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Original Research Article

Engineering a non-model yeast *Rhodotorula mucilaginosa* for terpenoids synthesis

Qiongqiong Chen [a,c,1], Liting Lyu [a,b,1,*], Haizhao Xue [a,c], Aabid Manzoor Shah [a], Zongbao Kent Zhao [a,b]

[a] Laboratory of Biotechnology, Dalian Institute of Chemical Physics, CAS, 457 Zhongshan Road, Dalian, 116023, China
[b] MOE Key Laboratory of Bio-Intelligent Manufacturing, School of Bioengineering, Dalian University of Technology, Dalian, 116024, China
[c] University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing, 100049, China

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ABSTRACT

Terpenoids have tremendous biological activities and are widely employed in food, healthcare and pharmaceutical industries. Using synthetic biology to product terpenoids from microbial cell factories presents a promising alternative route compared to conventional methods such as chemical synthesis or phytoextraction. The red yeast *Rhodotorula mucilaginosa* has been widely studied due to its natural production capacity of carotenoid and lipids, indicating a strong endogenous isoprene pathway with readily available metabolic intermediates. This study constructed several engineered strains of *R. mucilaginosa* with the aim of producing different terpenoids. Monoterpene α-terpineol was produced by expressing the α-terpineol synthase from *Vitis vinifera*. The titer of α-terpineol was further enhanced to 0.39 mg/L by overexpressing the endogenous rate-limiting gene of the MVA pathway. Overexpression of α-farnesene synthase from *Malus domestica,* in combination with MVA pathway rate-limiting gene resulted in significant increase in α-farnesene production, reaching a titer of 822 mg/L. The carotenoid degradation product β-ionone was produced at a titer of 0.87 mg/L by expressing the β-ionone synthase from *Petunia hybrida*. This study demonstrates the potential of *R. mucilaginosa* as a platform host for the direct biosynthesis of various terpenoids and provides insights for further development of such platforms.

1. Introduction

Terpenoids are naturally occurring compounds with diverse properties that have a wide range of potential applications, such as in pharmaceuticals [1], advanced biofuels [2], fragrances [3] and preservatives [4]. Nevertheless, the challenges of poor yields and complications in subsequent separation pose major barriers to the direct extraction of terpenoids from plants. Therefore, producing terpenoids through microbial cell factories emerges as an appealing alternative that may overcome the aforementioned bottleneck and facilitate more sustainable production to meet the growing market demands. In this regard, the synthetic biology approach has been shown to be successful in the production of terpenoids over the past decade [5–8].

Generally, two distinct biochemical pathways are known in nature for terpenoids biosynthesis: the mevalonate (MVA) pathway and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway [9]. There are significant differences between the MVA pathway and the MEP pathway with regards to the location of synthesis, synthetic precursors, and the enzymes involved. The MEP pathway is found mostly in prokaryotes and the plastids of plants, while the MVA pathway is mainly present in the cytoplasm of eukaryotes [10–12]. As universal precursors of isoprenoids, isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are derived from both pathways. Then, IPP and DMAPP are condensed to form pyrophosphate precursors with different chain lengths, including geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP). Terpenoids are synthesized through a series of enzymatic reactions, which include isomerization, cyclization, and modification of precursor molecules.

Red yeasts in the genus *Rhodotorula* have gained prominence in recent years as prospective eukaryotic hosts in industries such as

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* Corresponding author. Laboratory of Biotechnology, Dalian Institute of Chemical Physics, CAS, 457 Zhongshan Road, Dalian, 116023, China.
E-mail address: lyult@dlut.edu.cn (L. Lyu).
[1] The authors contributed equally to this work.

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medicine, agriculture, flavors, fragrances, cosmetics, and nutrition. In particular, *Rhodotorula mucilaginosa,* isolated from a variety of habitats, like soil, water and sea salt fields [13–16], exhibits the ability to utilize a wide range of substrates, including glycerol, sugarcane, wheat straw hydrolysate and various agricultural waste materials [17,18]. Moreover, *R. mucilaginosa* is capable of synthesizing a diverse range of bioactive compounds, including carotenoids, astaxanthin, polyunsaturated fatty acids, vitamin E and nucleotides [19–21]. Furthermore, the extracellular polysaccharides and special enzymes produced by *R. mucilaginosa* exhibit significant promise in several industries, such as pharmaceuticals, food, and biochemicals [14,16]. While wild-type *R. mucilaginosa* strains have been documented to synthesize carotenoids [19], to the best of our knowledge, genetic engineering of *R. mucilaginosa* has not been reported for overproduction of terpenoids. In this study, *R. mucilaginosa's* ability to produce terpenoids such as α-terpineol, α-farnesene and β-ionone was investigated through heterologous expression of different terpenoid biosynthesis genes. This is a first study that focuses on engineering the MVA pathway along with terpenoid enzymes in *R. mucilaginosa*.

2. Materials and methods

2.1. Strains, media, and culture conditions

The details of all the strains used in this study are given in Table S1. *R. mucilaginosa* JY1105 (CGMCC 2.6826) was used as parent strain for genetic engineering. *R. mucilaginosa* was cultured in YPD medium (20 g/L glucose, 10 g/L yeast extract and 20 g/L peptone) at 30 °C and 200 rpm. *Escherichia coli* DH10B (or Fast-T1) and *Agrobacterium tumefaciens* AGL1 were cultivated in Luria-Bertani medium (10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract) at 37 °C and 30 °C, respectively. Kanamycin (50 μg/mL) was added to LB medium when necessary. Antibiotics were used at the following concentrations: hygromycin of 50 μg/mL, nourseothricin of 50 μg/mL and cefotaxime of 300 μg/mL. For shake-flask culturing, the engineered strains were cultured in YTD_{50} medium (25 g/L tryptone, 10 g/L yeast extract and 50 g/L glucose, pH 6.0).

2.2. Construction of plasmids and strains

Plasmids used in this study are shown in Table S2. The α-terpineol synthase gene (*αTS,*GenBank: AAS79352.1) from *Vitis vinifera*, the α-farnesene synthase gene (*FS*, GenBank: NM_001293893) from *Malus domestica*, and the β-ionone synthase gene (*CCD1*, GenBank: AAT68189.1) from *Petunia hybrida* were subjected to codon optimization according to the codon preference of the closely related red yeast *Rhodosporidium toruloides* [22] and synthesized by SynBio Technologies (Suzhou, China). These optimized synthetic genes have been deposited into the database of NCBI (*αTS,* Accession Number OQ983656; *FS1*, Accession Number PP092216; *FS2*, Accession Number PP092217; *CCD1*, Accession Number PP092218). DNA sequences of codon-optimized terpene synthases were shown in Table S4. The truncated 3-hydroxy-3--methylglutaryl-CoA reductase gene (*tHMG1*) was amplified from the plasmid p424-tHMG1 [23]. *ERG20* (GenBank: CP046090) was amplified from the *Saccharomyces cerevisiae* genome and then mutated to $ERG20^{ww}$ (F96 W/N127W). DNA fragments were amplified by polymerase chain reaction (PCR) using PrimeSTAR Max DNA Polymerase (Takara). All plasmids were constructed by restriction-free cloning method [24]. The genotypes of all transformed strains were confirmed by colony PCR, followed by sequencing. All primers used in this study were listed in Table S3 (Synbio Technologies, Suzhou, China). Sangon Biothech Co. Ltd. (Shanghai, China) provided the DNA gel purification and plasmid extraction kits. The genes of interest were transformed into *R. mucilaginosa* using the *Agrobacterium tumefaciens*-mediated transformation method, according to a previously reported protocol [25].

2.3. Preliminary screening and shake-flask culturing

For preliminary screening, pure transformed colonies were inoculated into 24-well plates with 3 mL of YPD medium and cultivated for 24 h to prepare seed cultures. 2 mL of seed cultures were inoculated into a 6-well deep-hole plate with 18 mL of YTD_{50} medium and cultivated at 30 °C, 200 rpm for 96 h.

For shake-flask culturing, a single transformed colony was inoculated into 20 mL of YPD medium and cultured in 100-mL shake flask for 24 h to prepare the seed culture. Then, 5 mL of the seed culture was inoculated into 45 mL of YTD_{50} medium in 250-mL shake flask, and cultured at 30 °C and 200 rpm until the total glucose was used in fermentation media.

2.4. Western blot analysis

Cells were taken from 2 mL of overnight culture of each transformant in YPD medium, then the cell mixture was broken using high-speed shaking at 4.0 m/s for 45 s, and the process was repeated 5 times with 3-min intervals of an ice bath between each round of shaking. The supernatants were recovered by centrifuging the suspensions at 12,000 g for 3 min. The total proteins were separated using SDS-PAGE with 12 % polyacrylamide gels and transferred to the BioTrace™ NT nitrocellulose membrane (Pall, Beijing, China). P2A antibody was used as the primary antibody to probe the antigens, and secondary goat anti-mouse IgG (H + L) that was HRP-labeled was used. Finally, a DAB horseradish peroxidase color development kit was used to visualize the blotting results [26].

2.5. Terpene quantification

The concentration of terpene was identified by GC-MS system (Agilent Technologies 7890 A GC equipped with a 5975C insert 143 XL EI/CI MSD Detector) with a DB-WAX column (30 m × 0.32 mm × 0.25 μm) (Fig. S2).

For α-terpineol quantification, 2 mL of fermented broth was taken, and the same amount of hexane was added to it. The organic solvent and medium mixture were rotated at 200 rpm for 2 h at 25 °C, followed by 5 min of centrifugation at 4000 rpm. 500 μL of the hexane layer were transferred to a darkened glass vial for examination after the extraction. The amount of α-terpineol was determined by linear calibration curves. The standard α-terpineol was purchased from Solarbio (China, GC ≥ 99.0 %). For GC analysis, 1 μL of sample was injected, with a split ratio of 10:1, and nitrogen was used as the carrier gas with a flow rate of 1 mL/min. The injector and the detector temperatures were maintained at 250 °C and 260 °C, respectively. The oven temperature was as follows: 80 °C for 1 min and sequentially increased at the rate of 10 °C/min to 180 °C and 10 °C/min to 250 °C.

For α-farnesene quantification, 10 % (v/v) dodecane overlay was added to medium to capture α-farnesene before culturing. The supernatant of the fermentation broth was centrifuged at 4000 rpm for 5 min, and the dodecane layer was taken for detection. The amount of α-farnesene was determined by linear calibration curves. The standard α-farnesene was purchased from Toronto Research Chemicals (Canada, GC ≥ 99.0 %). The temperatures of the injector and the detector were set at 280 °C and 290 °C, respectively. The oven temperature was kept at 80 °C for 1 min, and then ramped to 250 °C at 10 °C/min and held for 1 min, then to 280 °C at 10 °C/min and held for 2 min.

For β-ionone quantification, 10 % (v/v) dodecane overlay was added to medium to capture β-ionone before culturing. The supernatant of the fermentation broth was centrifuged at 4000 rpm for 5 min, and the dodecane layer was taken for detection. The organic phase was carefully pipetted from the culture sample and centrifuged for 5 min at 12,000 rpm. The amount of β-ionone was determined by linear calibration curves. The standard β-ionone was purchased from Shanghai Yuanye Bio-Technology Co. Ltd. (China, GC ≥ 97.0 %). The temperatures of the

injector and the detector were set at 250 °C and 300 °C, respectively. The oven program was set as follows: start at 45 °C for 1 min, then raise the temperature up 8 °C/min to 300 °C and keep it constant for 5 min.

2.6. Analysis of glucose and OD_{600}

The culture samples were collected directly to determine glucose concentration and cell density. Glucose was measured by Bioanalyzer (SBA-40D, Shandong Academy of Sciences, China) following the manufacturer's instructions. Optical density (OD) was measured at 600 nm with a spectrophotometer (Evolution 220, Thermo Fisher Scientific, USA).

2.7. Dry cell weight measurement, lipid and carotenoid quantification

Dry cell weight quantification was conducted in triplicate. Samples of 5 mL taken from the cultivated medium were centrifuged for 5 min at 5500 g at room temperature. The cell pellets were washed twice with distilled water and dried at 105 °C for 24 h to a consistent weight.

Lipid concentration was determined from the wet cell pellets obtained after washing with distilled water using a sulfo-phospho-vanillin colorimetric assay [27]. Absorbance was measured with a spectrophotometer (Evolution 220, Thermo Fisher Scientific, USA) at 530 nm. A calibration curve ranging between 0 and 2.5 mg oil/mL was set using soybean oil dissolved in chloroform as the standard.

For carotenoid quantification, take 4 mL of culture solution, centrifuge at 3500 rpm for 15 min to obtain wet cells, add 6 mL of 3 mol/L HCl solution, oscillate and soak for 1 h at room temperature, boil in boiling water for 4 min, cool rapidly, centrifuge at 3500 rpm for 15 min, discard the supernatant, and precipitate with purified water. After 2–3 times, 5 mL of acetone was added, shaking at room temperature to extract carotenoids, and then centrifugation at 3500 rpm for 15 min. The resulting supernatant was the extract of carotenoids, which was repeated three times to ensure the complete extraction of pigments. Following the appropriate dilution, the absorbance at 475 nm was measured, and the carotenoid yield was calculated as follows: Carotenoids yield (g/mL) = $A \times D \times V/0.16 \times W$.

Where:

A: absorbance of the maximum wave strength of carotenoids; D: dilution ratio of pigment extract; V: total volume of solvent used for extraction (mL); W: volume of liquid (mL) used for extraction; 0.16: molar extinction coefficient of carotene.

3. Results

3.1. Engineering R. mucilaginosa for the synthesis of various terpenoids

The red yeast *R. mucilaginosa* has been widely studied due to its

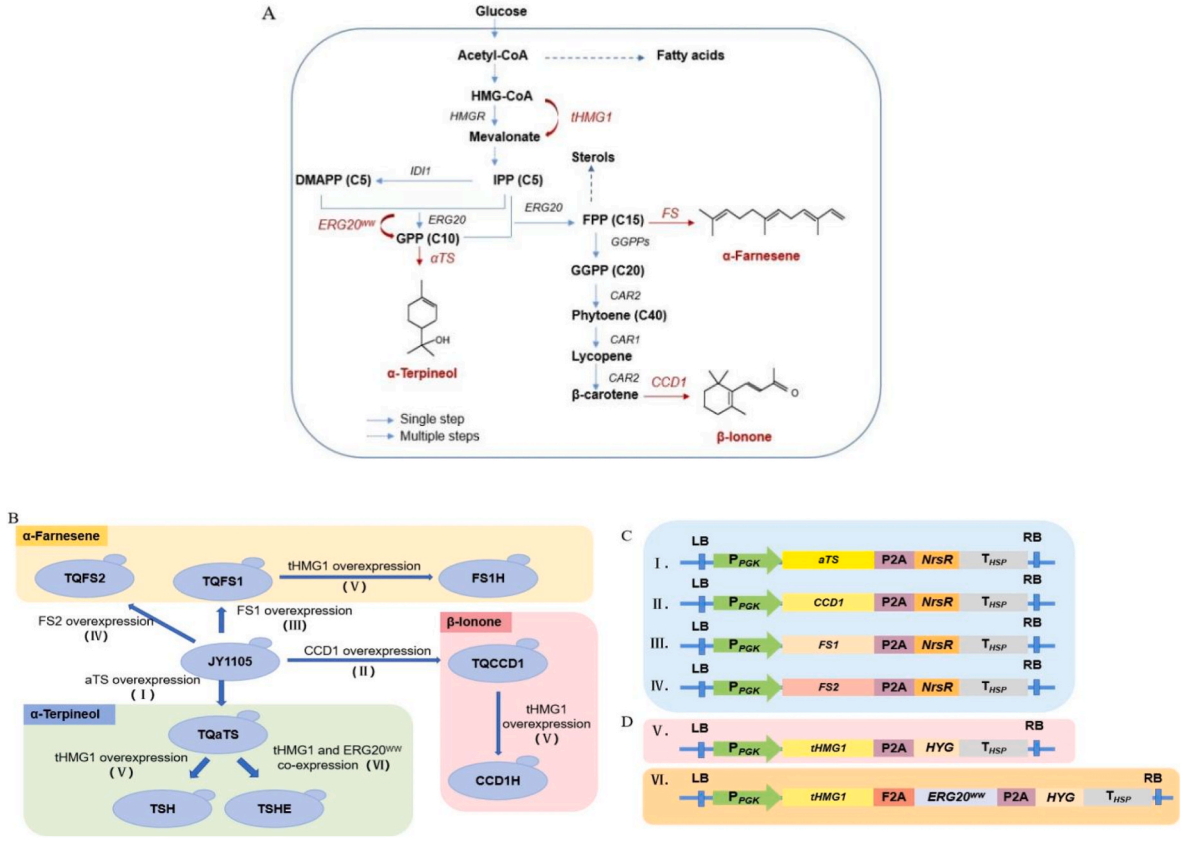

Fig. 1. Engineering strategy of various terpenes' biosynthesis in *Rhodotorula mucilaginosa* JY1105. (**A**) Schematic diagram of the mevalonate (MVA) pathway for terpene biosynthesis in *R. mucilaginosa* JY1105. HMG-CoA, 3-hydroxy-3-methylglutharyl-CoA; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; *HMG1*, 3-hydroxy-3-methylglutaryl-CoA reductase gene; *tHMG1*, the truncated *HMG1* gene; *IDI1*, isoprene diphosphate isomerase gene; *ERG20*, bifunctional FPP synthetase gene; *ERG20^{ww}*, *ERG20* mutant (F96 W/N127W) gene; *CAR2*, phytoene synthase/lycopene cyclase gene; *CAR1*, phytoene dehydrogenase gene; *αTS*, α-terpineol synthetase gene; *CCD1*, β-ionone synthetase gene; *FS*, α-farnesene synthetase gene. The blue arrows are the endogenous pathway in *R. mucilaginosa*. The red italic font indicates the expression of heterologous genes. Red bold font indicates the final products. (**B**) The derived relationships between wild-type and engineered strains. According to the preliminary screening results, TQaTS-3 was the starting strain of TSH strains and TSHE strains, and the starting strains of FSH strains and CCD1H strains were TQFS1-8 and TQCCD1-3, respectively. (**C**) Binary vectors (I-IV) containing the terpene synthase genes designed for genome integration in *R. mucilaginosa*. (**D**) Binary vectors (V and VI) to increase the precursor supply.

natural capacity for carotenoid and lipid production, indicating its strong endogenous isoprene pathway with readily available metabolic intermediates. Albeit draft genomes of *R. mucilaginosa* JY1105 (Bioproject: PRJNA855278) and a few *R. mucilaginosa* strains have been released, a reliable annotation remains to be documented. According to the gene annotation involved in the MVA pathway of *R. mucilaginosa* C2.5t1 [19], this study gave the possible MVA pathway of *R. mucilaginosa* JY1105 (Fig. 1A). Based on this hypothesis, three target products (α-terpineol, α-farnesene and β-ionone), which represented monoterpene, sesquiterpene and C13-norisoprenoid, were chosen to reveal the potential of heterologous terpenoids production by *R. mucilaginosa*.

α-Terpineol is a type of monoterpene compound that plays an important role in enhancing the flavor and aroma of grapes and wine [3]. α-Terpineol synthase (aTs) is an enzyme found in grapes (*Vitis vinifera*) that facilitates the conversion of GPP into α-terpineol. To produce α-terpineol, the optimized *aTS* gene was used to construct the desired plasmid pZPK-aTS-N (Fig. 1C). α-Farnesene is a member of sesquiterpene, which is highly valuable in fields such as medicine, cosmetics and bioenergy [2]. α-Farnesene synthase (Fs) from *M. domestica* catalyzes the conversion of FPP to α-farnesene. The gDNA and the cDNA of *FS* gene were both used to evaluate the ability of Fs to synthesize α-farnesene in *R. mucilaginosa*. The gDNA (named *FS1*) and the cDNA (named *FS2*) sequences were optimized to construct expression cassettes pZPK-FS1-N and pZPK-FS2-N, respectively (Fig. 1C). β-Ionone also has important pharmacological activities and has been wildly used in anti-inflammatory, cancer-preventing, antibacterial, antifungal and antileishmanial [28]. β-Ionone is naturally synthesized from the specific breakdown of β-carotene. This process is facilitated by carotenoid cleavage dioxygenase 1 (Ccd1), which catalyzes the cleavage of β-carotenoids at the 9,10 and 9′,10′ positions in the presence of oxygen. To produce β-ionone, the optimized *CCD1* gene was used to construct the expression cassette pZPK-CCD1-N (Fig. 1C).

The engineered strains coded as TQaTS, TQFS1, TQFS2, and TQCCD1 were obtained by transferring the selected terpenoid genes from the respective constructed binary plasmids pZPK-aTS-N, pZPK-FS1-N, pZPK-FS2-N, and pZPK-CCD1-N to *R. mucilaginosa* JY1105 by ATMT method (Fig. 1B). Since this method causes the random integration of heterologous DNA into the genome via non-homologous end-joining (NHEJ), multiple transformants were selected and identified by colony PCR to confirm the successful integration of exogenous genes into the genome (Fig. S1A). The selected transformants for each genotype were used for further screening.

When synthesizing terpenoids, YTD medium (peptone replaced by tryptone to speed up the fermentation process) with a high glucose concentration is commonly used for culturing [29]. Thus, YTD_{50} as production medium was used for culturing engineered strains. Dodecane was normally used as an extraction agent to capture terpene products *in situ*. However, the GC-MS equipment was unable to discriminate between α-terpineol and dodecane due to their total retention time overlap. In this experiment, strains JY1105, TQFS1, TQFS2, and TQCCD1 were cultured in YTD_{50} medium with a dodecane overlay. On the other hand, the TQaTS strains were grown in YTD_{50} medium without dodecane, and hexane was used to extract α-terpineol at the end of the culturing process. From the preliminary screening results, α-terpineol was detected in two transformants coded as TQaTS-3 and TQaTS-4, while α-farnesene and β-ionone were detected in all of the strains (Fig. 2). As all these products are not observed in the wild-type *R. mucilaginosa* JY1105 (data were not shown), this indicates that *R. mucilaginosa* has the potential to divert its natural MVA route to produce other plant-derived terpenoids. According to these results, three strains with the highest production of α-terpineol, α-farnesene and β-ionone, coded as TQaTS-3, TQCCD1-3 and TQFS1-8, respectively, were selected for further engineering.

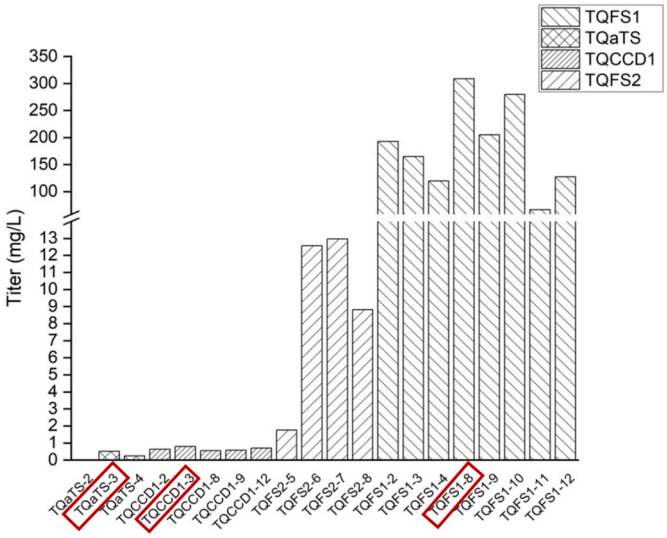

Fig. 2. Screening of the engineered strains. Titers of products in various transformants (the α-terpineol synthesizing strains coded as TQaTS, the α-farnesene synthesizing strains coded as TQFS1 and TQFS2, and the β-ionone synthesizing strains coded as TQCCD1) were detected by GC-MS after 96 h of culturing. Red boxes indicate the selected strains.

3.2. Culturing performance of engineered strains

For α-farnesene and β-ionone extraction, selected strains coded as TQFS1-8 and TQCCD1-3 were cultivated by shake flask method in 50 mL of YTD_{50} medium supplemented with dodecane overlay. The samples were collected after every 24 h, centrifuged, and the supernatant dodecane layer was used for compound detection. Another engineered strain coded as TQaTS-3, selected for α-terpineol production was cultivated in 50 mL of the YTD_{50} medium and extracted with hexane after sampling (as described in material and methods). Although the strain TQaTS-3 exhibited slower growth and glucose consumption as compared to other strains (Fig. 3A and B), its cell dry weight was similar to theirs (Fig. 3D). The final concentrations of α-terpineol, α-farnesene and β-ionone were 0.24 mg/L, 522.5 mg/L and 0.87 mg/L, respectively (Fig. 3C).

Carotenoids are considered as the end products of the MVA pathway in *R. mucilaginosa*. To verify the impact of heterologous terpenoid synthesis on carotenoid content in engineered strains, the carotenoids were extracted and quantified. Compared to the JY1105 strain, the levels of carotenoids in TQaTS-3 and TQFS1-8 showed a smaller drop, but no significant variations were detected (Fig. 3D). These results indicate an adequate supply of precursors for the synthesis of carotenoids, with only a minor fraction of these precursors being utilized for the synthesis of α-terpineol and α-farnesene. On the other hand, the integration of the *CCD1* gene (TQCCD1-3) changed the cell color from orange (JY1105 strain) to pale yellow (Fig. S3). Different from the TQaTS-3 and TQFS1-8 strains, the TQCCD1-3 strain accumulates minimal carotenoids (27.2 ± 4.2 μg/g dry weight), suggesting that the recombinant β-ionone synthetase Ccd1 might be active enough to consume carotenes. Lipid production profiles were also measured to verify if terpenoid synthesis had any impact on lipid accumulation. Results showed that lipid production was unaffected in all of the engineered strains (Fig. 3D), suggesting an unpreferred metabolic flow for the MVA pathway.

3.3. Enhancement of precursor supply to promote the terpene production

Enzymes involved in terpenoids biosynthesis are highly regulated at both the transcriptional and post-translational levels to ensure a balanced metabolite flux. The 3-hydroxy-3-methylglutaryl-CoA reductase (Hmg1), a rate-limiting enzyme in the MVA pathway, catalyzes the

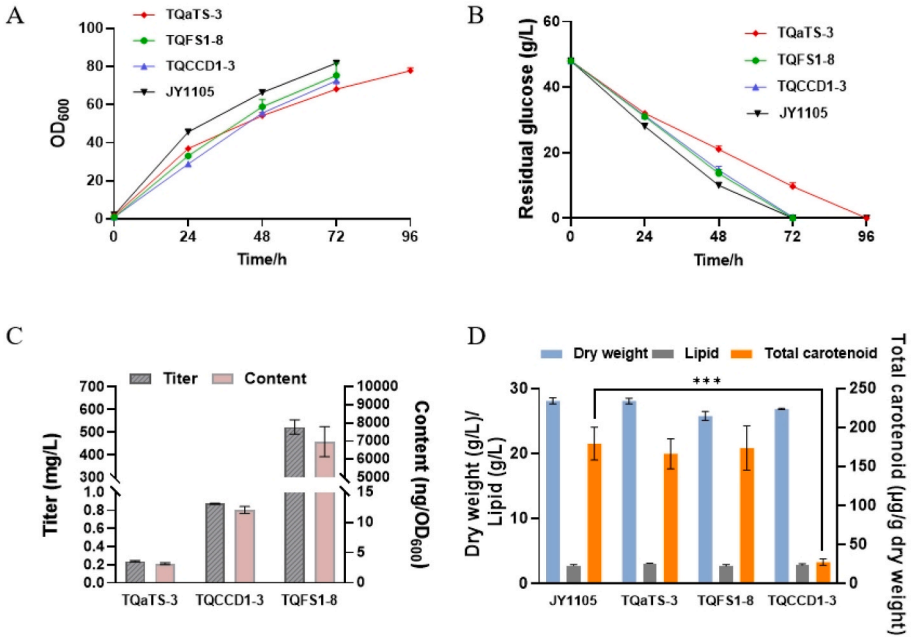

Fig. 3. Performance of engineered strains. (**A**) Cell growth; (**B**) Glucose consumption; (**C**) Product accumulation at the culturing endpoint; (**D**) Dry weight of biomass, lipid and total carotenoids at the culturing endpoint. Experiments were performed in triplicate. Error bars represent the standard deviation of three independent experiments. Where not visible, bars lie within the symbols. Significantly different values were performed by independent-samples T test. *** means $p < 0.001$.

conversion of HMG-CoA to MVA [30,31]. Early studies [23,32] showed that a truncated version of Hmg1 (tHmg1) could increase the production of isoprenoids and thus increase the production of terpenes. Deletion of its *N*-terminus transmembrane domain relieved the feedback inhibition at high levels of sterol. The overexpression of the *tHMG1* gene resulted in a 50 % increase in taxadiene in a recombinant *S. cerevisiae* strain [33]. Therefore, in this study, the *tHMG1* gene from *S. cerevisiae* was used to construct the expression cassette pZPK-tHMG1 to increase the supply of MVA. The selected gene *tHMG1* from binary plasmid pZPK-tHMG1 was transferred and integrated into the genome of engineered strains coded as TQaTS-3, TQFS1-8 and TQCCD1-3 through the ATMT method, achieving TSH strains, FS1H strains and CCD1H strains, respectively (Fig. 1B and D). Besides, GPP, as the intermediate product of FPP synthesis, is often rapidly consumed. The native FPP synthase (Erg20), possessing both GPP and FPP synthase activities, released few GPP from its catalytic site [34]. It has been reported that the Erg20 mutation ($Erg20^{F96W\_N127W}$) preferred GPP synthesis over FPP, thus achieving a significant increase in monoterpene titers in *S. cerevisiae* [35–37]. Therefore, the plasmid pZPK-tHMG1-$ERG20^{WW}$ was constructed and used to integrate selected *tHMG1* and $ERG20^{WW}$ genes into the genome of strain TQaTS-3 by the ATMT method to construct the TSHE strains (Fig. 1B and D). For gene identification, the colony PCR analysis clearly indicated the presence of *tHMG1* or $ERG20^{WW}$ genes for selected transformants (Fig. S1B). The protein expression was further verified by Western blot analysis. Bands of terpene synthase and tHmg1 proteins were detected at their respective protein sizes except of $Erg20^{WW}$ (Fig. 4A).

Initially, many transformants were picked and evaluated for their growth and terpenoid content. Among the TSH strains, 2 strains coded as TSH2 and TSH3 were found with higher α-terpineol titer (Fig. 4C). Similarly, among the series of strains co-expressing *tHMG1* and $ERG20^{WW}$, two strains coded as TSHE2 and TSHE3 were selected for

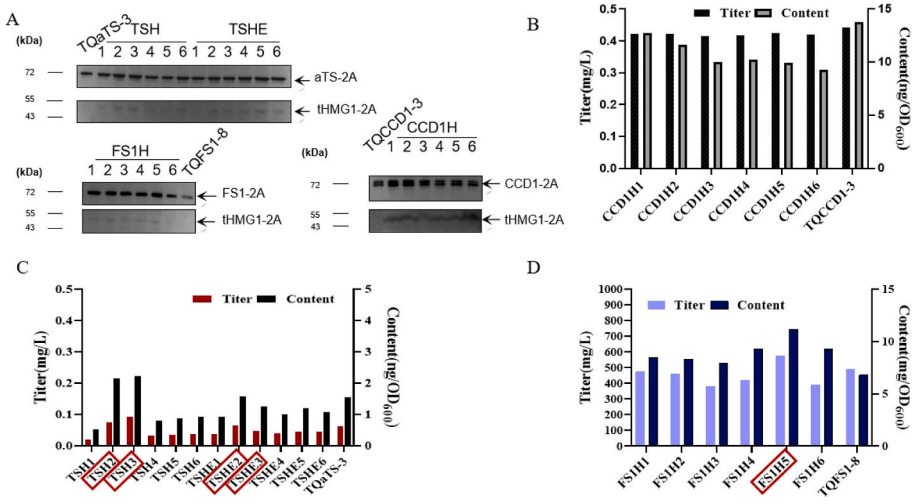

Fig. 4. Screening of the engineered strains with enhancement of precursor supply. (**A**) Western blot analysis, **(B)** the titer and the content of α-terpineol, (**C**) the titer and the content of α-farnesene and (**D**) the titer and the content of β-ionone. Red boxes indicate the selected strains.

further investigation. Then the performance of the above strains was detected again by shake-flask culturing. In comparison to the control strain TQaTS-3, strains TSH2 and TSH3 showed a small increment in α-terpineol titer without significant difference, while strains TSHE2 and TSHE3 exhibited a 50.8 % and a 62.5 % increase in this product, respectively (Fig. 5A). The titer of α-terpineol was quantified to be 0.39 mg/L in TSHE3. The results suggested that enhancing metabolic flux from MVA to GPP could effectively increase the production of α-terpineol.

The α-farnesene content of FS1H strains were increased to varying degrees compared with the control strain TQFS1-8 (Fig. 4D), and FS1H5 displayed the highest content and titer. The α-farnesene titer in strain FS1H5 showed a 1.3-fold increase compared to the control strain TQFS1-8, reaching to 822 mg/L (Fig. 5B and S2B). These observations were in accordance with earlier studies and suggest that the limiting factor in increasing terpenoid titers lies partly in insufficient precursor availability. Inconsistent with our expectations, there was no further improvement observed in the production of β-ionone in the CCD1H strain (Fig. 4B).

4. Discussion

Yeasts have gained significant attention for their biotechnological applications, as they also offer a versatile and efficient platform for facilitating terpenoid synthesis. *R. mucilaginosa* is regarded as a natural producer of carotenoids, including β-carotene, torulene and torularhodin. Terpenoids are not often synthesized by yeast. However, when plant-derived terpene synthases were introduced into yeast cells, it was observed that these enzymes could utilize natural precursors of the isoprenoid pathway and synthesize various terpenoids [32,38]. This study used metabolic engineering approach to produce a variety of terpenes, including monoterpene α-terpineol, sesquiterpene α-farnesene and β-ionone in *R. mucilaginosa*. Genes encoding terpene synthetase and MVA pathway rate-limiting enzymes were integrated into *R. mucilaginosa* genome to produce and increase terpene synthesis. The results obtained contribute to improving the understanding of the versatile metabolism of *R. mucilaginosa*.

It was found that engineered strain TQaTS-3 (expressing the α-terpineol synthase from *V. vinifera*) produced α-terpineol lower than the engineered *S. cerevisiae* strain [39]. The relative low production of α-terpineol was probably due to a shortage of precursors. Thus, co-expression of *tHMG1* and $ERG20^{WW}$ in TQaTS-3 resulted in an increase in α-terpineol production. Based on the data, it appears that the requirement for GPP is of more significance in the process of synthesizing monoterpenes in comparison to the requirement for mevalonic acid to be present. However, Western blot failed to show proper expression of $Erg20^{WW}$, but the TSHE strains produced more α-terpineol than the TSH strains (Fig. 5A). It implies that $Erg20^{WW}$ is expressed at low levels. In order to effectively tackle the problem of insufficient protein production, it is necessary to explore additional optimization options, such as the utilization of more potent promoters or the implementation of codon optimization techniques for the $ERG20^{WW}$ gene.

In recent times, farnesene has been produced through heterologous gene expression by various hosts, such as *S. cerevisiae* and *Y. lipolytica* [40,41]. In this study, the expression of α-farnesene synthase gene achieved the α-farnesene titer of 0.52 g/L in *R. mucilaginosa* (the TQFS1-8 strain), which was four times higher than that in *Y. lipolytica* [2]. Furthermore, we also examined the effect of *tHMG1* overexpression on α-farnesene biosynthesis in engineered *R. mucilaginosa*. As expected, overexpression of the *tHMG1* gene significantly enhanced the conversion of cytoplasmic acetyl-CoA to α-farnesene. These results suggested that *R. mucilaginosa* has the great potential for α-farnesene synthesis**.** It is noteworthy that the production titers of TQFS1 strains (expressing the gDNA version of the *FS* gene) consistently exceeded those of TQFS2 strains (expressing the cDNA version of the *FS* gene). This disparity could potentially be attributed to higher gene expression levels due to the influence of introns on mRNA stability or the presence of introns facilitates mRNA export from the nucleus to the cytoplasm [42,43].

The ionone family contains some of the most intriguing apocarotenoids. The cleavage of the C40-compound β-carotene produces β-ionone, which is highly regarded for its woody-violet aroma [44]. In accordance with the findings of this work, a significant majority of the carotenoids in *R. mucilaginosa* are converted upon expression of the *CCD1* gene (Fig. 3D and Fig. S5). The Ccd1 enzyme in carotenoid-accumulating microorganisms can produce several compounds (like geranylacetone, pseudoionone and β-ionone) using multiple carotenoids (e.g., ζ-carotene, γ-carotene, lycopene and β-carotene) as substrates [45]. Additionally, genome-scale stoichiometric analysis identified the unspecific cleavage activity of *PhCCD1* as responsible for the observed reduction in β-ionone yields [46]. Therefore, the amount of carotenoids consumed by the microorganism does not correspond to the amount of β-ionone produced. In the engineered *Y. lipolytica* strain, the rise of carotenoids synthesis led to an increase in β-ionone production [47]. However, it is noteworthy to mention that the overexpression of *tHMG1* did not lead to increased β-ionone production in *R. mucilaginosa*. According to the above analysis, the possible reason may be the poor activity or the unspecific cleavage activity of Ccd1 rather than the low MVA pathway flux or carotene synthesis. To tackle these limitations, the next step can focus on site-directed mutagenesis to improve the selectivity and activity of Ccd1, thereby avoiding unwanted by-products.

While observing the growth of all engineered strains, it was found that TQaTS-3 exhibited slower cell mass accumulation and glucose consumption (Fig. 3A and B). Due to the similar growth rates exhibited by the above strains (Fig. S4), the presence of dodecane might be the main reason for this phenomenon. Experiments were carried out to verity this hypothesis. As shown in Fig. S5, in the presence of 10 % (v/v) of dodecane, the engineered strains displayed similar cell growth patterns compared to the control strain JY1105. It showed the same result when cultured these strains without dodecane. Moreover, the culturing process was shortened with the dodecane addition. These results were

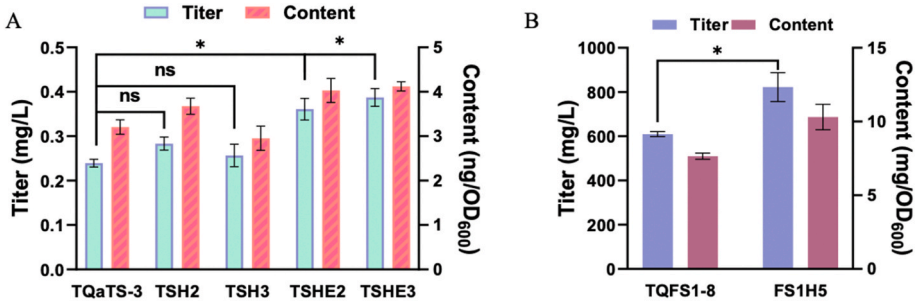

Fig. 5. Culturing performance of the engineered strains with enhancement of precursor supply. (**A**) α-Terpineol accumulation at the culturing endpointand; (**B**) α-Farnesene accumulation at the culturing endpoint. Significantly different values were performed by independent-samples T test. * and ns mean $p < 0.05$ and no significant difference, respectively. Experiments were performed in triplicate.

consistent with our suppose. During microbial culturing, oxygen mass transfer would be an important factor affecting cell growth, and dodecane was reported to be an oxygen-vector, with around 15-fold higher oxygen solubilization capacity than water [48–50]. It also mentioned that dodecane has shown fewer air bubbles and less shear stress to protect cells. Thus, the addition of dodecane may protect the cells and promote the ability of oxygen mass transfer to shorten the culturing process.

Normally, isoprenoid biosynthesis competes with lipid biosynthesis for the substrate acetyl-CoA in microorganisms. *R. mucilaginosa* is an oleaginous yeast that possesses the capability to accumulate lipids over 20 % of its cell dry weight [51,52]. Results showed that lipid production was unaffected in all of the engineered strains (Fig. 3D), suggesting a potential of downregulating lipid synthetic pathway to increase the precursor supply for isoprenoid synthesis in *R. mucilaginosa.* Meanwhile, considering the benefit of lipid droplets for storing hydrophobic compounds, it is necessary to balance the biosynthesis of lipid and isoprenoid [53–55].

In conclusion, our results demonstrated that *R. mucilaginosa* exhibits promising potential as a viable host to produce terpenes. While the construction of advanced cell factories in *R. mucilaginosa* encounters difficulties mostly attributed to less annotated genome and ineffective genetic tools, with the rapid development of genome sequencing technologies, CRISPR-Cas9 gene editing platforms and synthetic biology components, the applications of this yeast are going to be broader in the near future.

CRediT authorship contribution statement

Qiongqiong Chen: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Liting Lyu:** Conceptualization, Investigation, Writing – review & editing, Project administration. **Haizhao Xue:** Resources, Writing – review & editing. **Aabid Manzoor Shah:** Writing – review & editing. **Zongbao Kent Zhao:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.04.015.

References

[1] Zhao L, Zhu YH, Jia HY, Han YG, Zheng XK, Wang M, et al. From plant to yeast-advances in biosynthesis of artemisinin. Molecules 2022;27:6888.
[2] Liu YH, Jiang X, Cui ZY, Wang ZX, Qi QS, Hou J. Engineering the oleaginous yeast *Yarrowia lipolytica* for production of α-farnesene. Biotechnol Biofuels 2019;12:296.
[3] Martin DM, Bohlmann J. Identification of Vitis vinifera (-)-alpha-terpineol synthase by in silico screening of full-length cDNA ESTs and functional characterization of recombinant terpene synthase. Phytochemistry 2004;65:1223–9.
[4] Jing YW, Wang YX, Zhou DW, Wang JN, Li JW, Sun JX, et al. Advances in the synthesis of three typical tetraterpenoids including β-carotene, lycopene and astaxanthin. Biotechnol Adv 2022;61:108033.
[5] Jiang H, Wang X. Biosynthesis of monoterpenoid and sesquiterpenoid as natural flavors and fragrances. Biotechnol Adv 2023;65:108151.
[6] Li WJ, Cui LW, Mai J, Shi TQ, Lin L, Zhang ZG, et al. Advances in metabolic engineering paving the way for the efficient of terpenes in yeasts. J Agric Food Chem 2022;70:9246–61.
[7] Yang LM, Liu H, Jin YH, Liu JF, Deng L, Wang F. Recent advances in multiple strategies for the synthesis of terpenes by engineered yeast. Fermentation 2022;8:615.
[8] Park D, Swayambhu G, Lyga T, Pfeifer BA. Complex natural product production methods and options. Synth Syst Biotechnol 2021;6:1–11.
[9] Vranová E, Coman D, Gruissem W. Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu Rev Plant Biol 2013;64:665–700.
[10] Yang CGX, Jiang Y, Sun BB, Gao F, Yang S. Synergy between methylerythritol phosphate pathway and mevalonate pathway for isoprene production in *Escherichia coli*. Metab Eng 2016;37:79–91.
[11] Liao P, Hemmerlin A, Bach TJ, Chye ML. The potential of the mevalonate pathway for enhanced isoprenoid production. Biotechnol Adv 2016;34:697–713.
[12] Zhao L, Chang WC, Xiao Y, Liu HW, Liu P. Methylerythritol phosphate pathway of isoprenoid biosynthesis. Annu Rev Biochem 2013;82:497–530.
[13] Leyton A, Flores L, Mäki-Arvela P, Lienqueo ME, Shene C. Macrocystis pyrifera source of nutrients for the production of carotenoids by a marine yeast *Rhodotorula mucilaginosa*. J Appl Microbiol 2019;127:1069–79.
[14] Li HF, Huang LF, Zhang Y, Yan Y. Production, characterization and immunomodulatory activity of an extracellular polysaccharide from *Rhodotorula mucilaginosa* YL-1 isolated from sea salt field. Mar Drugs 2020;18:595.
[15] Benmessaoud S, Anissi J, Kara M, Assouguem A, AL Huqail AA, Germoush MO, et al. Isolation and characterization of three new crude oil degrading yeast strains, *Candida parapsilosis* SK1, *Rhodotorula mucilaginosa* SK2 and SK3. Sustainability 2022;14:3465.
[16] Lario LD, Chaud L, Almeida MD, Converti A, Sette LD, Pessoa A. Production, purification, and characterization of an extracellular acid protease from the marine antarctic yeast *Rhodotorula mucilaginosa* L7. Fungal Biol 2015;119:1129–36.
[17] Kot AM, Błażejak S, Kieliszek M, Gientka I, Bryś J. Simultaneous production of lipids and carotenoids by the red yeast *Rhodotorula* from waste glycerol fraction and potato wastewater. Appl Biochem Biotechnol 2019;189:589–607.
[18] Costa WA, Padilha CEA, Oliveira Júnior SD, Silva FLH, Silva J, Ancântara MA, et al. Oil-lipids, carotenoids and fatty acids simultaneous production by *Rhodotorula mucilaginosa* CCT3892 using sugarcane molasses as carbon source. Braz J Food Technol 2020;23:e2019064.
[19] Landolfo S, Ianiri G, Camiolo S, Porceddu A, Mulas G, Chessa R, et al. CAR gene cluster and transcript levels of carotenogenic genes in *Rhodotorula mucilaginosa*. Microbiology 2018;164:78–87.
[20] Li ZH, Li CJ, Cheng P, Yu GH. *Rhodotorula mucilaginosa*-alternative sources of natural carotenoids, lipids, and enzymes for industrial use. Heliyon 2022;8:e11505.
[21] Zhuang RY, Wang RC, Qiu XT, Che JH, Li Y, Zhang WX, et al. Bioactive substances produced by *Rhodotorula mucilaginosa*: a comprehensive review. Food Sci (N Y) 2020;41:318–29.
[22] Zhang Q, Jiao X, Liu XJ, Zhang Y, Zhang SF, Zhao ZB. Analysis of codon usage in *Rhodosporidium toruloides*. Mygosystema 2018;37:1454–65.
[23] Zhou YJ, Gao W, Rong Q, Jin G, Chu H, Liu W, et al. Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. J Am Chem Soc 2012;134:3234–41.
[24] van den Ent F, Lowe J. RF cloning: a restriction-free method for inserting target genes into plasmids. J Biochem Biophys Methods 2006;67:67–74.
[25] Sun W, Zhang S, Lin X, Luan Y. Application of a transformation in *Rhodotorula mucilaginosa* genetic engineering. China Biotechnol 2016;36:81–6.
[26] Lin X, Wang Y, Zhang S, Zhu Z, Zhou YJ, Yang F, et al. Functional integration of multiple genes into the genome of the oleaginous yeast *Rhodosporidium toruloides*. FEMS Yeast Res 2014;14:547–55.
[27] Izard J, Limberger RJ. Rapid screening method for quantitation of bacterial cell lipids from whole cells. J Microbiol Methods 2003;55:411–8.
[28] Simkin AJ, Underwood BA, Auldridge M, Loucas HM, Shibuya K, Schmelz E, et al. Circadian regulation of the PhCCD1 carotenoid cleavage dioxygenase controls emission of β-ionone, a fragrance volatile of petunia flowers. Plant Physiol 2004;136:3504–14.
[29] Chen G, Fan KW, Lu FP, Li Q, Aki T, Chen F, et al. Optimization of nitrogen source for enhanced production of squalene from thraustochytrid *Aurantiochytrium sp*. Nat Biotechnol 2010;27:382–9.
[30] Rodwell VW, Nordstrom JL, Mitschelen JJ. Regulation of HMG-CoA reductase. Adv Lipid Res 1976;14:1–74.
[31] Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990;343:425–30.
[32] Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 2006;440:940–3.
[33] Engels B, Dahm P, Jennewein S. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab Eng 2008;10:201–6.
[34] Fischer MJC, Meyer S, Claudel P, Bergdoll M, Karst F. Metabolic engineering of monoterpene synthesis in yeast. Biotechnol Bioeng 2011;108:1883–92.
[35] 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.
[36] Zhao J, Bao X, Li C, Shen Y, Hou J. Improving monoterpene geraniol production through geranyl diphosphate synthesis regulation in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 2016;100:4561–71.

[37] Jiang G, Yao M, Wang Y, Xiao W, Yuan YA. "push-pull-restrain" strategy to improve citronellol production in *Saccharomyces cerevisiae*. Metab Eng 2021;66:51–9.
[38] Li C, Qi Y, Sun Z, Jiang M, Li C. Way to efficient microbial paclitaxel mass production. Synth Syst Biotechnol 2023;8:673–81.
[39] Zhang C, Li M, Zhao GR, Lu W. Alpha-Terpineol production from an engineered *Saccharomyces cerevisiae* cell factory. Microb Cell Factories 2019;18:160.
[40] Shi T, Li Y, Zhu L, Tong Y, Yang J, Fang Y, et al. Engineering the oleaginous yeast *Yarrowia lipolytica* for β-farnesene overproduction. Biotechnol J 2021;16(7): e2100097.
[41] Meadows AL, Hawkins KM, Tsegaye Y, Antipov E, Kim Y, Raetz L, et al. Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature 2016; 537(7622):694–7.
[42] Moabbi AM, Agarwal N, El Kaderi B, Ansari A. Role for gene looping in intron-mediated enhancement of transcription. Proc Natl Acad Sci U S A 2012;109: 8505–10.
[43] Le Hir H, Nott A, Moore MJ. How introns influence and enhance eukaryotic gene expression. Trends Biochem Sci 2003;28:215–20.
[44] Gonzalez Verdejo CI, Obrero A, Roman B, Gomez P. Expression profile of carotenoid cleavage dioxygenase genes in summer squash (*Cucurbita pepo L.*). Plant Foods Hum Nutr 2015;70:200–6.
[45] Vogel JT, Tan BC, Mccarty DR, Klee HJ. The carotenoid cleavage dioxygenase 1 enzyme has broad substrate specificity, cleaving multiple carotenoids at two different bond positions. J Biol Chem 2008;283:11364–73.
[46] Lopez J, Bustos D, Camilo C, Arenas N, Saa PA, Agosin E. Engineering *Saccharomyces cerevisiae* for the overproduction of β-ionone and its precursor β-carotene. Front Bioeng Biotechnol 2020;8:578793.
[47] Lu YP, Yang QY, Lin ZL, Yang XF. A modular pathway engineering strategy for the high-level production of beta-ionone in *Yarrowia lipolytica*. Microb Cell Factories 2020;19:49.
[48] Wei DZ, Zhang LY, Song QX. Studies on a novel carbon source and cosolvent for lipase production by *Candida rugosa*. J Ind Microbiol Biotechnol 2004;31:133–6.
[49] Zhu P, Dong S, Li S, Xu X, Xu H. Improvement of welan gum biosynthesis and transcriptional analysis of the genes responding to enhanced oxygen transfer by oxygen vectors in *Sphingomonas sp*. Biochem Eng J 2014;90:264–71.
[50] Rols JL, Condoret JS, Fonade C, Goma G. Mechanism of enhanced oxygen transfer in fermentation using emulsified oxygen-vectors. Biotechnol Bioeng 1990;35: 427–35.
[51] Khot M, Ghosh D. Lipids of *Rhodotorula mucilaginosa* IIPL32 with biodiesel potential: oil yield, fatty acid profile, fuel properties. J Basic Microbiol 2017;57: 345–52.
[52] Enshaeieh M, Abdoli A, Madani M. Single cell oil (SCO) production by *Rhodotorula mucilaginosa* and its environmental benefits. J Agric Sci 2015;17:387–400.
[53] Cao X, Yang S, Cao C, Zhou YJ. Harnessing sub-organelle metabolism for biosynthesis of isoprenoids in yeast. Synth Syst Biotechnol 2020;5:179–86.
[54] Jaramillo Madrid AC, Lacchini E, Goossens A. Within and beyond organelle engineering: strategies for increased terpene production in yeasts and plants. Curr Opin Green Sustainable Chem 2022;33:100572.
[55] Basiony M, Ouyang L, Wang D, Yu J, Zhou L, Zhu M, et al. Optimization of microbial cell factories for astaxanthin production: biosynthesis and regulations, engineering strategies and fermentation optimization strategies. Synth Syst Biotechnol 2022;7:689–704.