Microbial Production of Food Bioactive Compounds

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MICROBIAL PRODUCTION OF TERPENES

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Abstract (approximately 250 words)

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Sustainability is one of the main challenges facing humankind in the 21st century due to the continuous increase in the demand for energy and resources that characterises our current industrial activity, driven in part by exponential population growth. This problem can be tackled from different strategies and among them the search for biomolecules, either recovered through processes based on the circular economy or through synthetic biology, are highly promising. In this sense, terpenes, the largest family of secondary metabolites in the plant kingdom, have attracted much of the research in recent decades in pursuit of alternative, more ecological and sustainable ways for their obtention. The reasons for this interest are due to their extensive structural diversity and the possibility of gaining new functionalities, simply by chemical modification, which also makes them excellent candidates in areas such as biomaterials and pharmaceuticals. But also, the fact that the natural biosynthetic pathways of terpenes are well known from the point of view of metabolites and enzymes facilitates their industrial production using genetically modified microorganisms.

This chapter aims to give the reader a broad but at the same time comprehensive view of the production of microbial terpenes in a sustainability context. Starting by the circumstances that lead to the need to look for renewable sources of biomolecules and following by why terpenes represent a very promising opportunity even if only their characteristics from a chemical and bioactivity point of view were considered. Finally, it will be discussed which microorganisms can produce these unique lipids and how, the main option followed nowadays is using synthetic biology strategies, involving modified organisms that are already being used on an industrial scale for applications ranging from biofuels to pharmaceuticals.

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Keywords (please provide around 4-8 keywords)

Sustainability, Terpenes, Synthetic biology, Saccharomyces cerevisiae, Farnesene, Squalene, Artemisinin

Terpenes & Sustainability

Throughout this and the following sections, the aim is to discuss how terpenes, among all the available options of biomolecules, have been identified and become a relevant avenue in the framework of sustainability and circular economy strategies. To this end, it is first necessary to review the circumstances that have led to the need to find biomolecules, as well as their sources, that fit into the paradigm of sustainability on an industrial scale and then to delve into the possibilities offered by terpenes.

Framework of a resource crisis: A sustainability problem

The 21st century has inherited a number of problems from our recent history, namely challenges resulting from lack of resource availability as well as environmental. They are rooted in the recent industrial/technological development and the consequent increase of human population in addition to our limited capacity of adaptation to the impact of such changes.

Our population growth rate was low during the first 18th century, only reaching 1 billion people by 1850 (1) but it was followed afterward by a 6-fold exponential increment until the 2000s. According to predictions, it is expected that by the year 2030, the planet Earth will have 8.5 billion humans, increasing up to 9.7 billion by 2050 (2). This situation will come associated with a steadily raise in the demand for both energy and natural resources.

The first (1760-1840) and second (1870-1914) industrial revolutions led to the automatization of commodities production, thanks to the development of technologies for the utilization of fossil fuels to obtain energy. Coal, oil and natural gas are the three main sources of fossil fuels: when burned, they deliver their stored energy but also greenhouse gases (i.e., CO_2, CH_4, N_2O and fluorinates gases). In fact, the release of these chemicals into the atmosphere is directly associated with human activity: CO_2 represents 74.4% of total global emissions (30.4% from electricity and heat and 15.9% from road transportation), CH_4 and NO_2 (17.3% and 6.2% respectively of total emissions) are mainly produced from agricultural activities, while fluorinated gases (6.1%) are coming from chemical and petrochemical processes (3). The cumulative anthropogenic carbon flow related to fossil CO_2 during the period 1850-2019 was 440 gigatonnes (GtC) being that from the 2010 to 2019 period, the annual flow was 9.4 GtC/yr (4) meaning a total of 94 GtC. This points toward a great acceleration of the emissions in the last years.

Furthermore, it must be considered that the formation of fossil fuels requires millions of years (i.e., they arise from geological processes) and mining rates largely exceed that of restock, they are not renewable sources, and shall be depleted during the following years. Hence, humankind requires new sources of energy with reliable production and, ideally, zero environmental impact. All current human activities follow the pattern extract-make-use-dispose in a linear economic model, but new approaches have arisen in the last years focussing on the reutilization of biological waste from land and sea to produce food, energy and various materials while also promoting the rationale use of all available resources. Nevertheless, this brings new challenges, since bio-waste is subjected to geographical diversity, season-dependent supply, and variability as well as complex composition that may compromise their utilization at a large scale. This, therefore, needs new technologies and strategies.

For example, in the search for sustainable alternatives to oil, bioethanol and biodiesel have currently emerged as solutions. Thus, bioethanol used as an octane additive (5), can be obtained from the fermentation of sugar or other starch-containing raw materials (first generation) (6) or hemicellulose and cellulose from

lignocellulosic biomass (i.e. bagasse or straw; second generation) (7) or other carbohydrates-containing biomass as marine algae (8).

The evolution of the technologies for bioethanol production aims to avoid the use of sources that may compete with food production as well as minimize as much as possible land utilization. Accordingly, biomass is, at this moment, the preferred feedstock (9). For example, biodiesel research, as this biofuel is composed of long-chain fatty acid esters, is focused since the last years on the utilization of waste plant oils and animal fats or oleaginous yeasts and fungi (10).

Other biofuels approaches are based on the anaerobic fermentation of bio-waste (i.e., from agriculture and food) resulting in the production of both CH_4 and CO_2. This strategy is highly promising since, only in the EU, 86 million tonnes of biowaste were generated during 2017 (11). Nevertheless, this type of biomass is disposed in landfills or by incineration, and 3% of total greenhouse emissions in the EU comes from this latter. However, the main drawbacks for its worldwide utilization are quality requirements related to further purification steps for the elimination of CO_2 and other gases (e.g. hydrogen sulfide, ammonia, siloxanes or oxygen) (10). The presence of such gases other than CH_4 may result in deterioration of engines and equipment.

As discussed above, most of the current strategies to obtain sustainable biofuels have moved from fossil to edible sources. This is bringing a high concern regarding the future availability of fertile cropland, pure water, and energy worsened by the climate change scenario that will disrupt society, environment and economics (12). Also, considering that, according to FAO, every year 1.3 billion tonnes of food (one-third of total global production) becomes into waste (i.e., as a result of decisions and actions by retailers) or losses (i.e., any food that is discarded, incinerated, or otherwise disposed) there is an urgent need to improve the efficiency of the food chain as part of the solution for the global hunger crisis. This may decrease the availability of biowaste compromising the production of sustainable energy.

Consequently, the solution seems to involve the use of molecules that can be replenished, once they must therefore exist in nature and be of biological origin (biomolecule), preferentially microbiological, since they have fast growth, but not primarily intended as food. Ideally, the metabolic pathways for these biomolecules should be fully understood or at least well known, with enough information regarding the enzymes and metabolites involved in their synthesis. Then, it would be possible to optimize microbiological production or modified suitable organisms (i.e., synthetic biology).

Terpenes as promising sustainable biomolecules

The technological and industrial breakthroughs since the XVII century have been supported by access to abundant or cheap energy sources and materials. For instance, it happened with coal during the first industrial revolution, with oil after the Second World War II and silicon at the beginning of the informatics era. In this recent scenario of seeking sustainable biomolecules with a reliable supply, the logic strategy seems to be looking at plant sources as there is a high number and diversity of species and they can be cultivated if needed. In fact, since ever, land plants have provided us with fats, carbohydrates, and proteins for food (some of them later crops after a domestication process), lignocellulosic feedstocks that we have used as materials (e.g., wood for houses, straw for adobe or mudbricks) as well as extracts and ingredients able to exert medicinal properties in what it is known as phytopharmaceuticals.

As part of their strategy to adapt and colonize as many ecosystems as possible, plants rely on an amazing synthetic capacity to be able of using inorganic precursors to build organic molecules (13). Such compounds fit into the category of primary metabolites (crucial for normal growth, development, and reproduction) or phytohormones (to regulate plant growth and development). Finally, those not involved in the growth or

reproduction but bringing selective advantages are known as secondary metabolites (SMs) although they also can have regulatory functions or be precursors of primary metabolites (13).

The SMs can be classified according to their chemical structure (terpenes, phenolics, glycosides and alkaloids), their biosynthetic origin (terpenoids, nitrogen and sulfur-containing compounds and phenolics) or their physiological activity (phytoalexin, growth-stimulating hormones, vitamins, pigments, alkaloids, and plant toxins) (14). It has been estimated that 100000 compounds hold functions as SMs, being that 8000 molecular structures are phenolics, 12000 are alkaloids but terpenes and terpenoids are the main integrating members of SMs since there are over 55000 structures and identified compounds (15). Therefore, from the point of view of finding sources of biomolecules, terpenes pose a great opportunity.

Humankind has already made use of natural products rich in terpenes since time immemorial, and this has helped us in our (still ongoing) journey towards technological development. For example, conifers provided us with pine tar, which is excellent for caulking hulls of wooden ships, like water repellent coating for ropes or adhesive while pine resin was an embalming or mummification fluid (16). Moreover, pine resin (i.e., oleoresin that plays an important role in tree defence) can be also distilled to obtain turpentine (its essential oil) and rosin, the solid non-volatile fraction. Just to put into context the importance of terpene-derived commodities, it has to be considered that all materials derived from pine resin (i.e., tar, pitch, turpentine, pine oil, rosin) were so relevant for the elaboration of products related to building and maintenance of wooden vessels that this group was commonly known as naval stores.

In fact, the name terpene was first proposed in 1866 by the German organic chemist August Kekulé as an abbreviation of terpentine, an obsolete term for turpentine, a word derived from the Greek, "τερεβινθίνη", terebinthine. Such material, which is also known by other names as gum turpentine, spirits of turpentine or terpenes oil, is a mixture of alpha and beta-pinene, 3-carene, camphene and limonene. This later compound, 1-Methyl-4-(prop-1-en-2-yl)cyclohex-1-ene by its IUPAC name, is a monoterpene containing two double bonds that can be easily oxidized to obtain esters, aldehydes, ethers and epoxides. Limonene can be exploited therefore as a building block for further applications and is attracting a high interest in recent years in research studies focusing on biopolymers (17). Furthermore, this is not the single one terpene that can be assayed for obtaining polyolefins, since pinene, myrcene, β-farnesene among others are also suitable for this purpose.

The use of terpenes for the manufacture of polymers is not new: the rubber tree or *Hevea brasiliensis* has been always highly appreciated by its latex. This is a suspension of cis-1,4 polyisoprene that after coagulation with formic acid and, depending on the application, vulcanization, the process invented by Charles Goodyear in 1839, yields natural rubber that is widely used nowadays, mainly in automobile tires and in other consumables, from toys to clothes. Anecdotally, the name was coined by the British physicist Joseph Priestley in 1770, after he observed that it could be used to erase pencil marks. Moreover, Aztecs made rubber balls for games played during ritual ceremonies.

As discussed in this section, terpenes are molecules with a wide diversity not only of sources but also of structures. They are easily functionalised compounds, by chemical transformation, adapting to the physicochemical characteristics required for the product to be manufactured. Throughout history, humankind has made use of different natural sources, mainly those obtained from conifer resins, which have had a great industrial and economic impact in the transportation and materials sectors.

This makes them biomolecules with a great utility for the circular economy, as they are sustainable and already exist in nature, since they come from plants, and their use so far is not related to food production.

Since the relevance of terpenes as biomolecules for circular economy is established, the following sections will deal with the chemical characteristics of this group of compounds, effects on human health as well as industrial applications.

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Terpenes: Definition, Structure and Sources

In this subsection, it will deepen into general concepts about terpenes. Thus, the definition of terpenes and terpenoids, their structure and main sources are discussed, as well as some of their biological activities.

Definition and Structure

Terpenes are hydrocarbons containing one or more double bonds in their structure. Also, the oxygenated derivatives of terpenes (comprising functional groups that include alcohols, aldehydes, ketones and carboxylic acids) are designated as terpenoids. These two families of compounds are divided according to their isoprene (2-methyl-1,3-butadiene, C_5H_8) number (n) in hemiterpenes (n=1, C_5), monoterpenes (n=2, C_{10}), sesquiterpenes (n=3, C_{15}), diterpenes (n=4, C_{20}), sesterterpenes (n=5, C_{25}), triterpenes (n=6, C_{30}), tetraterpenes (n=8, C_{40}) and finally, those with more than 8 isoprene units (i.e n>8) are named as polyterpenes (18). Accordingly, the general formula of terpenes is $(C_5H_8)_n$ (Figure 1).

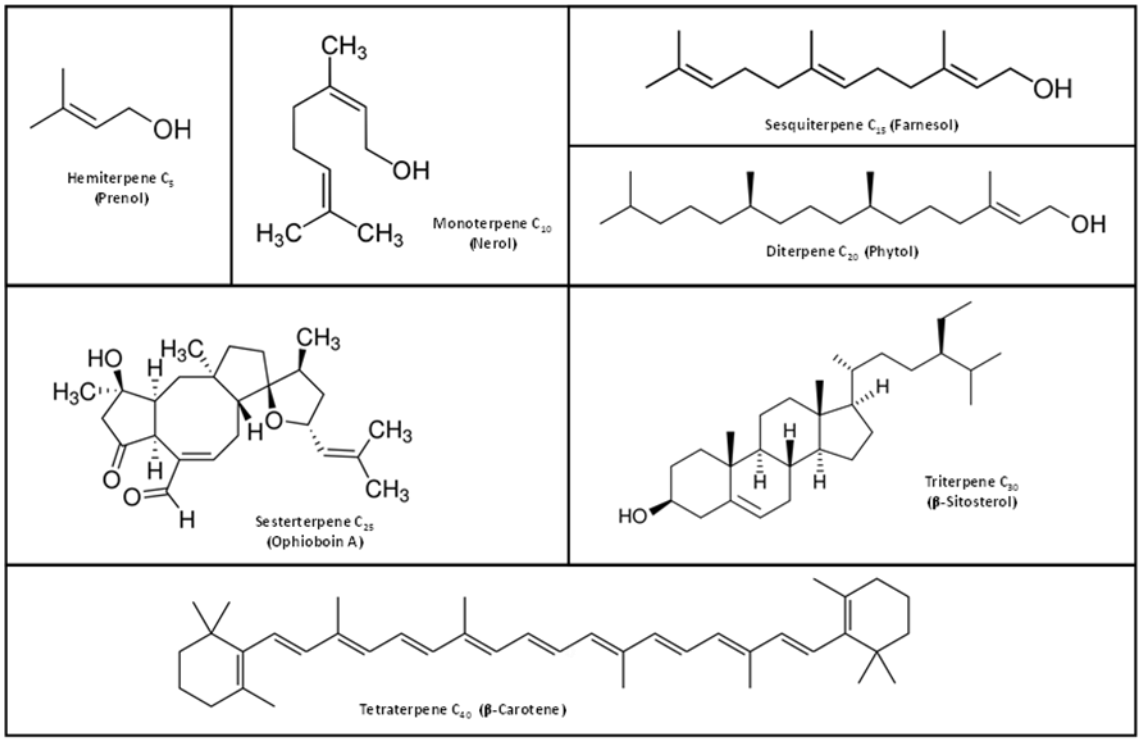

Figure 1. Chemical structure and classification of terpenes according to the number of isoprene units and number of carbon atoms with examples.

The isoprene units, where the first carbon is usually termed as "head" and carbon 4 as "tail", are generally assembled in a "tail-to-head" scheme (i.e., as a general rule 1-4 and 4-4 bonds does not occur in nature) although it can be found in some compounds as β-Carotene being an exception since a "tail-to-tail" link can be observed. Thus, terpenes show significant structural variability, including linear, cyclic, monocyclic, bicyclic or tetracyclic compounds (19). In this latter, links are not designed as crosslinks as they do not also follow the general rule. Terpenes with a larger number of isoprene units can present more complex structures, like macrocyclic forms (20). At the same time, stereoisomers can also be formed (21), increasing the variability of these compounds.

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Hemiterpenes (C_5H_8) are the simplest terpenes because they are formed by only one isoprene unit. Isoprene, the main hemiterpene, is a volatile compound, since it has a boiling point of 307.217 K or 34.067 °C, resulting from plant metabolism, and released to the atmosphere by the leaves, as a natural by-product. Animals and bacteria can also produce it (22). Other hemiterpenes such as acids and isoamyl alcohol that are synthesized by plants are used in industry for flavour and fragrances (22). Just as one more example of the structural diversity and application potential of this family of compounds, recently, 3,3-dimethylallyl alcohol (prenol) has demonstrated neuroprotective and longevity-promoting potential in *C. elegans* (23).

Monoterpenes ($C_{10}H_{16}$), composed of two isoprene units, are known to be compounds with aroma that play an important role in the scent of many flowers and fruits, being the main components of essential oils (i.e. the plant concentrated hydrophobic extract containing their volatile compounds), with agricultural, pharmaceutical, cosmetic and food applications (19,20,24). They also have biological activities namely in cardiovascular protection (25). Furthermore, sensory characteristics of wine products such as citrus, fruity and floral flavours are due to the presence of these terpenes derived from grapes such as linalool, nerol, citronellol, α-terpineol (26).

Sesquiterpenes ($C_{15}H_{24}$), composed of three isoprene units, are larger and more stable than monoterpenes and are naturally found in plants, fungi and insects (19,24). Applications in cosmetics, fragrance and food industries have been described for these compounds (22). Several sesquiterpenes have been isolated from grapes, contributing to the wine's aroma (27). They have many uses in traditional medicine since varied biological activities have been described: anticancer, anti-inflammatory, as well as antimalaria activities (i.e artemisinin) (28).

Diterpenes ($C_{20}H_{32}$), composed of four isoprene units, have been mainly isolated from plants and have been demonstrated to exert biological activities such as antitumor, anti-inflammatory activities (24) as well as antimicrobial and antifungal activities (18,22). Moreover, the cardiovascular effect has also been described for these terpenes as anti-hypertensive agents (29,30).

Sesterterpenes ($C_{25}H_{40}$) are a rare terpene class composed of five isoprene units and have been isolated from marine sponges, fungi, plants and insects (31) but those better characterized are from marine sponges and terrestrial fungi (32). Sesterterpenes are also biologically active with antimicrobial, antifungal, anti-inflammatory and anticancer effects (22,31,33). Immunosuppressive activity of sesterterpenes has also been observed (34).

Triterpenes ($C_{30}H_{48}$) are composed of six isoprene units and comprise steroids and sterols. They are biologically derived from squalene and are produced by animals, plants and fungi. Several biological activities such as anticancer, antioxidant, antiviral and anti-atherosclerotic have been described for this class of terpenes(24).

Tetraterpenes ($C_{40}H_{64}$) or carotenoids are composed of eight isoprene units. They are found in fungi, cyanobacteria, algae and plants and are mainly responsible for the yellow, red or orange fat-soluble plant and animal pigments (24). They present antioxidant and antitumor activities and are used in food, pharmaceutical and cosmetic industries as active ingredients (35).

Polyterpenes are composed of more than eight isoprene units. Examples of this class of terpenes are γ/δ-Tocopherol and as the main biological activity, they have been found as anticancer agents (18).

Some examples of terpenes included in each class, their role as well as their sources are summarized in Table 1.

Plant terpenes are synthesized via the mevalonate (MVA) and methylerythritol-phosphate (MEP) pathways from the precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). Yeasts and other fungi only harbor MVA pathway while bacteria uses exclusively the MEP pathway to produce terpenes (36). These pathways will be further described and discussed in detail in subsection "**Error! Reference source not found.**".

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As above mentioned, the basic building unit of the terpenes is isoprene. Terpene biosynthesis is obtained by condensation of isoprene units. This reaction can be performed in a head to head or head to tail orientation, as a "regular" obtention for a terpene whereas the "irregular" synthesis is applied to a "head to middle" linkage of the isoprene units (37). Examples of these irregular monoterpenes are lavandulol (37) and may also occur in tri- and tetraterpene molecules (38). The head-to-tail method to link the isoprene units occurs in mono-, sesqui-, di-, and sesterterpenes (38). Isoprene units conformation in chains can be arranged to form rings (22).

Terpenes and terpenoids are a vast family with a high and complex structural diversity and therefore humankind has found, since a long time ago different natural sources, that have been the starting point for further applications of the terpene molecules that they provided.

Oleoresins are synthesized from pine trees as a defence mechanism against bark beetles and their associated pathogenic fungi and are mainly composed of monoterpenes and diterpenes but have sesquiterpenes as minor components(39).

Several applications of the resin are known, such as in their use in paints, waxes, adhesives, soaps, pharmaceutical products, etc. Two main fractions can be extracted from pine resin: rosin (approx. 75%) and turpentine (approx. 15%) (40). Turpentine is the volatile fraction obtained from the pine resin (21) and rosin is the solid fraction that remains after the evaporation of turpentine (40).

Turpentine is mainly composed of the monoterpenes α-pinene, β-pinene and δ-carene and has been used as a solvent, fragrance and flavour compounds (40).

Rosin is mostly composed of 80-90% diterpenic acids (primarily abietic, pimaric and labdanic) and 10% of a combination of esters, alcohols, aldehydes and hydrocarbons (40).

In summary, terpenes are natural compounds that can be isolated from plants, animals, insects, fungi and marine organisms. They are more abundant in higher plants, citrus, conifers and eucalyptus, being widely distributed by the leaves, flowers, stems and roots. Among these, sesterterpenes are rare in nature, however, they can be isolated from insects, fungi and marine sponges.

Although these compounds occur in nature, they are present in low concentrations. Due to their diversity, there is the requirement for specific extraction protocols. Furthermore, the yield of these approaches is usually low and at the same time, obtained compounds are very unstable. Then the need to devise strategies to obtain terpenes urges, an alternative might be the use of microbial engineering to obtain compounds of interest.

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Table 1. Sources of terpenes and terpenes of interest

Classification	Source	Role	Examples	Reference
Monoterpenes (C10)	Flowers, fruits and leaves	Attract pollinators, repelling microorganisms from feeding off of plants, components of essential oils Cardiovascular effects	α-pinene (pine trees), limonene (citrus plants) geraniol, nerol, Δ9-Tetrahydrocannabinol	(18,24,25)
Sesquiterpenes (C15)	Plants, fungi and insects	Defensive mechanism, attract mates with pheromones in insects, plant growth hormones, signaling properties, Antitumor, anti-inflammatory, antioxidant activities	Farnesans, artemisinin, Elemene, farnesol, β-nerolidol, β-santalol, Bisabolene, patchoulol	(18,22,24,28)
Diterpenes (C20)	*Euphorbia* plants	Antitumor, anti-inflammatory, antimicrobial, antifungal, anti-hypertensive activities	Phytol, retinoic acid, sclareol, steviol	(18,22,24,30)
Sesterterpenes (C25)	Marine sponges, plants, fungi, insects	Anticancer, anti-inflammatory, antimicrobial, antifungal, immunosuppressive activities	Heteronemin, tetronic acid, manoalide, ophiobolin A	(22,31,33,34)
Triterpenes (C30)	Animals, plants and fungi	Anticancer, antioxidant, antiviral, anti-atherosclerotic activities	Steroids, sterols (β-Sitosterol, campesterol), betulin	(18,22,24)
Tetraterpenes (C40)	Plants, cyanobacteria, algae and fungi	Natural pigments, antioxidant, anticancer activities	β-carotene, lycopene, astaxanthin	(18,22,24,35)
Polyterpenes		Anticancer activity	γ/δ-Tocopherol	(18)

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Role of terpenes in human health

In the previous sections, it has been discussed the relevance of terpenes as biomolecules from an economical and environmental point of view and how their potential is related to their structural diversity.

Terpenes found in natural sources as distillates from herbal and plant extracts have been appreciated for their health-promoting properties. This subsection will bring to the reader a summary of the bioactivities of this family of compounds.

Suitability as Bioactive Agents

According to WHO, obesity is the second most prevalent metabolic disease, together with blood pressure, in the development of non-communicable conditions [i.e., cardiovascular diseases (CVD), cancer, chronic respiratory diseases, and diabetes]. This highlights that the prevalence of impaired metabolic pathways related to cholesterol biosynthesis or glucose uptake requires new approaches to control hypercholesterolemia and diabetes diseases. In fact, there is also an increasing prevalence of diabetes mellitus over the past decades and it is estimated that the number of diabetic patients will reach up to 366 million by 2030 (41). Moreover, fatty liver disease (FAD) is also an emerging important health condition due to its association with obesity and diabetes. Accordingly, new approaches to control these metabolic disorders may reside in potential therapeutic target molecules that can prevent or reverse the intracellular accumulation of lipids in the hepatocytes, as well as control the pro-inflammatory mediator's expression (i.e. minimizing cytokine stress)(42,43).

In this section, the bioactive effects of terpenes will be discussed, especially when applied as therapeutic drugs in diseases such as antidiabetic, hypocholesterolemic as well as in fatty liver pathological conditions. Terpenes can also exert anti-inflammatory activity, which can be explained by modification on the production of arachidonic acid metabolism and control of the cytokines production.

Anti-diabetic and Hypocholesterolemic Studies

The synergic bioactive effects of extracts composed of fatty alcohols and other phytochemicals such as terpenes and phytosterols have revealed high potential and therefore attracted great interest. There is an increasing awareness of triterpenoids in pharmaceutical, nutraceutical and cosmetic applications as functional molecules and their activity has been demonstrated through *in vitro* and *in vivo* studies, predominantly on mice models. These properties can be studied by evaluating the effect on body fat gain, decrease in hepatic lipid content and lower insulin resistance. Therefore, the increase of brown tissue activity and the improvement of hepatic lipid metabolism, as well as the hypolipidemic activity, are some reported effects of terpenes and triterpene acids considered as phytonutrients that are present in fruits and vegetables cuticular waxes (grape berry, apple, olive, and tomato peel). On the way to control type II diabetes, the following therapeutic approach can be considered: the inhibition of α-glucosidase and α-amylase promoted by phytonutrients, which can impact in digestion stage by delaying the absorption of glucose in the intestine, impacting on the decrease in the insulin levels and preventing postprandial hyperglycemia. These phytochemicals (i.e. friedelin, β-amyrin, ursolic acid, lupeol among others) can be found in several agro-industrial wastes, bringing a novel opportunity to obtain new sources of bioactive compounds with pharmacological properties (42,43).

Disrupted insulin secretion and insulin sensitivity cause high blood glucose levels, which is the main problem associated with diabetic patients. Type I diabetes is an autoimmune disease that destroys pancreatic cells, while type II diabetes is associated with insulin resistance and metabolic impairment resulting from obesity. Pentacyclic triterpenes such as ursolic acid ($C_{30}H_{48}O_3$), commonly present in the herbal plant

Phyllanthus Amarus, has been reported as being able of increasing plasma and pancreatic insulin concentrations in a diabetic mice model, resulting in the preservation of β-pancreatic cells when assayed at 0.5 g/kg rich diet during four weeks (43). Through the interaction and therefore activity control with the insulin Receptors (IR), the antidiabetic function of a certain biomolecule, or in this case terpene, can be predicted. Therefore, Jung et al. (44) demonstrated that bioactive extracts from *C. grandiflor* plant, mainly composed of pentacyclic triterpenoids, led to the activation of insulin-mediated tyrosine phosphorylation of the IR β-subunit. Furthermore, these authors also observed that ursolic acid was an insulin-sensitizer at doses as low as 1 μg/mL in a cellular model of insulin-sensitive adipose tissue.

Anti-inflammatory Properties

During the last two decades, our understanding of lipid biochemistry has drastically changed since several research investigations have reported the ability of these compounds to interact with both membrane and nuclear receptors. The study of the cellular signaling where lipids are involved and their mediation in inflammation pathways is a promising research field that allows unraveling such mechanism, obtaining, therefore, a deeper biological understanding, which can lead to the development of new future therapies for the treatment of various diseases, from cardiovascular to brain disorders. Molecules biologically synthesized from arachidonic acid (AA), belonging to the eicosanoids family, are involved in acute immune responses, pain perception, blood pressure regulation, coagulation and reproduction. The endogenous bioactive lipids such as eicosanoids, pro-resolving lipid mediators, lyso-glycerophospholipids/sphingolipids and endocannabinoids (ECs) have been largely related to cellular and molecular mechanisms connected to the pathogenesis of chronic disorders (45).

For instance, ECs that derive from AA such as N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are endogenous ligands for cannabinoid receptors (CRs) with impact in various physiological, pathological and pharmacological conditions. In fact, these CRs are expressed in brain and have been associated with Δ9-tetrahydrocannabinol (THC) psychoactive effect and can be present in immune cells and control inflammation as well as cardiovascular, respiratory and reproductive functions (46).

A vast number of phytocannabinoids isolated from *Cannabis sativa* such as cannabidivarin (CBDV), cannabigerol (CBG) and cannabidiol (CBD) have been widely assayed due to their anti-inflammatory properties and viability as epilepsy, Parkinson's and Alzheimer's diseases treatment agents, since their lipophilicity and therefore ability to cross the blood-brain barrier (47). Hence, the interaction between phytocannabinoids and the regulation of the endocannabinoid system has research gaps that need to be fulfilled, namely between the retrograde signaling of AEA and 2-AG and the attenuation of neurotransmitter release through CRs (CB1R), which influence the synaptic plasticity. (48).

Eicosanoids structurally are 20-carbon oxidized fatty acids derived from AA, which is a polyunsaturated fatty acid that is present in phospholipids of cell membranes. The release of AA from the cell membrane is driven by activation of specific receptors that promote the lipolytic enzyme phospholipase A_2 (PLA_2) activation that ultimately hydrolyzes the sn-2 ester bond in the phospholipid and releases AA as a free fatty acid, consequently leading to the formation of numerous lipidic mediators associated to inflammation via nonenzymatic (isoprostanes) or enzymatic pathways (epoxyeicosatrienoic acids, hydroxyeicosatetranoic acids, leukotrienes, thromboxanes, prostacyclin and prostaglandin) (49). Arachidonic acid is metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) in different eicosanoids such as prostaglandins (PGs) and leukotrienes (LTs) in diverse cells, and thromboxane A_2 in platelets (50). From these mediators, PGs and LTB_4 are involved in the initial steps of acute inflammation responses that promote changes in blood flow, edema and leukocytes recruitment, specifically neutrophils, in the local of injury. Macrophage cells are major producers of eicosanoids and other related lipid mediators during inflammation, receiving particular focus for understanding immunity and inflammation because of its central role and dynamic functionality (51).

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The high number of applications of essential oils (e.g., *Alpinia murdochii*, *Alpinia scabra*) can be due to the presence of terpenes (e.g., β-pinene, α-pinene) known to exert anti-inflammatory effects that have been intensively discussed. Existing data elsewhere associate these properties to the effect of terpenes in the regulation of the AA pathway (Figure 1). The terpene oxide 1,8-cineole and the monoterpene (-)-linalool have been proposed to inhibit leukotriene LTB_4 and prostaglandin PGE_2 production (50). Moreover, linalool also inhibited Nitric oxide (NO) release by the regulation of nitric oxide synthase (iNOS) enzyme activity. Nitric oxide is synthesized from L-arginine by the enzyme iNOS, contributing to vasodilation and as a determinant role in the inflammatory response as interleukin IL-1, tumour necrosis factor-α (TNF-α) or lipopolysaccharides (LPS) stimulate iNOS expression in macrophages. Nitric oxide production and iNOS expression are regulated by inflammatory intermediates in mammalian cells and NO is considered as a reactive oxygen species (ROS) because it can be converted in the peroxynitrite anion. Furthermore, there are existing shreds of evidence that molecules derived from terpenes can affect cytokine production by decreasing interleukin-1β and tumour necrosis factor-α on mouse macrophages (50).

Example of Squalene as a broad-spectrum Bioactive Agent

Squalene [(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene], an unsaturated triterpenic hydrocarbon, known to be an intermediate product in the human cholesterol biosynthesis, although its intake does not increase the serum cholesterol levels. Statins are widely known drugs with the capacity to reduce liver cholesterol biosynthesis by inhibition of HMG-CoA reductase. In fact, it is demonstrated that the synergic combination of 860 mg of squalene and a low dose (10 mg) of pravastatin is a safe and effective method to decrease total cholesterol, LDL cholesterol and triglyceride levels, as well as to increase HDL cholesterol in patients with high plasmatic cholesterol levels. Theoretically, this mechanism by which dietary squalene can reduce serum cholesterol levels can be explained due to the downregulation of HMG-CoA reductase activity (52).

Regarding antioxidant activity, the six unconjugated double bonds that form squalene chemical structure, make it a highly suitable oxygen scavenging agent. In humans, squalene is present in skin at about 500 μg/g, where it can absorb up to a quarter of its weight in oxygen, preventing the development of oxidation and proposing that the chain reaction of lipid peroxidation is unlikely to occur (53).

Mudiyanselage and colleagues (54) studied the skin lipid oxidation by collecting its sebum and then performing irradiation with defined doses of UVB and UVA. The photo-oxidation products were then identified by HPLC analysis as lipid squalene mono-hydroperoxide (lipid breakdown products) associated with the pathology of acne. In conclusion, this physiological effect in human skin is related to the formation of squalene mono-hydroperoxide and strongly induced by UVA radiation (54).

From an immunomodulatory point, emulsions performed with the terpene squalene improved the effectiveness of some drugs. One example of such an application is MF59, an oil in water (o/w) emulsion with squalene (4.3% dispersed phase), span 85 and tween 80 that was ultimately designed by Novartis® as a widely used safe and effective vaccine adjuvant. Research suggests that when the adjuvant is administered, the immune response is increased by producing an influx of phagocytic cells in the injection site, which increases the release of chemokines, recruiting more immune cells from the blood into the site of vaccination, leading to an amplification loop (55). Thus, there is an unfilled need that requires more research to provide further scientific evidence to describe the full potential of squalene and other terpenes as lipophilic carriers of drugs and vaccines and simultaneously as enhancers to their effectiveness.

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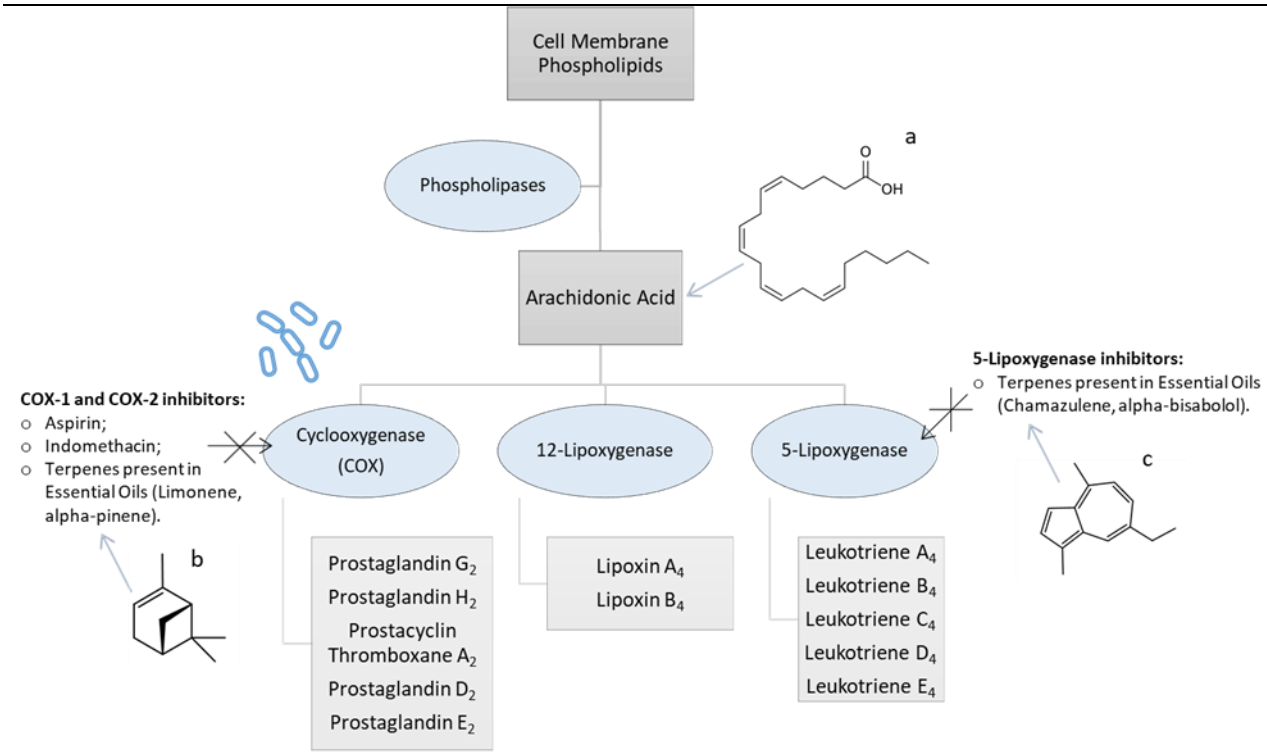

Figure 2. Arachidonic acid and inflammation pathway inhibitors [(structures: a) Arachidonic acid; b) alpha-pinene; c) chamazulene] (adapted from Kumar et al. 2021 (56) and Miguel 2010 (50). Chemical structures: The LIPID MAPS Lipidomics Gateway, 2020.

Synthetic biology to produce terpenes

In this subsection, terpene biosynthesis in plants and their classification according to structural units are overviewed. Two native terpene metabolic pathways and the regulation and functional diversification of their genes and enzymes are detailed. Furthermore, general terpene biosynthesis in *Saccharomyces cerevisiae*, relevant metabolic engineering strategies performed in this yeast for recombinant expression of terpenes and its advantages as a production platform over other host microorganisms are discussed.

Terpene biosynthesis in plants

Plant terpenes are derived from multiple isoprene building blocks (structural units) synthesised from the primarily cytosolic mevalonic acid (MVA) pathway and the plastidial 2-C-methyl-d-erythritol 4-phosphate (MEP) (the non-mevalonate) pathway. These structural units are then condensed to form various bulky and more complex terpenes (57). Based on the number of C5 isoprene units involved during their synthesis, terpenes are categorised as hemiterpene (C5), monoterpene (C10), sesquiterpene (C15), diterpene (C20), triterpene (C30), tetraterpene (C40), and polyterpene (C > 40). When these compounds have oxygen-containing functional groups, such as hydroxyl or carboxyl, they receive the name of terpenoids or isoprenoids. The MVA pathway in plants mainly supplies the precursor's compounds for the biosynthesis of sesquiterpenoids, polyprenols, phytosterols, brassinosteroids and triterpenoids in cytoplasm, and other terpenes such as ubiquinones and polyprenols in mitochondria, while MEP pathway principally provides isoprene units for the biosynthesis of hemiterpenoids, monoterpenoids, diterpenoids, carotenoids and their derivatives, such as tocopherols, chlorophyll, gibberellins, cytokinins and plastoquinones. These two different pathways are regulated at multiple levels. Besides the transcriptional regulation of MVA and MEP pathway

genes, isoprenoid-pathway fluxes are controlled at post-transcriptional/translational levels and by feedback regulation (58).

Biosynthesis of terpenes can be divided into four main steps: 1) building block formation, 2) condensation (polymerization), 3) cyclization or rearrangement, and 4) tailoring and post-modification (59). In the first step, universal precursors, a five-carbon monomer isopentenyl diphosphate (IPP), with its isomer dimethylallyl diphosphate (DMAPP), are synthesized through MVA and MEP pathways. In the second step, polymerization of one molecule DMAPP with one, two, or three molecules of IPP under the action of isopentenyl transferase leads to the formation of the linear prenyl diphosphate precursors geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP), which are the precursors of monoterpenoids, sesquiterpenoids, or diterpenoids. Similarly, long-chain C30 triterpenoids are generated via condensation reaction of two molecules of FPP. In the third step, primary terpene carbon skeleton is constructed via cyclization or rearrangement of linear prenyl diphosphate precursors under catalysis of terpene synthases. Finally, this carbon skeleton is subjected to a series of post-modifications and tailoring reactions (oxidations, acetylation, esterification, alkylation, etc.) under the action of cytochrome P450s (CYPs) to form biologically active terpenes (59,60).

Native pathways in plants: MEP and MVA

The MVA pathway in plants consists of six steps and initiates with the Claisen type condensation of two molecules acetyl-CoA to acetoacetyl-CoA under the action of acetoacetyl-CoA thiolase (AACT) (58) (Figure 3). Synthesized acetoacetyl-CoA is then combined with a third molecule of acetyl-CoA to form the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in aldol condensation reaction catalysed by 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS). In the subsequent rate-limiting step, HMG-CoA is converted to mevalonate (MVA) under the action of HMG-CoA reductase (HMGR) in two NADPH-dependent reduction steps. HMGR has a critical regulatory role in the biosynthesis of several terpenes (phytosterols, triterpenoids, and sesquiterpenoid phytoalexins) as it limits the accumulation of end products such as sterols. However, flux control often involves additional downstream enzymes such as sesquiterpene synthases (58). Finally, the mevalonate produced under the action of HMGR is converted into IPP through three enzymatic steps: two ATP-dependent phosphorylation steps, catalysed by mevalonate kinase (MK) and phosphomevalonate kinase (PMK), and one ATP-driven decarboxylation clearance catalysed by mevalonate diphosphate decarboxylase (MVD or MPDC) (58). The formation of IPP and DMAPP under the action of isopentenyl diphosphate isomerase (IDI) is also a crucial step for the MVA pathway (61).

Besides MVA pathway, plants also use MEP pathway for the synthesis of isoprenoid precursors. The MEP pathway (Figure 3), which occurs in all photosynthetic eukaryotes and cyanobacteria, apicomplexan protozoa, and most eubacteria, consists of seven enzymatic steps (58). The first step starts with the pyruvate and glyceraldehyde-3-phosphate (GAP) which form 1-deoxy-D-xylulose 5-phosphate (DXP) under decarboxylation reaction of deoxyxylulose-5-phosphate synthase (DXS). Similar to HMGR function in MVA pathway, DXS in MEP pathway has important regulatory and rate-limiting functions in the biosynthesis of plastidial terpenes (58). In the second step of MEP pathway, 2-C-methyl-Derythritol 4-phosphate (MEP) is formed from DXP catalysed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) through molecular rearrangement of DXP into 2-Cmethyl- D-erythrose 4-phosphate, followed by subsequent NADPH-dependent reduction. In some cases, the reaction catalysed by DXR is also considered as a rate-limiting step depending on the species, tissue, and developmental stage of plants (58). In the third step, MEP is transformed into 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) under the action of 4-diphosphocytidyl-2-C-methyl-Derythritol synthase (MCT or IpsD). Then, phosphorylation reaction of CDP-ME catalysed by 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK, IspE) leads to the formation of 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDPME2P) in the fourth step. And in the

subsequent step, thus, formed CDPME2P is cyclized into 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP), which is the only known cyclic diphosphate in nature, under the action of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS, IspF) by releasing the compound CMP. In the final two steps of MEP pathway, MEcPP is converted into 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) by the action of the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS, IspG). A mixture of IPP and DMAPP with a ratio of 5 to 6:1 is produced from HMBPP by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR, IspH) (for review see: (58,62)).

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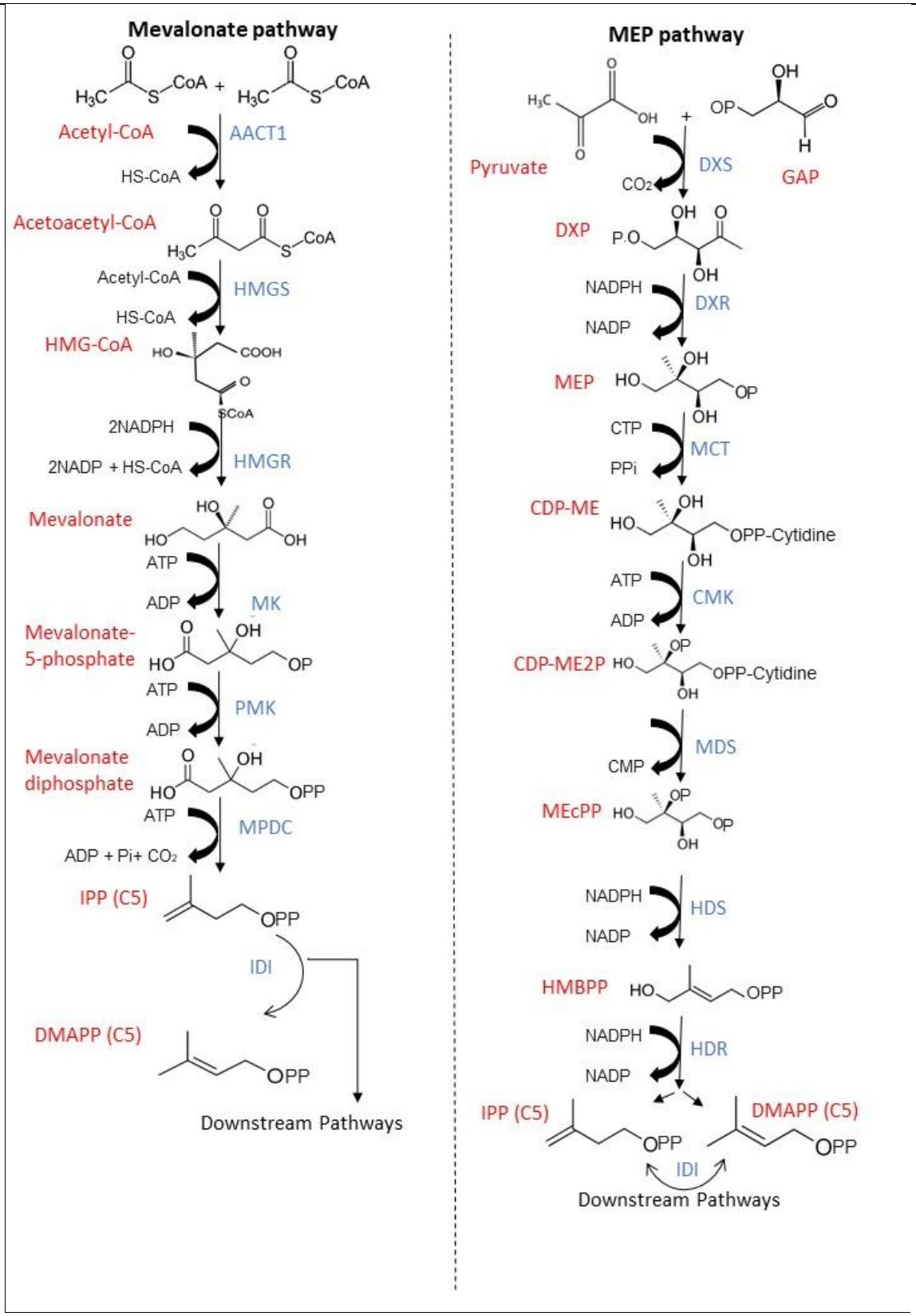

Figure 3. The MVA and MEP pathways in Plants. Adapted from Tholl 2015 (58). Abbreviations for enzymes (blue) and metabolites (red) are as described in the text.

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Terpene biosynthesis in *Saccharomyces cerevisiae* – an industrially powerful microbial platform

Since ancient times, the baker's yeast, *Saccharomyces cerevisiae*, has been extensively used in the production of alcoholic fermentation products, such as bakery products, bioethanol and alcoholic beverages (wine, beer and distilled spirits). However, with the recent advances in synthetic biology, it became one of the most widely industrially used cell factories for production of a wide number of products, such as amino acids, enzymes, proteins, organic acids, alcohols, chemicals and metabolites (57). This yeast has been chosen as the most suited microbial host by many biotechnology companies for the production of a large variety of recombinant products due to several advantages over other host microorganisms, such as its well-known genetics and physiological background, the availability of genetic tools, ease of manipulation, high genetic tractability, well-known biochemistry, and suitability for high density and large scale fermentation (57). Thus, *S. cerevisiae* has a wide range of additional advantages namely in terms of industrial-scale terpene production, including generally regarded as safe (GRAS) status (which facilitates approval of products obtained through this cell factory), possessing universal endogenous MVA pathway, ability to express eukaryotic cytochrome P450 enzymes, robustness, high sugar catabolic rate, relative absence of secondary metabolites, fast growth rate, and high tolerance against harsh industrial conditions (low pH, osmotic stress, and resistance towards phage infections)(57).

Besides yeast *S. cerevisiae*, alternative microorganisms have also been explored for terpenes production. Among them, *Escherichia coli* is one of the most studied bacteria. It produces a limited amount of endogenous terpenes (e.g., quinones) and, therefore, the improvement of MEP pathway by engineering enzymes for IPP and DMAPP synthesis, or the introduction of heterologous MVA pathway, are required. *Saccharomyces cerevisiae* has a native MVA pathway capable of accumulating a high amount of ergosterol as well as redox systems that allow cytochrome P450 enzymes to modify terpenes structure, whereas *E. coli* does not have either (57). Moreover, *S. cerevisiae* can produce value-added terpenes with greater structural complexity than *E. coli*. Non-conventional yeast *Yarrowia lipolytica* has been also considered as another good alternative in terpene production since it can produce a large amount of acetyl-CoA, the initial substrate of the MVA pathway. This non-conventional yeast has been successfully engineered mainly to produce carotenoids. However, the advances in metabolic engineering of *Y. lipolytica* are still in their early stages and therefore not fully developed, existing a number of challenges that need to be addressed before commercial success (57). Another yeast, *Rhodosporidium toruloides*, carotenogenic red yeast, has been also considered as a microbial platform for terpene production because it can naturally synthesize many carotenoids (C40 terpenes) as torularhodin, torulene and β-carotene, indicating that it may have a high carbon flux via its MVA pathway, providing pools of intermediates like IPP and DMAPP for the production of a wide variety of terpenes (57). This yeast has also advantages over the above mentioned host microorganisms in the metabolization of both xylose and glucose, and toleration to high osmotic stress, enabling the use of lignocellulosic hydrolysates as feedstock. However, terpene titers achieved with this yeast are not at industrial levels yet (i.e., <g/L). Further improvements in MVA pathway and limitation of driving carbon from the central metabolite acetyl-CoA towards lipid biosynthesis are therefore required. Photosynthetic microorganism *Cyanobacteria* has also potential for sustainable production of terpenes using light and CO_2, although further studies are required to improve productivity for industrial scale-up. The regulation of MEP pathway, a partition of carbon flux, and balancing carotenoid and monoterpene synthesis should be enhanced (57).

To date, rather than *S. cerevisiae*, none of these mentioned microorganisms reached commercial production levels of important terpenes. As an example, it has been demonstrated that it is possible to reach titers of more than 130 g/L of farnesene and 25 g/L of artemisinic acid (precursor of artemisinin, antimalarial drug) from sugar cane feedstock through optimized fed-batch fermentation using engineered yeast *S. cerevisiae* (Table 2). In summary, *S. cerevisiae* has been demonstrated of being capable of better performance when

compared to the other candidate microorganisms in terms of higher process robustness, fermentation capacity, plenty of available genetic tools in pathway engineering and genome editing, and proven capacity to attain industrial levels of relevant terpenes (57).

Native *S. cerevisiae* strains (most known laboratory strains used for system biology: CENPK, S288C, BY4741 and W303) can synthesize ergosterol by using their endogenous MVA pathway. All enzymes and genes in upstream MVA pathway and downstream ergosterol synthesis of *S. cerevisiae* have been discovered by using molecular biology techniques (57). Without genetic alterations, wild-type strains of *S. cerevisiae* cannot directly synthesize terpenes. Specifically, this yeast lacks the precursors for transferring intermediates between compartments (i.e. the cytoplasm and endoplasmic reticulum) and diverse classes of Cytochrome P450 (CYPs) enzymes for the oxygenation reactions to produce diverse terpenoids (63). Moreover, in order to reach industrially meaningful metric titers (g product/ L broth) of terpenes, several genetic modifications such as deletion, mutations and knockout of genes, promoter alterations (overexpression or repression of genes) and expression of heterologous genes need to be performed in yeast (63). Intermediary compounds (i.e. the direct precursors) in sterol pathway, such as FPP, GPP, GGPP, and squalene epoxide, can be diverted into specific terpenes through a series of enzymes designated as terpene synthases. However, these enzymes are not codified in the genome of *S. cerevisiae* in contrast to plants and other organisms. Due to this reason, most of the heterologous genes for the functional expression of terpene synthases obtained from plant sources (e.g., taxadiene synthases from *Taxus brevifolia*) or generated from synthetic sources (e.g. from NCBI gene accession number) are candidates to assayed for the transformation of this yeast genome (63). In addition, as it already mentioned, *S. cerevisiae* does not have all CYPs enzymes required for the modification of the hydrocarbon products from terpene synthases. These CYPs enzymes catalyse a diverse range of metabolic reactions, such as decarboxylation, oxygenations, dealkylation, deamination, and C–C cleavage(57). There are only three CYPs in *S. cerevisiae*: Erg5, Erg11 and Dit2 (putative cytochrome P450 involved in the synthesis of N,N-bisformyl dityrosine). Accordingly, in order to produce non-native terpenes, heterologous expression of CYPs in *S. cerevisiae* is also required (57). Most of CYPs successfully expressed in *S. cerevisiae* are from plant origin. As an example, CYP71AV1, NADPH dependent cytochrome P450 oxidoreductases (CPR1) and cytochrome b5 (CYB5) from *Artemisia annua* have been assayed in *S. cerevisiae* resulting in the production of 25 g/L of artemisinic acid in an optimized fed-batch fermentation (65). These modifications allow the production of specific terpenes, however other alterations are necessary to improve titers and reach industrially meaningful values. One of them is to increase flux into the MVA pathway of *S. cere*visiae, by identifying the rate-limiting reactions and overexpressing the gene, or set of genes, responsible for these reactions. HMG-CoA reduction to MVA was reported as the rate-limiting step in the sterol biosynthesis in *S. cerevisiae*. Two HMG-CoA reductases, Hmg1p and Hmg2p, are involved in this reduction reaction. Overexpression of N-terminal truncated HMG1 (tHMG1) and Hmg2p increased squalene production in *S. cere*visiae(63). Similarly, overexpression of the transcriptional factor Upc2-1, constitutively active mutant of Upc2 led to a significant improvement in the sterol uptake and expressions of genes involved in the MVA pathway. Terpene production was also enhanced by overexpression of all structural genes (ERG10, ERG13, tHMG1, ERG12, ERG8, IDI1, and ERG20) in MVA pathway (66). Another strategy is to increase precursor pool by down-regulation of downstream genes in competitive pathways. For instance, ERG9, which is an important gene, encoding squalene synthase, competes with sesquiterpenoid and diterpenoid synthase for the FPP pool. Downregulation of ERG9 via HTX1 promoter increased the FPP pool when the glucose concentration in the fermentation was low in fed-batch fermentation (57,67).

On the other hand, overexpression of all structural genes in MVA pathway of *S. cerevisiae* can cause depletion of acetyl-CoA and NADPH pools, which are the essential cofactors and precursors for the biosynthesis of various products, such as fatty acids, sterols, polyketides and also terpenes. Thus, depletion of acetyl-CoA and NADPH can negatively affect yeast growth and, in parallel with this consequence, a decrease

in terpene production. Therefore, a boost in acetyl-CoA and NADPH pools is a factor to be considered. It has been elsewhere reported that the most effective way for achieving this was to create a synthetic route with lower energy demand (63). Meadows et al. (66), could substitute the PDH-bypass, increase the cytosolic acetyl-CoA levels and lower ATP cost by establishing a new synthetic metabolic pathway. These authors combined acetaldehyde dehydrogenase, which converts acetaldehyde to acetyl-CoA, with xylulose-5-phosphate specific phosphoketolase and phosphotransacetylase, which convert xylulose-5-phosphate to acetyl-CoA. In the same study, an NADH-consuming HMG-CoA reductase was also used to improve the NADPH pool. With these combined modifications, β-farnesene production increased along with 75% less oxygen requirement in fed-batch fermentation (57).

In nature, terpenes structures are generally restricted to molecules that differ by increments of five carbon atoms (C5, C15, C20, etc.). However, with new developments in metabolic engineering of *S. cerevisiae*, it was possible to achieve the synthesis of noncanonical building block with 11 carbons. Thus, C11 terpene scaffolds were produced by engineering dedicated synthases. In order to obtain these specific enzymes, a single residue switch that converts C10 plant monoterpene synthases to C11 specific enzymes was identified (57). Moreover, as a way to improve the production of industrially relevant monoterpenes in *S. cerevisiae*, a new synthetic orthogonal monoterpenoid pathway based on an alternative precursor, neryl diphosphate, was established (57). Five different enzymes to accept the alternative substrate with high efficiency and selectivity were engineered and combined with dynamic regulation of metabolic flux to harness the potential of the orthogonal substrate (57). Recently, the highest geranylgeraniol titer to date of 5.07 g/L in a 5 L bioreactor by overexpression of exogenous geranylgeranyl diphosphate synthase (with stronger catalytic ability) and deletion of ROX1 gene, and using isoprenol as a substrate in *S. cerevisiae* had been achieved (68). Additionally, in another study, 2.23 g/L of limonene by combining the engineered PDH (pyruvate dehydrogenase) bypass with the deletion of CIT2 gene in fed-batch shake-flask fermentation had been produced (69). Some relevant metabolic engineering strategies performed in *S. cerevisiae* for terpene production are presented in Table 2.

Table 2. Relevant engineering strategies performed in *Saccharomyces cerevisiae* for terpene production (adapted from Carsanba et al. 2021 (57)).

Compound (titer)	**Strategy**	**References**
Amorpha-4,11-diene (>40 g/L)	Overexpression: ADS, upc2-1 Integration of genes copies: 3 copies of tHMG1, and ERG10, 13, 12, 8, and IDI1 Deletion: gal80Δ Downregulation: ERG9	(70)
Artemisinic acid (25 g/L)	Overexpression: ADS, CYP71AV1, CPR1, CYB5, ALDH1 and ADH1 from *A. annua,* HEM1, and CTT1 Deletion: gal80Δ Downregulation: ERG9	(65)
β-Farnesene (130 g/L)	Overexpression: ADA, xPK, PTA, NADH-HMGr, Farnesene synthase Deletion: adh1 Δ, ald4 Δ, ald6Δ, gpp1Δ, gal2Δ, bdh1Δ Overexpression: enzymes of the MVA pathway to Erg20 Downregulation: ERG9	(66)
Bisabolone (>900 mg/L)	Overexpression: tHMG1, ERG20, upc2-1 and bisabolene synthase Downregulation: ERG9	(71)
Alpha-Santalene (92 mg/L)	Overexpression: tHMG1 Deletion: Ipp1Δ and dpp1Δ Downregulation: ERG9	(67)
Patchoulol (42.1 mg/L)	Overexpression: ERG20 and PatTps177 Downregulation: ERG9	(72)
(S)-Linalool (0.26 mg/L)	Overexpression: Erg20 and (S)-linalool synthase Diploid	(73)
Geraniol (1.69 g/L)	2μ plasmid of PTEF1-tVoGES-(GGGS)-ERG20WW fusion protein 2μ plasmid of PTEF1-tHMG1, PPGK1-IDI1, PTEF1- upc2.1 PHXT1-ERG20, oye2Δ	(74)
Sabinene (1.75 mg/L)	2μ plasmid of PTDH3-ERG20 (F96W-N127W)- sabinene synthase (*Salvia pomifera*) fusion protein Diploid ERG9/erg9, ERG20/erg20, PGal1-HMG2 (K6R), PTDH3-HMG2 (K6R) × 2	(75)
Limonene (2.23 g/L)	Engineering the PDH bypass to directly push the acetyl-CoA flux into MVA pathway Deletion: CIT2	(69)
Geranylgeraniol (5.07 g/L)	Overexpression: geranylgeranyl diphosphate synthase Deletion: ROX1	(68)

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Current applications of microbial terpenes

In the previous subsection, general information for terpene biosynthesis in plants and metabolic engineering strategies applied in yeast *S. cerevisiae* for efficient terpene production were presented. Most of the terpene titers obtained by engineered *S. cerevisiae* in lab-scale experiments were in very low metric titers (mg/L). However, in order to produce terpene in commercially meaningful levels (i.e., g/L), besides targeted metabolic engineering, yeast fermentation should be also optimised. Therefore, this subsection highlights the determinant fermentation factors affecting the industrial production of terpenes. Furthermore, some commercially successful examples of terpenes production by yeast fermentation are overviewed. Production status, market size and demand, growth drivers and key players for the most relevant terpenes are summarised.

Large-scale production of terpenes by microbial fermentation

Terpenes which are extensively used in foods, pharmaceuticals, cosmetics, and agricultural fields, are generally recovered from natural resources like plant origin via extraction but also obtained by chemical synthesis. However, these methods are not convenient for commercial terpene production (57). The obtained titers and productivity levels are relatively low and process of extraction or chemical synthesis are technically challenging while wasting valuable resources and producing contaminants. Although many of the commercially used terpenes are still extracted from natural resources like plants (e.g., taxol from yew tree and artemisinin from the plant *Artemisia annua*), in most cases this process fails with regards to supply and quality management because of geographical and seasonal changes. Moreover, metabolic engineering approaches in plant resources are quite challenging and problematic because of tissue-specific expression and loss of volatile products by evaporation (57). The most promising alternative method is the use of plant cell cultures, which has been currently tested for the production of ginseng, taxol and anthocyanin. However, this method has also its drawbacks since there are high risks of culture heterogeneity, sensitivity to stress factors, aggregation, and possibility of obtaining low growth rates and variability in yields. For these reasons, research has explored alternative production methods. Microbial production of terpenes has received increasing attention. This is because this approach provides several advantages, such as a very promising terpene yield and productivity, batch-to-batch consistence, low production cost and more sustainability compared to natural production methods (57).

The fermentation processes employed in terpene production studies are batch, fed-batch and continuous types. There are important differences between these three approaches: in batch mode all medium components, feedstock and microorganisms are added to bioreactor at the beginning of fermentation, while in fed-batch mode the necessary nutrients and ingredients are added, and some of culture broth is removed from bioreactor, at certain intervals of time. On the other hand, in continuous fermentation mode, these required nutrients and ingredients are continuously fed and culture broth is simultaneously removed from bioreactor (6,57). The selection of fermentation process for efficient terpene production depends mostly on the yeast fermentative properties and the nature of the feedstock used. Fermentation kinetics, toxicity tolerance and lifespan of yeast are important parameters in the selection, as well as other criteria such as preparation, concentration and sterilization of feedstock also play a decisive role (57). High density cultivation through growing cells to high concentrations while maintaining high specific cell productivity is required to achieve high production, yield and productivity in the industrial big scale processes (in 200,000 litre bioreactors). The selection of an optimum bioreactor operating mode is important for the high-density cultivation process as environmental conditions affect growth and product formation (57). Among these conditions, feeding profile (at a slow or fast rate), medium optimization (nitrogen and/or substrate

limitations), and feedstock concentration are essential to be kept at optimum levels to prevent overfeeding or underfeeding (57).

Fed-batch mode is the preferred fermentation process in the industrial production of terpenes as it brings advantages from both batch and continuous modes. It has some benefits over conventional batch process, such as maintenance of long term high cell viability, extended life span of yeast cells, higher product formation, less inhibitory effect of higher feedstock and product concentrations and easy management of several critical parameters, such as pH, temperature, and dissolved oxygen at an optimum level via the feedback activities (57). Besides these, one of the important advantages of using this process is to ensure high cell density, which provides high product formation. The fed-batch mode, in which product is removed intermittently and feedstock is fed when residual nutrients are depleted, is generally considered the most effective mode of operation for terpenes production as they become less toxic to cells owing to broth dilution. Moreover, the optimization of feeding plays a critical role in increasing terpene productivity and yield (6).

In the available scientific literature, many terpenes (monoterpenes, sesquiterpenes, triterpenes, etc.) have been produced by using fed-batch fermentation (57). Here, some commercially successful cases will be presented as examples. For instance, artemisinin precursors amorpha-4,11-diene and artemisinic acid have been produced at concentrations of >40 g/L (70) and 25 g/L (65), respectively, in fed-batch fermentations using highly engineered *S. cerevisiae*. Two different feeding processes were applied in these studies: glucose/ethanol mixture substrate feeding with exponential feeding rate and ethanol substrate with pulse feeding (10 g/L). Feeding algorithms were designed to perform by automatically triggered via responses of DO (Dissolved Oxygen) percentage, evaluation of CO_2 rate and stir rate (65,70). Isopropyl myristate and methyl oleate were used as extracting agents for artemisinic acid and amorpha-4,11-diene removal from fermentation broth, respectively (65,70). With these approaches, a significant improvement in titer and productivity levels was obtained by removing highly concentrated acid from aqueous phase, since artemisinic acid accumulated in fermentation broth negatively affects cells viability. Moreover, ethanol-restricted fed-batch fermentation was performed to lower oxygen requirement of yeast cells (i.e. lower levels of oxygen uptake rate) in amorpha-4,11-diene production, which is more feasible for industrial-scale fermentation (65). Another method applied by the same authors was the phosphate limited fed-batch fermentation, which could restrict cell growth, while can direct flux to product formation, resulting in more than 2-fold higher amorpha-4,11-diene production. Another important example is β-farnesene production. Elsewhere it has been reported titers of more than 130 g/L of β-farnesene from sugar-cane syrup through optimised fed-batch fermentation using engineered yeast *S. cerevisiae* (66). At the beginning of the fed-batch process, the bioreactor was aerated at 30 L/h and DO was maintained at 30% by agitation ramp. After the initial sugar was consumed, the DO level increased, which triggered feeding of sugar cane syrup at 10 g TRS/L broth/h feed rate in pulses of 10 g TRS/L broth doses. Between pulses, feed rate was reduced to 5 g TRS/L broth/h. As biomass increased, DO levels decreased to 0%, reaching microaerophilic condition and pulse doses increased to 50 g TRS/L broth/h. Thereafter, feed rate was adjusted dynamically to meet sugar demand of cells using an algorithm, alternating a high feed rate and low feed rate (66). Besides these successful examples, higher titers of 5.2 g/L bisabolone and 5.5 g/L nerolidol by engineered *S. cerevisiae* strains in fed-batch fermentations had been also demonstrated to be feasible. In bisabolene production, a constant glucose feeding rate triggered by pH rise was used, while in nerolidol production, pulse feeding (initial glucose and then sucrose feeding) triggered by DO spikes was applied. Furthermore, in another study, triterpene ginsenoside Rh2 reaching concentrations of 2.25 g/L had been successfully generated in fed-batch fermentation where glucose was fed when ethanol concentration was less than 0.5 g/L. More recently, by applying a two-stage feeding strategy in high cell density fermentation, 2.99 g/L of ergosterol and 29.5 mg/g DCW (dry cell weight) ergosterol content in *S. cerevisiae* had been accumulated. At the first stage, in order to achieve fast cell growth glucose with vitamin

solution was fed to the fermenter using pseudo-exponential feeding model, while at the last stage, ethanol was fed to the fermenter to increase the intracellular ergosterol accumulation. Moreover, recombinant *S. cerevisiae* strain: LWG003-CaNES2 produced 7.01 g/L of trans-nerolidol through fed-batch fermentation in 5 L bioreactor. In this work, the mixed feeding solution was added automatically by controlling the pH and DO. When DO was higher than 50% and the pH was above 5.5, the feed was fed at a federate of 2 mL/h. For the pH adjustment, $NH_3 \cdot H_2O$ and for the extraction agent, dodecane (10%) was used (57).

The concentrations of terpenes obtained in batch fermentations are generally at mg/L levels, which is commercially inadequate. For example, (S)-linalool (73), limonene, geraniol, polpunonic acid and bisabolone titers obtained in batch type fermentations, mainly in flask assays, were lower than 1 g/L (57). On the other hand, fed-batch fermentation can yield titers at g/L levels, and reach high volumetric productivities by alleviating toxicity of final product, being the most convenient and effective method for industrial production of terpenes (57). Other relevant factors affecting the fermentation of terpenes include pH, DO, temperature, and medium composition. Yeast has optimum growth at a pH range between 4 and 6, and it can reach high cell density within these values (57). However, several terpenes are insoluble at this pH range and thus, may complicate the downstream processing by requiring more effort in separation of product from fermentation broth. This problem can be solved by increasing the pH of culture medium by addition of a base solution, as terpenes are generally soluble at high pH (57). Extraction agents, which were mentioned above, can also be used to decrease the toxicity of specific terpenes, however, this makes fermentation process more expensive. The use of fed-batch fermentation is another option to solve this issue since it can moderate toxicity of product by feedstock addition and product draw in certain intervals. The DO is frequently used as a trigger for the delivery of feedstock into culture medium to control yeast growth and product formation in fed-bath fermentation (57). The maintenance of optimum oxygen levels during fermentation plays a critical role in terpene production since low oxygen levels can reduce yeast productive capacity, while high levels can cause formation of reactive oxygen species (ROS), which can lead to oxidative stress on yeast cells and negatively affects their health status. Similarly, low temperatures can delay reaching high cell density, on the other hand high temperatures can reduce yeast cell viability (57). Besides these physiochemical factors, optimization of medium composition, namely selection of carbon, nitrogen, vitamins and trace metals sources, and their final concentrations, has also big importance as these ingredients constitute one of the main parts of the production costs. Among carbon sources, ethanol, glucose, galactose, sucrose, dextrose and raffinose were commonly used for terpene production. But these sources are expensive feedstocks and the use of low cost carbon sources, namely agricultural by-products such as sugar cane bagasse or straw, and by-product of biodiesel production, crude glycerol, could significantly decrease the feedstock cost. However, further improvements in the metabolism of *S. cerevisiae* are needed, since this microorganism is unable to directly metabolise these type of feedstocks (57).

Successful examples in microbial terpene production

Trans-β-farnesene, or simply β-farnesene [(6E)-7,11-dimethyl-3-methylidenedodeca- 1,6,10-triene; CAS RN No. 18794-84-8)], is a sesquiterpene alkene with many uses as a specialty chemical and petroleum substitute. β-farnesene has been commercially produced from Brazilian sugar cane by fermentation using engineered *S. cerevisiae* strains developed. It has been used in a wide variety of industrial applications as specialty and commodity chemicals have very low price, generally under $10 per kg (76). Because of its low price, it is important that microbial β-farnesene production could be as efficient as possible with a high titer, yields and productivities. The development of yeast strains capable of reaching high productivities requires comprehensive studies, which has been recently provided by synthetic biology technics (76). In this context, Amyris Inc has strongly invested in strain design development (namely: design automation, i.e., the use of hardware, software and robotics) and fermentation process optimization for reaching the commercial

production of β-farnesene (76). With the strain improvement using the tools of synthetic biology along with fermentation and downstream process optimization, the cost of β-farnesene production decreased to $1.75 per litre while efficiency of the fermentation increased (76). Beside these developments, finding sustainable and low-cost carbon source for fermentation played also an important role in the reduction of the β-farnesene production cost (76). Sugar cane was selected as fermentation feedstock since it combines properties of lower cost and life-cycle greenhouse gas (GHG) emissions for a product that will replace petroleum products as both specialty chemicals and fuels (76). Prior to microbial production, β-farnesene was produced through derivation from isoprene or the degradation of sesquiterpene alcohols, which are expensive chemical processes and produce low purity mixture of final product (β-farnesene, α-farnesene and other molecules). However, β-farnesene produced by engineered yeast is a pure enantiomer (76).

As it was stated before, β-farnesene has been used as a specialty feedstock for chemical transformations, for producing numerous industrial products, such as cosmetic oils (squalene), polymers, lubricants, surfactants and fuels (76). These wide varieties of markets stand opportunities to use β-farnesene to replace petroleum-derived ingredients in numerous products that consumers use worldwide. The 2,6,10-trimethyl-dodecane (also known as farnesene), which is the hydrogenated derivative of β-farnesene, has been used as a renewable hydrocarbon for diesel and jet fuel. The blends of farnesene with petroleum diesel meet the ASTM D975-11 Standard Specifications for Diesel Fuel Oils. It has been accepted to be commercially used as renewable diesel by the Brazilian National Agency for Petroleum, Natural Gas and Biofuels (ANP) (76). Another advantage to using farnasene instead of petroleum fuels is that it reduces at least 80% of greenhouse gas emissions and tailpipe emissions such as NO, carbon monoxide and particulate matter, and provides zero sulfur content. It is certain that with these numerous extras, β-farnesene supports maintaining healthier and cleaner planet and presents global biofuel solution for a longer-term (76).

Apart from its use as fuel, β-farnesene is also used as a lubricant feedstock. Considering the need for environmentally friendly lubricants that can be used as drop substitutes for petroleum-derived products, the use of β-farnesene as a lubricant raw material is of particular interest. β-farnesene is a pure hydrocarbon olefin which makes it a unique biologically-derived lubricant feedstock. The lubricant industry relies on petroleum-derived hydrocarbons. However, β-farnesene can be used in the lubricant industry and the production of β-farnesene-derived base oils does not require the development of new process technology and infrastructure, which is a significant gain for the commercialization (76). Thus, obtained β-farnesene-derived base oils has 50% renewable property since they are chemically produced by reacting petroleum-based linear alpha olefins with β-farnesene. Besides renewability, this type of lubricant produced from sugar has ecological and biodegradability properties and meets the requirements of the European Union (EU) Ecolabel (76). Additionally, they are non-toxic and categorised by the National Sanitation Foundation (NSF) as white oils suitable for incidental food contact (76). With these environmental and performance advantages, β-farnesene-derived base oils have been formulated into greases, compressor oils and high-performance hydraulic fluids, industrial gear oils, automotive engine oils, transformer oils, and two-cycle air and marine engine oils (76).

β-farnesene from sugar cane is the renewable building block chemical for the market. Its molecular structure makes it unique and attractive as a scaffold for specialty chemical applications, such as solvents, emollients and vitamins. Apart from these applications, it has been formulated into performance materials, adhesives, fragrances, surfactants, stabilizers, oligomers and polymers, resins, foams, coatings and sealants, emulsifiers, and crop protection (77). From a commercial point of view, the farnesene market size exceeded $315 million in 2020 and it is estimated to grow at over 6% Compound Annual Growth Rate (CAGR) (over $485 million) between 2021 and 2027, with major revenues expected from the personal care and cosmetic products (78). The key rollers operating in the global farnesene market include Amyris Inc., Precigen Inc., Tate and Lyle Plc, Santa Cruz Biotechnology Inc., ADL Biopharma, Toronto Research Chemicals Inc.,

Triveni chemicals, Antai Fine Chemical, Glentham Life Sciences, U.S. Biological Life Sciences, American Enzyme Company, and ALFA (78).

Another important commercial terpene is squalene ($C_{30}H_{50}$; (6E,10E,14E,18E)-2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene), which is found in deep-sea shark liver as primary source. However, it can be also obtained after the chemical reactions of bio-based farnesene, recovered as a fermentation product of the engineered yeast *S. cerevisiae*, namely dimerization (farnesene to isosqualene) and hydrogenation reactions (isosqualene to squalane). Squalane is mainly used as a skin moisturizer in cosmetics and immunologic adjuvant in vaccines (79). Apart from these applications, it plays diverse biological roles as an anti-oxidant, anti-cancer agent, age defyer, chemopreventive agent, anti-bacterial agent, drug carrier, as well as a detoxifier, which promotes its use in a variety of commercial areas, such as chemical, food, cosmetic, and pharmaceutical industries (55). In recent years, global squalene demand has increased and reported about 2.67 kilotons in 2014, with a total market size projected to reach $ 184 million by 2025 from $ 129 million in 2020 (80), with major revenues expected from the personal care and cosmetic products (55). The key market players in squalene production (plant-derived, shark-liver-derived and bio-based) are reported as Sophim (France), Amyris (US), Arista Industries Inc. (US), SeaDragon Marine Oils Limited (New Zealand), Nucelis LLC (US), Ekiz Olive Oil & Soap Inc. (Turkey), Kishimoto Special Liver Oil Co. Ltd. (Japan), Empresa Figueirense De Pesca, Lda (Portugal), Arbee Biomarine Extracts Pvt. Ltd. (India), and New Zealand Green Health Limited (New Zealand) (80)(Table 3).

Another important example of the commercial importance of microbial terpene production is semi-synthetic artemisinin, which is an essential antimalarial drug currently manufactured by the pharmaceutical company Sanofi, using an engineered *S. cerevisiae* strain developed by Amyris Inc (76). Artemisinin is a sesquiterpene lactone endoperoxide with anti-malarial activity found mainly in the plant *Artemisia annua* (81). Its use has been recommended for the first-line treatment of malaria by the World Health Organization (WHO) in 2004 and following years, the price of plant-derived artemisinin significantly increased and changed between around $1,000 per kg and less than $200 per kg, depending on seasonal and geographical limitations for this plant in some years (82). Moreover, *A. annua* just yields <0.8% of artemisinin by dry biomass weight. An alternative to plant-derived production was required to supply the market and reduce the price of this anti-malarial drug. For this reason, the approach of producing semi-synthetic artemisinin by fermentation using engineered yeast rose (82). After the series of development in engineered yeast by Amyris, optimization of fermentation (photo-Schenk ene-chemistry with specially designed and constructed large-scale photoreactors) and downstream process by Sanofi, it could be possible to produce pure artemisinin with 55% yield and 600 kg of pure fermentation-derived artemisinic acid (76). Thirty-five tons of semi-synthetic artemisinin in 2013 and 60 tons in 2014 were produced by Sanofi with a price point comparable to plant-derived artemisinin, in that above $150 per kg (83). The artemisinin combination therapy market size was $ 363.8 million in 2017 and is projected to grow at a CAGR of 8.5% during the period between 2018 and 2025 (84). Some of the major players in the artemisinin market are Novartis, Sanofi, Cipla, Ajanta Pharma, Ipca Laboratories, Guilin Pharmaceuticals, and KPC Pharmaceuticals (84) (Table 3).

With the latest developments in synthetic biology and metabolic engineering strategies in *S. cerevisiae*, different and more complex types of terpenes, especially the trending products cannabinoids and taxol, will be possible to be produced in this host in near future (57). Today, the use of cannabinoids in medical applications has globally increased and legislation is becoming more permissive in many countries, such as the USA, Canada, Israel, and several European countries including the Netherlands, Germany, and the Czech Republic, which promotes more research on alternative cannabinoid production (57). These compounds are mainly used as therapeutic agents for the treatment of several medical conditions due to their unique interaction with the human endocannabinoid system (57). They have been often used in the treatment of

pain-relieving conditions (in cancer chemotherapy, Acquired Immune Deficiency Syndrome (AIDS), and multiple sclerosis) (57). Due to these unique properties in health and wellness applications, demand for cannabinoids, especially cannabidiol (CBD) is quite high, driving the market growth (85). CBD production is expected to boost by acceptance and approvals by the governments to use this product for health purposes (85). The global cannabinoids market size was valued at $ 2.8 billion in 2020 and is expected to grow at CAGR of 21.2% from 2021 to 2028 (85). Key companies profiled for cannabidiol producers are Mile High Labs International, Rhizo Sciences, Global Cannabinoids, SPARKCBD, CBD Inc Group, GenCanna, Maricann Group Inc (85) (Table 3).

Taxol (Paclitaxel) is another very important and valuable tricyclic diterpenoid compound used as a chemotherapy drug in the treatment of several cancers, such as ovarian, colorectal, head and neck, small-cell and non-small-cell lung (NSCLCs), and breast cancers (57). It is naturally produced in the bark and needles of the Pacific yew tree (*Taxus brevifolia*) which are the main sources of this compound (57). The price of paclitaxel is very high at $ 600,000 per kilogram due to its relatively low concentration (0.001–0.05% in *T. brevifolia)* found in original plant source (i.e yew tree) (57). Production is dependent on the epigenetic and environmental factors and the slow growth rate of *Taxus* plants and yew trees (57). Moreover, natural ecological balance could be destroyed due to excessive use of these plants for this purpose and there is a risk of yew trees extinction (57). Because of these, alternative methods to obtain paclitaxel should be developed. One possibility is through microbial production. However, commercially meaningful metric titers could not be achieved yet by this method (57). The highest titer of oxygenated taxanes obtained was only 570 mg/L in engineered *E. coli* by optimizing the P450 expression of taxanes and other related enzymes (86). There are still challenges of expressing cP450's in microbial systems for biosynthesis of paclitaxel (87). For the commercial success, further improvement and research on the biosynthetic taxane pathway, namely efficient expression of taxadiene synthase and other specific enzymes like cP450s, is still required (88). In the last decades, global market size of paclitaxel increased rapidly, with an average growth rate of 12.3%. In 2017, global revenue of paclitaxel and actual production were at $ 80 million and 2600 kg, respectively (89). On the other hand, market size was reported as $ 114.8 million in 2020 and is expected to reach $ 162.3 million by 2026 with a CAGR of 9% from 2021 to 2026. Market size report for paclitaxel only includes two types of paclitaxel: natural and semi-synthetic paclitaxels. Major manufacturers of paclitaxel are Phyton, ScinoPharm, Novasep, Samyang, Polymed, TAPI (Teva), Fresenius-kabi, Huiang biopharma, Southpharma, Yunnan Hande, Hainan Yew Pharm and Jiangsu Yew Biotechnology (90) (Table 3).

Microbial Production of Food Bioactive Compounds

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Table 3 Market size reports for the most relevant terpene products.

Compound	Market Size	CAGR (%)	Growth drivers	Key rollers	References
Farnesene	$315.8 million (2020)	$485 million by 2027 6% CAGR (2021-2027)	The growing awareness toward natural and organic cosmetic products and ingredients	Amyris Inc., Precigen Inc., Tate and Lyle Plc, Santa Cruz Biotechnology Inc., ADL Biopharma, Toronto Research Chemicals Inc., Triveni chemicals, Antai Fine Chemical, Glentham Life Sciences, U.S. Biological Life Sciences, American Enzyme Company, and ALFA	(78)
Artemisinin	$363.8 million (2017)	8.5% CAGR (2018- 2025)	Recognition as first-line treatment for malaria by the WHO	Novartis, Sanofi, Cipla, Ajanta Pharma, Ipca Laboratories, Guilin Pharmaceuticals, and KPC Pharmaceuticals	(84)
Squalene	$129 million (2020)	$184 million by 2025 7.3% CAGR (2020-2025)	The growth in cosmetics and pharmaceuticals industry coupled with the awareness regarding beneficial properties of squalene on human health	Sophim, Amyris, Arista Industries Inc., SeaDragon Marine Oils Limited, Nucelis LLC, Ekiz Olive Oil & Soap Inc., Kishimoto Special Liver Oil Co. Ltd., Empresa Figueirense De Pesca, Lda, Arbee BiomarineExtracts Pvt. Ltd., and New Zealand GreenHealth Limited	(55)
Cannabinoid	$2.8 billion (2020)	21.2% CAGR (2021- 2028)	Acceptance and approvals by governments to use CBD for health purposes	Mile High Labs International, Rhizo Sciences, Global Cannabinoids, SPARKCBD, CBD Inc Group, GenCanna and Maricann Group Inc	(85)
Taxol	$114,8 million (2020)	$162.3 million by 2026 9% CAGR (2021-2026)	Pharma& Healthcare (Cancer treatments)	Phyton, ScinoPharm, Novasep, Samyang, Polymed, TAPI (Teva), Fresenius-kabi, Huiang biopharma, Southpharma, Yunnan Hande, Hainan Yew Pharm and Jiangsu Yew Biotechnology	(90)

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Concluding remarks

Humankind has reached a critical point in its development, probably some kind of "Great filter", where finding solutions to the current problems of technological and population development involves designing new forms of sustainable production. In this sense, it is essential that the proposals do not involve the use of arable land, involve as little water as possible and do not compete in any way with food production. In this sense, synthetic biology supported by fermentative processes represents an opportunity of great potential: all the factors mentioned above can be fully controlled and it offers an "à la carte" production of compounds.

It seems clear that the best approach is the production of biomolecules, as it ensures that they already exist in nature, and thus ecosystems and living beings will be adapted to their presence, we have a large number of options and we can also get to know if we do not already know, their biosynthetic routes, which can be adapted according to needs.

Terpenes fit exceptionally well into all these characteristics as they are the most abundant secondary metabolites of plant origin in nature. Furthermore, their chemical structure offers a great diversity of linear and cyclic compounds, which can be adjusted in a matter of application simply by the addition of oxygen-containing functional groups (alcohols, aldehydes, ketones and carboxylic), giving rise to a subfamily known as terpenoids. Thus, terpenes and terpenoids have been used since ancient times by our species: from the resins obtained from conifers for caulking ships, to essential oils prized for their aroma or their antiseptic, soothing, or anti-inflammatory properties. It is therefore not surprising that its two main synthesis pathways, the mevalonic acid (MVA) and the plastidial 2-C-methyl-d-erythritol 4-phosphate (MEP) pathways, have been studied in-depth and both the metabolites and enzymes involved are now completely described. Based on this knowledge, the next logical step is to search for microorganisms capable of producing terpenes of interest. Given the difficulties arising from the small number of candidates, which generally produce a mixture of compounds in quantities that are not suitable for industrial production, the logical step is to obtain genetically modified organisms. This makes it possible first to select the terpene to be produced and afterward to design the route to maximize the amount obtained. In this sense, *Saccharomyces cerevisiae* and *Escherichia coli* have shown a very good capacity to modify their genomes and produce new biomolecules. Currently, genetically engineered *S. cerevisiae* has shown a higher production capacity for specific terpenes than engineered *E. coli* and in the field of industrial production it is the most preferred host microorganism.

Thus, there are currently microbiologically produced terpenes on the market such as β-farnesene (with applications in biofuels), squalene (cosmetic and adjuvant), cannabinoids (cosmetic and pharmacological) and artemisinin (antimalarial). This shows that this strategy is viable, and the list of terpenes obtained in this way will probably continue to grow in the coming years.

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