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Developing fermentative terpenoid production for commercial usage

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Terpenoids comprise a large (>55 000) family of compounds, very few of which have been used commercially due to low and economically unpractical production in their native hosts (generally plants and microorganisms). Two examples of natural terpenoid production are described (rubber and astaxanthin), but the advent of metabolic engineering has allowed the development of fermentative production processes using heterologous microorganisms. The two biochemical pathways responsible for terpenoid production are described, along with manipulations that enable production of terpenoids at economically viable levels. Finally, this article reviews some terpenoids that are currently in commercial production or development, ranging from semisynthetic production of the antimalarial drug artemisinin, through fragrance molecules, to commodity chemicals such as isoprene and β-farnesene.

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Introduction

The terpenoids constitute a diverse family of compounds with over 55 000 known members [1]. They are composed of 5-carbon isoprene (2-methyl-1,3-butadiene) units and are successively assembled into terpenes according to the biogenic isoprene rule [2]. Their common precursor is the 5-carbon intermediate isopentyl diphosphate (IPP). The resulting terpenoids are classified by the number of isoprene units contained within the structure (Table 1: the term 'terpenoid' is used to denote any compound biologically derived from isoprene units. 'Terpene' denotes an alkene.). There are two major pathways for the biosynthesis of terpenoids: the mevalonate pathway (MVA) [3] and the methylerythritol phosphate (MEP) pathway [4]. Recently a modified MVA pathway was also proposed [5].

There are few examples of commercial terpenoid production from natural host organisms. An example is astaxanthin, a high value tetraterpenoid ketocarotenoid responsible for the red color of salmon and cooked crustaneans produced commercially as an aquaculture feed supplement by cultivation of natural astaxanthin-producing microbes, including the alga *Haematococcus pluvialis* [6•], the yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) [7], and the bacterium *Paracoccus carotinifaciens* [8]. Possibly the largest-scale industrial production of a terpenoid from its native producer is natural rubber, a high molecular weight polymer harvested from the rubber tree (*Hevea brasiliensis*). Efforts are underway to understand rubber biosynthesis and transfer it to heterologous organisms, but alternative commercially viable production has not been reported yet [9,10].

Metabolic engineering of higher plants and algae for terpenoid production has been recently reviewed [11]. Plant engineering for terpene production presents many opportunities, but also many complications such as tissue-specific expression and loss of volatile products to evaporation. Although heterologous production of terpenoids has been demonstrated in plants (e.g., artemisinin production in tobacco [12]) the production levels are too low for economic feasibility, thus commercial development of terpenoid production is predominantly focused on engineered microbes. Considerable effort is required to develop terpenoid production from the titers described in the scientific literature, typically at the mg/L level (e.g., premnispirodiene and botryococcene at 170 mg/L and 60 mg/L, respectively [13]) to economically viable performance metrics of titer (g product per L fermentation broth), yield (g product per g substrate) and productivity (g/L h) [14], along with satisfactory downstream processing and recovery. This review describes terpenoid products for which such development is either envisaged, ongoing, or production is already occurring at an industrial scale.

Both the MVA and MEP pathways are attractive for use in the heterologous microbial production of terpene-based chemicals and fuels. There has been success in engineering the MVA pathway in *Saccharomyces cerevisiae* [15••] and in *Escherichia coli* [16], and the MEP pathway in *E. coli* [17•], but with little success in *S. cerevisiae* [18,19•]. It is attractive to use *S. cerevisiae* as a chassis for the production of terpenes as is classified by the U.S. Food and Drug Administration as a 'generally recognized as safe' (GRAS)

Table 1

Terpenoid classification based on number of isoprene units.

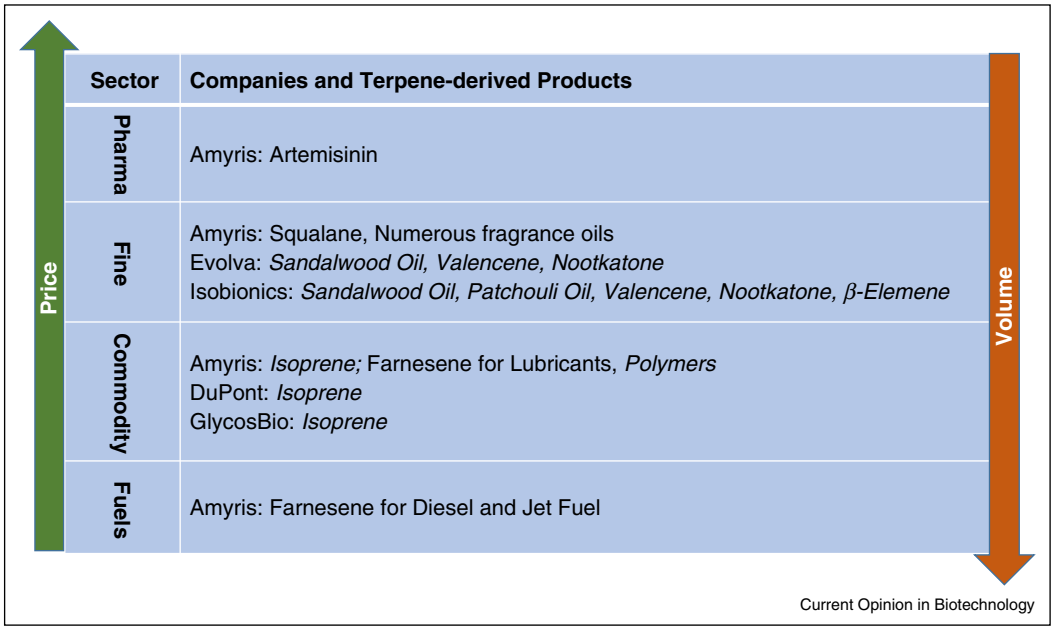
Class	Isoprene units	Molecular formula
Hemiterpenoids	1	C_5H_8
Monoterpenoids	2	$C_{10}H_{16}$
Sesquiterpenoids	3	$C_{15}H_{24}$
Diterpenoids	4	$C_{20}H_{32}$
Sesterterpenoids	5	$C_{25}H_{40}$
Triterpenoids	6	$C_{30}H_{48}$
Tetraterpenoids	8	$C_{40}H_{64}$
Polyterpenoids	>8	$(C_5H_8)_n$

organism. To date, the MVA pathway has proved to be superior for the production of terpenes at high yields and titers [15••,16]. However, the MEP pathway is theoretically superior to the MVA pathway due to higher maximum theoretical stoichiometric yield from glucose to IPP (30% for MEP and 25% for MVA), it is redox balanced, and requires less oxygen [16,20••]. In spite of this, the MEP pathway has been challenging to engineer in yeast, and has only achieved modest levels of product in *E. coli* [17•]. Challenges to engineering the MEP pathway in yeast include the engineering of two iron-sulfur cluster proteins and identification of the appropriate redox partners for these enzymes [21]. Another approach to achieving high yield and productivity remodels central carbon metabolism and the MVA pathway to create a synthetic metabolic pathway in *S. cerevisiae*, with a carbon yield equivalent to the MEP pathway, is redox balanced, and requires 75% less oxygen [22–24].

Terpenes of commercial interest

With the exception of petroleum-derived isoprene and natural rubber, no terpenoids are currently produced or used at 100 000 tons/year or more. Terpenoids are used as solvents and ingredients in perfumery, foods, cosmetics and personal care products, medicines, and a few other consumer applications, spanning the whole volume/price range of pharmaceutical, fine, commodity chemicals, and fuels (Figure 1). The total combined volume of all terpenoids used as fragrance ingredients is about 50 000 tons/year, with a limited number of monoterpenes accounting for more than 5000 tons/year [25]. Terpenoid supplies and prices can be very volatile because of the very small market size and the fact most are extracted directly from plant sources; these factors make the development of alternative stable production by fermentation attractive as such production has the potential to both reduce supply volatility and price (Figure 1). Pharmaceutical and fine chemicals have attractive price targets, so it should not be surprising that three of the five metabolic engineering companies mentioned in Figure 1 are targeting terpenes currently used in the pharmaceutical [15••,26] and fine chemicals (e.g., flavor and fragrances [27], and cosmetics [28]) sectors. Three companies are also targeting commodity chemical products and fuels (Figure 1). Both of these markets will be discussed in the following sections.

Figure 1



Companies producing or developing fermentative terpene production, their products, and market category (to the best of our knowledge, products in italics are not yet in commercial production). Companies include: Amyris: www.amyris.com; DuPont: www.biosciences.dupont.com; Evolva: www.evolva.com; GlycosBio: www.glycosbio.com; Isobionics: www.isobionics.com.

Terpenes as pharmaceuticals and fine chemicals

Of the known terpenoids extracted from plant materials for commercial use, most are used as ingredients in the fragrance and food industries [25], or as building blocks in the synthesis of more complex molecules. Metabolic engineering companies have targeted terpenoids that have or are predicted to have limited supply, and/or there is an environmental cost for their use. Microbial production using large-scale fermentation of organisms engineered to manufacture these compounds will result in a lower cost, more sustainable source. Examples discussed in the following text include currently produced terpenoid products, artemisinin and squalane, and products targeted for future production, like sandalwood and patchouli oil.

The highest published heterologous production titer of terpenoids to date derives from a program to develop semi-synthesis of the potent anti-malarial drug artemisinin [15••,29]. The semi-synthetic artemisinin project aimed to provide an assured second source of the drug immune from the supply and price fluctuations that have characterized plant-derived artemisinin [30•]. The sesquiterpene hydrocarbon artemisinin precursor, amorphadiene, was produced at 40 g/L [31•], and the oxidized precursor, artemisinic acid, at 25 g/L [15••], in yeast (*S. cerevisiae*) engineered to overexpress the mevalonate pathway enzymes for farnesyl diphosphate production. The entire semi-synthetic artemisinin program has recently been summarized [30•]. By way of comparison, the highest recorded production of taxadiene (the hydrocarbon diterpenoid precursor to the anticancer drug paclitaxel) to date uses *E. coli* with an optimized MEP pathway to increase terpenoid flux, producing 1 g/L taxadiene [17•]. As plant cytochromes P450 do not express well in *E. coli* [32], oxidized taxadiene was generated by co-culturing taxadiene-producing *E. coli* with P450-expressing yeast, producing 33 mg/L oxygenated taxanes [33]. A rationale for this co-culture was that the MEP pathway has potentially higher yield, yet it is unclear how such a co-culture could be stably conducted on an industrial scale, and high yield is of minimal importance when producing high value products such as paclitaxel [14].

Squalane, produced by the catalytic hydrogenation of squalene, a natural triterpene hydrocarbon isolated from shark liver or olive oil waste, is a valued cosmetic oil. In its pure form, it is a colorless, odorless, tasteless, and highly-stable hydrocarbon oil that is biocompatible with skin, non-toxic and non-irritant, and prevents moisture loss, restoring skin's suppleness and flexibility [34–36]. Like other natural ingredients, squalene supplies are subject to natural supply uncertainties; however due to the shark source, it has become the focus of campaigns against the harvesting of endangered sharks [37,38]. Squalane is currently produced by Amyris by dimerizing the fermentation product β-farnesene (see following section), followed by a chemical hydrogenation step to create the saturated hydrocarbon [28].

Sandalwood oil, an essential fragrance oil that is under development as a target for microbial production at Evolva and Isobionics, is currently obtained by steam distillation of the heartwood of *Santalum album*. The oil consists almost entirely of the isomeric sequiterpenes, α-santalol and β-santalol, of which the latter is mainly responsible for the odor. It is used almost exclusively in perfumery as a valuable, stable, woody note fixative. Its scarcity comes from the fact that only mature trees produce enough oil for its exploitation to be profitable. Due to over-harvesting, disease, and grazing animals, the *S. album tree* is now threatened, and production of the oil is unsustainable. The sesquiterpene synthases responsible for biosynthesis of the hydrocarbon santalene precursors have been identified [39,40], and cytochrome P450 CYP76F has been demonstrated to be responsible for the oxidation of santalenes to α-santalol and β-santalol and bergamotol [41].

Patchouli oil, produced by steam distillation of dried *Pogistemon cablin* leaves, has a characteristic odor described as camphoraceous, woody and balsamic. Its main component is (−) patchchoulenol (27–35%), with many other sesquiterpene components. Used in perfumery for its oriental and masculine notes, it is also widely employed in consumer products (fabric softeners) for its substantivity [42]. A single multi-functional sesquiterpene synthase has been identified that is responsible for the production of patchouli oil and its product profile has been characterized [43]. Production of patchoulol, the key ingredient of patchouli oil, has been reported in yeast, albeit it at fairly low titers (~20 mg/L) [44,45].

A final example of a fragrance molecule for which commercial fermentative production would be of interest is sclareol, the precursor to synthetic ambroxides which are used as replacements for ambergris, a waxy high value fixative (compounds used to extend the longevity of volatile scents in fragrances) from endangered sperm whales. Diterpene synthases responsible for ambroxide-related diterpenoid biosynthesis have been identified [46], and production of ~1.5 g/L sclareol from engineered *E. coli* demonstrated [47].

A prerequisite for heterologous production of fragrance oils is synthase identification and characterization. With the synthases in hand, microorganism engineering and optimization can commence, making production economical and sustainable.

Terpenes as commodity chemicals and fuels

The replacement of commodity chemicals currently derived from petrochemical sources with microbial

fermentation products will have the environmental benefits of reducing greenhouse gas emissions, producing less waste, and reducing dependence on oil. Two such products, isoprene and β-farnesene, are targets of metabolic engineering companies.

β-Farnesene, produced by Amyris, is a product that is unique in that it was not previously commercially available except as an expensive specialty chemical. It was developed on the basis of its many prospective commercial uses upon increasing supply and decreasing cost to commodity levels. It is a 15-carbon branched-chain alkene with a conjugated double bond system at its terminus that makes it amenable to chemical modification, and is produced as a pure enantiomer. Farnesene has many uses as a feedstock molecule for the production of cosmetic oils [28], polymers [48,49•], surfactants [49•], lubricants [50] and fuels [49•], among others. This substance is a natural product used as an alarm pheromone emitted by aphids [51], a natural coating of apples [52], or as a component of essential oils. The microbial fermentation product is obtained as a high-purity single isomer directly from the fermentation process, and a simple flash distillation results in high molecular purity (~98%). Amyris' production platform is fully scaled and β-farnesene is currently produced at their manufacturing plant operating in Brotas, Brazil (https://amyris.com/innovation/industrial-production/). This ready availability and high purity have allowed Amyris to develop a wide range of novel β-farnesene derivatives. Each of the uses of β-farnesene has been verified by Amyris and its partners, and approved for use by the various regulatory bodies (e.g., American Society for Testing and Materials, US EPA, the Brazilian Fuels Regulator ANP) that govern the use of the product in each of these markets. Amyris is currently producing and selling squalane (www.biossance.com), and diesel and jet fuel (https://amyris.com/media-center/ and press releases therein).

Isoprene, the target for fermentative production by Amyris, DuPont [16] and GlycosBio, is used in the production of various types of rubbers and is expected to face a limited supply/high price scenario in the future due to both increasing consumer demand and a shift in petroleum feedstocks from heavy crude to shale gas which has limited the availability of the C5 fraction from which it is obtained [53]. Some 0.8–1 million metric tons of isoprene are currently made and used annually, mainly (~95%) for the production of synthetic rubber (polyisoprene, isoprene rubber, IR), which is similar in structure and properties to natural rubber. Polyisoprene is mostly used in the manufacture of tires, with the remainder going into other polymers and as a raw material in the manufacture of other specialty chemicals [53].

Conclusions

Terpenoids have traditionally been supply limited, but the development of metabolic engineering technology has enabled their production at commercial scale. To date, only a few terpenoids have been produced commercially by fermentation of engineered microbes. The most notable examples are the anti-malarial drug precursor artemisinic acid, and the fuel and chemical feedstock molecule β-farnesene. Although both these products are sesquiterpenes produced by yeast, there are substantial differences in the challenges required to be overcome to make them commercially viable. Artemisinic acid is a high value pharmaceutical precursor to the active ingredient artemisinin [30•] that required considerable effort to obtain sufficient production of the oxidized precursor, artemisinic acid [15••], while β-farnesene is a low price-point commodity chemical that required significant effort to increase its production yield to reduce cost of production [22–24]. These products also differ in that one has a single use (production of a drug), while the other is converted into a wide array of products including an emollient (squalane [28]), polymers [48], and fuels [54]). The successful engineering of yeast metabolic pathways to enable commercially-viable production of farnesene also provides a base strain for the production of a virtually limitless array of other terpenoids. Replacement of the terminal enzyme of the pathway, farnesene synthase, with other sesquiterpene synthases leverages previous work that has maximized the conversion of sugar to terpenoid precursors. Discovery of relevant terpene synthases and modification enzymes should open the door to commercial production of fragrance molecules, and perhaps other terpenoids with currently undreamt of uses.

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