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Mini-Review

From flavors and pharmaceuticals to advanced biofuels: Production of isoprenoids in *Saccharomyces cerevisiae*

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Isoprenoids denote the largest group of chemicals in the plant kingdom and are employed for a wide range of applications in the food and pharmaceutical industry. In recent years, isoprenoids have additionally been recognized as suitable replacements for petroleum-derived fuels and could thus promote the transition towards a more sustainable society. To realize the biofuel potential of isoprenoids, a very efficient production system is required. While complex chemical structures as well as the low abundance in nature demonstrate the shortcomings of chemical synthesis and plant extraction, isoprenoids can be produced by genetically engineered microorganisms from renewable carbon sources. In this article, we summarize the development of isoprenoid applications from flavors and pharmaceuticals to advanced biofuels and review the strategies to design microbial cell factories, focusing on *Saccharomyces cerevisiae* for the production of these compounds. While the high complexity of biosynthetic pathways and the toxicity of certain isoprenoids still denote challenges that need to be addressed, metabolic engineering has enabled large-scale production of several terpenoids and thus, the utilization of these compounds is likely to expand in the future.

Keywords: Biofuels · Isoprenoids · Metabolic engineering · Microbial cell factories · *Saccharomyces cerevisiae*

1 Introduction

Isoprenoids are known as secondary metabolites, which provide plant oils and resins with characteristic smells. In addition, they comprise crucial photosynthetic pigments such as carotenoids, are assumed to be involved in fruit ripening processes and serve plants as defense against herbivores [1–3]. Isoprenoids also include metabolites important for cellular function such as dolichols, ubiquinones, growth regulators, and sterols [4]. For instance, the triterpene squalene is converted in a sequence of consecutive reactions into ergosterol, which is vital for membrane integrity in fungi.

Isoprenoids have received great attention in research owing to several reasons. First and foremost, isoprenoids have a considerable societal relevance due to a broad spectrum of applications ranging from food products, pharmaceuticals, and cosmetics to fuels. Second, with more than 40 000 compounds [5], isoprenoids are not only the largest, but also one of the structurally and functionally most diverse group of chemicals in the plant kingdom.

This review attempts to forge a bridge over the diverse applications of isoprenoids with a main focus on those considered to be advanced biofuel precursors. For this purpose, some of the most eminent examples from each field will be presented as well as the strategies for commercial production of isoprenoids in the microbial host *Saccharomyces cerevisiae*. Therefore, isoprenoids' underlying biosynthetic pathway will be elucidated to illustrate

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Abbreviations: CVS, citrus valencene synthase; **DMAPP,** dimethylallyl pyrophosphate; **FPP,** farnesyl pyrophosphate; **GGPP,** geranylgeranyl pyrophosphate; **GPP,** geranyl pyrophosphate; **HMG-CoA,** hydroxymethylglutaryl-CoA; **IPP,** isopentenyl pyrophosphate; **MEP,** 2-C-methyl-D-erythritol-4-phosphate; **MVA,** mevalonate

the complexity and challenges of developing microbial cell factories.

2 Production of isoprenoids

The production of isoprenoids can be realized by several different means. Considering their natural occurrence in plants, the most obvious method is isolation. Hereby, parts of the plant such as the peel or tree bark are collected and the target isoprenoid is extracted by mincing and subsequent hydro- or steam distillation. However, even though isoprenoids are ubiquitous in nature, many of them are present in low quantities in the plant source. The leaves of *Artemisia annua* were reported to contain the largest amounts of the antimalarial drug artemisinin with 0.44% per dry weight [6]. Considering over 200 million infections of malaria in 2010 [7], plant extraction is not sustainable as it cannot be employed for large-scale production. Furthermore, slow plant growth and yield dependency on seasonal changes as well as geographical conditions highlight further shortcomings of the extraction from plant material.

Likewise plant extraction, chemical synthesis is characterized by a number of drawbacks. First and foremost, the stereochemistry of pharmaceuticals and flavors, which is essential for their functionality, often complicates enantioselective synthesis and reduces the overall yield. As an example, for the efficient synthesis of the complex diterpenoid taxol, which possesses 11 stereogenic centers, 37 steps are required, while yields of 0.4% are attained [8]. Furthermore, hazardous solvents, which are often required for chemical synthesis, as for the allylic oxidation of (+)-valencene for the production of nootkatone, pose health risks, and raise environmental concerns [9].

Besides plant extraction and chemical synthesis, biotechnology offers alternative production strategies for isoprenoids. Most promising is the application of engineered microbes, which has several advantages over the previous strategies. Microorganisms feature fast growth, can be cultivated easily and production by microorganisms is easy to scale. Most importantly, microbes are able to couple a sequence of enzymatic reactions to specifically produce a desired chemical from inexpensive and renewable carbon sources such as glucose [10]. In addition, biological systems can be altered, redesigned, and even completely new pathways can be established using synthetic biology tools, which allows for the production of a wide range of chemicals [11].

The development of microbial cell factories is a complex task, which not only requires extensive knowledge about cellular metabolism and recombinant DNA technologies, but also the integration of other engineering disciplines. However, first and foremost, an appropriate host organism has to be selected with regard to the desired chemical. For production of functional isoprenoids, mainly *S. cerevisiae* and *Escherichia coli* are employed, since they are amenable to genetic manipulations with extensive molecular resources. The comparison of isoprenoid production presented in Table 1 shows that the titers achieved by metabolic engineering of *E. coli* are in most cases superior. Besides, slower growth of *S. cerevisiae* and its lacking ability to utilize alternative carbon sources such as xylose, which is abundant in plant biomass, denote economical disadvantages, and obstacles regarding its use in prospective industrial applications [12]. On the other hand, it allows for a facilitated expression of functional cytochrome P450 enzymes, which are essential for the modification of many isoprenoids and thereby responsible for their structural diversity. In addition, *S. cerevisiae* is more robust in large-scale fermentations compared to *E. coli*. It is relatively tolerant to low pH and high concentrations of sugars, as well as fairly resistant to inhibitors [13, 14]. Furthermore, a number of advanced molecular biology tools have been developed for precision

Table 1. Examples of isoprenoids produced in *S. cerevisiae* and *E. coli*

Isoprenoid	*S. cerevisiae*		*E. coli*	
	Titer	**References**	**Titer**	**References**
Monoterpenes				
Limonene	–	–	~60 mg/L	[68]
Sesquiterpenes				
Farnesol	4.63 g/L	[83]	135.5 mg/L	[76]
α-Farnesene	9.8 mg/L	[57]	400 mg/L	[57]
β-Farnesene	762 mg/L	[57]	1100 mg/L	[57]
Bisabolene	>900 mg/L	[77]	>900 mg/L	[77]
Amorphadiene	40 g/L	[47]	25 g/L	[48]
Artemisinic Acid	25 g/L	[46]	–	–
Valencene	1.5 mg/L	[35]	–	–
Diterpenes				
Taxadiene	8.7 mg/L	[51]	1 g/L	[52]

engineering of yeast [15, 16] as well as much information about regulation of its metabolism is available [17–19]. It is therefore the preferred cell factory for industrial production and in this review, we therefore focus on the production of isoprenoids in *S. cerevisiae*.

3 Biosynthesis of isoprenoids

Isoprenoids are all assembled from activated forms of isoprene, namely isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). These two precursors are made via two different pathways: the mevalonate (MVA) and the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. The MVA pathway was first discovered in the 1960s [20, 21] and was assumed to be the only pathway leading to IPP in all living organisms for almost 40 years. However, in the 1990s, the MEP pathway was found in bacteria, green algae, and higher plants as an alternative pathway [22]. With some exceptions, the MVA pathway is utilized by most eukaryotes as well as archaea, whereas the MEP pathway is typically found in prokaryotes and the plastids of photosynthetic organisms [23].

DMAPP is a reactive primer which undergoes elongation by head-to-tail condensation with one or more IPP molecules, to form geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP) (Fig. 1). By terpene synthases, the precursors GPP, FPP, and GGPP can be cyclized and/or rearranged to form

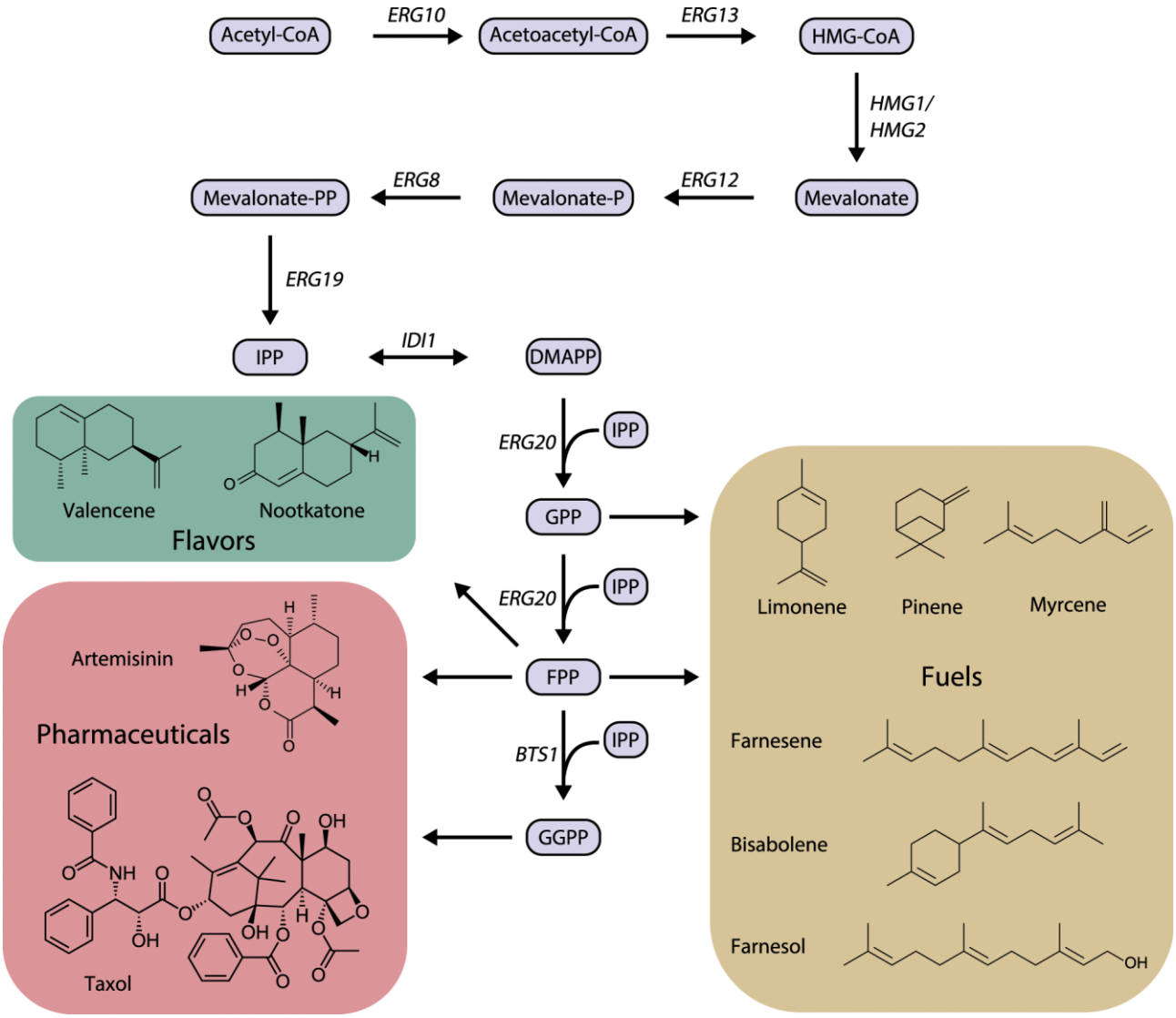

Figure 1. Production of isoprenoids in *Saccharomyces cerevisiae*: Overview of the mevalonate pathway and products that can be derived from it. Gene names are in italics. *ERG10*, acetoacetyl-CoA thiolase; *ERG13*, HMG-CoA synthase; *HMG1/HMG2*, HMG-CoA reductases; *ERG12*, mevalonate kinase; *ERG8*, phosphomevalonate kinase; *ERG19*, mevalonate pyrophosphate decarboxylase; *IDI1*, IPP:DMAPP isomerase; *ERG20*, FPP synthase; *BTS1*, GGPP synthase.

monoterpenes, sesquiterpenes, and diterpenes, respectively. Once the basic skeletons are formed, they are often further modified by terpene modifying enzymes, particularly cytochrome P450 monooxygenases, to generate functional products constituting the enormous diversity of isoprenoid families.

3.1 The MVA pathway

The yeast *S. cerevisiae* uses the MVA pathway to generate the precursors IPP and DMAPP from acetyl-CoA through seven enzymatic reactions (Fig. 1). This involves the conversion of three molecules of acetyl-CoA to MVA via acetoacetyl-CoA and hydroxymethylglutaryl-CoA (HMG-CoA). MVA subsequently undergoes phosphorylation and decarboxylation to form IPP. A stereospecific isomerization reaction converts IPP to its isomer DMAPP.

Several enzymes, especially HMG-CoA reductase, IPP isomerase, and FPP synthase, have been elucidated as key enzymes for engineering isoprenoid biosynthesis in *S. cerevisiae*. Two isozymes, Hmg1p and Hmg2p, both possess HMG-CoA reductase function, Hmg1p being responsible for about 83% of the enzyme activity in wild type yeasts, depending on the cultivation conditions [24]. On the post-translational level, Hmg2p was shown to undergo endoplasmic reticulum-associated degradation (ERAD) depending on ubiquitination [25], while Hmg1p was found to be relatively stable. *ERG20* encodes GPP synthase/FPP synthase, which combines IPP and DMAPP to GPP and catalyzes the subsequent addition of another IPP to yield FPP. A study in which Erg20p was overexpressed revealed an increased ergosterol production which indicates that FPP synthase may be a flux controlling enzyme [26].

Furthermore, FPP is situated at an important intersection building the connection to numerous compounds and primary metabolism. It is further condensed to squalene and subsequently undergoes nineteen conversion steps to form ergosterol, which is essential for cell growth and has a great impact on the regulation of membrane permeability and fluidity. FPP is also an important precursor for biosynthesis of many primary metabolites, such as dolichols, ubiquinone, carotenoids, and prenylated proteins [27].

3.2 The MEP pathway

The MEP pathway generates IPP and DMAPP in eight reactions based on pyruvate and glyceraldehyde 3-phosphate. In the first part, which requires the enzymes Dxs and Dxr, the two precursors are condensed to 1-deoxy-D-xylulose 5-phosphate, which is subsequently reduced to MEP. In a sequence of further reactions catalyzed by enzymes specified as IspD, IspE, and IspF, MEP is converted to 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate. The following reduction to 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-pyrophosphate and final conversion to IPP/DMAPP are catalyzed by IspG and IspH.

A comparative study of the two distinct biosynthetic pathways has shown that the MEP pathway is stoichiometrically more efficient than the MVA pathway [28]. For this reason and in order to bypass endogenous regulation, recent efforts have addressed the heterologous expression of the MEP pathway in *S. cerevisiae* [29, 30]. However, the strains were unable to grow and could not compensate for the loss of the endogenous MVA pathway, which was inhibited using lovastatin or by deletion of *ERG13*. Labeling experiments revealed that the heterologous pathway was only active until 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate, since *S. cerevisiae* failed to functionally express the iron sulfur cluster proteins that catalyze the last two reactions [29].

4 From flavors and pharmaceuticals to biofuels

4.1 Flavors

Two major families of isoprenoids, monoterpenoids (10 carbons) and sesquiterpenoids (15 carbons) are traditionally valued as fragrances and flavors, as they are the primary constituents of essential oils from flowers. They have been commercialized for ages but depend on plant extractions which are considered expensive and unreliable. Recently, two sesquiterpenoids, named nootkatone and valencene have been made available in commercial quantities by Allylix using a microbial fermentation process [31]. Nootkatone is a high-value flavorant used in perfumery and the flavor industry. It is a natural constituent of citrus oils, and stands out as a distinguished flavor and aroma of grapefruit. Valencene is also a characteristic fruit flavor and aroma component, which is currently used in beverage and chewing gum flavors, as well as in the production of nootkatone.

Valencene is commonly identified in nature but the corresponding synthase gene had not been cloned until Sharon-Asa et al. [32], Greenhagen [33], and Lücker et al. [34] isolated and characterized different *Citrus* valencene synthase (CVS) genes, the product of which catalyzes the cyclization of FPP to valencene. Since then, some efforts have specifically addressed valencene biosynthesis, but also other fragrances and flavors. All strategies described are clustered into four.

(1) *Enhancing flux through the MVA pathway*. Co-expression of a heterologous Arabidopsis or human FPP synthase (AsFPPS or HsFPPS) with truncated Hmg1p lacking the N-terminal regulatory domain (tHmg1) improved valencene production four-fold [35]. In a recent study, a multi-step engineering approach was taken to increase the flux through the MVA pathway in order to enhance the production of the sesquiterpene santal-

ene [36]. This involved overexpression of *tHMG1* as well as *ERG20* (encoding FPP synthase) and *GDH2* (NADH dependent glutamate dehydrogenase). Additionally, a point-mutated version of the transcription factor Upc2p was introduced to upregulate the expression of MVA pathway genes.

(2) *Limiting the use of the FPP pool*. Down-regulation of squalene synthase in yeast by replacing the native *ERG9* promoter with the tunable *MET3* promoter increased valencene production by 50% [37], and this strategy was further pursued for production of santalene [38]. In another study, by introducing a knockout mutation of the squalene synthase gene (*erg9Δ*) and simultaneously obtaining a mutant capable of efficient aerobic uptake of ergosterol from the culture media, accumulation of farnesol (the dephosphorylated form of FPP) was significantly increased, indicating an enhancement in the FPP pool [39]. Similarly, the use of a defective squalene synthase (dErg9) allowing more FPP to be available for isoprenoid production while still producing sufficient squalene to allow cell growth is beneficial, especially as this does not require the addition of nutrients such as ergosterol or methionine [40]. On the other hand, Farhi et al. [35] found that neither eliminating geranylgeranyl diphosphate synthase (Bts1p) nor two endogenous lipid phosphatases (diacylglycerol diphosphate phosphatase [Dpp1p] and lipid phosphate phosphatase [Lpp1p], both involved in dephosphorylation of FPP) could enhance valencene biosynthesis, which was similar to the finding that a single *DPP1* knock-out did not exhibit improved valencene production [39].

(3) *Spatial subcellular arrangement of metabolic enzymes*. Mitochondrial targeting of a valencene synthase led to a three-fold rise in valencene titers compared to the ones generated by the corresponding cytosolic forms of the synthase. Combination of this approach with mitochondrial targeting of FPP synthase led to an additional 40% improvement [35].

(4) *Synthase engineering*. Greenhagen elucidated the catalytic mechanism of terpene synthases leading to formation of valencene and other compounds [33, 41]. A transition between valencene and germacrene A production was found at approximately pH 8.2, which is close to the pK_a value of cysteine (pH 8.4). This is consistent with the fact that germacrene A synthases only differ from CVS by the presence of C440 in the active site. Either the single mutant CVS-I516V or the double mutant CVS-C402S/V516I exhibited a significant increase in the proportion of germacrene A. Although there has been no report on improving the synthase activity so far, this structure-function analysis would facilitate engineering activities and specificities of terpene synthases.

4.2 Pharmaceuticals

The potential of isoprenoids in the treatment of diseases is widely acknowledged. Since ancient times plant oils and herbal medicines have been used as antifungal and antibacterial agents. Many of them contain monoterpenoids, which are known for their cytotoxicity [42]. In the following some of the large efforts that have been made to enable microbial production of artemisinin and taxol are presented, which were addressed among others in several reviews in the recent past [43, 44].

4.2.1 Artemisinin

The discovery of artemisinin denotes a landmark in the treatment of malaria and dates far back into Chinese history, as its ability to efficiently inhibit parasite growth was first identified during the Jin Dynasty [45]. Nowadays, this sesquiterpene lactone is still utilized as the first-line treatment against malaria in artemisinin-based combination therapies (ACTs). Following the biosynthetic pathway, FPP is converted to amorpha-4,11-diene, which is subsequently oxidized via three reactions to artemisinic acid, the immediate precursor of artemisinin. As the final conversion is not yet fully understood, semi-synthetic production of artemisinin aims at providing its biochemical precursor artemisinic acid, which can subsequently be converted chemically to artemisinin via dihydroartemisinic acid at a yield between 40 and 45% [46]. Amorphadiene and artemisinic acid have both been successfully produced in *S. cerevisiae*. Overexpression of all MVA pathway genes to *ERG20* together with an amorphadiene synthase derived from *A. annua* led to final amorphadiene titers of 40 g/L in optimized fed-batch fermentations with pure ethanol feed [47]. In comparison, amorphadiene titers of 25 g/L were attained in a nitrogen and glucose limited fed-batch process with engineered *E. coli* [48]. Likewise, the authors reported successful conversion to artemisinic acid by overexpressing the involved cytochrome P450 oxidase (CYP71AV1) and its cognate reductase (CPR1), which was, however, manifold lower. The efficient conversion to artemisinic acid was recently achieved by introduction of the complete oxidation pathway from *A. annua* using the artemisinic alcohol and aldehyde dehydrogenase (ADH1/ALDH1) in combination with cytochrome b_5 (CYB5) together with CYP71AV1 and CPR1. Using this approach, final artemisinic acid titers of 25 g/L were attained in fed-batch fermentations demonstrating the crucial importance of the dehydrogenases [46].

4.2.2 Taxol

The complex diterpenoid Taxol is an effective antineoplastic drug in the treatment of different cancer types such as ovarian, breast, and colon cancer since it prevents microtubule deploymerization and thereby blocks the cell cycle [49].

Table 2. Comparison of conventional diesel and jet fuel with isoprenoid based biofuels[a)]

Property	Unit	D2 diesel	Farnesane	Bisabolane (wt 50 %)	Limonane (wt 10 % in diesel)	Myrcane (wt 10 % in diesel)	Jet A	Limonene (dimers)
Density	g/mL	0.865	0.774	0.820	n.a.	n.a.	0.811	0.914
Cloud point	°C	–21	<–50	<–78	–11	–11	n.a.	n.a.
Flash point	°C	73	109	111	58.9	60	43	n.a.
Cetane number	–	41.6	58.6	41.9	42.8	44.7	n.a.	n.a.
Net heat of combustion	MJ/kg	42.4	44.2	n.a.	n.a.	n.a.	43.4	41.906
Viscosity	mm^2/s (at °C)	2.440 (at 40°C)	2.325 (at 40°C)	2.91	2.311 (at 40°C)	3.899 (at 40°C)	4.1 (at 20°C)	n.a.
References		[57]	[57]	[77]	[63]	[63]	[59, 86]	[61]

a) Farnesane, bisabolane, limonene, and myrcane refer to the hydrogenated forms of farnesene, bisabolene, limonene and myrcene, respectively. N.a., not available. Similar values were also presented in [64, 84, 85].

Taxol production is still strongly dependent on primary resources, but can be realized semi-synthetically from 10-deacetylbaccatin III and baccatin III, its biosynthetic precursors, which are extracted from the needles of various taxus species. The biosynthetic production is equally complicated and not yet fully understood. In 19 enzymatic reactions, including 8 cytochrome P450-mediated oxygenations, taxol is derived from GGPP [50]. Approaches for microbial production of taxol have mostly focused on three aspects: the supply of GGPP for diterpene production, overexpression of taxadiene synthase for an increased conversion of GGPP to the committed intermediate taxa-4(5),11(12)-diene [51], and engineering of the cytochrome P450-mediated oxidation of taxadiene to taxa-4(20),11(12)-dien-5α-ol [52]. Taxadiene titers of 8.7 mg/L were attained in *S. cerevisiae* by using a codon optimized taxadiene synthase from *Taxus chinensis*, *upc2-1*, a mutant allele of the transcriptional sterol regulator, and a GGPP synthase from *Sulfolobus acidocaldarius* in combination with a truncated HMG-CoA reductase (tHmg1) [51]. In comparison, taxadiene levels could already be raised up to 1 g/L in engineered *E. coli* [52], which is still over 100-fold higher. It is especially the high pathway complexity that demands for the integration of systems biology tools to promote microbial production of taxol and its precursors. Recently, a computational approach (a variation of the minimization of metabolic adjustment [MoMA] algorithm) was applied to enhance taxa-4(5),11(12)-diene production in *E. coli* [53]. As a result, four genetic engineering targets outside of the native isoprenoid precursor pathway were identified, which would improve cofactor availability and could thereby increase taxadiene accumulation.

4.3 Fuels

The ongoing search for alternative transportation fuels is complicated by demands for fuels that fit the current infrastructure and that can be produced at low cost and at extremely high volumes. While the primary objective is to produce low cost and environmentally friendly fuels from renewable sources, they additionally have to fit narrow constraints in terms of density, chain length, combustion heat and efficiency, lubricity, and stability [54]. Within the past years, much research efforts have been dedicated to the use of terpenes for fuel applications [55, 56]. While monoterpenes have properties similar to conventional aviation fuels such as Jet A and Jet A-1, sesquiterpenes have potential applications as diesel. Table 2 lists properties of some terpene-derived fuels in comparison with conventional jet fuel and petroleum based diesel. The most promising examples include the monoterpene limonene, the sesquiterpenes farnesene and bisabolene and the sesquiterpene alcohol farnesol, which can either be used as fuel additives or directly as replacements for diesel and jet fuels in their hydrogenated form [57–59]. Other cyclized monoterpenes, which can be used as fuel precursors or additives are pinene, cymene, myrcene, camphene, and terpinene. Furthermore, valuable gasoline additives such as isopentanol and isoamylacetate can be produced from the isoprenoid pathway [56], and were shown to possess beneficial blend properties as they increase the octane number [60].

4.3.1 Monoterpene fuels

Due to low densities, which limit the heating value, jet fuels produced from cellulosic butanol (Biojet) are considered to be deficient. On the other hand, limonene, pinene, and camphene are regarded as suitable raw material for high-density renewable fuels [61]. Pinene was reported to have a net heat of combustion comparable to JP-10 and dimerization of these isomers results in a higher density, which is beneficial for an additional increase of the combustion heat [62]. Since many liquid catalysts are hazardous, corrosive, and require large efforts for waste treatment and recycling, recent studies have focused on the

investigation of different catalysts to approach sustainable dimerization of pinenes and other monoterpenes [61, 62]. In addition, full hydrogenation of the reactive double bonds in the olefinic structures of limonene and myrcene was performed successfully using palladium and platinum catalysts, which increases their value as fuel [63].

The microbial production of monoterpenes is facing a fundamental challenge. Contrary to other isoprenoids, monoterpenes have been reported to be highly toxic, which makes them interesting antimicrobial agents. The mechanism of toxicity is not yet fully understood and it remains unclear if it is either derived directly from molecular interactions or from phase toxicity, which was recently discussed by Brennan et al. [64]. Based on their hydrophobicity, monoterpenes interact with cellular and mitochondrial membranes and dismantle membrane integrity. Microarray analysis of gene expression profiles of *S. cerevisiae* under α-terpinene exposition revealed the up-regulation of genes associated with lipid and fatty acid metabolism, detoxification, and cell wall structure [65]. Based on these observations, the authors concluded that ergosterol synthesis is inhibited during the treatment with terpinene. Severe growth restrictions for *S. cerevisiae* were also observed under the impact of other monoterpenes. β-Pinene was shown to inhibit respiration and transport of H^+ and K^+, which is essential for ATP generation [66]. Limonene was reported to inhibit growth completely at concentrations between 0.5 and 0.8 g/L [64, 67]. Under these circumstances, one possibility to realize microbial production of toxic biofuel precursors is to employ an extractive two-phase fermentation. By adding an immiscible organic layer onto the cultivation medium, the product can be harvested in situ and the harmful impact on the host organism can be reduced. Using dibutyl phthalate, the minimum inhibitory concentration (MIC) of limonene, an indicator of its toxicity, could be increased up to 42.1 g/L for *S. cerevisiae* [64]. However, the selection of an appropriate layer is elaborate, since organic solvents have to be adapted to product and host. On the other hand, efflux pumps are considered to be a promising opportunity to enhance biofuel tolerance. Recently, a computational approach was used to identify new efflux pumps, which improved resistance to toxic biofuels. For this purpose, a library of 43 efflux pumps from sequenced bacterial genomes was created and heterologously expressed in *E. coli*. As a result, strains expressing an efflux pump from *Alcanivorax borkumensis* revealed significantly increased limonene production [68]. Similarly, the pleiotropic drug resistance (PDR) network, a subgroup of ABC transporters, which serves the efflux of cytotoxic compounds and is therefore essential for detoxification, was expected to enhance limonene tolerance of *S. cerevisiae*. However, overexpression of several PDR transporters, which appeared to be upregulated under limonene stress, did not attain the desired effect [67].

4.3.2 Sesquiterpene fuels

Since all sesquiterpenes originate from FPP, microbial production poses a challenge in terms of redirecting metabolic fluxes from this branch point. Regarding the production of the sesquiterpene alcohol farnesol in *S. cerevisiae*, deletion of squalene synthase and adjusting the pH to 7 elevated the final concentrations up to 102.8 mg/L, whereas ergosterol had to be added to the medium to maintain viability [69]. Besides, the abundance of the direct precursor FPP and the converting enzymes are considered to have the greatest impact on the production levels of this fuel compound. By overexpressing a modified Hmg1 reductase, a significant increase of farnesol production in *S. cerevisiae* was recorded at pH 7 with 145.7 mg/L [70]. Depyrophosphorylation of FPP can be realized by several enzymes. In *S. cerevisiae*, the native alkaline phosphatase Pho8p as well as the lipid phosphatases Dpp1p and Lpp1p were reported to hydrolyze FPP to farnesol in two reactions [71, 72]. On the other hand, pyrophosphatases are able to catalyze this reaction directly in one step and may denote a potential engineering target. However, no pyrophosphatase has been characterized with regard to farnesol formation until now [73]. Furthermore, the promiscuity of phosphatases in terms of their substrate specificity complicates the selection of an appropriate engineering target. Another possibility is the utilization of terpene synthases. Heterologous expression of the *OsTPS13* gene in *E. coli*, which encodes a farnesol synthase from *Oryza sativa*, showed that 84.2% of FPP could be converted to farnesol [74]. One last critical aspect for farnesol production in *S. cerevisiae* originates from its regulatory function. Farnesol causes degradation of HMG-CoA reductase and thereby inhibits its own production [75] – a fact that needs to be addressed in further engineering strategies.

In comparison, farnesol production in *E. coli* remains superior so far. Heterologous expression of the MVA pathway together with the overexpression of *ispA* led to final farnesol titers of 135.5 mg/L in only 48 h of cultivation time [76].

Further sesquiterpenes that can be used as fuel precursors comprise farnesene and bisbolene. The latter was recently identified as a biosynthetic precursor of D2 diesel, since its hydrogenated derivative bisabolane was presumed to be a potential fuel due to its chemical structure [77]. The authors, who used an existing microbial platform for amorphadiene production, were able to attain final titers above 900 mg/L of bisabolene in *E. coli* and *S. cerevisiae*, which imparted no toxic effects on the microbial hosts used in this study.

Farnesene was first identified in apple peel [78]. Besides its high energy density and low hygroscopicity, its hydrogenated form farnesane is characterized by a cetane number of 58, which is advantageous over conventional diesel [57, 79]. Stepwise optimization of α-farnesene production has been performed successfully in *E. coli*. For this purpose, a codon optimized gene of the

plant synthase from *Malus domestica* was used and the rate limiting enzymes of the MEP pathway, Dxs and Idi, were overexpressed. In combination with the heterologously expressed MVA pathway and a fusion of the FPP synthase with the α-farnesene synthase, this led to α-farnesene accumulation up to 380 mg/L [79].

5 Future perspectives

The group of isoprenoids has been studied for many years and includes valuable products that are required in large quantities by the food and pharmaceutical industry. Additionally, several isoprenoids were assigned with fuel properties in the recent past, which has elevated the interest for these compounds. Even though chemical finishing is required, which increases the price of these fuels, they combine several advantageous properties and are beneficial over conventional biodiesel and jet fuel. Associated with the structural and functional diversity of this group of chemicals, the product range is likely to expand further in the future. However, the overall success will strongly depend on the production process, which is demanding in terms of productivity, cost efficiency and sustainability. While chemical synthesis and plant extraction suffer from numerous drawbacks, microbial cell factories have emerged as a platform technology for the supply of bulk and specialty chemicals. To this day, metabolic engineering has successfully enabled industrial production of for example valencene and farnesene in large-scale fermentation processes and there are bright prospects for further biobased isoprenoids. However, several challenges remain, one of them being engineering of the terpene synthases due to their often observed product promiscuity and low activities. While several studies succeeded in altering product specificity (e.g. [80, 81]) examples of increasing enzyme activity (e.g. [82]) remain scarce. As well-established cell factories, *S. cerevisiae* and *E. coli* have predominantly been employed. While the endogenous MEP pathway of *E. coli* is energetically more efficient than the MVA pathway, the expression of cytochrome P450 oxygenases is facilitated in yeast, which can be crucial for the production of active pharmaceutical ingredients such as taxol. Numerous strategies have been investigated ranging from overexpression of different plant synthases to heterologous expression of complete pathways. Yet the functional and regulatory complexity that is comprised by metabolic networks demands for the integration of other engineering disciplines in many cases. These may also be beneficial in establishing a functional MEP pathway in *S. cerevisiae*, an approach that is still to be demonstrated. It is especially the available tools from systems biology, which will progressively contribute to this field and have the potential to accelerate the design of future cell factories for isoprenoid production.

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