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Ashbya gossypii as a versatile platform to produce sabinene from agro-industrial wastes

Gloria Muñoz-Fernández^{1,2}, Javier-Fernando Montero-Bullón¹, José Luis Martínez², Rubén M. Buey¹ and Alberto Jiménez^{1*} 

Abstract

Background *Ashbya gossypii* is a filamentous fungus widely utilized for industrial riboflavin production and has a great potential as a microbial chassis for synthesizing other valuable metabolites such as folates, biolipids, and limonene. Engineered strains of *A. gossypii* can effectively use various waste streams, including xylose-rich feedstocks. Notably, *A. gossypii* has been identified as a proficient biocatalyst for producing limonene from xylose-rich sources. This study aims to investigate the capability of engineered *A. gossypii* strains to produce various plant monoterpenes using agro-industrial waste as carbon sources.

Results We overexpressed heterologous terpene synthases to produce acyclic, monocyclic, and bicyclic monoterpenes in two genetic backgrounds of *A. gossypii*. These backgrounds included an NPP synthase orthogonal pathway and a mutant *erg20^{F95IW}* allele with reduced FPP synthase activity. Our findings demonstrate that *A. gossypii* can synthesize linalool, limonene, pinene, and sabinene, with terpene synthases showing differential substrate selectivity for NPP or GPP precursors. Additionally, co-overexpression of endogenous HMG1 and ERG12 with heterologous NPP synthase and terpene synthases significantly increased sabinene yields from xylose-containing media. Using mixed formulations of corn-cob lignocellulosic hydrolysates and either sugarcane or beet molasses, we achieved limonene and sabinene productions of 383 mg/L and 684.5 mg/L, respectively, the latter representing a significant improvement compared to other organisms in flask culture mode.

Conclusions Engineered *A. gossypii* strains serve as a suitable platform for assessing plant terpene synthase functionality and substrate selectivity *in vivo*, which are crucial to understand monoterpene bioproduction. The NPP synthase pathway markedly enhances limonene and sabinene production in *A. gossypii*, achieving levels comparable to those of other industrial microbial producers. Furthermore, these engineered strains offer a novel approach for producing monoterpenes through the valorization of agro-industrial wastes.

Keywords *Ashbya gossypii*, Terpene synthase, Limonene, Sabinene, Monoterpene, Xylose, Waste valorization, Metabolic engineering

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Background

Terpenes represent the largest category of plant secondary metabolites, encompassing over 80,000 different structures that have been identified [1]. Terpenes utilize two C5 units of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) as building blocks, which in archaea and eukaryotes are synthesized from acetyl-CoA through the mevalonate (MVA) pathway (Fig. 1). IPP and DMAPP can be condensed by various prenyltransferases to generate different prenyl diphosphate precursors, including geranyl diphosphate (GPP, trans-isomer; C10), neryl diphosphate (NPP, cis-isomer; C10), farnesyl diphosphate (FPP; C15), and geranylgeranyl diphosphate (GGPP; C20). These precursors are the substrates of several terpene synthases that produce hemiterpenes ($n=1$), monoterpenes ($n=2$), sesquiterpenes ($n=3$), diterpenes ($n=4$), triterpenes ($n=5$), tetraterpenes ($n=8$, C40) and polyterpenes ($n>8$). (Fig. 1). Terpenes are also grouped according to the structural re-arrangement that undergo the isoprene scaffolds, thus including acyclic, monocyclic, bicyclic, or tetracyclic compounds [2].

Terpenes play significant ecological roles by mediating interactions among organisms [3]. In addition, terpenes have also important and extensive applications as pharmaceuticals, cosmetics, food additives, pesticides and biofuels [4], and, thereby, there is considerable interest in their production. Nevertheless, conventional methods of agricultural production or chemical synthesis are not

viaible due to their high costs and significant environmental pollution. Consequently, these biomolecules present an attractive alternative for integration into microbial bioprocessing applications [5]. Indeed, the microbial production of terpenes has experienced considerable expansion during the last years, primarily through the utilization of bacterial and fungal microbial platforms [1, 2, 6].

One of the main advantages of the microbial production of terpenes is the use of agro-industrial wastes and by-products as cheap carbon sources for microbial fermentation, allowing for the implementation of cost-effective and environmentally-friendly bioprocesses [7]. In this regard, many examples of waste valorization to produce different terpenes have been reported [8], including the utilization of lignocellulosic hydrolysates [9–11], glycerol [12–14], waste cooking oil [10, 15], olive mill waste [16] or textile and cardboard waste [17].

Recently, *A. gossypii*, a filamentous hemiascomycete that is currently used for the industrial production of riboflavin [18], has been presented as an efficient biocatalyst for the production of limonene from xylose as the carbon source [19], which could enable the exploitation of xylose-rich feedstocks such as lignocellulosic hydrolysates. *A. gossypii* has additional benefits in its fermentation characteristics, including the utilization of inexpensive carbon sources and the ease of mycelial harvesting through simple filtration, thus circumventing costly aspects of its bioprocessing [20]. This advantages,

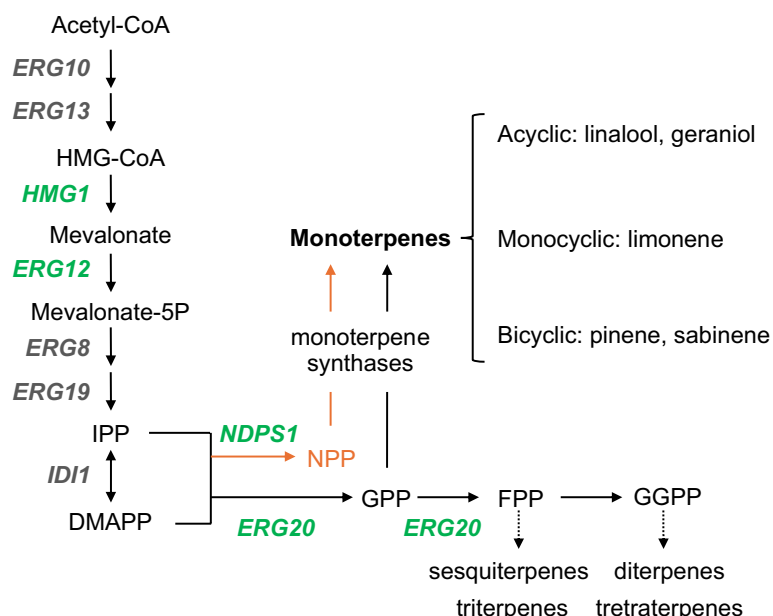


Fig. 1 Schematic representation of the terpenes pathway. Genes controlling the MVA pathway are indicated. The genes engineered in this work are highlighted in green. IPP isopentenyl diphosphate, DMAPP dimethylallyl diphosphate, NPP neryl diphosphate, GPP geranyl diphosphate, FPP farnesyl diphosphate, GGPP geranyl geranyl diphosphate. The NPP orthogonal pathway is colored in orange

together with the availability of an extensive molecular toolbox for its genomic manipulation [21–23] make of *A. gossypii* a very convenient biotechnological chassis with high capacities for the production of a variety of industrially relevant metabolites such as folates, biolipids and monoterpenes [19, 24, 25], among others.

The present work aims at exploring the ability of engineered strains of *A. gossypii* to produce different classes of plant monoterpenes using agro-industrial wastes as carbon sources. For this, the heterologous overexpression of monoterpene synthases from different organisms were carried out to examine the production of industrially-relevant monoterpenes, including the acyclic linalool and geraniol, the monocyclic limonene and the bicyclic α - and β -pinene and sabinene. Beyond obtaining preliminary production data for all monoterpene synthases, we selected the most suitable ones for the production of either limonene or sabinene. We found that limonene and sabinene can be efficiently produced in *A. gossypii* using mixed formulations of corn-cob lignocellulosic hydrolysates (Cch) plus either sugarcane or beet molasses, thus providing a novel platform for their sustainable production.

Materials and methods

***A. gossypii* strains and growth conditions.** All the *A. gossypii* strains used in this study are described in the Additional file 1. MA2 (2% glucose as carbon source) and MX2 (2% xylose plus 0.5% glucose as carbon sources) media contained 20 g/L bactopectone (Condalab), 2 g/L yeast extract (Condalab) and 0.6 g/L myo-inositol (Thermo); SPA media was used for sporulation and contained 20 g/L corn steep liquor (Sigma), 3 g/L yeast extract (Condalab), 3 g/L soytone (Gibco), 3 g/L malt extract (Gibco) and 10 g/L glucose; SPAX media formulation was the same as SPA, but contained 10 g/L xylose instead of glucose. The pH was adjusted to 6.8 for all culture media. *A. gossypii* flask liquid cultures were carried out at 28°C in an orbital shaker at 200 r.p.m. The *A. gossypii* transformation protocol as well as the sporulation conditions and spore isolation method were performed as described previously [26]. Concentrations of 250 mg/L for Geneticin (G418) (ChemCruz) were used for isolation of G418-resistant clones.

Corn cob lignocellulosic hydrolysates (Cch) preparation. Corn cob was soaked in 2.5% (w/v) diluted sulfuric acid and subjected to hydrothermal treatment in an autoclave for 140 min at 121 °C. After cooling, the mixture was neutralized with CaCO₃ and clear hydrolysate (Cch) recovered by vacuum filtration through cellulose paper. For detoxification, the Cch was treated with activated charcoal (Sigma), in proportion 1:5 (w/v), under constant agitation for 1 h. This step allows to remove,

by subsequent filtration, some apolar compounds with antifungal effect such as furfural, hydroxymethylfurfural or phenols. Remaining excess of salts and ions were similarly cleansed by stepwise use of both cationic—Amberlite IR-120 (Fisher), and anionic—Amberlite IRA-96 (Thermo), exchange resins. Incubations of 45 min with ratio 1:20 (w/v) and 1:10 (w/v) to the volume of Cch, were respectively performed for each resin. Final solution was adjusted to neutral pH with KOH and analysed in a 1260 Infinity II HPLC system (Agilent Technologies, CA, USA), equipped with a REZEX ROA Organic Acid H+ (8%) column (Phenomenex, CA, USA), upon isocratic gradient of 0.005N H₂SO₄. Sugars concentrations were determined, based on pure external standards calibration, averaging titers circa 30 g/L xylose and 5 g/L glucose.

Gene overexpression. Transformation cassettes for genomic integration were used for the overexpression of either endogenous or heterologous genes. The xylose-utilizing strain comprised the overexpression of the endogenous XR-XDH-XK pathway (*GRE3*, *XYL2* and *XKS1* genes) together with the heterologous PKT pathway (*pta* gene from *Bacillus subtilis* and *xpkA* gene from *Aspergillus nidulans*) [27]. The overexpression of endogenous genes was performed by promoter replacement, using integrative overexpression cassettes that comprised recombinogenic flanks, loxP-kanMX-loxP selection marker and the constitutive strong promoter *P_{GPD1}* as described elsewhere [19]. The overexpression of heterologous genes was carried out with integrative cassettes that were assembled using a Golden Gate method as described previously [28]. The integrative cassettes for heterologous overexpression comprised recombinogenic flanks, loxP-kanMX-loxP selection marker, and the transcriptional unit with the indicated promoter and terminator sequences. For the overexpression of monoterpene synthases, synthetic codon-optimized sequences of the different enzymes (Additional file 2) were assembled with the strong promoter *P_{GPD1}* and the terminator *T_{PGKI}*. The recombinogenic flanks targeting the *AFR171W* locus were used. For the overexpression of the synthetic codon-optimized *tNDPS1* gene from *S. lycopersicum* (Additional file 2), recombinogenic flanks targeting the *ABR025C* locus were used, and the regulatory sequences were the strong promoter *P_{TSA1}* and the terminator *T_{ENO1}*. All the synthetic codon-optimized sequences were obtained from Integrated DNA Technologies (USA).

The corresponding integrative cassettes were used to transform spores (germlings) of *A. gossypii*. Primary heterokaryon clones were isolated in G418-containing medium. Homokaryon clones were selected after the sporulation of the primary transformants. The genomic integration of each overexpression cassette was

confirmed by analytical PCR followed by DNA sequencing (see Additional file 3 for primer sequences). The loxP inverted sequences of the kanMX marker enabled its elimination by expressing a Cre recombinase, as previously described [29].

Monoterpenes extraction and quantification. Flask cultures for monoterpenes production were initiated with either spores (10^6) or mycelium preinocula (exclusively for Cch-molasses cultures) in a total volume of 40 mL of the indicated culture media with a 5% dodecane overlay. The cultures were harvested at the indicated time points and centrifuged for 10 min at 4400 r.p.m. The upper dodecane phase was collected and the quantification of different monoterpenes was carried out by GC–MS analysis using 10 μ L of the dodecane phase as described previously [19]. In brief, dodecane was diluted in ethyl acetate and injected in an EI-MS 220 Ion Trap spectrometer coupled to a 7890A GC System (Agilent Technologies, CA, USA), bearing a DB-5 column—30 m long, 0.25 mm internal diameter, 25 μ m film (Agilent Technologies, CA, USA). Separation was performed in a typical increasing temperature gradient with helium as carrier. Identification and quantification of monoterpenes was performed upon external calibration with linalool, geraniol, limonene, sabinene and α - and β -pinene standards (Sigma).

Determination of lipid profiles. 10 μ L of interphase between culture and dodecane overlay were collected and diluted 1:100 (v/v) in MeOH for injection in an Orbitrap Q-Exactive Focus spectrometer hyphenated to liquid chromatography in a Vanquish Flex UPLC (Thermo Fisher Scientific, MA, USA). A C18 Poroshell column—4.6 \times 50 mm, 2.7 μ m (Agilent Technologies, CA, USA) was used for lipids separation upon a binary gradient (0.1% formic acid:acetonitrile) at 0.3 mL/min with increasing proportions of organic solvent. A non-targeted lipidomics analysis was performed in switching positive and negative polarities relying on HRAM spectra (resolution 70,000 and 1 ppm accuracy) for m/z range 120–1500 and data dependent top intensity MS2 acquisition. The 10 most height-intense signals for each polarity along the full chromatogram were annotated, queried for identification against the LipidMaps database (<https://www.lipidmaps.org/>)—accuracy tolerance 2.5 ppm, and further confirmed examining fragmentation pattern in the corresponding MS2 spectra.

Results

Functional analysis of different plant monoterpene synthases in *A. gossypii*

The overexpression of the limonene synthase from *Citrus limon*, coupled to an NPP synthase orthogonal pathway (Fig. 1), enabled the efficient production of limonene in

A. gossypii [19]. The highest limonene titer was obtained with a xylose-utilizing strain (see Materials and Methods for details), which also comprised the overexpression of the endogenous *HMG1* and *ERG12* genes. In this work, we aimed at exploring the functionality of different monoterpene synthases in two different *A. gossypii* genetic backgrounds: (i) *tNDPS1*, an NPP synthase overexpressing strain (using the heterologous *NDPS1* gene from *Solanum lycopersicum*), and (ii) *erg20mut*, a mutant strain expressing an *erg20*^{F95W} allele with a reduced FPP synthase activity [19]. Hence, two different approaches for metabolic flux redirection were assayed with each monoterpene synthase: *tNDPS1* and *erg20mut*, designed to increase the precursors NPP and GPP, respectively (Fig. 1).

Additionally, both parental strains were equipped with a functional endogenous xylose-utilizing pathway, an heterologous phosphoketolase pathway, and the overexpression of the native *HMG1* gene [19].

Six different truncated monoterpene synthases, lacking the plastid targeting signal, were selected for gene overexpression in *A. gossypii*: limonene synthase from *C. limon* (tLS) used as a control, linalool synthase from *Actinidia arguta* (tLoS), geraniol synthase from *Valeriana officinalis* (tGS), pinene synthases from *Abies grandis* (tPS-Ag) and *Pinus taeda* (tPS-Pt), and sabinene synthase from *Salvia pomifera* (tSS). Integrative overexpression modules were assembled using codon-optimized sequences of the six terpene synthases (Additional file 2) and used for transformation of the two parental strains (*tNDPS1* and *erg20mut*). The engineered strains were grown in xylose-containing media for 72 h and the production of monoterpenes was analyzed. Our results revealed a great heterogeneity of the terpene synthases functionality (Fig. 2).

The strains expressing tLS were previously described and used as controls for limonene production [19]. Geraniol was not detected in any genetic background, suggesting that tGS from *V. officinalis* is not active in *A. gossypii*. Sabinene was produced at high levels (above 40 mg/L) in the *tNDPS1* genetic background, which also supported the production of linalool and pinene (only from tPS-Pt) (Fig. 2). In contrast, the *erg20mut* genetic background allowed the production of lower levels (under 10 mg/L) of linalool, limonene and pinene (from both tPS-Pt and tPS-Ag). However, while limonene and pinene (from tPS-Pt) were significantly higher in the *tNDPS1* genetic background, the production of linalool was favored in the *erg20mut* background, thereby reflecting different substrate selectivity (NPP vs GPP) among the terpene synthases. However, the linalool titer produced by *A. arguta* tLoS suggests the need to explore for additional heterologous genes that may provide

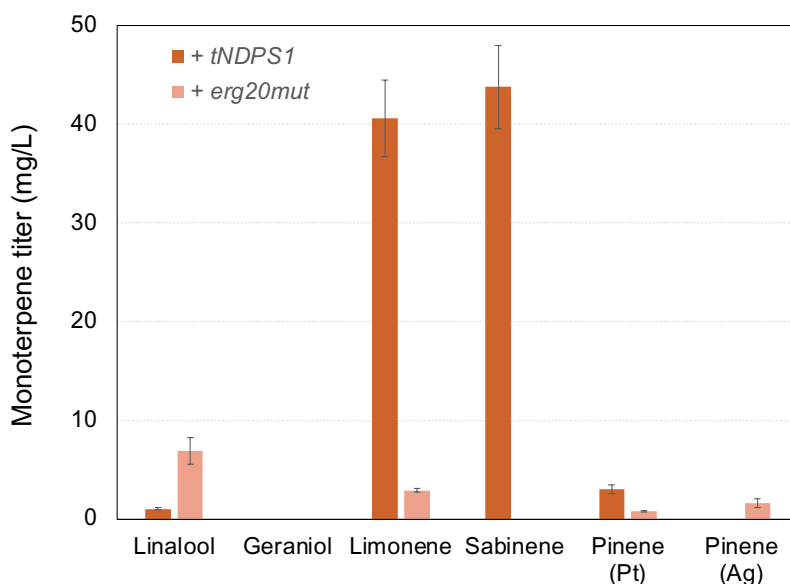


Fig. 2 Functional analysis of plant terpene synthases in *A. gossypii*. Engineered strains expressing different monoterpene synthases were analyzed for their ability to produce the corresponding monoterpene. The results show the monoterpene titer in two different genetic backgrounds: *tNDPS1*, overexpressing the heterologous NPP synthase pathway; *erg20mut*, expressing a mutant *erg20*^{F95W} allele. Flask cultures were performed using MX2 as the culture media for 72 h. Data are the means of two independent experiments performed in duplicate. Error bars represent the standard deviation

higher linalool synthase activity in *A. gossypii*. Hence, we next focused on the limonene and sabinene producing strains in the *tNDPS1* genetic background for further experiments.

Sabinene is efficiently produced in *A. gossypii* from xylose as the carbon source

Our previous work showed that the overexpression of the endogenous *ERG12*, encoding mevalonate kinase (Fig. 1), provided a significant increase in the production of limonene in *A. gossypii* [19]. Consequently, the overexpression of *ERG12* in the sabinene producing strain was conducted using the strong constitutive promoter *P_{SEDI}* [23]. This was done to evaluate the impact of mevalonate kinase overproduction on both the growth and sabinene production capacities of the sabinene producing strain (A1554), using the limonene producing strain (A1308) as a control.

Liquid cultures of both strains were grown for 240 h using MX2 media (containing 0.5% glucose plus 2% xylose as the carbon sources). Aliquots were taken at the indicated time points to evaluate the biomass production (Fig. 3). An exponential growth phase was observed from 24 h to 96–120 h, depending on the strain. Both strains initiated the exponential growth after 24 h of culture; however, the sabinene producing strain (A1554) showed a higher xylose consumption rate, a more extended exponential growth phase, and

the ability to generate more biomass than the limonene producing strain (A1308) (Fig. 3).

The production of monoterpenes was quantified from 48 to 120 h in both strains using MX2, as a simple defined medium; and SPAX, a complex medium containing a higher (not defined) sugar content from soytone, malt extract and corn steep liquor (see Material and Methods for details). Increasing concentrations of limonene (Fig. 4A) and sabinene (Fig. 4B) were obtained in both culture media. In MX2 media at 120 h, the production of limonene was 325 ± 2 mg/L (yield = 13 mg/g of carbon source), and the sabinene titer reached 407 ± 25 mg/L (yield = 16.3 mg/g). However, the highest titers of limonene and sabinene were obtained at 120 h in SPAX media. Specifically, the A1308 strain produced 367 ± 10.5 mg/L of limonene, while the A1554 strain produced 470.5 ± 25.5 mg/L of sabinene, thus demonstrating that both terpenes can be efficiently produced in the xylose-utilizing engineered strains of *A. gossypii* and that a higher sugar concentration in the culture media helps to increase the production of both limonene and sabinene. Interestingly, the A1308 cultures showed a lipidic interphase (between the aqueous culture media and the dodecane overlay), that was particularly visible at 48 h in MX2 media (Additional file 4A), and was mostly composed by free fatty acids (FAs) and phospholipids (PLs) (Additional file 4B-F).

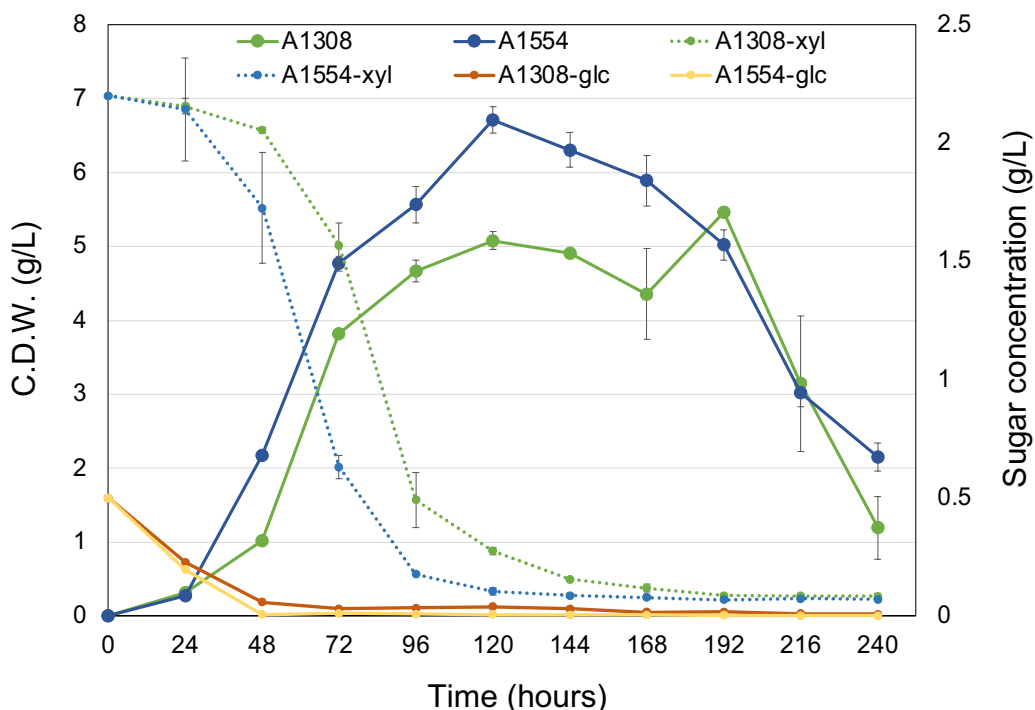


Fig. 3 Growth analysis of terpene-producing strains. Biomass production and sugar consumption (glc, glucose; xyl, xylose) of the A1308 (limonene producer) and A1554 (sabinene producer) strains grown in MX2 flask culture media. Data are the means of two independent experiments performed in duplicate. Error bars represent the standard deviation

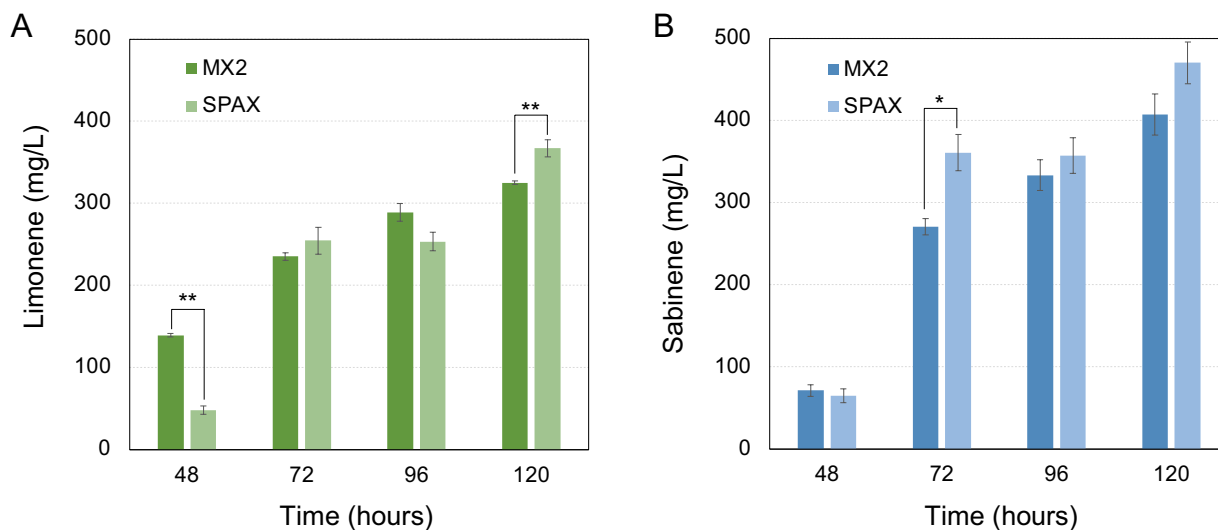


Fig. 4 Monoterpene production of engineered *A. gossypii* strains in synthetic culture media. A, limonene titers of the A1308 strain grown in MX2 and SPAX culture media. B, sabinene titers of the A1554 strain grown in MX2 and SPAX culture media. Data are the means of two independent experiments performed in duplicate. Error bars represent the standard deviation. The Student's t test was performed to determine significant differences ($P < 0.05$)

Valorization of xylose-rich wastes to produce limonene and sabinene in *A. gossypii*

We have previously described that the utilization of mixed formulations of Cch plus either sugarcane or

beet molasses represents an excellent strategy for the production of microbial lipids in *A. gossypii* [25]. Prompted by these results, we decided to analyze the production of monoterpenes using these xylose-rich

waste streams. The sugar composition of these waste by-products was analyzed to calculate an adequate dilution of sugars in the culture media for flask fermentations (Table 1). Hence, culture media was prepared using 25% Cch plus 4% of either sugarcane or beet molasses, to use a final concentration of 10 g/L of xylose and approximately 25 g/L of sucrose.

Flask liquid cultures were grown using 25% Cch plus 4% of either sugarcane (SM) or beet molasses (BM) as the carbon sources. Cultures were grown for 120 h and the concentration of monoterpenes in the dodecane layer was analyzed. The A1308 strain showed almost identical titers of limonene among the two culture media, reaching near 400 mg/L of limonene (Fig. 5A). The conversion yields were, in this case, 9.6 mg/g and 10.9 mg/g for Cch+SM and Cch+BM, respectively. In contrast, the A1554 strain displayed large differences in sabinene production among the culture media: while Cch+BM produced a sabinene titer of 398.5 ± 28.5 mg/L (yield = 11.3 mg/g), the utilization of Cch+SM provided a significantly higher titer of sabinene reaching 684.5 ± 19.5 mg/L (yield = 17.1 mg/g).

Table 1 Sugar composition of waste by-products used in this work

	Sugar composition (g/L)			
	Sucrose	Glucose	Fructose	Xylose
Cch	–	3.8	–	40
Sugarcane molasses	680.6	23.2	21.3	–
Beet molasses	597.9	1.3	9.1	–

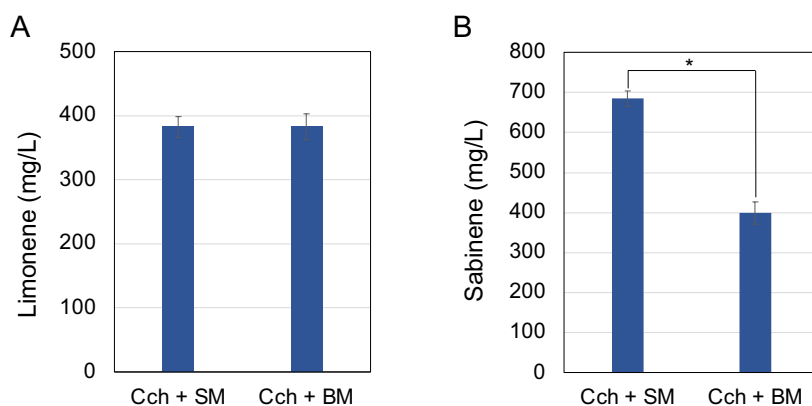


Fig. 5 Monoterpene production of engineered *A. gossypii* strains using wastes as carbon sources. Mixed formulations of Cch plus sugarcane molasses (Cch + SM) and Cch plus beet molasses (Cch + BM) were used as the sole carbon sources for flask cultures. Limonene (A) and sabinene (B) were quantified from A1308 and A1554 cultures, respectively. Data are the means of two independent experiments performed in duplicate. Error bars represent the standard deviation. The Student's t test was performed to determine significant differences ($P < 0.05$)

Discussion

Our results showed that *A. gossypii* can afford the functional expression of different terpene synthases leading to the production of acyclic (linalool), monocyclic (limonene) and bicyclic (pinene and sabinene) monoterpenes, thus supporting the versatility of *A. gossypii* as an efficient microbial factory.

The utilization of two different genetic backgrounds revealed significant differences of substrate selectivity and functionality among the assayed terpene synthases, which might be critical for the engineering of new biocatalysts. In agreement, diverse substrate selectivity of terpene synthases has been previously described and it can largely determine the productivity of monoterpenes in heterologous hosts such as *A. gossypii* [30]. Hence, our model could serve to discriminate the substrate selectivity of different monoterpene synthases in vivo as a first approach for the development of optimized engineering bioprocesses. The present study identified the sabinene synthase from *S. pomifera* as a promising candidate for sabinene production in *A. gossypii*. In contrast, other monoterpene synthases did not exhibit comparable activity, underscoring the importance of our model in assessing both substrate selectivity and functionality. For example, the production of high levels of linalool in eukaryotic cell factories has not been reported [31], including the use of tLoS from *A. rugosa* in *A. gossypii* (this study). This suggests that further exploration is needed to identify suitable candidates among other linalool synthases from different organisms.

The growth kinetics of the limonene (A1308) and sabinene (A1554) producing strains showed remarkable differences that can reflect a dissimilar utilization of xylose and, consequently, explain the differences observed in

biomass and production of monoterpenes. Apparently, the production of both limonene and sabinene are coupled to the mycelial growth in *A. gossypii*. In microorganisms, isoprenoids are essential for cell growth and, therefore, their volumetric concentration correlates with microbial growth due to growth-coupled biosynthesis [32]. However, a growth-uncoupled production would be an advantageous option since the less biomass is generated, the more substrate would be available for the synthesis of bioproducts, as previously described for isoprenoid biosynthesis in *Rhodobacter sphaeroides* [33]. This approach is especially suited to produce non-native compounds, which are not required for growth. In this regard, the production of limonene in the A1308 strain correlated with an enhanced accumulation of lipids (mostly free FAs and PLs), which can compete with the production of limonene for the utilization of acetyl-CoA as the immediate precursor. An alternative hypothesis is that increased lipid production may facilitate the dissolution of limonene, sequestering it from the host and thereby enhancing its overall productivity. Why this correlation is not observed in the A1544 sabinene producing strain remains however unknown.

The utilization of SPAX culture media provided a higher titer of both limonene and sabinene at 120 h of culture, showing that a higher concentration of carbon and nitrogen sources (from corn steep liquor, soytone and malt extract) correlates with an enhanced production of monoterpenes. In addition, the higher level of mycelial lysis that occurred in SPAX media at 120 h of culture (Additional file 5) can also help to increase the accumulation of monoterpenes in the dodecane phase. In fact,

the autolysis of the *A. gossypii* mycelium at later stages of the growth culture represents a significant advantage in terms of bioproduct recovery, thereby contributing to save downstream costs.

The highest limonene and sabinene titers were obtained using mixed formulations of waste streams, i. e. Cch plus molasses. This was particularly evident for the production of sabinene by the strain A1554. In this regard, the production of limonene was almost identical in Cch + SM and Cch + BM, despite the higher sugar concentration in the Cch + SM medium (Table 1). However, the titer of sabinene was significantly enhanced in the Cch + SM medium. These differences can be attributed to the superior growth and sugar consumption abilities of the sabinene producing strain (Fig. 3). Alternatively, the increased accumulation of lipids in the strain A1304 may also influence the biosynthesis of monoterpenes. Overall, these results underpin the idea that the utilization of mixed formulations of xylose-rich hydrolysates together with molasses is a suitable approach for the valorization of waste by-products using *A. gossypii*, as previously reported for the production of biolipids [25]. A similar strategy using paddy straw hydrolysate as a diluent of molasses was recently described to increase ethanol production in *S. cerevisiae* [34].

Gram-scale titers for limonene and sabinene have been obtained with the biotechnological workhorses *E. coli* and *S. cerevisiae* (Table 2). Li et al. have recently reviewed the state of the art of terpene production using different microbial chassis, showing that the carbon source, the extraction phase and the fermentation mode and scale strongly influence the process performance [31].

Table 2 Limonene and sabinene titers in different microbial cell factories

	Organism	Fermentation mode	Titer (mg/L)	Carbon source	Extraction phase	Ref
Limonene	<i>E. coli</i>	Flask	605	Glucose	Dodecane	[43]
		Flask (fb) ^a	1290	Glucose	Isopropyl myristate	[40]
		Bioreactor (fb)	3630	Glycerol	Diisononyl phthalate	[41]
	<i>S. cerevisiae</i>	Flask (fb)	2580	Galactose/raffinose	Isopropyl myristate	[39]
		Flask (fb)	2230	Glucose/ethanol	Isopropyl myristate	[42]
		Bioreactor (fb)	2630	Soytone/sucrose/glycerol	Dodecane	[44]
	<i>Rodosporidium toruloides</i>	Flask	358.1	Glucose	Dodecane	[45]
	<i>Yarrowia lipolytica</i>	Flask	23.56	Glucose/pyruvate	Dodecane	[46]
		Bioreactor (fb)	165.3	Glycerol/citrate	Dodecane	[14]
<i>A. gossypii</i>	Flask	383	Cch/BM	Dodecane	This study	
Sabinene	<i>E. coli</i>	Flask	150	Glucose/citrate	None	[47]
		Bioreactor (fb)	2650	Glycerol	None	[13]
	<i>S. cerevisiae</i>	Flask	17.5	Galactose/raffinose	Dodecane	[48]
	<i>A. gossypii</i>	Flask	684.5	Cch/SM	Dodecane	This study

^a fb fed-batch mode

The A1554 strain presented in this work reached about 700 mg/L of sabinene using agro-industrial wastes in flask cultures, which represents a significant improvement compared to other organisms in flask culture mode [13, 31, 35]. In any case, further optimization of fermentation parameters is expected to increase the sabinene titers up to the gram-scale in *A. gossypii*. These parameters might include the organellar compartmentalization of the enzymatic machinery that has been successfully employed to significantly increase the production of limonene, squalene and other terpenes in yeast [36–39]. Also, the use of organic solvents, different from dodecane, to extract these volatile bioproducts might increase monoterpene titers (Table 2) [40–42]. Finally, the implementation of optimized bioreactor conditions for *A. gossypii* cultures could also benefit the productivity of the bioprocess, as previously demonstrated for other microbial cell factories [1, 31].

Altogether, this work represents a proof of concept for sabinene production near to the gram-scale in *A. gossypii* through agro-industrial waste valorization, a finding with obvious industrial relevance that deserves further investigation.

Conclusions

The development of novel microbial biocatalysts for monoterpene production is a valuable strategy in industrial biotechnology. In this study, we report engineered strains of *A. gossypii* that serve as an ideal platform to investigate the substrate selectivity and functionality of plant terpene synthases in vivo. Furthermore, we demonstrate that the filamentous fungus *A. gossypii*, traditionally used for industrial riboflavin production, can be effectively repurposed as a novel microbial factory for monoterpene biosynthesis. Although further optimization of metabolic and fermentative parameters may be required to increase limonene and sabinene titers beyond the gram-scale, *A. gossypii* offers a promising alternative for the valorization of agro-industrial wastes such as corn cobs and molasses.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40694-024-00186-1>.

Additional file 1. Ashbya gossypii strains used in this work
 Additional file 2. Synthetic DNA sequences used in this work
 Additional file 3. List of primers used in this work
 Additional file 4. Lipid profile of interphase from A1308 MX2 cultures. A, lipidic interphase in A1308 MX2 flask cultures at 48 h. B, average MS spectrum of signals in positive ESI. C, average FTMS spectrum of signal in negative ESI. D, most abundant m/z signals identified in data analysis and relative intensity versus maximum, in positive ESI. E, most abundant m/z signals identified and relative intensity versus maximum, in negative ESI.

PC, phosphatidylcholine; FA, fatty acid. F, list of identified lipid species by query of LipidMaps Structure Databases with data from both positive and negative ESI signals. The top 25 signals for both ionization modes were selected and identified with accuracy threshold of ± 0.010 amu and further MS2 confirmation for ambiguity

Additional file 5. Biomass production of A1308 and A1554 strains

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Author contributions

GM-F, design of the work, investigation, acquisition, analysis and interpretation of data; J-FM-B, investigation and acquisition of data; JLM, analysis and interpretation of data; RMB, funding acquisition, analysis and interpretation of data; AJ, conceptualization, design of the work, funding acquisition, analysis and interpretation of data, original draft preparation. All authors participated in the review/editing process of the original draft and approved the submitted version.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Fordjour E, Mensah EO, Hao Y, Yang Y, Liu X, Li Y, et al. Toward improved terpenoids biosynthesis: strategies to enhance the capabilities of cell factories. *Bioresour Bioprocess*. 2022;9:6. <https://doi.org/10.1186/s40643-022-00493-8>.
2. Pimentel L, Carsanba E, Teixeira F, Vidigal S, Pintado M, Oliveira C, et al. Microbial production of terpenes. In: *Microbial production of food bioactive compounds*. Cham: Springer International Publishing; 2022. p. 1–38.
3. Gershenzon J, Dudareva N. The function of terpene natural products in the natural world. *Nat Chem Biol*. 2007;3:408–14.
4. Vickers CE, Williams TC, Peng B, Cherry J. Recent advances in synthetic biology for engineering isoprenoid production in yeast. *Curr Opin Chem Biol*. 2017;40:47–56. <https://doi.org/10.1016/j.cbpa.2017.05.017>.
5. Zhang C, Hong K. Production of terpenoids by synthetic biology approaches. *Front Bioeng Biotechnol*. 2020;8:347.
6. Wu W, Maravelias CT. Synthesis and techno-economic assessment of microbial-based processes for terpenes production. *Biotechnol Biofuels*. 2018;11:294. <https://doi.org/10.1186/s13068-018-1285-7>.

7. Lad BC, Coleman SM, Alper HS. Microbial valorization of underutilized and nonconventional waste streams. *J Ind Microbiol Biotechnol*. 2022;49:1–15. <https://doi.org/10.1093/jimb/kuab056/6371101>.
8. Arnesen JA, Borodina I. Engineering of *Yarrowia lipolytica* for terpenoid production. *Metab Eng Commun*. 2022;15: e00213. <https://doi.org/10.1016/j.mec.2022.e00213>.
9. Yao F, Liu SC, Wang DN, Liu ZJ, Hua Q, Wei LJ. Engineering oleaginous yeast *Yarrowia lipolytica* for enhanced limonene production from xylose and lignocellulosic hydrolysate. *FEMS Yeast Res*. 2020;20:1–9.
10. Li Z-J, Wang Y-Z, Wang L-R, Shi T-Q, Sun X-M, Huang H. Advanced strategies for the synthesis of terpenoids in *Yarrowia lipolytica*. *J Agric Food Chem*. 2021;69:2367–81. <https://doi.org/10.1021/acs.jafc.1c00350>.
11. Kirby J, Geiselman GM, Yaegashi J, Kim J, Zhuang X, Tran-Gyamfi MB, et al. Further engineering of *R. toruloides* for the production of terpenes from lignocellulosic biomass. *Biotechnol Biofuels*. 2021;14:101. <https://doi.org/10.1186/s13068-021-01950-w>.
12. Willrodt C, David C, Cornelissen S, Bühler B, Julsing MK, Schmid A. Engineering the productivity of recombinant *Escherichia coli* for limonene formation from glycerol in minimal media. *Biotechnol J*. 2014;9:1000–12.
13. Zhang H, Liu Q, Cao Y, Feng X, Zheng Y, Zou H, et al. Microbial production of sabinene—a new terpene-based precursor of advanced biofuel. *Microb Cell Fact*. 2014;13:1–10.
14. Cheng BQ, Wei LJ, Lv YB, Chen J, Hua Q. Elevating limonene production in oleaginous yeast *Yarrowia lipolytica* via genetic engineering of limonene biosynthesis pathway and optimization of medium composition. *Biotechnol Bioprocess Eng*. 2019;24:500–6.
15. Li S, Rong L, Wang S, Liu S, Lu Z, Miao L, et al. Enhanced limonene production by metabolically engineered *Yarrowia lipolytica* from cheap carbon sources. *Chem Eng Sci*. 2022;249:117342. <https://doi.org/10.1016/j.ces.2021.117342>.
16. Guner O, Demirkol A, Yuceer YK, Toğay SO, Hosoglu Mİ, Elibol M. Production of flavor compounds from olive mill waste by *Rhizopus oryzae* and *Candida tropicalis*. *Braz J Microbiol*. 2017;48:275–85.
17. Zebec Ž, Poberžnik M, Lobnik A. Enzymatic hydrolysis of textile and cardboard waste as a glucose source for the production of limonene in *Escherichia coli*. *Life*. 2022;12:1423.
18. Revuelta JL, Ledesma-Amaro R, Lozano-Martínez P, Díaz-Fernández D, Buey RM, Jiménez A. Bioproduction of riboflavin: a bright yellow history. *J Ind Microbiol Biotechnol*. 2017;44:659–65.
19. Muñoz-Fernández G, Martínez-Buey R, Revuelta JL, Jiménez A. Metabolic engineering of *Ashbya gossypii* for limonene production from xylose. *Biotechnol Biofuels Bioprod*. 2022;15:1–13. <https://doi.org/10.1186/s13068-022-02176-0>.
20. Schwachheimer SK, Park EY, Revuelta JL, Becker J, Wittmann C. Biotechnology of riboflavin. *Appl Microbiol Biotechnol*. 2016;100:2107–19. <https://doi.org/10.1007/s00253-015-7256-z>.
21. Aguiar TQ, Silva R, Domingues L. *Ashbya gossypii* beyond industrial riboflavin production: a historical perspective and emerging biotechnological applications. *Biotechnol Adv*. 2015;33:1774–86. <https://doi.org/10.1016/j.biotechadv.2015.10.001>.
22. Jiménez A, Hoff B, Revuelta JL. Multiplex genome editing in *Ashbya gossypii* using CRISPR-Cpf1. *N Biotechnol*. 2020;57:29–33.
23. Muñoz-Fernández G, Montero-Bullón J-F, Revuelta JL, Jiménez A. New promoters for metabolic engineering of *Ashbya gossypii*. *J Fungi*. 2021;7:906.
24. Serrano-Amatriain C, Ledesma-Amaro R, López-Nicolás R, Ros G, Jiménez A, Revuelta JL. Folic acid production by engineered *Ashbya gossypii*. *Metab Eng*. 2016;38:473–82.
25. Díaz-Fernández D, Aguiar TQ, Martín VI, Romání A, Silva R, Domingues L, et al. Microbial lipids from industrial wastes using xylose-utilizing *Ashbya gossypii* strains. *Bioresour Technol*. 2019;293:122054.
26. Jiménez A, Santos MA, Pompejus M, Revuelta JL. Metabolic engineering of the purine pathway for riboflavin production in *Ashbya gossypii*. *Appl Environ Microbiol*. 2005;71:5743–51. <https://doi.org/10.1128/AEM.71.10.5743-5751.2005>.
27. Díaz-Fernández D, Lozano-Martínez P, Buey RM, Revuelta JL, Jiménez A. Utilization of xylose by engineered strains of *Ashbya gossypii* for the production of microbial oils. *Biotechnol Biofuels*. 2017;10:3. <https://doi.org/10.1186/s13068-016-0685-9>.
28. Ledesma-Amaro R, Jiménez A, Revuelta JL. Pathway grafting for polyunsaturated fatty acids production in *Ashbya gossypii* through golden gate rapid assembly. *ACS Synth Biol*. 2018;7:2340–7. <https://doi.org/10.1021/acssynbio.8b00287>.
29. Aguiar TQ, Dinis C, Domingues L. Cre-loxP-based system for removal and reuse of selection markers in *Ashbya gossypii* targeted engineering. *Fungal Genet Biol*. 2014;68:1–8.
30. Lei D, Qiu Z, Qiao J, Zhao G-R. Plasticity engineering of plant monoterpene synthases and application for microbial production of monoterpenoids. *Biotechnol Biofuels*. 2021;14:147. <https://doi.org/10.1186/s13068-021-01998-8>.
31. Li T, Liu X, Xiang H, Zhu H, Lu X, Feng B. Two-phase fermentation systems for microbial production of plant-derived terpenes. *Molecules*. 2024;29:1127.
32. Von Kamp A, Klamt S. Growth-coupled overproduction is feasible for almost all metabolites in five major production organisms. *Nat Commun*. 2017;8:1–10.
33. Orsi E, Mouggiakos I, Post W, Beekwilder J, Dompè M, Eggink G, et al. Growth-uncoupled isoprenoid synthesis in *Rhodobacter sphaeroides*. *Biotechnol Biofuels*. 2020;13:1–13. <https://doi.org/10.1186/s13068-020-01765-1>.
34. Pandey AK, Kumar M, Kumari S, Gaur NA. Integration of acid pre-treated paddy straw hydrolysate to molasses as a diluent enhances ethanol production using a robust *Saccharomyces cerevisiae* NGY10 strain. *Renew Energy*. 2022;186:790–801.
35. Cao Y, Zhang H, Liu H, Liu W, Zhang R, Xian M, et al. Biosynthesis and production of sabinene: current state and perspectives. *Appl Microbiol Biotechnol*. 2018;102:1535–44.
36. Zhu ZT, Du MM, Gao B, Tao XY, Zhao M, Ren YH, et al. Metabolic compartmentalization in yeast mitochondria: burden and solution for squalene overproduction. *Metab Eng*. 2021;68:232–45.
37. Liu GS, Li T, Zhou W, Jiang M, Tao XY, Liu M, et al. The yeast peroxisome: a dynamic storage depot and subcellular factory for squalene overproduction. *Metab Eng*. 2020;57:151–61.
38. Yang L, Liu H, Jin Y, Liu J, Deng L, Wang F. Recent Advances in multiple strategies for the synthesis of terpenes by engineered yeast. *Fermentation*. 2022;8:615.
39. Dusséaux S, Wajn WT, Liu Y, Ignea C, Kampranis SC. Transforming yeast peroxisomes into microfactories for the efficient production of high-value isoprenoids. *Proc Natl Acad Sci U S A*. 2020;117:31789–99.
40. Wu J, Cheng S, Cao J, Qiao J, Zhao G-R. Systematic optimization of limonene production in engineered *Escherichia coli*. *J Agric Food Chem*. 2019;67:7087–97. <https://doi.org/10.1021/acs.jafc.9b01427>.
41. Rolf J, Julsing MK, Rosenthal K, Lütz S. A gram-scale limonene production process with engineered *Escherichia coli*. *Molecules*. 2020;25:1881.
42. Zhang X, Liu X, Meng Y, Zhang L, Qiao J, Zhao G-R. Combinatorial engineering of *Saccharomyces cerevisiae* for improving limonene production. *Biochem Eng J*. 2021;176:108155. <https://doi.org/10.1016/j.bej.2021.108155>.
43. Alonso-Gutiérrez J, Kim EM, Batth TS, Cho N, Hu Q, Chan LJJ, et al. Principal component analysis of proteomics (PCAP) as a tool to direct metabolic engineering. *Metab Eng*. 2015;28:123–33.
44. Kong X, Wu Y, Yu W, Liu Y, Li J, Du G, et al. Efficient synthesis of limonene in *saccharomyces cerevisiae* using combinatorial metabolic engineering strategies. *J Agric Food Chem*. 2023;71:7752–64. <https://doi.org/10.1021/acs.jafc.3c02076>.
45. Zhao D, Gao Q, Zheng X, Liu S, Qi Q, Wang X, et al. Optimization of fermentation conditions for elevating limonene production with engineered *Rhodospiridium toruloides*. *Fermentation*. 2023;9:431.
46. Cao X, Lv Y-B, Chen J, Imanaka T, Wei L-J, Hua Q. Metabolic engineering of oleaginous yeast *Yarrowia lipolytica* for limonene overproduction. *Biotechnol Biofuels*. 2016;9:214. <https://doi.org/10.1186/s13068-016-0626-7>.
47. Liu H, Cheng T, Zou H, Zhang H, Xu X, Sun C, et al. High titer mevalonate fermentation and its feeding as a building block for isoprenoids (isoprene and sabinene) production in engineered *Escherichia coli*. *Process Biochem*. 2017;62:1–9.
48. Ignea C, Pontini M, Maffei ME, Makris AM, Kampranis SC. Engineering monoterpene production in yeast using a synthetic dominant negative geranyl diphosphate synthase. *ACS Synth Biol*. 2014;3:298–306. <https://doi.org/10.1021/sb400115e>.

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