showed improved geraniol, linalool and citranello production and the levels were improved by 10–20-folds when geraniol synthases obtained from *Ocimum basilicum* was heterologously expressed in conjunction. The K197G strategy was also adopted in other studies to improve monoterpene in *S. cerevisiae* [46,47] (Table S2). According to patent US008481286B2 [48], several point mutations were generated in squalene synthase gene (*ERG9*) to improve FPP levels and also screened for viability so as to avoid the external addition of ERG for cultivation (Table S3). Amongst the isozymes, Hmg1p is quite stable contributing to 83% of reductase activity whereas its counterpart Hmg2p undergoes regulated degradation [29,49,50]. Essentially, it was lysine (K) at position 6 and 357 on Hmgp2 were found to be critical for degradation and these lysines were replaced to observe the stability of Hmgp2 [49]. Thus, the K6R (lysine to arginine replacement) mutant of Hmgp2 was utilized for improved reductase activity as a strategy for improved terpene production [32,42,51,52] (Table S2).

Expression of cytochrome P450 (CYP-CPR) enzymes

Cytochrome P450 (CYP) catalyze diverse metabolic reactions ranging from region- and specific- oxygenations to structural alterations such as ring modifications, deamination, dealkylation, decarboxylation and C-C cleavage. In the case of terpene synthesis, they carry

out several changes in intermediary terpene backbones and P450 monooxygenase is one of the major enzymes involved in such activities. The transfer of electrons from NADPH is via NADPH-dependent cytochrome P450 oxidoreductases (CPR) which acts as electron donors that colocalize along with monooxygenase [53]. CPR is membrane bound and is localized in the ER-membrane in the case of *S. cerevisiae*. The CYPs are classified into different clans, among which CYP71 comprises more than half of CYPs in higher plants [54]. Prominent clans involved during terpene synthesis in plants belong to CYP51, CYP71, CYP72, CYP76, CYP85 and CYP97 [55]. Ro et al. [37], Shiba et al. [56], and Paddon et al. [57] have successfully expressed CYP71AV1 and CPR from *Artemisia annua* in *S. cerevisiae* for the production of artemisinic acid, a sesquiterpene (Table S2). CYP71AV1 catalyzes three successive oxidations that take place from amorphadiene to artemisinic acid [58]. Co-expression of CYP71AV1, along with NADPH-dependent CPR and cytochrome b5, has improved artemisinic acid production to 25 g/l after process optimization in fed-batch cultivation [57]. Overexpression of CPR causes electron transfer coupling thereby leading to toxicity and also leads to reactive oxygen species (ROS) formation, whereas cytochrome b5 prevents reactive oxygen formation and toxic intermediates accumulation. Hence, cytochrome b5 is co-expressed along with CPR in yeast host cells. A similar strategy has been adopted where CYB5 and CPR from *Catharanthus roseus* were expressed in *S. cerevisiae* during the production of strictosidine [59] (Table S2). Selecting the right CYP-CPR system either from plants or of microbial origin to successfully express in *S. cerevisiae* is a challenge [60]. For oxygenation of (+)-valencene at the second position to produce (+)-nootkatone, Gavira et al. [60] expressed CYP71D4 from *Solanum tuberosum*, CYP71D51v2 from *Nicotiana tabacum*, CYP71D1 from *C. roseus* and CYP71D326 from *Ricinus communis* separately in *S. cerevisiae* to observe the efficacy of this CYP clan in a gene mining technique. They have also observed that the synthesis of the alcohol derivative (nootkatol in their case) causes inhibition of CYP. Fukushima et al. [61] used a combinatorial approach based on [62] to heterologously express CPR, CYP93E2 and CYP72A61v2 from *Medicago truncatula* to produce soyasapogenol B and CPR, CYP716A12 and CYP72A68v2 from the same source to produce gypsogenic acid in *S. cerevisiae* (Table S2). They have also observed that such combinations of CYP-CPR produce native as well as rare triterpenes (that are not produced in *M. truncatula*) in *S. cerevisiae*. Thus, heterologous expression of P450 enzymes in *S. cerevisiae* might be a critical step

in metabolic engineering to produce non-native terpenes.

Miscellaneous metabolic strategies

Some of the distinct metabolic engineering strategies that have been established to improve terpene production in *S. cerevisiae* are described in this section and apparently does not fall into generic categories as described in the above sub-sections. The native mitochondrial signal peptide of COX4 of *S. cerevisiae* was fused to *A. annua* amorphadiene synthase, farnesyl diphosphate synthase from *Arabidopsis thaliana* and valencene synthase (TPS) from *Camellia sinensis* to be successfully expressed in *S. cerevisiae* [63]. This strategy has substantially increased the valencene and amorphadiene production (Table S2) indicating the presence of the viable precursor pool in mitochondria. The same strategy was also later patented [64]. Heterologous expression of structural genes in the sterol pathway of *S. cerevisiae* could also be a potential strategy and Evolva Inc. adopted this in their patent where all the enzymes upstream of the DMAPP node starting from acetoacetyl transferase were heterologously expressed from a diverse class of sources to improve the target terpene, β -carotene [65] (Table S3). This strategy has essentially enabled them to improve the inherent availability of prenyl phosphate precursor pools. In another patent by KRIBB, South Korea [66], *ispA* (geranyl diphosphate synthase/FDS) from *E. coli* was heterologously expressed as a substitute *ERG20* to achieve a squalene titer of 1.24 g/l (Table S3).

One study recently reported that mutagenesis was selectively carried out to produce premnaspirodiene (sesquiterpene), farnesol (sesquiterpene), botryococcene (triterpene) and squalene (triterpene) [67]. In their study, the BY4741 haploid laboratory strain was subjected to *ethyl methanesulfonate* mutagenesis to select colonies grown under a selective pressure of nystatin (selection for mutants which do not produce ERG), squalastatin (inhibition of squalene synthase, Erg9p) and cholesterol (for the restoration of growth). Here, the surviving colonies indicate that the cells are not producing ERG (due to squalastatin impairment of Erg9p) and ERG producing colonies do not survive as nystatin binds to ERG on the cell membrane eventually leading to their death. However, for the survivability of yeast, ERG is replaced with cholesterol (to which nystatin cannot bind). The viable colonies were further screened for growth in the presence of ERG and ERG + squalastatin. A final selection was based on farnesol response and the colonies accumulated more than 50 mg/l farnesol. Thus, generated mutant strains were

used as chassis for further rational design procedures to produce premnaspirodien, botryococcene and squalene (Table S2). Ignea et al. [51] attempted to identify new genetic targets for improving sesquiterpene production, by analyzing the interaction proteins Hmg1p and Hmg2p. Positive interactors of Hmg1p and Hmg2p has been identified for genetic perturbations based on the effects of digenic deletions on cell viability. Deletion of those positive interactors improved the stability of their interactor proteins. The targets identified include: ubiquitin ligase Ubc7p, Ssm4p and the ER resident protein Pho86p. These proteins are involved in degradation of endoplasmic reticulum transmembrane protein. Hence, deletion of these proteins has improved the stabilization of Hmg1p and Hmg2p. Tandem heterologous deletion of three of the identified target genes, along with the over-expression of a degradation stabilized variant of Hmg2p with caryophyllene synthase from *Salvia fruticosa* has improved caryophyllene production to 125 mg/l in a shake flask culture which is 11-fold higher compared to the wild-type strain (Table S2). The design of enzyme fusion chimera has been a successful strategy in metabolic engineering [68–71]. The fusion of two enzymes modulates the spatial organization of pathway enzymes; thus a terpene synthase fused to the structural gene of the sterol pathway for simultaneous heterologous expression and overexpression, respectively, could be a potential strategy. Miltiradiene, a diterpene, has been produced in *S. cerevisiae* via this approach where GGPPS, obtained from *Sulfolobus acidocaldarius*, was fused with *ERG20* and *BTS1* structural genes to generate an *ERG20-BTS1*-SaGGPPS product and this has resulted in a production titer of 433 mg/l in fed-batch cultivation [72]. Ignea et al. [73] exploited an interchangeable modular approach to design a plug-and-play technique to synthesize structurally distinct terpenes. Conceptually, they defined three modules via which labdane- diterpenes are heterologously synthesized in *S. cerevisiae*. The module 1 consists of a precursor enzyme platform (DMAPP, GPP, FPP and GGPP), module 2 involves the terpene synthases that are involved in cyclization of prenyl-diphosphates from module 1 and finally module 3 carries the terminal reactions involving modifying enzymes such as cytochrome P450 and transferases for deriving functionally distinct labdane terpenes. Having a variable component in each module and utilizing a combinatorial approach has enabled the unlocking of the diversity in labdane-diterpenes. The modules were interchanged to produce diterpenes including miltiradiene (26 mg/l), manoyl oxide (35 mg/l), sclareol (87 mg/l), cis-abienol (3.9 mg/l), manool (96 mg/l) and Z-biformene (1.3 mg/l). One of the challenges using the *S. cerevisiae* cell factory for

terpene synthesis is to improve the transport of precursors between the cellular compartments (such as mitochondria) and the cytosol to enhance their utilization levels. One approach to address this was by subcellular engineering of the terpene biosynthesis pathway inside the mitochondria for the utilization of mitochondrial acetyl-CoA in diterpene synthesis [74]. In another approach, farnesyl pyrophosphate synthase (FPPS) is targeted to mitochondria to move the flux toward terpene synthesis rather than in the production of ubiquinones, dolichols and prenylated proteins [63].

Manipulations outside the sterol pathway

Improvement of acetyl-CoA in cytoplasm

As the biosynthesis of specific compounds involves well-regulated multi-step reaction schemes inside the cell and the improvements in key upstream molecules which are formed due to catabolic reactions may have an impact on increasing downstream pools of anabolic reactions. Acetyl-CoA is one such upstream metabolite that participates in the tricarboxylic acid (TCA) cycle, biosynthesis of sterols, fatty acids and polyketides and also acts as an acetyl-group donor to proteins. In *S. cerevisiae* as it is compartmentalized, the cytoplasmic acetyl-CoA is generated through a PDH -bypass, which essentially drives the fatty acid and sterol metabolism [56,75]. Over-expression of sterol synthesis genes drains the acetyl-CoA pool inside *S. cerevisiae* cells compromising the cell functions. Increasing the availability of acetyl-CoA in the cytoplasm is one of the key strategies applied to improve the downstream products from the MVA pathway. One of the first studies conducted on this strategy was by Shiba et al. [56] where overexpression of aldehyde dehydrogenase (*ALD6*) and heterologous expression of *Salmonella enterica* acetyl-CoA synthetase variant (*ACS1*) with proline at 641 position substituted for leucine, this mutation removes inhibition due to acetylation and has significantly improved amoradiene production [76]. Push and pull is a metabolic strategy where increases in the precursor pool (pushing the flux) is paired with overexpression of genes toward the downstream of metabolic sink (pulling the flux) and has been a very successful strategy in several metabolic engineering studies [77,78]. This technique has been implemented by [79] where acetyl-CoA was improved similar to [56] with additional overexpression of alcohol dehydrogenase (*ADH2*) in order to re-convert the ethanol formed to acetaldehyde, removing other competitive sinks (knockout of peroxisomal citrate synthase, *CIT2* and the cytoplasmic malate synthase, *MLS1*) to direct the flux toward acetyl-CoA formation and

overexpression of *ERG10*, *tHMG1* genes to pull the flux into the sterol pathway for successful improvement in α -santalene production (Table S2). An interesting approach has been adopted by Lian et al. [80]. Initially, alcohol dehydrogenase genes (*ADH1* and *ADH4*), glycerol-3-phosphate dehydrogenase genes (*GPD1* and *GPD2*) were removed to eliminate endogenous ethanol and glycerol production thus improving acetate and acetyl-CoA levels in the cytoplasm. The competing peroxisomal *CIT2* and cytoplasmic *MLS1*, as a sink for acetyl-CoA, were also eliminated. It was hypothesized that in the absence of mitochondrial targeting sequences, (Mts) the PDH expression would convert the pyruvate to acetyl-CoA in the cytoplasm instead of mitochondria and thus it can substitute the low efficient and energy intensive PDH-bypass in *S. cerevisiae*. Hence, Mts-free PDH genes from *S. cerevisiae* and *E. coli* were expressed and it was found that PDH of *E. coli* along with heterologous expression of SeACSL641P and *clostridial* species genes has improved n-butanol significantly reaching a titer more than 100 mg/l. This strategy could also be applied to improve terpene synthesis as the acetyl-coA serves as a common precursor pool. In the patent filed by Evolva, Inc., Hansen [65] reported that heterologous expression of ATP citrate lyase, which converts cytosolic citrate to acetyl-CoA along with heterologous expression of MVA pathway genes in *S. cerevisiae*, improved β -carotene production (Table S3).

Cofactor engineering

In the previous section, the discussions were pertinent to either altering the substrate pool, the enzyme characteristics or introducing new reactions. However, it should be noted that in all biochemical reactions which are conducted by specific enzymes, more than 50% of them require a coenzyme or cofactors [81]. Thus, manipulation of these cofactors could be exploited in order to effect improvements in the reaction flux [82] and this outlines the cofactor engineering. However, in the case of *S. cerevisiae*, due to the compartmentalization of the cell and the lack of transhydrogenase activity for interconverting NADH to NADPH directly, this has furthered the complexity of the reactions [83]. In the sterol pathway (Table S1) of *S. cerevisiae*, several enzymes require NADPH as their cofactor. Hence, overexpression of structural genes *HMG1*, *HMG2*, *ERG9* in the sterol pathway would drain the cytosolic NADPH. Direct application for terpene synthesis can be seen in the study by Brown et al. [59] where *ZWF1*, *SAM2* was overexpressed in *S. cerevisiae* in order to improve NADPH and S-adenosyl methionine availability, respectively during the production of monoterpene indole alkaloid,

strictosidine (Table S2). *Pos5* and *Zwf1* have been overexpressed to enhance the NADPH production in the cells. It has been observed that *Zwf1* overexpression has improved lycopene and β -carotene production to 1.6- and 1.2-fold. Whereas, *Pos5* overexpression has improved lycopene and β -carotene production to 1.9- and 1.6-fold, respectively [84].

In silico analysis for identification of target manipulations

Conducting isolated alterations inside the cell based on the qualitative assessment (intuitive though) has not been a significantly successful strategy, and multiple alterations have become inevitable to effect the required changes [85]. However, in order to effect such quantitative assessments, there is need to have a holistic view of all possible interactions between cellular components [17]. Toward this extent, stoichiometric models derived from the genomic sequences have been successful prediction tools to make quantitative assessments. Stoichiometric modeling using flux balance analysis (FBA) has become a platform for several applications for a better understanding of the cell [86]. The genome-scale model for *S. cerevisiae* was published in 2003 [87], and since then it is possible to increase the robustness of model predictions. Several algorithms such as minimization of metabolic adjustment (MOMA) [88], OptKnock [89], OptStrain [90], OptGene [91], FSEOF [92] etc., have been developed to optimize the strain using FBA schema. Sun et al. [93] implemented MOMA using FBA on iMM904 to make *in silico* predictions to improve the IPP pool in *S. cerevisiae*. They have formulated an objective function (v_{diff}) as a difference between solutions of flux vectors when IPP and biomass are maximized separately for a specific input condition ($v_{IPP} - v_{biomass}$) and termed it as flux distribution comparison analysis. Later from the solution space, lethal genes were screened out to predict potential knockouts to improve the IPP pool toward amorphadiene production. These *in silico* predictions include the knockout of *HXK1*, *HXK2*, *SOR1* and *SOR2* in the glycolytic pathway; *SER3*, *SER33*, *SER1*, *SER2* and *ALT2* in amino acid synthesis; and *ALT2*, *CTP1* and *IDP1* in the TCA cycle. When each of these genes was knocked out separately in wild-type *S. cerevisiae* strains, there was a significant increase in amorphadiene production by 8–10-fold in all the knockout strains (excepting *SER2* deletion) when compared to the reference strain. However, double deletions of these predictions did not improve amorphadiene production. Although logical premise was attributed to finding these targets, such as improving the cofactor availability for *SER3*, *SOR1*, *IDP1* deletions

Table 2. List of various fed-batch conditions adopted for production of diverse terpenes.

Sl. No.	Fed-batch cultivation strategy	pH	Temp (°C)	DO (%)	Product	References
1.	DO-based feeding (i) Glucose feeding during growth phase (ii) Galactose feeding for growth and induction phase	5.0	30	DO >55 feed ON	Artemisinic acid 2.5 g/l	[95]
2	Pulsed ethanol feeding (95%) Feedback signal: CO ₂ measurement {DO is cascade controlled: agitation followed by pure oxygen}	5.0	30	40	Amorphadiene 40 g/l	[34]
3	Glucose exponential feeding Specific growth rate set value: 0.06 h ⁻¹	5.0	30	>30	α -Santalene 92 mg/l	[96]
4	Two stage variable feeding Stage 1: Glucose (500 g/l) Stage 2: Glucose (250 g/l) & Glycerol (250 g/l)	NR	NR	NR	Lycopene 1.61 g/l	[115]
		5.0	30	NR	Carotenoid 20.79 mg/g DCW	[40]
5	Glucose feeding Feedback signal: ethanol <0.5 g/l	5.0	NR	NR	Protopanaxadiol 1189 mg/l dammarenediol-II 1548 mg/l	[98]
6	Constant feeding at 5 ml/h Medium: Glucose (400 g/l), peptone (40 g/l) yeast extract (20 g/l)	5.5	30	NR	Miltiradiene 488 mg/l Squalene 852 mg/l	[72]
7	Constant glucose (15 g/h) feeding	5.3	23 or 30	>40	Kaurene 569 mg/l Abietadiene 207 mg/l	[116]
8	RQ-controlled exponential feeding $\mu = 0.1$ h ⁻¹ , RQ =1, RQ <1 feed pump ON; RQ >1 feed pump OFF in order to avoid ethanol formation	5.0	30	30	α -Farnesene 170 mg/l β -Farnesene 160 mg/l α -Santalene 163 mg/l	[99]

NR: not reported.

and improving glycolytic flux in case of *HXK1*, *HXK2* deletions, it is often difficult to assign specific reasoning as the *in silico* predictions are made by calibrating the designed mathematical function considering all possible reactions inside the cell. *GDH1* deletion for cubebol production was also predicted via MOMA using the iFF708 model [94].

Process strategies for improving terpene synthesis

Although the technology for the production of terpenes to high titers in fed-batch cultivation has not matured enough in comparison to other bulk chemicals and proteins in *S. cerevisiae*, some efforts have been made. Feeding strategies have been enumerated or sometimes a novel strategy has been designed for a specific terpene synthesis during fed-batch cultivation of *S. cerevisiae* (Table 2). The feeding strategies will depend on several factors and some of the critical factors are discussed below. The selection of the substrate to be fed can also dictate the precursor pool, for instance [34], selected ethanol as the substrate since it can be readily directed toward acetyl-CoA formation (Table 2). Choice of the promoter on the heterologously expressed plasmids also affects the selection of the feed substrate. In the case of cells harboring gal-promoter plasmids, galactose needs to be included in the nutritional source

that can serve as a carbon source as well as a gratuitous inducer for functional expression of cloned genes. However, gal-regulon is repressed in the presence of glucose [100], and galactose as a sole carbon source makes the process expensive. Westfall et al. [34] deleted the *GAL1* gene to generate a yeast strain where glucose can be used as a primary carbon source along with low galactose supplementation for induction (Table 2). It was further explained that perhaps galactose, would act as inducer during the diauxic phase when consumption of ethanol occurs. Constitutive promoters offer constant expression levels of the heterologous gene during the active cell growth phase. Comprehensive information on promoters and types of plasmids used for metabolic engineering of *S. cerevisiae* is provided by [41]. Constitutive promoters such as P_{GPD} (glyceraldehyde-3-phosphate), P_{TEF1} (translational elongation factor), P_{ADH1} (alcohol dehydrogenase), P_{PGK1} (phosphoglycerate kinase) etc., were selected in order to overcome repression-related challenges of inducible promoters during fed-batch cultivation to produce terpenes (Table 2). As metabolic engineering involves the expression of more than one gene, the bidirectional promoters on a single vector have been constructed and widely used [51,97,101]. Partow et al. [102] constructed bidirectional constitutive promoter-based integrative vectors pSP-G1 and pSP-G2 (TEF1-PGK1), and several variants of this have been used for metabolic engineering of

Table 3. General metabolic engineering considerations for selection of genetic perturbations in *S. cerevisiae* to produce terpenes^{a,b}.

Monoterpenes	Diterpenes	Sesquiterpenes	Triterpenes	Tetraterpenes
OE: <i>tHMG1</i> or <i>HMG2</i> (K6R) <i>ERG20</i> <i>upc2-1</i> SSM: <i>Upc2p</i> (G888A) <i>HMG2p</i> (K6R) <i>ERG20p</i> (K197G)	OE: <i>tHMG1</i> or <i>HMG2</i> (K6R) <i>ERG20</i> (F96C) <i>BTS1</i> <i>upc2-1</i> SSM: <i>ERG20</i> (F96C) <i>Upc2p</i> (G888A) <i>HMG2p</i> (K6R) R: <i>P_{MET3}ERG9</i>	OE: <i>tHMG1</i> or <i>HMG2</i> (K6R) <i>ERG20</i> SSM: <i>Upc2p</i> (G888A) <i>HMG2p</i> (K6R) R: <i>P_{MET3}ERG9</i>	OE: <i>tHMG1</i> or <i>HMG2</i> (K6R) <i>ERG20</i> <i>ERG9</i> HE: {CYP-CPR} system	OE: <i>tHMG1</i> <i>BTS1</i> HE: CrtYB CrtI CrtE
Strategy <i>tHMG1/HMG2</i> (K6R) <i>Upc2p</i> (G888A) <i>P_{MET3}ERG9</i> <i>ERG20</i>	Role of the perturbation Overexpression increases the precursor pool as HMGP is considered as the rate-limiting enzyme for sterol pathway. Global transcriptional regulator that upregulates sterol biosynthesis genes involved in ergosterol biosynthesis. <i>ERG9</i> , squalene synthase gene is repressed to lower the flux moving toward ergosterol biosynthesis. <i>ERG20p</i> is involved in FPP and GPP synthesis that in turn will be converted to terpenes.			

^aHE: heterologous expression; OE: overexpression; R: repression; SSM: site-specific mutagenesis.

^b*HMG2*(K6R) overexpression is possible only if the *HMG2* gene is subjected to site-specific mutagenesis (i.e. K6R).

S. cerevisiae. Vickers et al. [103] developed a pCEV vector based on the pSP background featuring antibiotic selection marker instead of an auxotrophic marker. Uptake control mutants (*upc*[−]) created on *Upc2p*, *upc2-1* has enabled *S. cerevisiae* to take up external sterols during aerobic cultivation and this protein along with *Ecm22p* are considered as sterol regulatory element binding proteins [104,105]. Thus *upc2-1* mutation has been introduced by several researchers in order to cultivate the strains aerobically whose metabolic path does not lead to the ERG formation however leads to production specific terpenes of interest [37,72,106, etc.) (Table S2).

The second important challenge following the production of these diverse terpene compounds is their recovery and purification. It has been reported that monoterpenes, being lipophilic in nature, interfere with cell membrane functionalities and hence are toxic to the yeast cells [107,108]. Therefore, their accumulation beyond a specific level during production may create end product inhibition to the producing cell. Brennan et al. [109] conducted an *in situ* biphasic extractive system to improve fermentation where an organic solvent was used to extract monoterpenes thus alleviating their detrimental effects to the cell. *In situ* product removal (ISPR) strategies have also been used for other terpenes. In studies by Asadollahi [110] and Scalcinati et al. [96] dodecane was selected based on log *Pow* coefficients (octanol- water partition coefficient) to remove cubebol and santalene (sesquiterpenes), respectively, in an ISPR strategy. Westfall et al. [34] used methylolate, whereas Paddon et al. [57] used isopropyl myristate to extract amorphadiene and artemisinic acid respectively as an ISPR strategy. The ISPR strategy can, in general, be applied to extracellular terpenes due to their low solubilities in an aqueous phase. Comprehensive details on ISPR techniques for bioprocessing can be reported by Freeman et al. [111] and Stark and von Stockar [112].

However, Schewe et al. [113] outlines the application of ISPR in the context of microbial terpene production.

Conclusion and prospects

Broad applications of terpenes and the demand-supply imbalance have led to developing platforms for the microbial production of these potential compounds. This review has attempted to collate strategies to build a *S. cerevisiae* cell factory for the production of a spectrum of terpene molecules. Initially, terpenes such as geraniol, taxadiene, β - amyrin, epicedrol, β -carotene have been successfully synthesized and became representational molecules for mono-, di-, tri-, sesqui- and tetraterpenes, respectively, for heterologous expression in *S. cerevisiae*. Later efforts have been made and continued by several researchers to span this diverse class of terpenes by understanding the specific modifications required to be incorporated in the precursor molecules. Although not all molecules are translated into commercial production, but the promising nascent research will eventually inspire commercial exploitation. The success stories of artemisinic acid and farnesene production would perhaps give a boost to the venture capitalists to broaden commercialization of microbial-based terpene production. Moreover, synthesis of derivative compounds, such as triterpene glycosides and monoterpene alkaloids, will expand the market for terpenes. General metabolic engineering strategies for strain improvement of *S. cerevisiae* for production of a different class of terpenes are compiled in Table 3. These considerations were generalized from studies carried so far by several researchers to observe significant increases in terpene production as described in Table S2. This compilation does not include the heterologous expression of terpene synthases and other explicit genetic perturbations. However, they describe the generic approach for improving terpene production focusing mainly on

the precursor pools in the sterol pathway. Hence, these strategies would be the starting point for production of the terpenes using *S. cerevisiae* as the microbial cell factory. The present review has tried to brief all the established molecular and process strategies described to date in order to build a chassis *S. cerevisiae* strain for successful production of a terpene molecule. The treatment of various strategies in this review was more generic and not restricted to a specific terpene. The underlining theme for successful production of a terpene discussed here was possible due to the combination of several metabolic engineering strategies but not to an isolated strategy alone. Moreover, the success of commercial exploitation depends on how these molecular strategies work in tandem with process development.

Acknowledgements

KP acknowledges the Department of Biotechnology, India, for the award of research fellowship. SM is most indebted to Prof. Gunnar Lidén, Chemical Engineering, Lund University. SM acknowledges Science Engineering and Research Board (SERB), India, for providing the financial assistance under Young Scientist Programme. The Director of CSIR-CFTRI, Mysore, India, is kindly acknowledged for supporting the work.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding

Science and Engineering Research Board [YSS/2014/000565].

References

- [1] Osbourn AE, Lanzotti V, editors. Plant-derived natural products: synthesis, function, and application. Heidelberg: Springer; 2009.
- [2] Dictionary of Natural Products. Supplement 1 (Dictionary of Natural Products, Vol 8). Chapman and Hall/CRC; 1994. ISBN: 9780412577802
- [3] Bution ML, Molina G, Abrahão MR, et al. Genetic and metabolic engineering of microorganisms for the development of new flavor compounds from terpenic substrates. *Crit Rev Biotechnol*. 2015;35: 313–325.
- [4] Flavor and fragrance industry BCC market research. 2014. [cited 2015 May 25]. Available from: <http://www.bccresearch.com/market-research/chemicals/flavors-fragrances-global-markets-chm034c.html>
- [5] Bloch K. Sterol molecule: structure, biosynthesis, and function. *Steroids*. 1992;57:378–383.
- [6] Rohmer M, Knani M, Simonin P, et al. Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochem J*. 1993;295:517–524.
- [7] Takahashi S, Kuzuyama T, Shimizu T, et al. Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. *Tetrahedron Lett*. 1998;39:7913–7916.
- [8] Liu L, Guan N, Li J, et al. Development of GRAS strains for nutraceutical production using systems and synthetic biology approaches: advances and prospects. *Crit Rev Biotechnol*. 2017;37:139–150.
- [9] Goffeau A, Barrell BG, Bussey H, et al. Life with 6000 genes. *Science*. 1996;274:563–567.
- [10] Tuite MF. Strategies for the genetic manipulation of *Saccharomyces cerevisiae*. *Crit Rev Biotechnol*. 1992; 12:157–188.
- [11] Annaluru N, Muller H, Mitchell LA, et al. Total synthesis of a functional designer eukaryotic chromosome. *Science*. 2014;344:55–58.
- [12] Juhas M. On the road to synthetic life: the minimal cell and genome-scale engineering. *Crit Rev Biotechnol*. 2015;8551:1–8.
- [13] Dugar D, Stephanopoulos G. Relative potential of biosynthetic pathways for biofuels and bio-based products. *Nat Biotechnol*. 2011;29:1074–1078.
- [14] Varman AM, Xiao Y, Leonard E, et al. Statistics-based model for prediction of chemical biosynthesis yield from *Saccharomyces cerevisiae*. *Microb Cell Fact*. 2011;10:45.
- [15] Bailey JE. Toward a science of metabolic engineering. *Science*. 1991;252:1668–1675.
- [16] Nielsen J. Metabolic engineering. *Appl Microbiol Biotechnol*. 2001;55:263–283.
- [17] Stephanopoulos GN, Aristidou AA, Nielsen J. Metabolic engineering: principles and methodologies. London (UK): Academic Press; 1998.
- [18] Pickens LB, Tang Y, Chooi Y-H. Metabolic engineering for the production of natural products. *Annu Rev Chem Biomol Eng*. 2014;2:211–236.
- [19] Dudareva N, Cseke L, Blanc VM, et al. Evolution of floral scent in *Clarkia*: novel patterns of S-linalool synthase gene expression in the *C. breweri* flower. *Plant Cell*. 1996;8:1137–1148.
- [20] Rico J, Pardo E, Orejas M. Enhanced production of a plant monoterpene by overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase catalytic domain in *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. 2010;76:6449–6454.
- [21] Degenhardt J, Köllner TG, Gershenzon J. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry*. 2009;70:1621–1637.
- [22] Gong HY, Zeng Y, Chen XY. Diterpene synthases and their responsible cyclic natural products. *Nat Prod Bioprospect*. 2014;4:59–72.
- [23] Dejong JM, Liu Y, Bollon AP, et al. Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. *Biotechnol Bioeng*. 2006;93:212–224.
- [24] Kirby J, Nishimoto M, Park JG, et al. Cloning of casbene and neocembrene synthases from Euphorbiaceae

- plants and expression in *Saccharomyces cerevisiae*. *Phytochemistry*. 2010;71:1466–1473.
- [25] Phillips DR, Rasbery JM, Bartel B, et al. Biosynthetic diversity in plant triterpene cyclization. *Curr Opin Plant Biol*. 2006;9:305–314.
- [26] Verwaal R, Wang J, Meijnen JP, et al. High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. *Appl Environ Microbiol*. 2007;73:4342–4350.
- [27] Fell D. Understanding the control of metabolism elements of metabolic control analysis (MCA). 1997. [cited 2016 Apr 4]. Available from: http://mudshark-static.brookes.ac.uk/Delhi2013/Slides/wshop2_mca1.pdf
- [28] Kacser H, Burns J. The control of flux. *Symp Soc Exp Biol*. 1973;27:65–104.
- [29] Basson ME, Thorsness M, Rine J. *Saccharomyces cerevisiae* contains two functional genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Proc Natl Acad Sci USA*. 1986;83:5563–5567.
- [30] Basson ME, Thorsness M, Finer-Moore J, et al. Structural and functional conservation between yeast and human 3-hydroxy-3-methylglutaryl coenzyme A reductases, the rate-limiting enzyme of sterol biosynthesis. *Mol Cell Biol*. 1988;8:3797–3808.
- [31] Polakowski T, Stahl U, Lang C. Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. *Appl Microbiol Biotechnol*. 1998;49:66–71.
- [32] Mantzouridou F, Tsimidou MZ. Observations on squalene accumulation in *Saccharomyces cerevisiae* due to the manipulation of HMG2 and ERG6. *FEMS Yeast Res*. 2010;10:699–707.
- [33] Ignea C, Trikkas FA, Nikolaidis AK, et al. Efficient diterpene production in yeast by engineering Erg20p into a geranylgeranyl diphosphate synthase. *Metab Eng*. 2015a;27:65–75.
- [34] Westfall PJ, Pitera DJ, Lenihan JR, et al. Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc Natl Acad Sci USA*. 2012;109:E111–E118.
- [35] Wu TK, Chang CH. Enzymatic formation of multiple triterpenes by mutation of tyrosine 510 of the oxidosqualene-lanosterol cyclase from *Saccharomyces cerevisiae*. *ChemBioChem*. 2004;5:1712–1715.
- [36] Kirby J, Romanini DW, Paradise EM, et al. Engineering triterpene production in *Saccharomyces cerevisiae*-beta-amyrin synthase from *Artemisia annua*. *FEBS J*. 2008;275:1852–1859. 5
- [37] Ro D-K, Paradise EM, Ouellet M, et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*. 2006;440:940–943.
- [38] Kampranis SC, Makris AM. Developing a yeast cell factory for the production of terpenoids. *Comput Struct Biotechnol J*. 2012;3:1–7.
- [39] Lv X, Xie W, Lu W, et al. Enhanced isoprene biosynthesis in *Saccharomyces cerevisiae* by engineering of the native acetyl-CoA and mevalonic acid pathways with a push-pull-restrain strategy. *J Biotechnol*. 2014;186:128–136.
- [40] Xie W, Ye L, Lv X, et al. Sequential control of biosynthetic pathways for balanced utilization of metabolic intermediates in *Saccharomyces cerevisiae*. *Metab Eng*. 2015;28:8–18.
- [41] Da Silva NA, Srikrishnan S. Introduction and expression of genes for metabolic engineering applications in *Saccharomyces cerevisiae*. *FEMS Yeast Res*. 2012;12:197–214.
- [42] Ignea C, Cvetkovic I, Loupassaki S, et al. Improving yeast strains using recyclable integration cassettes, for the production of plant terpenoids. *Microb Cell Fact*. 2011;10:4.
- [43] Kaur J, Sharma R. Directed evolution: an approach to engineer enzymes. *Crit Rev Biotechnol*. 2006;26:165–199.
- [44] Blanchard L, Karst F. Characterization of a lysine-to-glutamic acid mutation in a conservative sequence of farnesyl diphosphate synthase from *Saccharomyces cerevisiae*. *Gene*. 1993;125:185–189.
- [45] Fischer MJC, Meyer S, Claudel P, et al. Metabolic engineering of monoterpene synthesis in yeast. *Biotechnol Bioeng*. 2011;108:1883–1892.
- [46] Jongedijk E, Cankar K, Ranzijn J, et al. Capturing of the monoterpene olefin limonene produced in *Saccharomyces cerevisiae*. *Yeast*. 2015;32:159–171.
- [47] Liu J, Zhang W, Du G, et al. Overproduction of geraniol by enhanced precursor supply in *Saccharomyces cerevisiae*. *J Biotechnol*. 2013;168:446–451.
- [48] Julien B, Burlingame RP. Method for production of isoprenoid compounds. United States patent US 8,481,286; 2013 (patent issued 9 July 2013).
- [49] Gardner RG, Hampton RY. A highly conserved signal controls degradation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in eukaryotes. *J Biol Chem*. 1999;274:31671–31678.
- [50] Hampton RY, Rine J. Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. *J Cell Biol*. 1994;125:299–312.
- [51] Ignea C, Trikkas FA, Kourtzelis I, et al. Positive genetic interactors of HMG2 identify a new set of genetic perturbations for improving sesquiterpene production in *Saccharomyces cerevisiae*. *Microb Cell Fact*. 2012;11:162.
- [52] Ignea C, Pontini M, Maffei ME, et al. Engineering monoterpene production in yeast using a synthetic dominant negative geranyl diphosphate synthase. *ACS Synth Biol*. 2014;3:298–306.
- [53] Bak S, Beisson F, Bishop G, et al. Cytochromes p450. *Arabidopsis Book*. 2011;9:e0144.
- [54] Nelson D, Werck-Reichhart D. A P450-centric view of plant evolution. *Plant J*. 2011;66:194–211.
- [55] Schrader J, Bohlmann J. *Biotechnology of isoprenoids*. Switzerland: Springer International Publishing AG; 2015.
- [56] Shiba Y, Paradise EM, Kirby J, et al. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metab Eng*. 2007;9:160–168.
- [57] Paddon CJ, Westfall PJ, Pitera DJ, et al. High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature*. 2013;496:528–532.

- [58] Teoh KH, Polichuk DR, Reed DW, et al. *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. *FEBS Lett.* 2006;580:1411–1416.
- [59] Brown S, Clastre M, Courdavault V, et al. *De novo* production of the plant-derived alkaloid strictosidine in yeast. *Proc Natl Acad Sci USA.* 2015;112:3205–3210.
- [60] Gavira C, Höfer R, Lesot A, et al. Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by *Saccharomyces cerevisiae*. *Metab Eng.* 2013;18:25–35.
- [61] Fukushima EO, Seki H, Sawai S, et al. Combinatorial biosynthesis of legume natural and rare triterpenoids in engineered yeast. *Plant Cell Physiol.* 2013;54:740–749.
- [62] Pollier J, Moses T, Goossens A. Combinatorial biosynthesis in plants: a (p)review on its potential and future exploitation. *Nat Prod Rep.* 2011;28:1897–1916.
- [63] Farhi M, Marhevka E, Masci T, et al. Harnessing yeast subcellular compartments for the production of plant terpenoids. *Metab Eng.* 2011;13:474–481.
- [64] Alexander V, Elena M, Moran F, et al. Expression constructs and uses thereof in the production of terpenoids in yeast. United States patent US 0,302,861 A1; 2013 (patent issued on 14 November 2013).
- [65] Hansen J. Method of producing isoprenoid compounds in yeast. WO146833; 2011(patent issued on 24 November 2011).
- [66] Choi ES, Whang JI, Ryu K, et al. Modified yeast strain and a method for producing squalene using the same. United States patent US 0,322,129; 2012 (patent issued on 20 December 2012).
- [67] Zhuang X, Chappell J. Building terpene production platforms in yeast. *Biotechnol Bioeng.* 2015;112:1854–1864.
- [68] Aristidou AA, San KY, Bennett GN. Metabolic engineering of *Escherichia coli* to enhance recombinant protein production through acetate reduction. *Biotechnol Prog.* 1995;11:475–478.
- [69] Chen X, Dong X, Wang Y, et al. Mitochondrial engineering of the TCA cycle for fumarate production. *Metab Eng.* 2016;33:75–85.
- [70] George KW, Thompson MG, Kang A, et al. Metabolic engineering for the high-yield production of isoprenoid-based C5 alcohols in *E. coli*. *Sci Rep.* 2015;5:Art number 11128.
- [71] Wang C, Yoon S-H, Jang H-J, et al. Metabolic engineering of *Escherichia coli* for α -farnesene production. *Metab Eng.* 2011;13:648–655.
- [72] Dai Z, Liu Y, Huang L, et al. Production of miltiradiene by metabolically engineered *Saccharomyces cerevisiae*. *Biotechnol Bioeng.* 2012;109:2845–2853.
- [73] Ignea C, Ioannou E, Georgantea P, et al. Reconstructing the chemical diversity of labdane-type diterpene biosynthesis in yeast. *Metab Eng.* 2015b;28:91–103.
- [74] Yuan J, Ching C-B. Mitochondrial acetyl-CoA utilization pathway for terpenoid productions. *Metab Eng.* 2016;38:303–309.
- [75] Krivoruchko A, Zhang Y, Siewers V, et al. Microbial acetyl-CoA metabolism and metabolic engineering. *Metab Eng.* 2015;28:28–42.
- [76] Starai VJ, Celic I, Cole RN, et al. Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science.* 2002;298:2390–2392.
- [77] Huo Y-X, Cho KM, Rivera JGL, et al. Conversion of proteins into biofuels by engineering nitrogen flux. *Nat Biotechnol.* 2011;29:346–351.
- [78] Tai M, Stephanopoulos G. Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. *Metab Eng.* 2013;15:1–9.
- [79] Chen Y, Daviet L, Schalk M, et al. Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. *Metab Eng.* 2013;15:48–54.
- [80] Lian J, Si T, Nair NU, et al. Design and construction of acetyl-CoA overproducing *Saccharomyces cerevisiae* strains. *Metab Eng.* 2014;24:139–149.
- [81] Duine JA. Cofactor engineering. *Trends Biotechnol.* 1991;9:343–346.
- [82] Celton M, Goelzer A, Camarasa C, et al. A constraint-based model analysis of the metabolic consequences of increased NADPH oxidation in *Saccharomyces cerevisiae*. *Metab Eng.* 2012;14:366–379.
- [83] Moreira dos Santos M, Raghevedran V, Kötter P, et al. Manipulation of malic enzyme in *Saccharomyces cerevisiae* for increasing NADPH production capacity aerobically in different cellular compartments. *Metab Eng.* 2004;6:352–363.
- [84] Zhao X, Shi F, Zhan W. Overexpression of ZWF1 and POS5 improves carotenoid biosynthesis in recombinant *Saccharomyces cerevisiae*. *Lett Appl Microbiol.* 2015;61:354–360.
- [85] Visser D, Heijnen JJ. The mathematics of metabolic control analysis revisited. *Metab Eng.* 2002;4:114–123.
- [86] Lewis NE, Nagarajan H, Palsson BØ. Constraining the metabolic genotype-phenotype relationship using a phylogeny of *in silico* methods. *Nat Rev Microbiol.* 2012;10:291–305.
- [87] Förster J, Famili I, Fu P, et al. Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. *Genome Res.* 2003;13:244–253.
- [88] Segrè D, Vitkup D, Church GM. Analysis of optimality in natural and perturbed metabolic networks. *Proc Natl Acad Sci USA.* 2002;99:15112–15117.
- [89] Burgard AP, Pharkya P, Maranas CD. OptKnock: a bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. *Biotechnol Bioeng.* 2003;84:647–657.
- [90] Pharkya P, Burgard AP, Maranas CD. OptStrain: a computational framework for redesign of microbial production systems. *Genome Res.* 2004;14:2367–2376.
- [91] Patil KR, Rocha I, Förster J, et al. Evolutionary programming as a platform for *in silico* metabolic engineering. *BMC Bioinformatics.* 2005;6:308.
- [92] Choi HS, Lee SY, Kim TY, et al. *In silico* identification of gene amplification targets for improvement of lycopene production. *Appl Environ Microbiol.* 2010;76:3097–3105.
- [93] Sun Z, Meng H, Li J, et al. Identification of novel knockout targets for improving terpenoids

- biosynthesis in *Saccharomyces cerevisiae*. PLoS One. 2014;9:e112615.
- [94] Asadollahi MA, Maury J, Patil KR, et al. Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through *in silico* driven metabolic engineering. Metab Eng. 2009;11:328–334.
- [95] Lenihan JR, Tsuruta H, Diola D, et al. Developing an industrial artemisinic acid fermentation process to support the cost-effective production of antimalarial artemisinin-based combination therapies. Biotechnol Prog. 2008;24:1026–1032.
- [96] Scalcinati G, Partow S, Siewers V, et al. Combined metabolic engineering of precursor and co-factor supply to increase α -santalene production by *Saccharomyces cerevisiae*. Microb Cell Fact. 2012;11:117.
- [97] Xie W, Liu M, Lv X, et al. Construction of a controllable β -carotene biosynthetic pathway by decentralized assembly strategy in *Saccharomyces cerevisiae*. Biotechnol Bioeng. 2014;111:125–133.
- [98] Dai Z, Liu Y, Zhang X, et al. Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenosides. Metab Eng. 2013;20:146–156.
- [99] Tippmann S, Scalcinati G, Siewers V, et al. Production of farnesene and santalene by *Saccharomyces cerevisiae* using fed-batch cultivations with RQ-controlled feed. Biotechnol Bioeng. 2015;113:72–81.
- [100] Johnston M, Flick JS, Pexton T. Multiple mechanisms provide rapid and stringent glucose repression of GAL gene expression in *Saccharomyces cerevisiae*. Mol Cell Biol. 1994;14:3834–3841.
- [101] Kim SR, Park YC, Jin YS, et al. Strain engineering of *Saccharomyces cerevisiae* for enhanced xylose metabolism. Biotechnol Adv. 2013;31:851–861. Elsevier Inc.
- [102] Partow S, Siewers V, Bjørn S, et al. Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. Yeast. 2010;27:955–964.
- [103] Vickers CE, Bydder SF, Zhou Y, et al. Dual gene expression cassette vectors with antibiotic selection markers for engineering in *Saccharomyces cerevisiae*. Microb Cell Fact. 2013;12:96.
- [104] Lewis TL, Keesler GA, Fenner GP, et al. Pleiotropic mutations in *Saccharomyces cerevisiae* affecting sterol uptake and metabolism. Yeast. 1988;4:93–106.
- [105] Vik Å, Rine J. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. Mol Cell Biol. 2001;21:6395–6405.
- [106] Engels B, Dahm P, Jennewein S. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab Eng. 2008;10:201–206.
- [107] Andrews RE, Parks LW, Spence KD. Some effects of Douglas fir terpenes on certain microorganisms. Appl Environ Microbiol. 1980;40:301–304.
- [108] Uribe S, Ramirez J, Peña A. Effects of beta-pinene on yeast membrane functions. J Bacteriol. 1985;161:1195–1200.
- [109] Brennan TCR, Turner CD, Krömer JO, et al. Alleviating monoterpene toxicity using a two-phase extractive fermentation for the bioproduction of jet fuel mixtures in *Saccharomyces cerevisiae*. Biotechnol Bioeng. 2012;109:2513–2522.
- [110] Asadollahi MA. Establishment of yeast platform for isoprenoid production. Lyngby, Denmark: Technical University of Denmark; 2008.
- [111] Freeman A, Woodley JM, Lilly MD. *In situ* product removal as a tool for bioprocessing. Biotechnology (NY). 1993;11:1007–1012.
- [112] Stark D, von Stockar U. *In situ* product removal (ISPR) in whole cell biotechnology during the last twenty years. In: Scheper T, Series editor. Process Integration in Biochemical Engineering. Springer; 2003. p. 149–175.
- [113] Schewe H, Mirata MA, Schrader J. Bioprocess engineering for microbial synthesis and conversion of isoprenoids. In: Schrader J, Bohlmann J, editors. Biotechnol isoprenoids. Springer; 2015. p. 251–286.
- [114] Gooday GW. Cell membrane. In: Gow NAR, Geoffrey MG, editors. The growing fungus. London: Chapman & Hall; 1995. p. 63–74.
- [115] Xie W, Lv X, Ye L, et al. Construction of lycopene-overproducing *Saccharomyces cerevisiae* by combining directed evolution and metabolic engineering. Metab Eng. 2015;30:69–78.
- [116] Zhuang X. Engineering novel terpene production platforms in the yeast *Saccharomyces cerevisiae*. Ph.D Thesis. Lexington, Kentucky, USA: University of Kentucky; 2013.