Engineering the oleaginous yeast *Candida tropicalis* for α-humulene overproduction

Lihua Zhang, Haiquan Yang, Yuanyuan Xia, Wei Shen, Liming Liu, Qi Li* and Xianzhong Chen*

**Abstract**

**Background:** α-Humulene is a plant-derived monocyclic sesquiterpenoid with multiple pharmacological activities, and far-reaching potential for the development of new drugs. Currently, the production of α-humulene is typically achieved via plant extraction, which is not sustainable and limited by low yields. The oleaginous yeast *Candida tropicalis* has recently emerged as a valuable host for producing high-value-added chemicals. However, the potential of *C. tropicalis* for terpenoid production has not been exploited.

**Results:** In this study, *C. tropicalis* was engineered for de novo synthesis of α-humulene from glucose. To improve α-humulene production, the codon-optimised α-humulene synthase gene and the entire endogenous farnesyl diphosphate synthesis pathway were co-overexpressed. Furthermore, bottlenecks in the α-humulene synthase pathway were identified and relieved by overexpressing α-humulene synthase, acetoacetyl-CoA thiolase and NADH-dependent HMG-CoA reductase. Combined with fermentation medium optimisation, the engineered strain produced 195.31 mg/L of α-humulene in shake flasks and 4115.42 mg/L in a bioreactor through fed-batch fermentation, a 253- and 5345-fold increase over the initial production, respectively.

**Conclusions:** This study demonstrates the potential of *C. tropicalis* for α-humulene production, and presents a platform for the biosynthesis of other terpenoids.

**Keywords:** *Candida tropicalis*, α-Humulene, Rate-limiting enzymes, Metabolic engineering, Mevalonate pathway

---

**Background**

Terpenoids, the largest family of natural compounds (>55,000 members), are widely used in the fields of food processing, agriculture, medicine and industry [1]. α-Humulene is a highly valued monocyclic sesquiterpenoid generally found in plants associated with its analogues β-humulene and isocaryophyllene [2]. α-Humulene and its isomers possess anti-inflammatory, antimicrobial and antitumour activities [2, 3]. Additionally, α-humulene has an 11-membered-ring and is a key intermediate for chemoynthesis or biosynthesis of zerumbone and other bioactive compounds [4, 5]. Currently, the large-scale production of α-humulene is typically achieved via plant extraction, while the α-humulene content in plants is low and significant differences among varieties and regions [6, 7]. Considering the complicated process and depletion of natural resources, the traditional methods are limited by low yields (only 6.2 g of α-humulene per kilogram of dried unopened flower buds of *Syzygium aromaticum* [6]), and shortage of raw materials. On the other hand, the chemical synthesis of α-humulene suffers from the numerous steps using environmental hazardous catalysts [8]. Therefore, biotechnological process is expected to provide an environmental-friendly and economical alternative.

Recently, α-humulene has been produced by various hosts. Krieg et al. constructed an α-humulene-producing strain of *Cupriavidus necator*, which expressed the α-humulene synthase gene (ZSSI) from...
Zingiber zerumbet Smith and overexpressed the hetero-
ologous mevalonate (MVA) pathway from *Methylobac-
terium extorquens* to improve production [9]. The titre
of the engineered strain reached 17 mg/g dry cell weight (DCW) using CO₂ as carbon source and sunlight as energy source. In another study, *Methylotuvinicium alcaliphilum* 20Z was engineered to express ZSS1 in combination with optimisation of the native methyler-
ythritol phosphate (MEP) pathway, and the strain accum-
ulated 0.75 mg/g DCW of α-humulene from methane [10]. By comparison, engineered *Methylobacterium extorquens* (expressing ZSS1 in combination with a hetero-
ologous mevalonate pathway from *Myxococcus xanthus*) produced up to 1.65 g/L α-humulene in methanol-limited fed-batch fermentation [11].

Although these unconventional substrates (CO₂, meth-
ane, methanol and acetate) are considered promising sustain-
able carbon sources for industrial biomanufacturing, sugar is currently the dominant raw material. Current-
ly, *Saccharomyces cerevisiae*, *Yarrowia lipolytica* and *Escherichia coli* are the three main chassis hosts for natural products produced from sugars. *E. coli* was engi-
neered to express ZSS1 and a heterologous mevalonate
pathway to improve the titre of α-humulene to 1 g/L in
terric broths containing 0.5 g/L of mevalonolactone or
1 g/L of lithium acetoacetate [12]. In general, the MEP
pathway in bacteria has a theoretically higher mass yield, but the MVA pathway typically performs better in terms of precursor supply for terpenoid production [13, 14].

To optimise α-humulene production in *S. cerevisiae*, the α-humulene synthesis enzyme was packaged in peroxi-
somes combined with cytoplasmic engineering [15]. The engineered strain produced 1.73 g/L of α-humulene in a
5-L bioreactor with glucose as carbon source, the high-
est titre reported to date for microorganisms. A similar
strategy has been used for α-farnesene, isoprene, and
squalene overproduction [16–18].

*Candida tropicalis* is a diploid, oleaginous yeast, and
an invaluable host for industrial production due to its
robust tolerance to unfavourable conditions [19, 20],
and its ability to degrade cyanide [21] and utilise vari-
ous carbon sources [21, 22]. Moreover, lipid accumula-
tion in this yeast species can reach 58% of dry biomass
[23]. It has recently emerged as a valuable host for pro-
ducing high-value-added chemicals such as long-chain α,ω-dicarboxylic acids [24], α-hydroxy fatty acids [22] and xylitol [20]. However, inadequate genetic engineer-
ing tools for metabolic engineering of complex metabolic pathways have limited the application of *C. tropicalis*. There are few reports on the use of this yeast species in
the production of terpenoids. Recently, the CRISPR–
Cas9 system for multiple genome editing, pathway assembly [25] and gene interference (CRISPRi, Y) Li et al.
unpublished) was developed for *C. tropicalis*. By integrat-
ing *carB* (encoding phytoene dehydrogenase) and *carRP* (encoding bi-functional enzymes phytoene synthase and lycopene cyclase) genes from *Mucor circinelloides* into the chromosome of *C. tropicalis*, the resulting DRPB strain could accumulate 0.23 mg/g DCW of β-carotene [25]. This indicates that the terpenoid precursors farnesyl diphosphate (FPP) and geranylgeranyl diphosphate are present in *C. tropicalis* (biosynthesis via the MVA path-
way, Fig. 1a), hence the yeast is a potential host for terpe-
noid production. Acetyl-CoA is a common precursor of lipid biosynthetic pathway and terpenoid pathway. Gen-
erally, the oleaginous yeast has a rich acetyl-CoA pool
[26]. The oleaginous yeast *Y. lipolytica* has been metaboli-
cally engineered for the production of linalool [27], ginsenoxide compound K [28], β-carotene [29] and lycopene [30] in recent years. With the development of genetic engineering tools, *C. tropicalis* may become a useful plat-
form strain for terpenoid production.

In this study, we explored the potential of the oleagi-
 nous yeast *C. tropicalis* for α-humulene biosynthesis fol-
lowing metabolic engineering. The ZSS1 gene from
*Z. zerumbet* was codon-optimised and integrated into the genome of *C. tropicalis*, and a basal α-humulene-
producing strain was constructed. Further improvement in α-humulene production was achieved by overexpress-
ing the entire endogenous FPP synthesis pathway and
adjusting gene dosage. Finally, the fermentation condi-
tions for α-humulene production were optimised, and
an impressive yield of 4115.42 mg/L of α-humulene was
achieved using fed-batch fermentation.

**Results and discussion**

**Engineering *C. tropicalis* for α-humulene production**

Because *C. tropicalis* lacks an efficient autonomously replac-
ing plasmid, exogenous genes are usually inte-
grated into the genome for stable expression. Previous
studies showed that single deletion of *CAT* in *C. tropi-
calis* had no impact on cells growth [31], therefore the
*CtCas9* expression cassette was integrated at the *CAT*
locus to generate strain CU-207 for further facilitat-
ing genetic manipulation. After the *URA3* marker was
excised from *C. tropicalis* CU-207, the resulting uracil
auxotrophic strain CU-208 was used as the platform
strain for further metabolic engineering. Our previous
studies showed that it is challenging to express hetero-
ologous genes in *C. tropicalis* without codon optimi-
sation [25, 31]. Thus, the codon-optimised ZSS1 from
*Z. zerumbet* was integrated into the chromosome of
*C. tropicalis* CU-208 through CRISPR–Cas9, result-
ing in strain HC01 (possessing a single copy of ZSS1 at
the POX5 locus) and HC02 (possessing double copies
of ZSS1 at the POX5 loci). After 96 h of fermentation,
α-humulene production was detected by GC–MS (Fig. 1b and Additional file 2: Fig. S1). HC-02 produced 1.41 mg/L α-humulene, roughly double that of HC-01 (0.77 mg/L), suggesting that the ZSS1 gene can be successfully expressed in C. tropicalis, and the expression level of ZSS1 might be a key factor influencing α-humulene production. Compared with CU-207, the biomass of HC01 and HC02 was not markedly different, indicating that a low level of α-humulene had little or no effect on yeast growth. Nevertheless, the α-humulene concentration was lower than that reported for S. cerevisiae (2.32 mg/L, possessing a single copy of ZSS1) [15].

Previous studies have reported that the peroxisome was more appropriate for the synthesis of limonene, α-humulene and squalene in S. cerevisiae [15, 17, 32]. Therefore, it is necessary to evaluate whether the peroxisome could be benefit for producing α-humulene in C. tropicalis. However, no confirmed peroxisome targeting signals are presented in C. tropicalis. Firstly, the function of peroxisome targeting signal-1 (PTS1, SKL) was evaluated with ZSS1-GFP as a reporter. To label peroxisome, a red fluorescence protein was fused with peroxisome membrane protein (PEX3-mScarlet) and co-expressed with ZSS1-GFP-PTS1. Fluorescence microscopy results showed that the GFP and mScarlet signals colocalised (Fig. 1c), indicating that ZSS1-GFP-PTS1 could be transported into peroxisome. However, when α-humulene synthase was directed to peroxisomes by PTS1, only 0.06 mg/L of α-humulene accumulated in the transformant possessing double copies of ZSS1-PTS1 (HP02; 0.04 mg/L for HP01 possessing one copy of ZSS1-PTS1; Fig. 1b).

**Fig. 1** Biosynthesis of α-humulene in C. tropicalis. **a** Scheme showing the α-humulene biosynthesis pathway in C. tropicalis. ERG10, acetoacetyl-CoA thiolase; ERG13, hydroxymethylglutaryl-CoA synthase; hHMGR, truncated hydroxymethylglutaryl-CoA reductase; NADH-HMGR, NADH-dependent HMG-CoA reductase from Silicibacter pomeroyi; ERG12, mevalonate kinase; ERG8, phosphomevalonate kinase; ERG19, mevalonate diphosphate decarboxylase; IDI1, isopentenyl diphosphate isomerase; ERG20, geranyl/farnesyl diphosphate synthase; ZSS1, α-humulene synthase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate. **b** α-humulene production by C. tropicalis expressing ZSS1 in the cytoplasm (HC01 and HC02) and in peroxisomes (HP01 and HP02). Results are means ± standard deviations of biological triplicates. **c** Fluorescence microscopy of C. tropicalis 01 co-expressed the ZSS1-GFP-PTS1 fusion protein and the peroxisome marker PEX3-mScarlet.
Effects of HMGR and ERG10 overexpression and ERG9 repression on α-humulene production

The biosynthesis of α-humulene from acetyl-CoA in C. tropicalis requires multiple enzymes and complex metabolic regulation (Fig. 1a). Previous studies demonstrated that overexpression of HMGR and ERG10 and repression of ERG9 expression positively affect terpenoid production in S. cerevisiae and Y. lipolytica [27, 29, 33]. Therefore, the influence of these three genes on the production of α-humulene in C. tropicalis was evaluated.

First, HMGR and ERG10 were expressed in HC03 (a uracil auxotrophic derivative of HC02). The resulting strain HC05 (overexpressing HMGR) produced 1.88 mg/L α-humulene, 33.3% more than HC02 (Fig. 2, HC05 vs. HC02). By contrast, overexpressing ERG10 did not improve production of α-humulene (Fig. 2, HC04 vs. HC02).

It has been reported that the hydroxymethylglutaryl-CoA reductases of S. cerevisiae and Y. lipolytica share similar structures, with N-terminal multiple transmembrane domains and a C-terminal catalytically active domain [4, 29, 34]. Moreover, the N-terminal domain is a response element for signal regulation; its deletion can enhance protein stability. Thus, a truncated HMGR of C. tropicalis (tHMGR, lacking the N-terminal multiple transmembrane domains, Additional file 2: Fig. S2) was overexpressed in HC03. As expected, α-humulene production in HC06 (overexpressing tHMGR) was increased by 66.7% compared to HC02 (Fig. 2).

Squalene synthase (ERG9) catalyses the reductive dimerisation of two FPP moieties to form one molecule of squalene. FPP is a precursor of sesquiterpenoids in eukaryotes, while squalene plays an integral role in sterol synthesis (Fig. 1a). In order to increase the FPP flux towards α-humulene biosynthesis, a single copy of ERG9 was disrupted in HC03, generating strain HC07. Unexpectedly, the biomass and α-humulene titre of HC07 were decreased compared with HC02, although the α-humulene content was improved slightly (Fig. 2). In addition, the content of β-carotene in ERG9-disrupted C. tropicalis followed the same trend (YJ Li et al. unpublished). RT-qPCR analysis showed that ERG9 mRNA levels in strain HC07 were 43% lower than in HC02 (Additional file 2: Fig. S3), indicating that expression of ERG9 was repressed. Similarly, the lycopene production capacity of engineered Candida utilis could not be increased when two copies of ERG9 were deleted in tetraploid yeast [35]. In our previous study, one copy of the CAT gene was disrupted in C. tropicalis, and carnitine acetyltransferase mRNA levels and enzyme activity were decreased [31]. Meanwhile, the concentration of α,ω-dodecanedioic acid was not changed significantly. These data may indicate that the normal allele can completely (or mostly) cancel the mutant allele in diploid and polyploid yeast.

Overexpressing the entire α-humulene synthesis pathway to improve α-humulene production

Although strains HC02 and HP02 could produce α-humulene, production was very low. This might be due to the inefficiency of the native MVA pathway, which is tightly regulated in yeast. Firstly, a short synthetic terminator (Tsynth7, 32 bp) [36], which functions in S. cerevisiae and Y. lipolytica, was functionally verified in C. tropicalis by the GFP reporter system (Additional file 2: Fig. S4). Then the strong promoters P GAP1 and P FBA1 and terminators Tsynth7, TENDO, TPGK1 and TADH2 were used to control gene expression. To further enhance α-humulene production, genes encoding the entire α-humulene synthesis pathway (ERG10, ERG13, tHMGR, ERG12, ERG8, ERG19, IDI1, ERG20 and ZZSI) were constitutively overexpressed in cytoplasmic and peroxisomal fractions of CU-208. The α-humulene titre of the peroxisome engineered strain DP-H01 (expressing double copies of the nine genes) was 2.42 mg/L, 43.33-fold higher than HP02, and 1.70-fold higher than the strain expressing one copy of each of the nine genes in peroxisomes (Fig. 3a). These results indicate that the peroxisome-targeted α-humulene biosynthetic pathway could enhance production in C. tropicalis. Similarly, previous studies reported that targeting biosynthetic pathways to peroxisomes can enhance productivity.

**Fig. 2** Effects of ERG10, HMGR and tHMGR overexpression and ERG9 repression in C. tropicalis on biomass and α-humulene production: 2C represents the expression of double copies of genes in the cytoplasm and 1Δ represents the disruption one of the ERG9 alleles. Results are means ± standard deviations of biological triplicates.
and inhibit by-product formation [32, 37]. However, the α-humulene titre in the engineered C. tropicalis strain was much lower than that reported for S. cerevisiae [15]. Interestingly, strains overexpressing the entire α-humulene synthesis pathway in the cytoplasm exhibited a remarkable increase in α-humulene production. In strain DC-H01 expressing double copies of the α-humulene synthesis pathway genes in the cytoplasm, α-humulene production was improved more than five-fold compared with DP-H01, reaching 12.89 mg/L (Fig. 3a). A similar result was obtained for strain SC-H01 expressing only one copy of each α-humulene synthesis pathway gene in the cytoplasm (Fig. 3a). In addition, the cell growth of the engineered strains was significantly inhibited compared with the initial strain (Fig. 1b, a).

The peroxisome subcellular organelle is nonessential for yeast growth, and a series of studies have focused on peroxisome engineering of yeast for terpenoid production [15–17]. However, our current results showed that the peroxisome of C. tropicalis is not an ideal subcellular location for α-humulene production. Therefore, strain DC-H01 was chosen for further genetic modification to improve α-humulene biosynthesis.

Identifying rate-limiting steps in the α-humulene pathway

Many researchers have demonstrated that cytoplasmic-engineered S. cerevisiae can be used for terpenoid production with high efficiency [38, 39]. Moreover, β-carotene production in cytoplasmic-engineered Y. lipolytica reached 6.5 g/L [40]. Our current results showed that the α-humulene titre of DC-H01 was significantly higher than that of SC-H01, indicating a bottleneck in the α-humulene biosynthesis pathway of the SC-H01 strain. However, the α-humulene titre was significantly lower (12.89 mg/L, Fig. 3a). Therefore, we hypothesised that α-humulene biosynthesis in DC-H01 may be limited by one or several steps in the pathway.

To confirm this hypothesis, three gene expression cassettes (cassette 1 for ERG10, ERG13 and tHMGR expression; cassette 2 for ERG12, ERG8, ERG19 and IDI1 expression; cassette 3 for ERG20 and ZSS1 expression)
were constructed and transformed into strain DC-H02 (a *URA3* pop-out derivative of DC-H01), generating strain DC-H03S (expressing one copy of cassette 1), DC-H03D (expressing double copies of cassette 1), DC-H05S (expressing one copy of cassette 2), DC-H05D (expressing double copies of cassette 2), DC-H07S (expressing one copy of cassette 3) and DC-H07D (expressing double copies of cassette 3). Engineered strain DC-H07S produced nearly 50% more α-humulene compared than DC-H01 (19.33 mg/L vs 12.90 mg/L; Fig. 3b), whereas strain DC-H07D produced 32.68 mg/L of α-humulene, indicating that overexpression of *ERG20* and *ZSS1* enhanced α-humulene production. Further studies indicated that the increase in α-humulene titre was mainly due to expression of *ZSS1* (Fig. 3b, strain DC-H09D overexpressing *ZSS1* vs DC-H07D). However, the effects of co-expression of *ERG10*, *ERG13* and *tHMGR*, and *ERG12*, *ERG8*, *ERG19* and *IDII* were limited (DC-H03S, DC-H03D, DC-H05S and DC-H05D vs. DC-H01), indicating that the steps catalysed by these enzymes are not the bottlenecks for α-humulene production in strain DC-H01. Compared with DC-H01, expression of cassette 2 inhibited cell growth of strains DC-H05S and DC-H05D. It was previously reported that IPP and DMAPP are toxic to mitochondria, and higher levels of these pyrophosphorylated intermediates can inhibit the growth of cells [33]. Overexpression of *ERG12*, *ERG8*, *ERG19* and *IDII* genes can lead to accumulation of IPP and DMAPP in DC-H05S and DC-H05D, and they may be transported from the cytoplasm to the mitochondria [41], where they disrupt mitochondrial function and inhibit cell growth.

Considering that increasing the expression of the *ZSS1* gene can significantly increase the production of α-humulene (DC-H09D vs DC-H01 and DC-H07D vs DC-H01), we speculated that increasing the copy number of the *ZSS1* gene may further improve the yield of α-humulene. Since the *GAPI* promoter is one of the strongest promoters (more than twofold stronger than the *FBA1* promoter) [25], we chose this promoter to overexpress the *ZSS1* gene. The *ZSS1* expression cassette was integrated at the D-lactate dehydrogenase gene (*DLD1b*) and/or the lipid phosphate phosphatase gene (*LPP2*) locus of strain DC-H08 (a *URA3* pop-out derivative of DC-H07D) to increase the copy number of *ZSS1*, resulting in strains DC-H11S, DC-H11D, DC-H13S and DC-H13D. As shown in Fig. 3c, compared with DC-H07, the titre of α-humulene was significantly improved with increasing *ZSS1* copy number. The maximum α-humulene levels in DC-H11S, DC-H11D, DC-H13S and DC-H13D reached 55.25, 77.00, 92.87 and 99.62 mg/L, respectively (Fig. 3c). Moreover, the engineered strains showed a slight increase in cell growth compared with DC-H07.

Lipid phosphate phosphatase is one of the main contributors to phosphatase activity in yeast. Deleting the *LPP1* gene can increase sesquiterpene levels in *S. cerevisiae* [39, 42]. However, our results showed that deleting the *LPP2* gene did not increase the α-humulene titre in *C. tropicalis* (Fig. 3c, DC-H15D vs DC-H07D). Compared with DC-H07D, the farnesol titre of strain DC-H15D was not changed significantly (data not shown). Indeed, in addition to the *LPP2* alleles, there are other phosphate phosphatases (at least three isozymes of diacylglycerol pyrophosphate phosphatase) in *C. tropicalis*.

Previous studies have shown that HMGR is the first rate-limiting enzyme in the MVA pathway, and NADH-dependent HMG-CoA reductase (NADH-HMG) from *S. pomeroyi* has better performance for the production of sesquiterpenoid in yeast [38, 43]. To further investigate the rate-limiting step of DC-H13D for α-humulene synthesis, *tHMGR* and *NADH-HMG* from *S. pomeroyi* were overexpressed. Compared with *tHMGR*, *NADH-HMG* achieved a more significant increase in α-humulene titre (Fig. 3d, DC-H17D vs DC-H13D and DC-H19D vs. DC-H13D). When both *NADH-HMG* and *ERG10* genes were overexpressed in DC-H13D (generating strain DC-H21D), an α-humulene titre of 119.07 mg/L was achieved, ~19.5% higher than that of DC-H13D (Fig. 3d).

**Fed-batch fermentation for α-humulene production**

In order to improve α-humulene production of strain DC-H21D, three different types of medium, nitrogen stress medium [23] with 100 g/L glucose, YPD60 medium and Y20P40D60 medium, were tested in shake flasks prior to fed-batch fermentation. The α-humulene titre of DC-H21D was increased to 171.50 mg/L and 195.31 mg/L in YPD60 and Y20P40D60 medium, an increase of 44.0% and 64.0% compared with YPD medium (Fig. 4). Moreover, biomass was also improved. However, cell growth and α-humulene production in nitrogen stress medium were significantly lower than in YPD medium (Fig. 4).

To further characterise α-humulene production in *C. tropicalis*, strain DC-H21D was employed for fed-batch fermentation in a 5-L bioreactor (Bailun Co., Shanghai, China) with 2 L YPD60 or Y20P40D60 medium. As shown in Fig. 5, the strain DC-H21D grew continuously in both fermentations. Finally, the maximum titre of α-humulene reached 1957.28 mg/L and 3144.37 mg/L from the YPD60 and Y20P40D60 medium, respectively, at 216 h (Fig. 5a, b). In order to further increase the titre of α-humulene, scale-up experiment was performed in a 30-L bioreactor (INFORS, Switzerland) with 12 L Y20P40D60 medium (Fig. 5c). In this fed-batch culture, glucose was quickly consumed within 16 h, and feeding was initiated at ~16 h after fermentation. The biomass
The uracil auxotrophic *C. tropicalis* CU-206 strain derived from *C. tropicalis* ATCC20336 was used as the parent strain for metabolic engineering [25]. The medium (MM, SM, FOA-SM, 2 × YPD) and culture conditions used for genetic manipulation of *C. tropicalis* were as described previously [25]. YPD60 (10 g/L yeast extract, 20 g/L peptone, 60 g/L glucose, 0.5 g/L MgSO₄), Y20P40D60 (20 g/L yeast extract, 40 g/L peptone, 60 g/L glucose, 0.5 g/L MgSO₄) and nitrogen stress medium (NS100, 0.5 g/L yeast extract, 0.4 g/L MgSO₄-7H₂O, 0.5 g/L CaCl₂, 2 g/L KH₂PO₄, 0.05 g/L CuSO₄-5H₂O, 0.5 g/L (NH₄)₂SO₄, 100 g/L glucose) medium were used for fermentation.

Conclusions

In this work, the oleaginous yeast *C. tropicalis* was successfully engineered for de novo synthesis of α-humulene from glucose. Specifically, the entire α-humulene synthesis pathway was overexpressed in the cytoplasm, and the rate-limiting steps of α-humulene production were identified and relieved. And our results reveal that ZSS1 was the most crucial bottleneck enzyme of α-humulene synthesis, followed by NADH-HMGR and ERG10. The final titre of α-humulene was 195.31 mg/L and 4115.42 mg/L in shake flasks and fed-batch fermentation, respectively. This study is the first to report on terpenoid synthesis by *C. tropicalis* for metabolic engineering [25]. The construction of α-humulene and other terpenoids.

Materials and methods

**Strains, media and culture conditions**

The uracil auxotrophic *C. tropicalis* CU-206 strain derived from *C. tropicalis* ATCC20336 was used as the parent strain for metabolic engineering [25]. The medium (MM, SM, FOA-SM, 2 × YPD) and culture conditions used for genetic manipulation of *C. tropicalis* were as described previously [25]. YPD60 (10 g/L yeast extract, 20 g/L peptone, 60 g/L glucose, 0.5 g/L MgSO₄), Y20P40D60 (20 g/L yeast extract, 40 g/L peptone, 60 g/L glucose, 0.5 g/L MgSO₄) and nitrogen stress medium (NS100, 0.5 g/L yeast extract, 0.4 g/L MgSO₄-7H₂O, 0.5 g/L CaCl₂, 2 g/L KH₂PO₄, 0.05 g/L CuSO₄-5H₂O, 0.5 g/L (NH₄)₂SO₄, 100 g/L glucose) medium were used for fermentation.

**Construction of plasmids and strains**

The *Zingiber zerumbet* Smith α-humulene synthase gene (ZSSI; GenBank: AB247331.1) and the *Silicibacter pomeroyi* NADH-dependent HMG-CoA reductase gene (NADH-HMGR; NCBI Reference Sequence: WP_011241944.1) were codon-optimised and synthesised by Genewiz (Suzhou, China). The promoters (P<sub>gap1</sub> and P<sub>traa1</sub>), terminators (T<sub>ENO1</sub>, T<sub>PKG1</sub> and T<sub>ADH2</sub>) [25] and coding sequences of the eight genes involved in the FPP pathway (ERG10, encoding acetoacetoy-CoA thiolase; ERG13, encoding hydroxymethylglutaryl-CoA synthase; HMRG, encoding hydroxymethylglutaryl-CoA reductase; ERG12, encoding mevalonate kinase; ERG8, encoding phosphomevalonate kinase; ERG19, encoding mevalonate diphosphate decarboxylase; IDI1, encoding isopentenyl diphosphate isomerase; and ERG20, encoding geranyl/farnesyl diphosphate synthase) were amplified from *C. tropicalis* ATCC20336. The short synthetic terminator T<sub>synth7</sub> [36] was created by adding the sequence to primers. Previous studies found that knockout of POX5 (encoding acyl-CoA oxidase), ALD1 (encoding fatty aldehyde dehydrogenase), FAO1 (encoding alcohol oxidase), DLD1b and DLD1a (encoding D-lactate dehydrogenase), and LPP2 (encoding diacylglycerol pyrophosphatase phosphatase 2) have little effect on cells growth when cultured with glucose as the sole carbon source [44, 45], therefore we selected these loci for genomic integration of the α-humulene synthesis pathway. Single guide RNA expression cassettes targeting these genome sites were constructed using the method described in our previous work [25]. Plasmids were constructed according to standard restriction enzyme-based cloning or using a ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). The detailed process for plasmid construction is described in the Additional file 1.

All primers used in this study are listed in Additional file 3: Table S1. To obtain a higher strain construction efficiency, the codon-optimised Cas9 expression cassette CAT2-gda324-URA3-P<sub>gap1</sub>-CtCas9-T<sub>ENO1</sub>-CAT2 was first inserted into *C. tropicalis* CU-206 and integrated at the CAT locus via homologous recombination, resulting in strain CU-207, and the marker gene URA3 pop-out derivative strain was named CU-208. Detailed procedures for integrating gene expression cassettes were
**Fig. 5** Production of α-humulene through fed-batch fermentation by strain DC-H21D in bioreactor. α-Humulene production in a 5-L bioreactor with YPD60 medium (a) and Y20P40D60 medium (b). c α-Humulene production in a 30-L bioreactor with Y20P40D60 medium. The black arrow indicates the start of glucose concentration control.
Table 1  C. tropicalis strains used in this study

| Strains          | Genotypes                                                      | References |
|------------------|----------------------------------------------------------------|------------|
| C. tropicalis ATCC 20,336 | URA3/URA3, CAT/CAT, FAO1/FAO1, ALD1/ALD1, POX5/POX5, DLD1a/DLD1a, DLD1b/DLD1b, LPP2/LPP2, ERG9/ERG9 | ATCC       |
| C. tropicalis CU-206  | C. tropicalis ATCC 20,336, ura3/ura3                          | [25]       |
| C. tropicalis 05-3  | C. tropicalis ATCC 20,336, ura3/ura3, cat::gda324/cat::gda324-URA3-PGAP1-yeFP3-TGAP1 | [31]       |
| C. tropicalis 01  | C. tropicalis CU-206, Cat/cat::gda-PGAP1-ZSS1-yeFP3-TGAP1, ALD1/ald1::gda-URA3-PGAP1-PEX3-mScarlet-TGAP1 | This study |
| C. tropicalis 02  | C. tropicalis CU-206, Cat/cat::gda-URA3-PGAP1-yeFP3-Tsynth7  | This study |
| CU-207           | C. tropicalis CU-206, Cat/cat::gda-URA3-PGAP1-Cas9-3 × SV40-Tsynth7 | This study |
| CU-208           | CU-207, Cat/cat::gda-PGAP1-Cas9-3 × SV40-Tsynth7             | This study |
| HC01             | CU-208, POX5/pox5::gda-URA3-PBAT-ZSS1-TGAP1                  | This study |
| HC02             | CU-208, pox5::gda-URA3-PBAT-ZSS1-TGAP1/pox5::gda-URA3-PBAT-ZSS1-TGAP1 | This study |
| HC03             | CU-208, pox5::gda-URA3-PBAT-ZSS1-TGAP1/pox5::gda-URA3-PBAT-ZSS1-TGAP1 | This study |
| HP01             | CU-208, POX5/pox5::gda-URA3-PBAT-ZSS1-TGAP1                  | This study |
| HP02             | CU-208, pox5::gda-URA3-PBAT-ZSS1-TGAP1/pox5::gda-URA3-PBAT-ZSS1-TGAP1 | This study |
| HCO4             | HCO3, fa01::gda-URA3-PGAP1-ERG10-Tsynth7/pox5::gda-URA3-PGAP1-ERG10-Tsynth7 | This study |
| HCO5             | HCO3, fa01::gda-URA3-PGAP1-HMGRTsynth7/pox5::gda-URA3-PGAP1-HMGRTsynth7 | This study |
| HCO6             | HCO3, fa01::gda-URA3-PGAP1-HMGRTsynth7/pox5::gda-URA3-PGAP1-HMGRTsynth7 | This study |
| SC-H01           | SC-H01, ERG9/erg9::gda-URA3                                   | This study |
| DC-H01           | DC-H01, fa01::gda-TPOX-ERG13-PBAT-PGAP1-HMGRTsynth7-PGAP1-ERG10-Tsynth7/pox5::gda-URA3-PGAP1-ERG10-Tsynth7/ZSS1-PBAT | This study |
| DC-H02           | DC-H02, fa01::gda-TPOX-ERG13-PBAT-PGAP1-HMGRTsynth7-PGAP1-ERG10-Tsynth7/pox5::gda-URA3-PGAP1-ERG10-Tsynth7/ZSS1-PBAT | This study |
| SP-H01           | SP-H01, fa01::gda-TPOX-PTS1-ERG13-PBAT-PGAP1-HMGRT-PTS1-Tsynth7-PGAP1/ald1::gda-URA3-PGAP1-ERG10-Tsynth7 | This study |
| DP-H01           | DP-H01, fa01::gda-TPOX-PTS1-ERG13-PBAT-PGAP1-HMGRT-PTS1-Tsynth7-PGAP1/ald1::gda-URA3-PGAP1-ERG10-Tsynth7 | This study |
| DC-H03S          | DC-H02, DLD1a/dld1::gda-URA3-PGAP1-ERG10-Tsynth7/ERG10-Tsynth7 | This study |
| DC-H03D          | DC-H02, dld1::gda-URA3-PGAP1-ERG10-Tsynth7/ERG10-Tsynth7     | This study |
| DC-H05S          | DC-H02, DLD1a/dld1::gda-URA3-PGAP1-ERG10-Tsynth7/ERG10-Tsynth7 | This study |
| DC-H05D          | DC-H02, dld1::gda-URA3-PGAP1-ERG10-Tsynth7/ERG10-Tsynth7     | This study |
| DC-H07S          | DC-H02, dld1::gda-URA3-PGAP1-ERG10-Tsynth7/ERG10-Tsynth7     | This study |
| DC-H07D          | DC-H02, dld1::gda-URA3-PGAP1-ERG10-Tsynth7/ERG10-Tsynth7     | This study |
| DC-H08S          | DC-H02, dld1::gda-URA3-PGAP1-ERG10-Tsynth7/ERG10-Tsynth7     | This study |
| DC-H08D          | DC-H02, dld1::gda-URA3-PGAP1-ERG10-Tsynth7/ERG10-Tsynth7     | This study |
| DC-H11S          | DC-H11, LPP2/lpp2::gda-URA3-PGAP1-ZSS1-Tsynth7              | This study |
| DC-H11D          | DC-H11, LPP2/lpp2::gda-URA3-PGAP1-ZSS1-Tsynth7              | This study |
| DC-H12           | DC-H12, dld1b::gda-URA3-PGAP1-ZSS1-Tsynth7                  | This study |
| DC-H13S          | DC-H12, dld1b::gda-URA3-PGAP1-ZSS1-Tsynth7                  | This study |
| DC-H13D          | DC-H12, dld1b::gda-URA3-PGAP1-ZSS1-Tsynth7                  | This study |
| DC-H14           | DC-H12, dld1b::gda-URA3-PGAP1-ZSS1-Tsynth7                  | This study |
| DC-H15D          | DC-H12, dld1b::gda-URA3-PGAP1-ZSS1-Tsynth7                  | This study |
performed according to our previous work [25]. All C. tropicalis strains used in this study are listed in Table 1.

**α-Humulene fermentation in shake flasks**

To produce α-humulene, yeast cells were pre-cultured in 100-mL shake flasks containing 20 mL YPD medium with shaking at 200 rpm and 30 °C (until the OD$_{600}$ value reached 10–15). Logarithmic phase cultures were diluted to an initial OD$_{600}$ of 0.1 in 30 mL YPD medium and cultivated at 200 rpm and 30 °C. Next, 10% (v/v) n-dodecane was added to the culture aseptically after 10 h. After 96 h of fermentation, the liquid culture was centrifuged at 5000 g for 5 min. The upper organic layer was collected for volume measurement and α-humulene quantification. All experiments were performed in triplicate.

**Fed-batch fermentation of α-humulene**

Fed-batch fermentation was performed in a 30-L bioreactor (INFORS, Switzerland). Firstly, a single colony of C. tropicalis DC-H21D was pre-cultured in a 250-mL shake flask containing 50 mL YPD medium for 20 h. The resulting culture was diluted in 100 mL of fresh YPD medium to an initial OD$_{600}$ of 0.5 in a 500 mL shake flask and cultivated at 200 rpm and 30 °C for 12 h. The entire 1000 mL seed culture was inoculated into a 30-L bioreactor containing 12 L of Y20P40D60 medium. Fermentation for α-humulene production was performed at 30 °C and the pH was maintained at 5.5 using ammonium hydroxide. Dissolved oxygen was maintained above 20% by adjusting the agitation (300–900 rpm) and the air flow rate (2–4vvm). When the glucose concentration dipped below 5 g/L for the first time, concentrated glucose solution (80%, w/v) was added to the yeast culture to provide adequate carbon source (below 5 g/L). For two-phase extractive fermentation, 10% (v/v) n-dodecane was added to the culture after 12 h of fermentation.

**Analytical methods**

Glucose was quantified using a biosensor (Biologe Institute of Shandong Academy of Sciences, Shandong, China). The biomass (OD$_{600}$) of C. tropicalis was determined using a UV-2000 spectrophotometer (UNIC, Shanghai, China).

For qualitative analysis of α-humulene in fed-batch fermentation culture, 5 mL of fermentation broth was centrifuged at 5000 g for 5 min, and cell pellets were resuspended in ddH$_2$O and lysed with an ultrasonic cell disruptor. The culture supernatant and cell lyase were extracted with 10 mL of n-hexane for 5 min at room temperature with agitation. The n-hexane extract was appropriately diluted with n-hexane and residual water was removed with Na$_2$SO$_4$. α-Humulene was quantified with a Trace1310 Triple Quadrupole GC–MS system equipped with a gas chromatograph (Thermo Scientific, Waltham, MA, USA), and a TG-5 ms column (Thermo Scientific) connected to a TSQ8000 mass spectrometer (Thermo Scientific). Measurements were conducted with a helium flow maintained at 1.2 mL/min, split injections (10:1) at 280 °C, an initial column temperature of 50 °C maintained for 1 min, then increased to 180 °C at a rate of 10 °C/min, then increased to 280 °C at 25 °C/min and maintained for 6 min.

**Abbreviations**

DCW: Dry cell weight; ZSS1: α-Humulene synthase; MVA: Mevalonate; MEP: Methylerythritol phosphate; carB: Phytoene dehydrogenase; carRP: Phytoene synthase and lycopene cyclase; FPP: Farnesyl diphosphate; CRISPR–Cas9: Clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9; GC–MS: Gas chromatography–mass spectrometry; POX5: Acyl-CoA oxidase; ALD1: Fatty aldehyde dehydrogenase; FAO1: Alcohol oxidase; CAT: Carnitine acetyltransferase; IDL1: Fatty aldehyde dehydrogenase; GDH1: L-Lactate dehydrogenase 1a; DLD1b: L-Lactate dehydrogenase 1b; LPP1: Lipid phosphate phosphatase 1; LPP2: Lipid phosphate phosphatase 2; PFB1: Promoter of glyceraldehyde-3-phosphate dehydrogenase gene; P$_{BAD}$: Promoter of fructose bisphosphate aldolase gene; Tsynth7: Short synthetic terminator; Tendo1: Terminator of fructose bisphosphate aldolase gene; T_5′: Terminator of glyceraldehyde-3-phosphate dehydrogenase gene; T_3′: Terminator of glucose-6-phosphate transporter gene; T_5′: Terminator of thiamine pyrophosphate transporter gene; TENO1: Terminator of alcohol dehydrogenase gene; GFP: Green fluorescent protein; CRISPRi: CRISPR interference; HMG: Hydroxymethylglutaryl-CoA reductases; CRISPR–Cas9: Clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9; PTS1: Peroxisome targeting signal-1; HMGR: Hydroxymethylglutaryl-CoA reductase; GFP: Green fluorescent protein; RT-qPCR: Real-time quantitative PCR.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13068-022-02160-8.

Additional file 1: Additional methods for plasmid construction.

Additional file 2: Fig S1. GC-MS analysis of α-humulene from the dodecane of the cultures in engineered C. tropicalis. Fig S2 Bioinformatic analysis of HMGRI protein from C. tropicalis ATCC20336. Fig S3 Transcription levels of the ERG9 gene in HC02 and HC07 strains. Fig S4 Confirmation of the synthetic terminator Tsynth7. Using GFP as a reporter.

Additional file 3: Table S1. Primers used for plasmids construction.

Acknowledgements
Not applicable.

Author contribution
XC, QL and LL conceived and designed the research. LZ and HY performed the experiments, collected and analysed the data. LZ, YX and WS wrote and revised the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by the 111 Project (No. 111-2-06), National Natural Science Foundation of China (32001064), Key Research and Development Program of China (2021YFC200102-03), China Postdoctoral Science Foundation (20200617133), and Postgraduate Research and Practice Innovation Program of Jiangsu Province (No. KYCQD-1807).

Availability of data and materials
All data generated or analysed during this study are included in this published article and its Additional information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors agree to submit the work to the journal.

Competing interests
The authors declare no competing financial interests.

Received: 16 January 2022 Accepted: 17 May 2022
Published online: 26 May 2022

References
1. Zu Y, Prather KL, Stephanopoulos G. Metabolic engineering strategies to overcome precursor limitations in isoprenoid biosynthesis. Curr Opin Biotech. 2020;66:171–8.
2. de Lacerda Leite GM, de Oliveira BM, Lopes MIJ, de Araújo DG, Bezerra DS, Araújo IM, de Alencar CDC, Coutinho HDM, Penteiro LR, Barbosa Filho JM. Pharmacological and toxicological activities of α-humulene and its isomers: a systematic review. Trends Food Sci Tech. 2021;15:255–74.
3. Di Sotto A, Mancinelli R, Gulli M, Eufemi M, Mammola CL, Mazzanti G, Di Gaetano S. Chemopreventive potential of caryophyllane sesquiterpenes: a systematic review. Trends Food Sci Tech. 2021;15:255–74.
4. Zhang C, Liu J, Zhao F, Lu C, Zhao GR, Lu W. Production of sesquiterpene zebunene from metabolic engineered Saccharomyces cerevisiae. Metab Eng. 2018;49:28–35.
5. Kikuchi H, Nishimura T, Kwon E, Kawai J, Oshima Y. Development of a terpenoid-like compound library based on the humulene skeleton. Chemistry. 2016;22(44):15819–25.
6. Wei MC, Xiao J, Yang YC. Extraction of alpha-humulene-enriched oil from clove using ultrasound-assisted supercritical carbon dioxide extraction and studies of its fictitious solubility. Food Chem. 2016;210:172–81.
7. Abdollahi M, Sefidkon F, Calagari M, Mousavi A, Mahomoodally MF. Impact of four hemp (Cannabis sativa L) varieties and stage of plant growth on yield and composition of essential oils. Ind Crop Prod. 2020;155:112793.
8. Hu T, Corey EJ. Short syntheses of (+/-)-delta-aroaraneene and humulene utilizing a combination of four-component assembly and palladium-mediated cyclization. Org Lett. 2002;4(14):2441–3.
9. Krieg T, Sydow A, Faust S, Huth J, Holtmann D. C02 to terpenes: auto- trophic and electroautotrophic alpha-humulene production with Cupri- avidus necator. Angew Chem Int Ed. 2018;57(7):1879–82.
10. Nguyen AD, Kim D, Lee EY. Unlocking the biosynthesis of sesquiterpe- noids from methane via the methylythylitol phosphate pathway in methanotrophic bacteria, using alpha-humulene as a model compound. Metab Eng. 2020;61:69–78.
11. Sonntag F, Kroner C, Lubuta P, Peyraud R, Horst A, Buchhaupt M, Schrader J. Engineering Methylobacterium extorquens for de novo synthesis of the sesquiterpenoid alpha-humulene from methanol. Metab Eng. 2015;32:82–94.
12. Harada H, Yu FNA, Okamoto S, Kuzuyama T, Utsumi R, Misawa N. Efficient synthesis of functional isopenoids from acetoacetate through metabolic pathway-engineered Escherichia coli. Appl Microbiol. 2009;81(5):915–25.
13. Martin VJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. Engineering a malonate pathway in Escherichia coli for production of terpenoids. Nat Biotechnol. 2003;21(7):796–802.
14. Ma YR, Li WJ, Mai J, Wang JP, Wei YJ, Ledaesma-Amaro R, Ji XJ. Engineering Yarrowia lipolytica for sustainable production of the chamomile sesquiterpene (-)-alpha-bisabolol. Green Chem. 2021;23(2):780–7.
15. Zhang C, Li MW, Zhao GR, Lu W. Harnessing yeast peroxisomes and cytosol acetyl-CoA for sesquiterpene alpha-humulene production. J Agr Food Chem. 2020;68:1382–9.
16. Liu H, Chen SL, Xu JZ, Zhang WG. Dual regulation of cytoplasm and peroxisomes for improved alpha-farnesene production in recombinant Pichia pastoris. ACS Synth Biol. 2021;10(6):1563–73.
17. Liu GS, Li T, Zhou W, Jiang M, Tao XY, Liu M, Zhao M, Ren YH, Gao B, Wang FQ, Wei DZ. The yeast peroxisome: a dynamic storage depot and subcellular factory for squalene overproduction. Metab Eng. 2020;57:151–61.
18. Lv X, Wang F, Zhou P, Ye L, Xie W, Xu H, Yu H. Dual regulation of cyto- plasmic and mitochondrial acetyl-CoA utilization for improved isoprene production in Saccharomyces cerevisiae. Nat Commun. 2016;7:12851.
19. Wang S, Cheng G, Joshua C, He Z, Sun X, Li R, Liu L, Yuan Q. Furfural tolerance and detoxification mechanism in Candida tropicalis. Biotechnol Biofuels. 2016;9:250.
20. Zhang L, Chen Z, Wang J, Shen W, Li Q, Chen X. Stepwise metabolic engineering of Candida tropicalis for efficient xylitol production from xylose mother liquor. Microb Cell Fact. 2021;20:105.
21. Thangavelu K, Sundararaju P, Srinivasan N, Muniraj L, Uthandi S. Simulta- neous lipid production for biodiesel feedstock and desalination of sago processing wastewater using Candida tropicalis ASY2. Biotechnol Biofuels. 2020;13:35.
22. Lu W, Niess JE, Xie W, Zhang X, Minshull J, Gross RA. Biosynthesis of monomers for plastics from renewable oils. J Am Chem Soc. 2010;132:15451–5.
23. Dey P, Maiti MK. Molecular characterization of a novel isolate of Candida tropicalis for enhanced lipid production. J Appl Microbiol. 2013;114:1357–68.
24. Cao W, Li H, Luo J, Yin J, Wan Y. High-level productivity of alpha, omega-dodecanedioic acid with a newly isolated Candida viswanathi strain. J Ind Microbiol Biol. 2017;44:8.
25. Zhang L, Zhang H, Liu Y, Zhou J, Shen W, Liu L, Li Q, Chen X. A CRISPR-Cas9 system for multiple genome editing and pathway assembly in Candida tropicalis. Biotechnol Bioeng. 2020;117(2):531–42.
26. Chattopadhyay A, Mittra M, Maiti MK. Recent advances in lipid metabolic engineering of oleaginous yeasts. Biotechnol Adv. 2021;53:107722.
27. Cao X, Wei LJ, Lin JY, Hua Q. Enhancing linalool production by engi- neering oleaginous yeast Yarrowia lipolytica. Bioreourc Technol. 2017;245:1641–4.
28. Li J, Wu Y, Zhang C, Sun J, Zhou Z, Lu W. Production of triterpene ginse- noid compound K in the non-conventional yeast Yarrowia lipolytica. J Agr Food Chem. 2019;67(9):2581–8.
29. Gao S, Tong Y, Zhu L, Ge M, Zhang Y, Chen D, Jiang Y, Yang S. Iterative integration of multiple-copy pathway genes in Yarrowia lipolytica for heterologous beta-carotene production. Metab Eng. 2017;41:192–201.

30. Luo Z, Liu N, Lazar Z, Chatzivasileiou A, Ward V, Chen J, Zhou J, Stephanoopoulos G. Enhancing isoprenoid synthesis in Yarrowia lipolytica by expressing the isopentenol utilization pathway and modulating intracellular hydrophobicity. Metab Eng. 2020;61:344–51.

31. Zhang LH, Chen XZ, Chen Z, Wang ZZ, Jiang S, Li L, Potter M, Shen W, Fan Y. Development of an efficient genetic manipulation strategy for sequential gene disruption and expression of different heterologous GFP genes in Candida tropicalis. Appl Microbiol Biot. 2016;100(22):9567–80.

32. Dusseaux S, Wajn WT, Liu YX, Ignega C, Campranis SC. Transforming yeast peroxisomes into microfactories for the efficient production of high-value isoprenoids. P Natl Acad Sci USA. 2020;117(50):31789–99.

33. Zhu ZT, Du MM, Gao B, Tao XY, Zhao M, Ren YH, Wang FQ, Wei DZ. Metabolic compartmentalization in yeast mitochondria: Burden and solution for squalene overproduction. Metab Eng. 2021;68:232–45.

34. DeBose-Boyd RA. Feedback regulation of cholesterol synthesis: sterol-accelerated ubiquitination and degradation of HMG CoA reductase. Cell Res. 2008;18(6):609–21.

35. Shimada H, Kondo K, Fraser PD, Miura Y, Saito T, Misawa N. Increased carotenoid production by the food yeast Candida utilis through metabolic engineering of the isoprenoid pathway. Appl Environ Microb. 1998;64(7):2676–80.

36. Curran KA, Morse NJ, Markham KA, Wagman AM, Gupta A, Alper HS. Short synthetic terminators for improved heterologous gene expression in yeast. ACS Synth Biol. 2015;4(4):3824–32.

37. Zhou YJ, Buijs NA, Zhu ZW, Gomez DO, Boonsombut A, Siewers V, Nielsen J. Harnessing yeast peroxisomes for biosynthesis of fatty-acid-derived biofuels and chemicals with relieved side-pathway competition. J Am Chem Soc. 2016;138(47):15368–77.

38. Meadows AL, Hawkins KM, Tsegaye Y, Antipov E, Kim Y, Raetz R, Dahl RV, Tainer J, Liao G, Leng LS, Dasika MS, Murarka A, Lennihan J, Eng D, Leng JS, Liu CL, Bengt JW, Jiang HX, Chao LL, Westfall P, Lai J, Ganesan S, Jackson P, Mans R, Platt D, Reeves CD, Sajja PR, Wichmann G, Holmes BF, Benjamin K, Hill PW, Gardner TS, Tiom AE. Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature. 2016;537:694–7.

39. Wang JH, Jiang W, Liang CJ, Zhu LH, Li YR, Mo Q, Xu S, Chu A, Zhang L, Ding ZY, Shi GY. Overproduction of alpha-farnesene in Saccharomyces cerevisiae by farnesene synthase screening and metabolic engineering. J Agr Food Chem. 2021;69(10):3103–13.

40. Larroude M, Cellinska E, Back A, Thomas S, Malaussena M, Ledesma-Amaro R. A synthetic biology approach to transform Yarrowia lipolytica into a competitive biotechnological producer of beta-carotene. Biotechnol Bioeng. 2018;115(2):464–72.

41. Farhi M, Marhevka E, Masci T, Marcos E, Eyal Y, Ovadis M, Abeliovich H, Vainstein A. Harnessing yeast subcellular compartments for the production of plant terpenoids. Metab Eng. 2011;13(5):474–81.

42. Scalcinati G, Knuf C, Partow S, Chen Y, Maury J, Schall M, Daiwet K, Nielsen J, Sievers V. Dynamic control of gene expression in Saccharomyces cerevisiae engineered for the production of plant sesquiterpenes alpha-santalene in a fed-batch mode. Metab Eng. 2012;14(2):91–103.

43. Friesen JA, Rodwell VW. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. Genome Biol. 2004;5:248.

44. Wang ZZ, Zhang LH, Zhang MQ, Hu SY, Li L, Shen W, Fan Y, Chen XZ. Functional identification of fatty aldehyde dehydrogenase genes CtlA1 and CtlA2 from Candida tropicalis. Acta Microbiol Sin. 2018;19(1):23–31.

45. Zhang HB, Zhang LH, Chen XZ, Shen W, Fan Y. Effect of fatty alcohol oxidase genes disruption on physiological function of Candida tropicalis. Chin J Bioproc Eng. 2021;61:354–60.