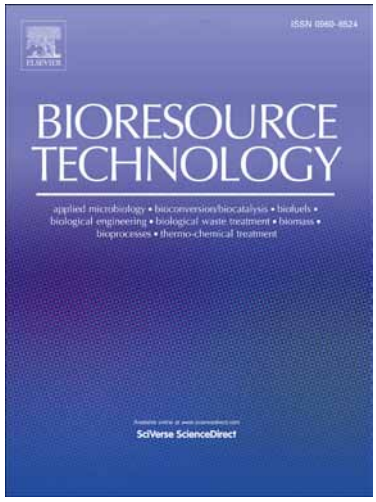

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Heterologous production of α-farnesene in metabolically engineered strains of *Yarrowia lipolytica*

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Abstract

Herein, we studied the heterologous production of α-farnesene, a valuable sesquiterpene with various biotechnological applications, by metabolic engineering of *Yarrowia lipolytica.* Different overexpression vectors harboring combinations of *tHMG1*, *IDI*, *ERG20* and codon-optimized α-farnesene synthase (*OptFS*) genes were constructed and integrated into the genome of *Y. lipolytica* Po1h. The engineered strain produced 57.08 ± 1.43 mg/L of α-farnesene corresponding to 20.8-fold increase over the initial production of 2.75 ± 0.29 mg/L in the YPD medium in shake flasks. Bioreactor scale-up in PM medium led to α-farnesene concentration of 259.98 ± 2.15 mg/L with α-farnesene to biomass ratio of 33.98 ± 1.51 mg/g, which was a 94.5-fold increase over the initial production. This first report on α-farnesene synthesis in *Y. lipolytica* lays a foundation for future research on production of sesquitepenes in *Y. lipolytica* and other closest yeast species and will potentially contribute in its industrial production.

Key words: biofuels; fed-batch; metabolic engineering; terpene synthases; *Yarrowia lipolytica*;

1. Introduction

Sesquiterpenes belong to a large and diverse isoprenoid family with important medical and industrial properties, such as their potent anticancer, antitumor, cytotoxic, antiviral, and antibiotic properties, as well as their characteristic flavors and aromas, and these properties have made them industrially relevant and interesting compounds(Groussin & Antoniotti, 2012). α-Farnesene, one of the simplest acyclic sesquiterpenes, is a compound abundantly located in apple peels with high-functional performance in plant defense (Nieuwenhuizen et al., 2014; Yang et al., 2011). In nature, α-farnesene isomers act as chemical signaling particles that are suggested to have diverse functions in plants and animals, including plant pollination, apple seed dispersal, and predation response to plant herbivores (Kollner et al., 2009; Pechous & Whitaker, 2004). Besides, α-farnesene also works as an alarm pheromone to signal danger in aphids and termites, and as a molecule implicated in the orientation of worker fire ants (Šobotník et al., 2008; Suckling et al., 2010). On the industrial level, α-Farnesene is being developed as a precursor for biofuel production owing to its characteristic low hygroscopicity and high energy density. For instance, α-fanesane, a compound derived from farnesene, is regarded as a potential bio-jet fuel candidate. However, despite their paramount profitable value, sesquiterpenes like α-farnesene are naturally produced in limited and infinitesimal amounts (Janssens et al., 1992; Renninger & Mcphee, 2010). Production of these compounds by plant extraction fails to meet industrial requirements and is often not sustainable or is dependent on seasonal and geographical changes (Howes et al., 2004; Wallaart et al., 2000). In the last decade, efforts of several research groups have been focused on microbial metabolic engineering as an alternative and attractive route for the improved production of these rare and precious compounds. At present, the heterologous

production of α-farnesene has been performed in hosts such as *Escherichia coli* and *Saccharomyces cerevisiae* using exogenous genes cloned from plants such as *Artemisia annua*. It was previously reported that high level of (E)-β-farnesene (23.8 g Farnesene/100 g glucose) can be attained by metabolic engineering of the FPP downstream pathway and the overexpression of farnesene synthase isolated from *A. annua* and enzymes of the mevalonate pathway in *S. cerevisiae* (Chandran et al., 2011). Recently, in the yeast *S. cerevisiae,* an α-farnesene titer of 170 mg/L in fed-batch fermentations with respiratory quotient (RQ)-controlled exponential feeding was achieved using three terpene synthases with distinct plant backgrounds (Tippmann et al., 2015). In *E. coli*, the heterologous expression of α-farnesene synthase from apple peel allowed bacterial production of 1.1 g/L of α-farnesene, while another study showed that only 380 mg/L of α-farnesene was obtained from the same source (Wang et al., 2011; Zhu et al., 2014).

Yarrowia lipolytica, the non-conventional oleaginous yeast of the *Yarrowia* clade, is regarded as a viable candidate platform for the production of terpenoids because of documented genomic, systems biology, genetic engineering, and transcriptomic information of the organism gathered in the last decade (Matthaus et al., 2014; Papanikolaou & Aggelis, 2002; Plácido & Capareda, 2015). In addition, *Y. lipolytica* has many advantages, such as high safety (non-pathogenicity), the ability to use a wide range of substrates, convenient genetic manipulation, and simple industrial scale-up operations (Groenewald et al., 2014). What's more, by using expression plasmid pINA1312, the overexpressed genes can be successively integrated into the yeast chromosome to improve strain stability. Besides, the synthetic promoter hp4d of plasmid pINA1312 for protein expression has a high transcriptional activity and this promoter is irrepressible by carbon or nitrogen sources and peptone is not essential for

its induction (Madzak et al., 2000). More recently, heterologous production of high amounts of lycopene was achieved in *Y. lipolytica*, which reinforced the hypothesis that this microorganism would be effective for the biosynthesis of other terpenoids such as α-farnesene. However, although heterologous expression of sesquiterpene synthase genes in yeast conducts the production of the corresponding sesquiterpenes, the produced amounts are insufficient to support a practical industrial process. Thus, it is essential to increase the supply of precursors for their robust production (Asadollahi et al., 2009; Mirzaee et al., 2016; Ro et al., 2006; Takahashi et al., 2007). α-Farnesene biosynthesis is initiated by the formation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) precursors issued from the mevalonate (MVA) pathway in eukaryotes, with hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and the FPP branch-point as the two main regulation sites in the pathway (Figure 1) (Withers & Keasling, 2007).

The present study was designed to engineer α-farnesene-producing strains of *Y. lipolytica* by cloning and overexpressing an artificial apple α-farnesene synthase gene and biochemically characterizing the effect of the overexpression of genes in the mevalonate pathway on α-farnesene biosynthesis.

2. Materials and methods

2.1. Media and culture conditions

In this study, the auxotrophic *Y. lipolytica* strain Po1h, which was kindly provided by Professor Catherine Madzak (Institut National de la Recherche Agronomique/AgroParisTech, France) along with the plasmid pINA1312 was used as the target gene expression host. Restriction enzymes, DNA polymerase and DNA ligase were both purchased from Takara Bio, Inc (Takara, Dalian, China).

Oligonuleotide primers were obtained from Shanghai Generay Biotech Co., Ltd. (Generay, Shanghai, China). *E. coli* strain JM109 was used as a host for plasmid proliferation and recombinant vectors construction. The cells were grown in Luria-Bertani (LB) complete medium (0.5% yeast extract, 1% tryptone, and 1% NaCl) at 37 ℃ for 12 h. When required, kanamycin (100 μg/ml) was added to the LB solid medium for selection of transformants. YPD and YNB media were prepared for *Y. lipolytica*. The YPD medium (1% yeast extract, 2% peptone, and 2% glucose) was used for strain activation, whereas the synthetic medium YNB (0.67% yeast nitrogen base without amino acids, 1% glucose, and 1.6% agar) lacking the appropriate nutrients was used for the screening of transformants. To harvest α-farnesene in culture broth, 1 mL of dodecane containing an internal standard carypohyllene was primarily overlaid on 50 mL of culture broth. Cell growth was determined by measuring the OD_{600}. All experiments were performed in triplicate.

2.2. Construction of plasmids

The primers used for constructing of plasmids and strains are shown in Table 1. The α-farnesene synthase gene (*FS*) was cloned from apple peels (*Malus x domestica*) cDNA using TRIzols Reagent (Invitrogen) and Reverse Transcriptase M-MLV (RNase H) (Takara). The artificial α-farnesene synthase gene (*OptFS*) was synthesized by Shanghai Generay Biotech Co., Ltd. (Generay, Shanghai, China), according to *Y. lipolytica* codon usage, The truncated Hmg1(*tHMG1*) gene was amplified from *S. cerevisiae* CEN.PK113-5D. Meanwhile, the *ERG20* and *IDI* genes were amplified from *Y. lipolytica* DMS3286. DNA manipulation was performed according to the standard protocols. The multi- but low-copy plasmid pINA1312, carrying the *URA3* gene, which can integrate at random into the genome of host strain. The above-mentioned genes were amplified using 5’ and 3’ primers, respectively,

such that both contained a *BamH1* restriction site; the genes were then ligated to the plasmids using seamless connection method (ClonExpress® II One Step Cloning Kit). To fuse *ERG20* and *OptFS* in two ways, two *ERG20* products and two *OptFS* products were amplified with primers respectively. One of the *ERG20* products with a linker after the gene *ERG20L*was amplified with primers ERG20L-F and ERG20L-R, and the other one with a linker before the gene *LERG20* was amplified with primers LERG20-F and LERG20-R. Similarly, we got two *OptFS* products. After this, these products were cloned into the BamHI sites of pINA1312 overlap extension PCR to generate pINA06 and pINA07 (containing the *ERG20* and *OptFS* with the TAA stop codon removed but a (GSG) linker introduced), respectively. For the final plasmid pFT06 construction, the *StuI-StuI* restriction sites were used for inserting DNA segments from pINA07 into pINA03, partly. Meanwhile, the *EcoRI-EcoRI* DNA fragment from pINA05 was inserted into pINA03 (Figure 2). All newly constructed vectors were screened by PCR and restriction enzyme digestion, and finally verified by DNA sequencing. All primers used in the present study were presented in Table 1. All plasmids constructed and used were listed in Table 1.

2.3. Transformation of Y. lipolytica and selection of α-farnesene -producing clones

The synthetic vectors constructed above, were linearized with *NotI* restriction enzyme, purified by gel recovery, and then transformed into *Y. lipolytica* Po1h cells using the Frozen-EZ Yeast Transformation II^{TM} kit. *Y. lipolytica* transformants were selected on YNB agar plates. The plates were incubated at 30 °C until colonies were visible (2-6 d). Initially, 5 colonies were picked from each plates. The colonies were subsequently incubated in 4 ml of YPD abundant medium for 24 h at 30 ℃ and 220 rpm. The PCR amplification step was used to further investigate the target genes integrating into the

Y. lipolytica chromosomes. All positive recombinant α-farnesene-accumulating strains obtained are presented in Table 1.

2.4. Fermentation for α-farnesene production at flask level

For α-farnesene production, the metabolically engineered *Y. lipolytica* were inoculated into 50 ml of YPD medium for 24 h at 30 °C and 200 rpm. These precultures were then transferred to a 250 ml shake flask containing 50 ml of PM medium (1.474% $MgSO_4 \cdot 7H_2O$, 0.0097% $NiCL_2 \cdot 6H_2O$, 0.0099% $ZnCL_2$, 0.04% $MnSO_4 \cdot 7H_2O$, 0.0012% KI, 0.02% $MnCL_2 \cdot 4H_2O$, 0.01% $Na_2MoO_4 \cdot 2H_2O$, 0.005KNO_3, 0.096% Yeast Extract, 0.051% $(NH_4)_2SO_4$, 1% KH_2PO_4, 0.001% Vitamin B1, 0.287%$FeCL_3$, 1.99% glucose, and 2% fructose); S2 medium (0.05% Yeast Extract, 0.05% $(NH_4)_2SO_4$, 0.15% $MgSO_4 \cdot 7H_2O$ and 3% glucose); KY medium (0.6% KH_2PO_4, 0.05% $(NH_4)_2SO_4$, 0.5% L-Lysine, 0.5% L-Tryptopham, 0.01% Ergosterol, 0.03% Ethanolamine, 0.1% L-Asparagine, 0.1% L-isoleucine, 0.0006% Thiamine, 2% glucose, and 2% fructose); and MP medium (0.6% KH_2PO_4, 0.05% $(NH_4)_2SO_4$, 0.5% L-Lysine, 0.5% L-Tryptophan, 0.01% Ergosterol, 0.03% Ethanolamine, 0.1% L-Asparagine, 1% L-isoleucine, 1% glucose, and 2% fructose) (Nambou et al., 2015; Nambou et al., 2014) in the initial cell OD_{600} of 0.01 and were grown for another 5 days under the same fermentation temperature and agitation speed.

2.5. Batch fermentation for α-farnesene production in the 1.5 L fermenter

Similar to the shake flask cultivations described above, the engineering strain P7 was inoculated in 5 mL of YPD medium and incubated for 24 h at 30 °C and 220 rpm. Cells were then transferred to 100 mL of YPD medium in 250-mL shake flasks and cultivated for 24 h. For α-farnesene production, cells were cultured in a 1.5-L batch

fermenter and 100 ml of the seed culture (OD_{600} nm = 1.0) was inoculated in 1.0 L each of PM medium and YPD medium with 5% dodecane. The fermentation temperature and pH were 30 °C and 7.0, respectively. Meanwhile, the DO was controlled above 50%.

2.6. Identification and quantification of α-farnesene

The dodecane phase of the two-phase culture was collected and centrifuged for 10 min at 12,000 rpm to remove cell debris, and subsequently subjected to GC–MS (gas chromatography–mass spectrometry) to quantify the concentration of produced α-farnesene using an Agilent 19091J–433 GC–MS apparatus (Agilent Technologies, Santa Clara, CA). Samples were injected at a split ratio of 1:20, and 0.4 μL samples were separated on a HP-5 ms column (30.0 m×0.25 mm×0.25 μm) (Agilent Technologies). The oven temperature was initially held at 160 °C for 2 min and was increased at a rate of 15 °C/min to 205 °C, and the post-run time was 5 min to 280 °C. Helium was used as the carrier gas with an inlet pressure of 14 psi. The detector temperature was maintained at 250 °C. Trans-β-farnesene (Sigma–Aldrich, US) was used as the standard compound to construct the standard curve (R240.99) for the estimation of α-farnesene production.

2.7. Analysis of glucose and fructose

The glucose and fructose contents of the medium were determined using an HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a HPX-87H column (Bio-Red, Laboratories, Hercules, CA) and an RI detector. The mobile phase consisted of 5 mM H_2SO_4 with a low rate of 0.6 mL/min at 50 °C.

2.8. Quantitative reverse transcription-PCR (qRT-PCR) studies

The cells were pelleted and total RNA was extracted as previously described (Verwaal et al., 2002). One thousand nanograms of RNA from each sample was used for a reverse transcription reaction with the ReverTra Ace qPCR RT Master Mix (TOYOBO Co., Osaka, Japan). Subsequently, a 2 µl sample from each reaction mixture was used to set up quantitative PCRs (qPCRs) (in triplicate) with SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) using primers described in Supplementary Table 1, with a non-template control. The reactions were performed in a Bio-Rad CFX Fast real-time PCR system.

To prevent from the variation of the starting materials amounts, *ACT1* was conserved and used as a reference gene.

2.9. Statistical analysis

The results are expressed as fractions of gene expression between the target gene (gene of interest) and the housekeeping gene, *ACT1* and data was analyzed via two-way ANOVA with statistical significance fixed for p value and r value using Graphpad software (Pfaffl, 2001).

3. Results and Discussion

3.1. OptFS codon optimization for α-farnesene synthesis in Y. lipolytica

To assess the ability of *Y. lipolytica* to synthesize α-farnesene, we constructed a recombinant vector (pINA01-*FS*) by inserting the *FS* gene isolated from wild-type apple peal into the pINA01 plasmid and transformed it into the host strain Po1h to generate the engineered strain P0. The engineered strain harboring the *FS* gene was cultured in 50 mL of YPD medium with an overlay of 1 mL of dodecane at 30 °C for 120 h after which, biomass and α-farnesene productions were evaluated. The results (Figure 3) showed that the P0 strain was unable to synthesize α-farnesene. This result

was likely due to the inexpression of α-farnesene synthase. To overcome the expression problem, a codon-optimized artificial α-farnesene synthase gene (*OptFS*) was designed and transformed into Po1h to generate the P1 strain. The results (Figure 3) showed that the optimization of the *OptFS* codon allowed the production of 2.75 ± 0.29 mg/L of α-farnesene, undoubtedly due to the improved expression of α-farnesene synthase. These results suggested that *Y. lipolytica* has the potential for α-farnesene synthesis. Nevertheless, the obtained concentration was lower than that reported for the heterologous production of α-farnesene in other species, such as the yeast *S. cerevisiae* and the bacteria *E. coli*. The low relative production of α-farnesene obtained in P1 was probably due to a shortage of essential precursors, such as IPP, DMAPP, and FPP. In fact, in our recent publication, using flux balance analysis, we have improved lycopene production in an engineered strain of *Y. lipolytica* by supplying media components which were screened according to the flux balance analysis (FBA) and other reference data. Subsequently, the Pareto chart was used to analyze the effects of different components on lycopene production to determine the amount and the type of components. The joint effect of all selected components essential for redirecting the carbon flux towards FFP pool realized the enhancement of lycopene production (Nambou et al., 2015). Therefore, we hypothesized that the enhancement of IPP, DMAPP, and FPP synthesis through further metabolic engineering was required for α-farnesene production.

3.2. The overexpression of tHmg1 improves α-farnesene biosynthesis in Y. lipolytica

We examined the effect of tHmg1 overexpression on α-farnesene biosynthesis in *Y. lipolytica.* As shown in Figure 3, we found that the overexpression of *tHMG1* in the P1 background (P2) resulted in a 12% increase in α-farnesene yield leading to α-farnesene production of 6.12 ± 0.32 mg/L (Figure 3). Meanwhile, overexpression of

tHmg1 allowed the enhancement of biomass production and that of the ratio of α-farnesene concentration/biomass production. These observations were in accordance with those of earlier studies, which showed that the overexpression of the catalytic domain of Hmg1 (tHmg1) can lead to an improved production of isoprenoids because Hmg1 regulation conducts proteolysis and overexpression of a truncated form of Hmg1 deficient of the sterol sensing domain (SSD) that evades post-transcriptional control and leads in a constitutively active non-membrane bound form (Scalcinati et al., 2012). In *S. cerevisiae*, it was also reported that one of the major regulatory control points of the MVA pathway is the formation of MVA from HMG-CoA catalyzed by HMG-CoA reductase (Hmg) 1 and 2 with Hmg1 contributing at least 83% of the activity (Zhou et al., 2012).

3.3. Redirection of FPP flux towards α-farnesene biosynthesis

FPP is a key precursor in the biosynthesis of α-farnesene, and high levels of FPP would determine increased production of α-farnesene synthase. In this study, we hypothesized that the fusion of α-farnesene synthase and FPP synthase could increase the production of α-farnesene. Therefore, we co-localized FPP synthase and α-farnesene synthase by in-frame protein fusion and constructed P3 and P4 strains as well as the strain expressing free FPPS and OptFS (P5) to determine the possibility to redirect FPP flux to α-farnesene (Figure 3). The engineered strain P3 produced nearly 30% higher α-farnesene (21.39 ± 0.61 mg/L) compared to that produced by strain P4 (16.50 ± 0.16 mg/L), whereas P5 produced 5.14 ± 0.61 mg/L of α-farnesene, indicating that the recombinant fusion protein may promote the conversion of FPP to α-farnesene. This result suggests that the expression of the FPP synthase–farnesene synthase fusion gene enables P3 to synthesize α-farnesene from IPP and DMAPP with little loss of the FPP intermediate. Physical fusion of the two enzymes optimized the

local concentration and spatial organization of the two enzymatic activities to redirect the metabolic flux toward the product more efficiently than that with free enzymes (Zhou et al., 2012). This may serve as a useful and simple alternative to strategies that employ enzyme-docking stations to achieve similar effects. The relative failure to improve α-farnesene production in P4 was hypothesized to be due to the fact that the *ERG20LOptFS* fusion probably triggered steric encumbrance, which jammed both structural configurations and enzyme activities (Albertsen et al., 2011). Unfortunately, with our current knowledge, the impact of this fusion on the cellular concentration of correctly folded chimeric proteins and the activity of single catalytic sites is not easy to predict, and the success may rely on intrinsic characteristics of the enzymes to be fused. The spatial position of both active sites in the fusion protein is the possible explanation for the enhanced product yields.

3.4. Enhancement of the IPP and DMAPP supply for α-farnesene production

P6 and P7 strains were constructed to enhance the expression of IDI, a rate-limiting enzyme in the MVA pathway. The P6 strain produced approximately 22.41 ± 0.22 mg/L of α-farnesene, which was almost equal to the concentration obtained with the fusion protein expression strain P3. This result suggests that the only overexpression of IDI in the strain P6 without tHmg1 overexpression could not improve α-farnesene production may be due to the shortage of precursors, like mevalonate. Therefore, on this basis, we constructed the strain P7 (Table 1), which resulted in a dramatic increase in α-farnesene production to 57.08 ± 1.43 mg/L (Figure 3) .The synthesis of one molecule of α-farnesene requires two molecules of IPP and one molecule of DMAPP. Overexpression of IDI would result in a compromise between the consumption and formation of IPP and DMAPP. Moreover, P6 and P7 alleviated the growth inhibition observed in P3. This result suggested that the balance between IPP

and DMAPP is of great importance for α-farnesene production. Furthermore, to find out the differences among engineered strains and explain the advantages of the using of *Y. lipolytica* as the host strain, we detect the fatty acid production and content in all the strains (Fig. S1). The results showed that the lipid content declined with the improvement of α-farnesene, which might suggest that the decreases of lipid content were favorable for the improvement of α-farnesene product in engineered strains. Although a marked increase in α-farnesene production was attained in this study, FPP accumulation suggested inefficiency in the bottom portion of the MVA pathway that needs to be reprogrammed. Farnesol and squalene are two main competitive products of the FPP pool, the precursor for farnesene biosynthesis (Albertsen et al., 2011; Du et al., 2014). We have previously tried to detect extracellular and intracellular farnesol by GC-MS. Surprisingly, farnesol was not detected in the engineered strain, indicating that the intracellular FPP pool might be not sufficient to produce farnesol, an unnecessary metabolite for the growth of *Y. lipolytica*. (Faulkner et al., 1999). Therefore, future goals include identifying the bottleneck in this pathway and maximizing the supply of IPP, DMAPP, and FPP. Furthermore, optimizing for α-farnesene production by repression of the *ERG9* gene could be utilized (Asadollahi et al., 2010). The main effect of *ERG9* gene repression may be a substantial increase in sesquiterpene production.

3.5. Gene expression analysis in engineered strains

In order to confirm that the observed difference on α-farnesene production was indeed related to the transcriptional level of the overexpressed genes as well as the possible relevance of MVA pathway, qPCR studies were also performed (Figure 4). The expression level of *OptFS* gene was increased following the increase in the number of successive overexpression of genes in the biosynthetic pathway, which was in

agreement with corresponding increased α-farnesene production. We found that transcriptional level of *tHMG1*, *ERG20*, and *IDI* genes were relatively high in corresponding engineered strains, indicating that the overexpression of these genes resulted in high α-farnesene production and that reactions catalyzed by the above genes might be the limiting steps during the biosynthetic pathway. Furthermore, the MVA pathway was significantly enhanced with these genetic modifications. Pearson correlation analysis (Supplementary Table 2 and Supplementary Table 3) revealed that *IDI* gene had a significant impact on *OptFS* gene and little effect on *tHMG1* gene. As terpenoids synthesis metabolic pathway, MVA pathway was correlated with the overexpressed genes, whereas *ERG20* and *IDI* genes were not found as the main factors affecting genes in MVA pathway.

3.6. α-Farnesene Production in Shake Flask Cultivations

In our previous studies, we have designed cost-effective culture media from *Y. lipolytica* for the production of lipid (basal S2 medium) and terpenoids (MP and KY media based on the FBA of genome-scale metabolic model network, and PM medium designed using Placket-Burman statistical design) production from *Y. lipolytica* (Nambou et al., 2015; Nambou et al., 2014). In the present study, we found that the engineered strains were able to accumulate α-farnesene when cultivated in these media. After 5 days of cultivation, the best engineered strain, P7, produced the highest concentration of α-farnesene compared with the other strains (Table 2). However, the production was still lower than in the YPD medium (Figure 3). The components in the modelling-assisted designed media might affect the intracellular chemical reactions and activate genes of the farnesene backbone, which might lead to different farnesene production in different media. Particularly, the production of α-farnesene by P7 was 43.78 ± 0.43 mg/L, and the α-farnesene to biomass ratio had increased up to 4.52 ±

0.33 mg/g with almost 6.5 g/L of residual glucose and 17.6 g/L of residual fructose (data not shown) in the PM medium in shake flasks. Meanwhile, we discovered that all the designed media had low glucose and fructose consumption.

3.7. Fed-Batch Fermentation of Y. lipolytica for α-Farnesene Production on Designed Media PM

In view of the results obtained in flaks cultivation using various media, we performed scale-up experiments in a 1.5 L fermenter with a working volume of 1 L in batch mode using the PM medium for cultivating of the engineered strain P7. The results of biomass and α-farnesene production over the fermentation course are shown in Figure 5. After 5 days of fermentation, we found a maximum biomass production of 7.65 ± 1.40 g/L with an α-farnesene concentration of 259.98 ± 13.81 mg/L from the PM medium with almost zero residual sugar content. The engineered strain could hardly consume fructose before 48 h due to carbon catabolite repression in the presence of glucose. In the YPD medium, a biomass of 10.08 g/L and an α-farnesene concentration of 43.45 mg/L were obtained after 3 days of fermentation, because the glucose was consumed so quickly that the cells were unable to grow well and produce more α-farnesene (data not shown). In particular, the insufficiency in the amount of glucose and fructose was a limitation for the engineered strain to grow well and produce more α-farnesene, thus the fed-batch fermentation might be a good method to yield a better result. The present findings show the PM medium might be suitable for large scale production of α-farnesene and further support our previous publication based on the designing of culture media using FBA for lycopene production (Nambou et al., 2015).

4. Conclusions

In summary, we have demonstrated for the first the feasibility of producing α-farnesene in metabolically engineered *Y. lipolytica*. The final α-farnesene production of 33.98 mg/g DCW (7.65 g/L) achieved in this study is the highest ever reported from an engineered yeast strain, demonstrating the efficiency of this combinational strategy of culture media optimization and metabolic engineering for the construction of unimpeded biosynthetic pathways. This study can serve as a foundation for the construction of much more robust strains and fermentation platforms for sesquiterpene production in the future.

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Figure Captions

Figure 1. Production of farnesene from glucose in *Y. lipolytica*. Isoprenoids are synthesized via the mevalonate pathway from cytosolic acetyl-CoA. The mevalonate pathway comprises seven steps and yields the isomers IPP and DMAPP. Condensations of the two isomers form the precursors for mono-, sesqui- or longer chain terpenoids. In the last step, FPP is converted by α-farnesene synthase to yield α-farnesene. Enzymes denote engineering targets used in this study with tHmg1: truncated HMG-CoA reductase, Erg20: FPP synthase, IDI: IPP isomerase, OptFS: α-farnesene codon-optimized α-farnesene synthase.

Figure 2. Design for construction of the plasmid pFT06. The pFT06 plasmid containing *tHMG1* and *OptFSLERG20* and *IDI* genes. The above-mentioned genes were amplified using 5' and 3' primers, respectively, such that both contained a *BamH1* restriction site; the genes were then ligated to the plasmids using seamless connection method. And then the *StuI-StuI* DNA fragment from pINA07 and the *EcoRI-EcoRI* fragment from pINA05 were inserted into pINA03 to generate pFT06 vector.

Figure 3. Comparison of yeast strains carrying different genes in shake flask cultivation. *Y. lipolytica* strain Po1h was transformed with plasmids pINA1312, pINA01, pINA02, pFT01, pFT02, pFT03, pFT04, pFT05, and pFT06 to compare α-farnesene production and to study the effect of truncated HMG-CoA reductase tHmg1, Erg20, and IDI. Cells were cultivated for 120 h and α-farnesene was extracted using a dodecane overlay. Values represent average concentrations normalized to the total culture volume of three biological replicates with standard deviation.

Figure 4. Quantitative PCR (qPCR) results for the determination of overexpression of biosynthetic genes and MVA pathway genes in eight engineered α-farnesene -producing yeast strains (P0-P7) and wild type strain Po1h and Po1h (pINA1312) strain with pINA1312 plasmid. *OptFS* represents α-farnesene synthase gene, *tHMG1* represents truncated HMG-CoA reductase gene, *ERG20* represents FPP synthase gene, *IDI* represents IPP isomerase gene, *YALI0F30481g* represents HMG-CoA synthase gene, *YALI0E04807g* represents HMG-CoA reductase gene, *YALI0B16038g* represents mevalonate kinase gene, *YALI0E06193g* represents phosphomevalonate kinase gene and *YALI0F05632g* represents MVA-diphosphate kinase decarboxylase gene. Error bars embody standard deviation among the average of two distinct reiterated experiments.

Figure 5. Fed-batch cultivation of the engineered strain, P7, for α-farnesene and biomass production in a 1.5-L fermenter at 30 °C and DO ≥ 50% using the designed medium (PM). The pH was controlled at 7.0. The aeration rate was fixed at 0.6 vvm (volume air per volume per minute) and the agitation rate was set to automatically respond to the dissolved oxygen (DO) concentration to maintain it at ≥ 50%. Error bars embody standard deviation among the average of two distinct reiterated experiments.

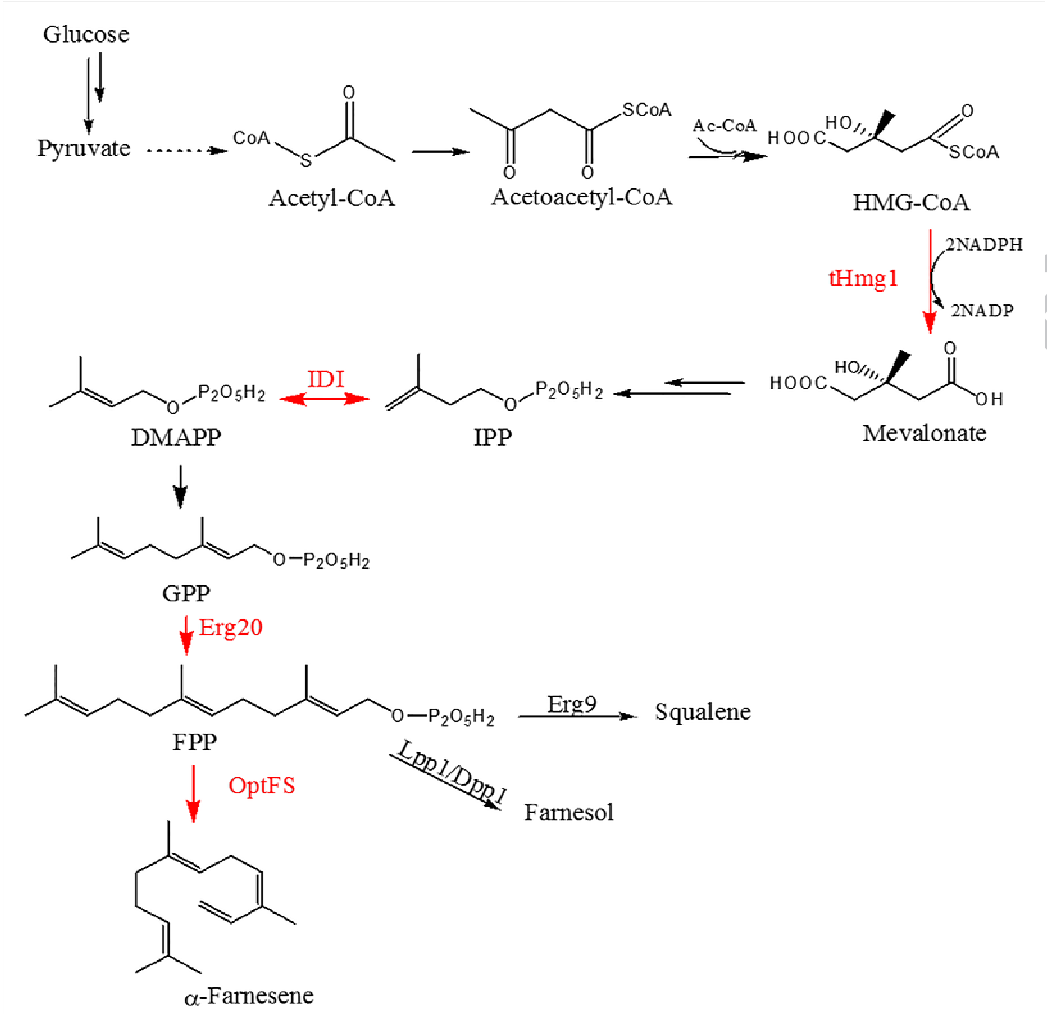

Fig. 1

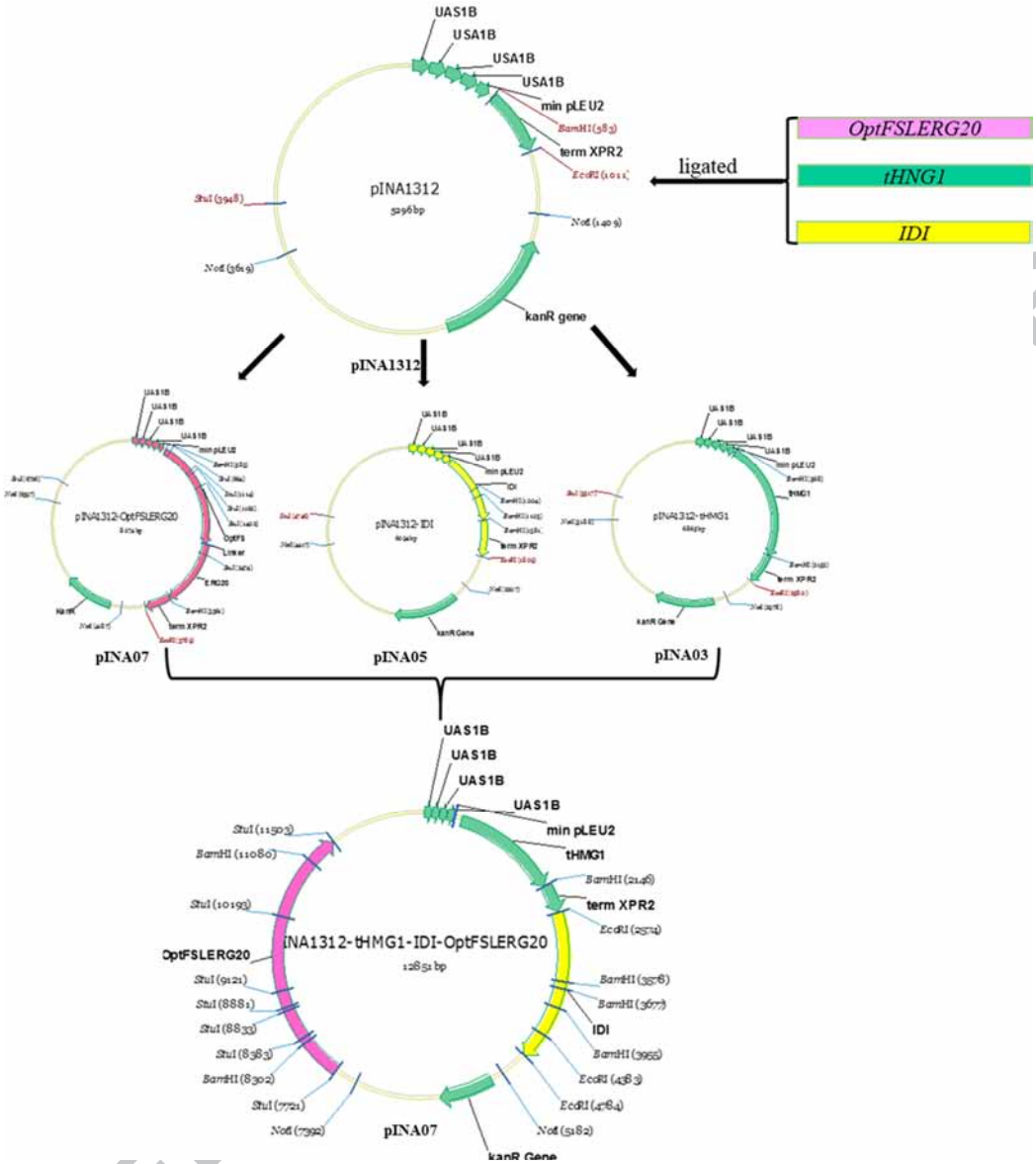

Fig. 2

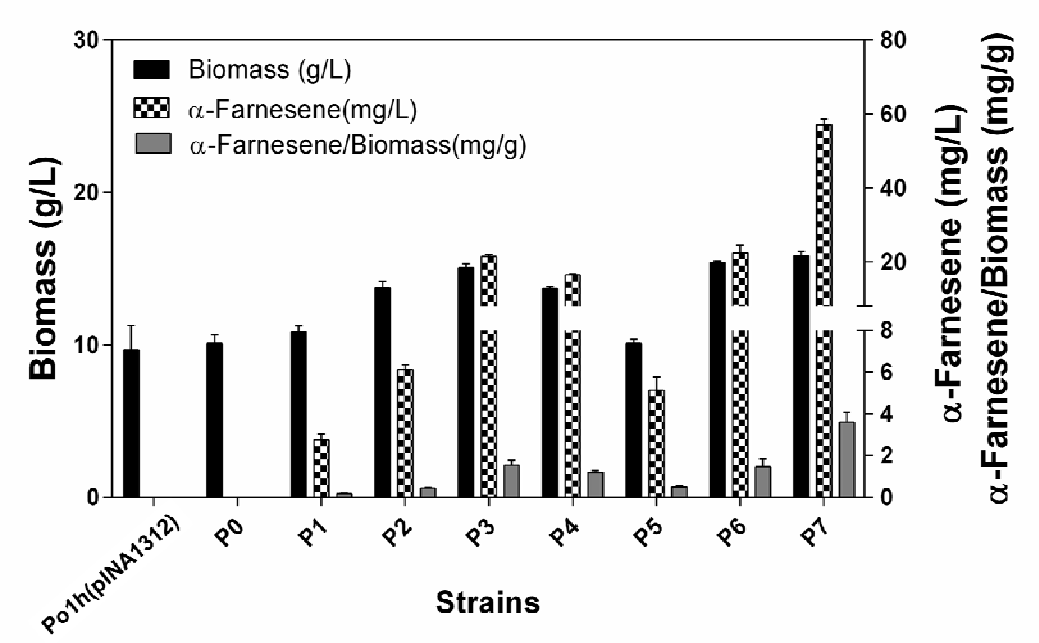

Fig. 3

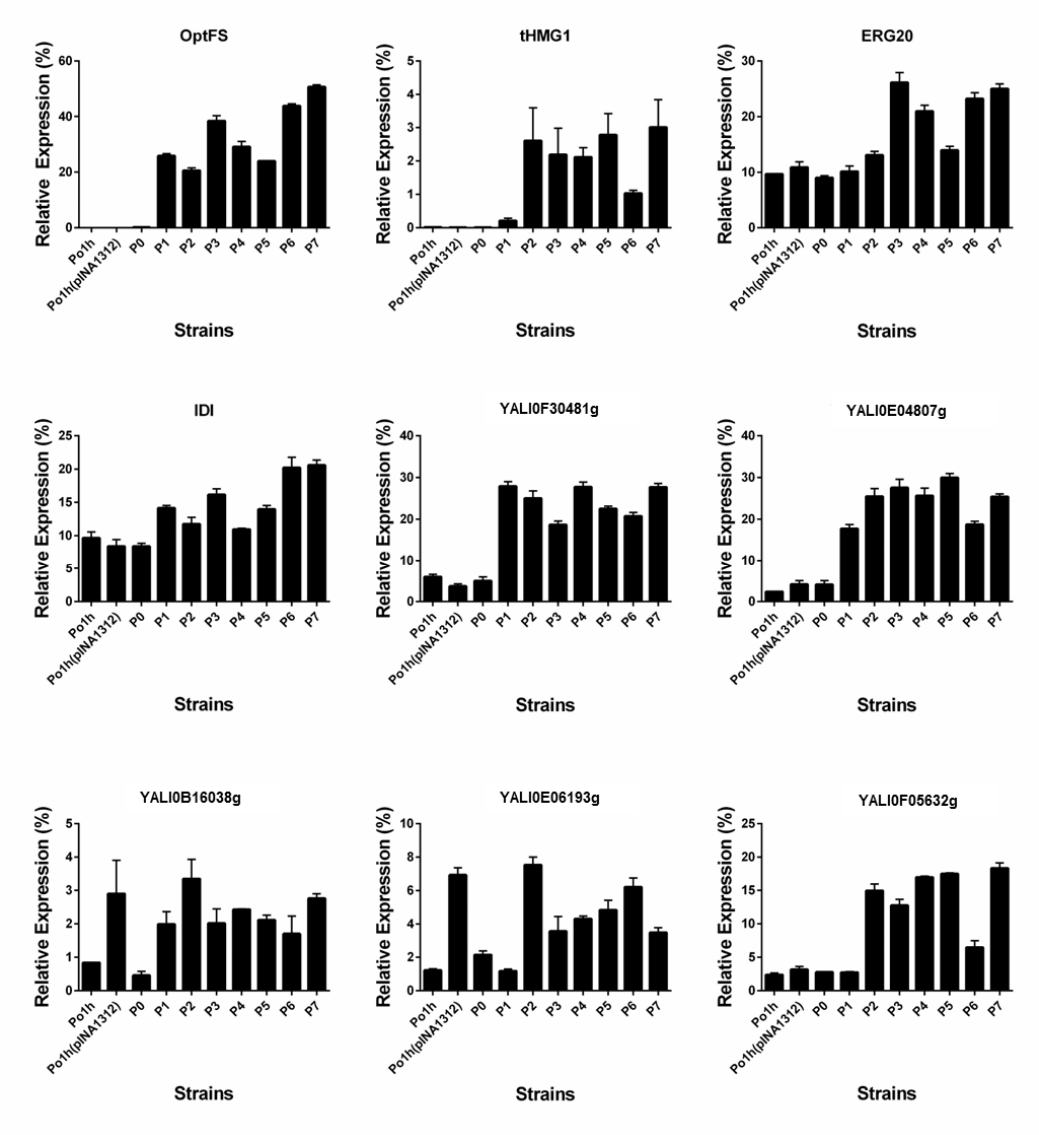

Fig. 4

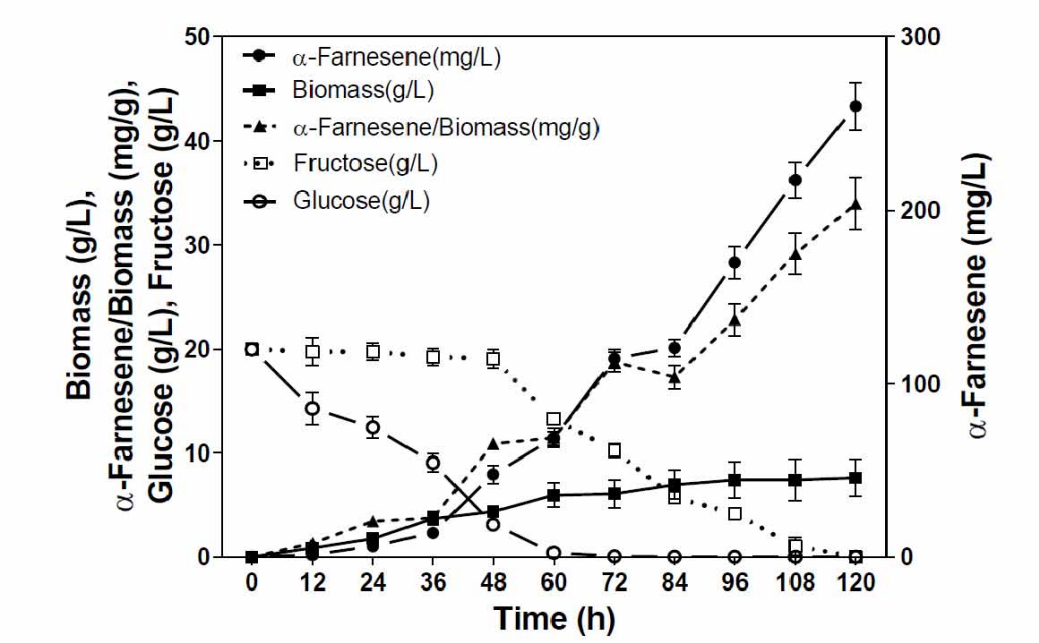

Fig. 5

Table 1. Primers, plasmids and strains used in this study.

Name	Description	Reference
Primers		
FS-F	GGAACCCGAAACTAAGGATCCATGGAATTCAGAGTTCACTT	This study
FS-R	GGCCATGGAGGTACCGGATCCCTAGTTTACAAGAGGTTGGA	This study
OptFS-F	GGAACCCGAAACTAAGGATCCATGGAGTTCCGAGTCCACCT	This study
OptFS-R	GGCCATGGAGGTACCGGATCCTTAGTTGACGAGGGGCTGGA	This study
tHMG1-F	ACAACCACACACATCGGATCCATGGACCAATTGGTGAAAAC	This study
tHMG1-R	GGCCATGGAGGTACCGGATCCTTAGGATTTAATGCAGGTGA	This study
ERG20-F	GGAACCCGAAACTAAGGATCCATGTCCAAGGCGAAATTCGA	This study
ERG20-R	GGCCATGGAGGTACCGGATCCCTACTTCTGTCGCTTGTAAA	This study
OptFSL-F	GGAACCCGAAACTAAGGATCCATGGAGTTCCGAGTCCACCT	This study
OptFSL-R	TTCGAATTTCGCCTTGGACATACCAGAACCGTTGACGAGGGGC	This study
LERG20-F	AGCCCCTCGTCAACGGTTCTGGTATGTCCAAGGCGAAATTCGAA	This study
LERG20-R	GGCCATGGAGGTACCGGATCCCTACTTCTGTCGCT	This

	TGTAAA	study
ERG20L-F	GGAACCCGAAACTAAGGATCCATGTCCAAGGCGA AATTCGA	This study
ERG20L-R	CTTGAAGGTGGACTCGGAACTCCATACCAGAACCC TTCTGTCGCTTGT	This study
LOptFS-F	CAAGCGACAGAAGGGTTCTGGTATGGAGTTCCGA GTCCACCT	This study
LOptFS-R	GGCCATGGAGGTACCGGATCCTTAGTTGACGAGGG GCTGGA	This study
IDI-F	ACAACCACACACATCCACGTGATGACGACGTCTTA CAGCGA	This study
IDI-R	GGCCATGGAGGTACCGGATCCCTACTTGATCCACC GCCGAA	This study
Plasmids		
pINA1312	*Y.lipolytica* integrative plasmid, hp4d promoter, *XPR2* terminator, *ura3d1* selection marker, Km^R	(Nicaud et al., 2002)
pINA01	pINA1312 vector containing *FS* from *Malus x domestica*	This study
pINA02	pINA1312 vector containing codon-optimized *FS*(*OptFS*)	This study
pINA03	pINA1312 vector containing *tHMG1* from *Saccharomyces cerevisiae*	This study
pINA04	pINA1312 vector containing *ERG20* from *Y.lipolytica*	This study
pINA05	pINA1312 vector containing *IDI* from *Y.lipolytica*	This study
pINA06	pINA1312 vector containing a fusion gene of *ERG20* and *OptFS* with a (GSG) linker between *ERG20* and *OptFS* (*ERG20LOptFS*)	This study
pINA07	pINA1312 vector containing a fusion gene of *OptFS* and	This

	ERG20 with a (GSG) linker between *OptFS* and *ERG20* (*OptFSLERG20*)	study
pFT01	pINA1312 vector containing *tHMG1* and *OptFS*	This study
pFT02	pINA1312 vector containing *tHMG1* and *OptFSLERG20*	This study
pFT03	pINA1312 vector containing tHMG1 and ERG20LOptFS	This study
pFT04	pINA1312 vector containing *tHMG1* and *ERG20* and *OptFS*	This study
pFT05	pINA1312 vector containing *OptFSLERG20* and *IDI*	This study
pFT06	pINA1312 vector containing *tHMG1* and *OptFSLERG20* and *IDI*	This study
pINA1312	*Y.lipolytica* integrative plasmid, hp4d promoter, *XPR2* terminator, *ura3d1* selection marker, Km^R	(Nicaud et al., 2002)
pINA01	pINA1312 vector containing *FS* from *Malus x domestica*	This study
pINA02	pINA1312 vector containing codon-optimized *FS*(*OptFS*)	This study
pINA03	pINA1312 vector containing *tHMG1* from *Saccharomyces cerevisiae*	This study
pINA04	pINA1312 vector containing *ERG20* from *Y.lipolytica*	This study
pINA05	pINA1312 vector containing *IDI* from *Y.lipolytica*	This study
pINA06	pINA1312 vector containing a fusion gene of *ERG20* and *OptFS* with a (GSG) linker between *ERG20* and *OptFS*(*ERG20LOptFS*)	This study
pINA07	pINA1312 vector containing a fusion gene of *OptFS* and *ERG20* with a (GSG) linker between *OptFS* and *ERG20*(*OptFSLERG20*)	This study
pFT01	pINA1312 vector containing *tHMG1* and *OptFS*	This study

pFT02	pINA1312 vector containing *tHMG1* and *OptFSLERG20*	This study
pFT03	pINA1312 vector containing tHMG1 and ERG20LOptFS	This study
pFT04	pINA1312 vector containing *tHMG1* and *ERG20* and *OptFS*	This study
pFT05	pINA1312 vector containing *OptFSLERG20* and *IDI*	This study
pFT06	pINA1312 vector containing *tHMG1* and *OptFSLERG20* and *IDI*	This study
Strains		
Po1h	CLIB882：Ura−，△AEP，△AXP，Suc+	(Barth, 1996)
Po1h(pINA1312)	Po1h-pINA1312	This study
P0	Po1h-pINA1312-*FS*	This study
P1	Po1h-pINA1312-*OptFS*	This study
P2	Po1h-pINA1312-*tHMG1-OptFS*	This study
P3	Po1h-pINA1312-*tHMG1-OptFSLERG20*	This study
P4	Po1h-pINA1312-*tHMG1- ERG20LOptFS*	This study
P5	Po1h-pINA1312-*tHMG1-OptFS-ERG20*	This study
P6	Po1h-pINA1312-*OptFSLERG20-IDI*	This study

P7	Po1h-pINA1312-*tHMG1-OptFSLERG20-IDI*	This study

Table 2. Effect of the different mediums (KY, MP, PM, and S2) on α-farnesene production and cell growth of the engineered strain.

Strains	α-Farnesene (mg/L)				Biomass (g/L)				α-Farnesene/ Biomass (mg/g)			
Medium	KY	MP	PM	S2	KY	MP	PM	S2	KY	MP	PM	S2
P1	1.3±0.1	0.8±0.7	3.6±0.5	1.5±0.1	2.2±0.2	1.6±0.2	9.8±0.3	4.4±0.2	0.6±0.1	4.4±0.3	0.4±0.0	0.3±0.0
P2	3.1±0.2	2.7±0.4	10.8±0.1	3.8±0.3	2.7±0.1	2.0±0.1	9.6±0.3	4.6±0.1	1.1±0.1	1.4±0.1	1.1±0.1	0.8±0.1
P3	14.5±0.5	15.2±2.1	29.2±1.4	13.8±2.6	1.4±0.2	2.1±0.4	8.4±0.4	4.5±0.1	10.6±0.3	7.2±0.5	3.5±0.3	3.1±0.3
P6	14.5±0.4	15.2±2.0	31.5±2.6	13.1±0.1	2.8±0.4	2.2±0.4	9.1±0.4	4.7±0.1	5.2±0.3	7.0±0.5	3.5±0.5	2.8±0.5
P7	22.0±2.5	18.5±3.0	43.8±4.3	25.4±3.6	2.9±0.1	2.1±0.4	9.7±1.6	4.2±0.2	7.7±0.5	8.9±0.7	4.5±0.5	6.0±0.5

Highlights

- Metabolic engineering of *Y. lipolytica* guaranteed α-farnesene production.
- Fusing *ERG20* and *OptFS* improved α-farnesene production.
- Overexpressing *tHMG1*, IDI and *OptFS-ERG20* fusion led to a high α-farnesene yield.
- Batch fermentation showed feasibility for large scale α-farnesene production.