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ORIGINAL ARTICLE

Boosting Geranyl Diphosphate Synthesis for Linalool Production in Engineered *Yarrowia lipolytica*

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Abstract

Linalool is a pleasant-smelling monoterpenoid widely found in the essential oils of most flowers. Due to its biologically active properties, linalool has considerable commercial potential, especially in the food and perfume industries. In this study, the oleaginous yeast *Yarrowia lipolytica* was successfully engineered to produce linalool de novo. The (S)-linalool synthase (*LIS*) gene from *Actinidia argute* was overexpressed to convert geranyl diphosphate (GPP) into linalool. Flux was diverted from farnesyl diphosphate (FPP) synthesis to GPP by introducing a mutated copy of the native $ERG20^{F88W-N119W}$ gene, and *CrGPPS* gene from *Catharanthus roseus* on its own and as part of a fusion with *LIS*. Disruption of native diacylglycerol kinase enzyme, *DGK1*, by oligo-mediated CRISPR-Cas9 inactivation further increased linalool production. The resulting strain accumulated 109.6 mg/L of linalool during cultivation in shake flasks with sucrose as a carbon source. *CrGPPS* expression in *Yarrowia lipolytica* increased linalool accumulation more efficiently than the $ERG20^{F88W-N119W}$ expression, suggesting that the increase in linalool production was predominantly influenced by the level of GPP precursor supply.

Keywords Linalool · Geranyl diphosphate synthase · *Yarrowia lipolytica* · Metabolic engineering · Monoterpenoids

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Introduction

Linalool is a terpene alcohol with a pleasant scent, found in the majority of popular essential oils [1]. Linalool has been demonstrated to have antibacterial, antifungal, anticancer, anti-inflammatory, and antioxidant properties, making it interesting for both pharmaceutical and cosmetic applications [2]. This volatile component is also a precursor in the biosynthesis of vitamin E [3]. The most popular use of linalool is as a fragrance, where the proportion of linalool among terpenoids in floral fragrances is up to 70% [4]. The two enantiomers of linalool possess differing odor characteristics. For one, the odor threshold value of the (R)-enantiomer is about nine times less than that of (S)-linalool [5, 6]. Furthermore, (R)-linalool has a smell reminiscent of lavender, while (S)-linalool has a citrus aroma [7]. Global linalool consumption exceeded 11,000 metric tons in 2018, and its global market is projected to reach $12.3 billion in 2024 [8].

At present, several microorganisms have been used for heterologous biosynthesis of linalool, such as *Pantoea ananatis*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Yarrowia lipolytica* [9–12]. Nitta et al. achieved the highest titer of linalool to date using a *P. ananatis* strain constructed a genetically modified strain by damaged endogenous crtEXYIB-crtZ operon responsive to carotenoid synthesis, knocked out glucose dehydrogenase *GCD* knocked out, and several heterologous genes overexpressed. Specifically, the authors introduced acetyl-CoA acetyltransferase/hydroxymethyl-CoA reductase (mvaE) and hydroxymethylglutaryl-CoA synthase (mvaS) from *Enterococcus faecalis*, mevalonate kinase *M. paludicola* (MVK), linalool synthase from *Actinidia arguta* (LIS), and linalool synthase (LIS) fused with halophilic β-lactamase from *Chromohalobacter* sp. 560 with hexahistidine at the N-terminus, which increases the solubility of the enzyme in the host organism [9]. This strain in dual-phase fed-batch fermentation produced linalool at a concentration of 10.9 g/L. In a previous study, *A. arguta* LIS was found to support the highest of 16 screened enzymes for (S)-linalool production in *P. ananatis* [13]. However, *P. ananatis* is pathogenic on a broad range of plant hosts as well as humans and so is not suitable for industrial production [14].

Y. lipolytica is a non-conventional oleaginous yeast that is generally recognized as safe (GRAS) [15]. It can accumulate high levels of neutral lipids and therefore both efficiently synthesize a massive amount of acetyl-CoA as the precursor of the acyl group in lipids, and support strong regeneration of NADPH [16]. The mevalonate (MVA) pathway for the synthesis of terpenoid begins with the condensation of two molecules of acetyl-CoA [17] and so *Y. lipolytica* is considered an attractive host for terpenoid biosynthesis [18, 19]. Nevertheless, the highest described linalool titer in *Y. lipolytica* was only 5.34 mg/L in YPD medium and 6.96 mg/L in YP medium supplemented with citrate and pyruvate in shake flasks [12].

The standard approach in metabolic engineering for bioproduction is to increase the supply of product precursors. The reaction catalyzed by 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG1) is irreversible and HMG1 is considered to be the key rate-limiting enzyme in the MVA pathway [20]. In *Y. lipolytica*, almost all terpenoid production efforts have used *HMG1* overexpression to improve terpenoid production [21]. Similarly, *HMG1* overexpression showed a significant (4.7-fold) increase in linalool production by *Y. lipolytica* [12]. The overexpression of both *HMG1* gene and the mevalonate kinase-encoding gene *ERG12* showed a 7.6-fold increase in linalool titer. The five-carbon universal terpenoid building blocks, isopropyl diphosphate (IPP), and its allylic isomer dimethylallyl diphosphate (DMAPP) can be ligated in a head-to-tail manner to form geranyl diphosphate

(GPP) via geranyl diphosphate synthase (GPPS) [21]. GPP must then be converted to linalool. However, the native *Y. lipolytica* enzyme ERG20 converts IPP and DMAP into both GPP and the side product FPP. Mutating *ERG20* to $ERG20^{F88W-N119W}$ allows GPP synthase activity while removing this side activity [22].

In this study, (S)-linalool synthase from *Actinida argute* was expressed in *Y. lipolitica*. Markerless gene integration and deletion was performed using a CRISPR-Cas9-based toolkit [23]. *DGK1* knockout, overexpression of GPPS from *Catharanthus roseus* (CrGPPS) by itself and in fusion with LIS in a *Y. lipolytica* strain with an optimized mevalonate pathway, resulted in the best titer yet reported (109.6 mg/L) for *Y. lipolytica*.

Materials and Methods

Strains and Culture Media

E. coli strain XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)]) was used for cloning and plasmid propagation. Cells were grown at 37 °C with constant shaking on 5 mL LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl), and ampicillin (100 µg/mL), chloramphenicol (120 µg/mL), or spectinomycin (50 µg/mL) were added for plasmid selection.

Y. lipolytica strains used in this study are derived from the wild-type *Y. lipolytica* W29 (ATCC 20460) strain. All the strains used in this study are listed in Supplementary Table S1. Rich media were based on YP containing 10 g/L yeast extract, 10 g/L bacto-peptone. YPsuc contained YP with 20 g/L sucrose. If necessary, nourseothricin (Nat, 250 µg/mL) was added to the media at a concentration of 250 µg/mL. Solid media for *E. coli* and *Y. lipolytica* were prepared by adding 20 g/L agar.

Construction of Backbone Vectors

The constructed plasmids and primers used in this study are presented in Supplementary Tables S2 and S3. All synthetic genes were codon-optimized for *Y. lipolytica* using the GenSmart™ Codon Optimization webtool (GenScript) and ordered from Twist Bioscience. The DNA sequences are presented in Supplementary Table S5. All described plasmids were assembled using standard cloning strategies [24], Gibson assembly [25], Golden Gate Assembly, and *E. coli* recombineering [23]. Enzymes for molecular biology were obtained from Thermo Fisher Scientific. The genes, homologous arms for chromosomal integration, promoters, and terminators were amplified using Phusion High-Fidelity DNA polymerase. All assembled products were verified by sequencing both strands.

The set of backbone plasmids for integration into IntA2, IntB1, IntD4, and IntF14 loci contains *Y. lipolytica* homology arms (IntUp and IntDn), promoters P_{TEFin} and P_{EXP1}, terminators T_{TEF} and T_{XPR2}, autonomously replicating sequence (ARS), and hygromycin (*Hyg*) resistance marker. The plasmids were assembled using Gibson assembly from seven fragments (Supplementary Table S3). All PCR-products were amplified from genomic DNA of W29 strain, except the chloramphenicol resistance gene (Cm), which was amplified from pMW-att-Cm [26] using primers Oligo21 and Oligo22, and the $ERG20^{F88W-N119W}$ gene, which was amplified from synthetic fragment $ERG20^{F88W-N119W}$ (Supplementary Table S5) using primers Oligo31 and Oligo32.

The set of backbone plasmids for integration into IntC2, IntC3, IntC13, IntC14, IntE12, and IntE16 loci contains *Y. lipolytica* homology arms (IntUp and IntDn) (Supplementary Table S5) and does not contain ARS or *Hyg* elements. Plasmids pT-flIntE12-LIS, pT-flIntE16-LIS, and pT-IntC14-LIS containing codon optimized synthetic gene encoding *A. argute* LIS (GenBank ID: GQ338153.1) and plasmid pT-IntC3-CrGPPS containing a truncated version (Met_{100}–CrGPPS) of codon optimized *CrGPPS* gene encoding *C. roseus* CrGPPS (GenBank ID: JX417185.1) were synthesized by Twist Bioscience. The assembly of plasmids for integration into IntC2 and IntC13 was performed by Golden Gate assembly [27]. To obtain the pC13US1.1-P_{TEF}-Rad52-T_{LIP} expression plasmid harboring codon optimized *ScRad52* gene encoding *S. cerevisiae* Rad52 (GenBank ID: CAA86623.1), Golden Gate assembly was used (Supplementary Table S3, S5).

Strain Construction

Y. lipolytica strains were engineered with a markerless CRISPR-Cas9 system. Guide sequences (sgRNA) and Cas9 proteins were expressed from an episomal pCasNA-series plasmid [23] (Supplementary Table S3) that was mixed with a repair cassette containing transcription unit or units flanked by arms homologous to the targeting chromosomal region. Summarized data about 10 integration loci, homology arms, and integration efficiency of the repair cassettes is presented in Supplementary Table S4. Yeast strains were transformed using a DTT/lithium acetate method [23]. All transformants were selected on agar YPsuc medium containing 250 µg/mL of nourseothricin. Six transformants per plate were tested by PCR for verification of transcription unit integration using primer sets (Int-chr-F and Int-chr-R) presented in Supplementary Table S2. Removal of an episomal pCasNA was performed by streaking the strain on agar YPsuc medium to get single colonies followed by the spot test on the media with and without nourseothricin. After the marker recovery procedure was completed, the marker-free integration cycle was restarted.

The 120 bp double-stranded oligos for *DGK1* and for *PAH1* genes knockout were obtained by annealing pairs of complementary single-stranded oligos Oligo35/Oligo36 and Oligo33/Oligo34 respectively, as described in oligonucleotide annealing protocol (https://www.sigmaaldrich.com/RU/en/technical-documents/protocol/genomics/pcr/annealing-oligos) (Supplementary Table S2). The resulting repair fragments for the *DGK1* or *PAH1* genes were co-transformed with helper plasmids pCNR-sgDGK1d/pCNR-sgDGK1f or pCNR-sgPAH1a/pCNR-sgPAH1b, respectively, into *Y. lipolytica* strains VKPM Y-3178, G0, G1, and G8 (Supplementary Table S1). Summarized data on efficiency of genes inactivation are presented in Table 1.

Table 1 The efficiency of oligo-mediated CRISPR-Cas9 *DGK1* and *PAH1* genes inactivation

Gene	*Strain*		
	W29	W29 Δ*ku70*	W29 *Rad52*
DGK1	0%	100%	67%
PAH1	0%	50%	17%

Culture Conditions

Strains were initially grown in 5 mL YPsuc medium in 50-mL test tubes and cultured overnight at 30 °C. For test tube cultivation, 10 mL of YP with sucrose 90 g/L was inoculated by the cell suspension to an OD_{600} of 0.2. The cultivation was run for 5 days at 28 °C and 275 rpm. One milliliter of isopropyl myristate (IPM) (ITW Reagents, Germany) was added to the test tubes at 48 h of cultivation. For shake-flask cultivation, 50 mL of YP with sucrose 90 g/L was inoculated to an OD_{600} of 0.2 and the cultured for 7 days at 28 °C and 275 rpm. Five milliliters of IPM was added to the test flasks at 48 h of cultivation.

Linalool Measurement

The culture broth was collected, and the cells were precipitated at 3841 × g for 5 min. The organic phase was taken for GC/MS analysis. The cell pellets were washed twice with ethanol and water (1:1 v/v) and centrifuged at 3841 × g for 5 min and lyophilized by a FreeZone 6 Plus system (Labconco, USA). Lyophilized cells were used for the dry-weight measurements (DCW). The concentrations of linalool in organic phase were determined using an GC/MS system on a "Maestro" (Interlab) (Agilent 7820A GC and 5977 MSD, Agilent Technologies). The carrier gas was helium. The oven temperature was maintained at 70 °C for 2 min after injection and then programmed at 15 °C/min to 260 °C, then at 30 °C/min to 320 °C and holding at 320 °C for 1 min. Sample injection was conducted with 1/5 split carrier gas. Registration of mass spectra was performed in full scanning mode of 35–300 AU masses. Evaporator temperature was 270 °C. Interface temperature kept 280 °C, quadrupole temperature was 150 °C, and source was 230 °C. Electron impact ionization, lionization energy was 70 eV. Injected sample volume was 1 µL. Twenty microliters of isopropyl myristate extract sample was added to 130 µL of hexane (dilution by a factor of 7.5).

Sugar Measurement

Supernatants obtained after removal of the cell pellets were used for analysis. The concentrations of glucose, sucrose, and fructose were determined using an HPLC system Dionex Ultimate 3000 (Thermo scientific) with a RID detector (temperature of 50 °C) and an YMC-Pack Polyamine II 250 × 4.6 mm I.D column (YMC, Japan). The mobile phase composition of ACN/H2O/EA (200:125:10, v/v) was used at a flow rate of 1.5 mL/min for 6–10 min at 50 °C.

Results and Discussion

Linalool Synthesis by *Y. lipolytica*

Y. lipolytica W29 (VKPM Y-4620), a lipid accumulating strain capable of growing on sucrose as the sole carbon source, was used as the platform strain for linalool production in this study. To increase the metabolic flux from MVA pathway, *HMG1* and *ERG12* genes were overexpressed (Fig. 1) [27]. Two copies of $ERG20^{F88W-N119W}$ gene encoding a mutated version of *Y. lipolytica* Erg20 with substitution mutations F88W and N119W, which shifts the activity of the enzyme to favor the generation of GPP, were introduced in

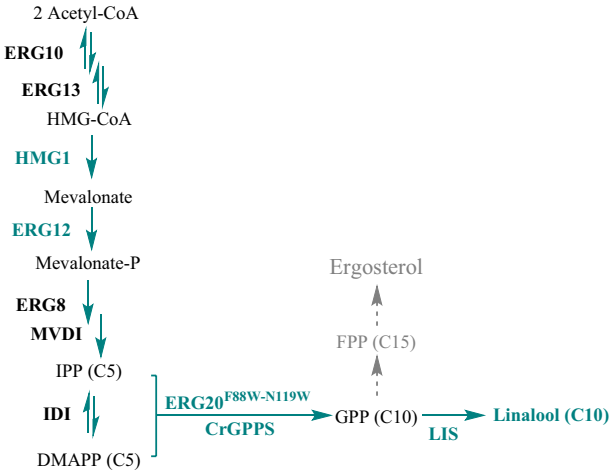


Fig. 1 Metabolic pathway involved in linalool biosynthesis. Enzymes expressed in this study are shown in green

GBK3 strain (Supplementary Table S1). Then, three individual copies each of *LIS* gene encoding linalool synthase from the plant *Actinidia arguta* were consecutively integrated into the genome of GBK3 strain resulted in G5, G6, and G7 strains, respectively (Supplementary Table S1, Fig. S1). Due to the fact that (S)-linalool is cytotoxic and semi-volatile [9], dual-phase flask cultivation in YP with 90 g/L sucrose and IPM was used. Enhancing the copy number of *LIS* gene led to increase linalool accumulation from 6.9 mg/L for one copy to 12.6 mg/L for three copies (Fig. 3).

Boosting GPP Synthesis by Expression of Exogenous GPPS

Low levels of monoterpene production are often associated with an insufficient precursor supply [28]. Therefore, to further increase linalool biosynthesis, an exogenous gene encoding a truncated version of geranyl diphosphate synthase, *CrGPPS* from *C. roseus*, was introduced in strain G7 (yielding strain G8, Supplementary Material Table S1). CrGPPS is a homomeric enzyme with two highly conserved aspartate-rich regions essential for catalytic function and substrate binding [29]. Unlike heterodimeric plant geranyl diphosphate synthases, CrGPPS lacks the conserved CxxxC motif critical for physical interaction between the two subunits forming a heterodimeric enzyme. In vitro analysis has shown that the single product of CrGPPS-catalyzed condensation of IPP and DMAPP was GPP, and only trace amount of GGPP was detected in the presence of either GPP or FPP along with IPP [29]. Indeed, G8 strain harboring *CrGPPS* gene had 2.5-fold increase in linalool titer (31.6 mg/L) compared to the parental G7 strain (Fig. 3).

Improving Linalool Production by Reducing Cytotoxicity

It has previously been shown that disruption of the phosphatidic acid phosphatase-encoding (PAH1) leads to enlargement of the endoplasmic reticulum (ER), which stimulates the production of recombinant triterpene biosynthesis enzymes and ultimately increases triterpenoid accumulation [30]. It was also noted that the cells lacking *PAH1* are defective in cell wall integrity [31], which can increase permeability and so decrease the concentration

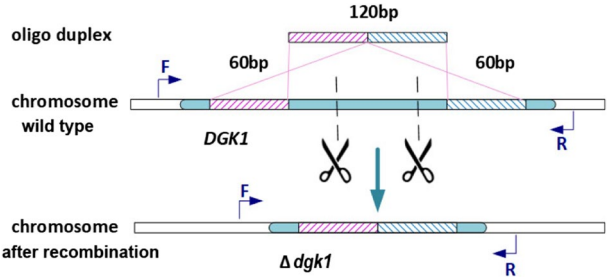
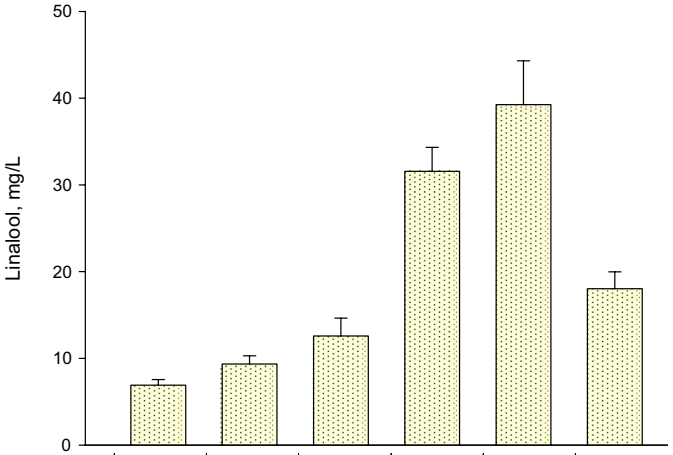


Fig. 2 Scheme of *DGK1* gene knockout via oligo-mediated CRISPR-Cas9 homologous recombination. The DNA strand is indicated by the colorless rectangle. The *DGK1* gene is indicated in blue. Two oligonucleotides, complementary to each other, were annealed to themselves to obtain an oligo duplex. The resulting DNA double strand, 120 base pairs long, was used as a patch for inactivation using CRISPR-Cas9. The double-stranded DNA breakage caused by the Cas9 protein are shown as scissors. The positions of primers annealing are depicted F and R



	G5	G6	G7	G8	G9	G10
HMG1	+	+	+	+	+	+
ERG12	+	+	+	+	+	+
ERG20$^{F88W-N119W}$	++	++	++	++	++	++
LIS	+	++	+++	+++	+++	+++
CrGPPS				+	+	+
Δdgk1					+	
Δpah1						+

Fig. 3 Linalool production by the engineered *Y. lipolytica* strains during cultivation with sucrose as a carbon source with in situ extraction in organic solvent (IPM) in flask for 5 days

of toxic linalool in cells. Previous reports have shown that deletions of *DGK1* and *PAH1* genes in *Y. lipolytica* also led to a distinctive elongated morphology of cell membranes, which in turn can cause terpene excretion and reduce cytotoxicity [28].

Y. lipolytica is characterized by a high efficiency of non-homologous end joining (NHEJ) for repair of DNA double-strand breaks (dsb) which hampers integration by homologous recombination (HR) [32]. Two strategies can be applied to improve the frequency and efficiency of homologous recombination (HR) in *Y. lipolytica*: disruption of the *KU70* gene responsible for double stranded break repair in the NHEJ pathway [33] and heterologous expression of *S. cerevisiae RAD52* gene, the key component of *S. cerevisiae* HR [34].

First, we tested the efficiency of oligo-mediated CRISPR-Cas9 gene inactivation in *Y. lipolytica* by disruption of *DGK1* and *PAH1* genes in three different strains: W29, W29 Δ*ku70*, and W29 *Rad52*. For *DGK1* and *PAH1* gene knockouts, synthetic 120 bp double-stranded oligos were used as repair templates together with two Cas9-helper episomal plasmids (Fig. 2) [23]. Gene disruption was confirmed by PCR from six isolated colonies for each variant (Supplementary Fig. S2).

As expected, there was no gene disruption by HR in W29 strain (Table 1). The *DGK1* gene knockout showed greater efficiency (100% in W29 Δ*ku70* and 67% in W29 *Rad52*) than *PAH1* (50% in W29 Δ*ku70* and 17% in W29 *Rad52*). In both cases, better performance was observed in the Δ*ku70* strain.

We then aimed to apply this technique to perform *DGK1* and *PAH1* knockouts in the G8 strain. *PAH1* null mutant strain (G10) was characterized by reduced growth (9.1 g DCW/L biomass accumulated) as well as a 1.8-fold decrease in linalool titer (18.0 mg/L) (Fig. 3). On the contrary, *DGK1* gene inactivation resulted in 1.2 higher linalool production (39.3 mg/L) compared to the parental G8 strain (Fig. 3).

Constructing Fusion Proteins

One way to improve the utilization of substrates between proteins that catalyze sequential steps in a pathway is fusing two or more genes with a linker region [35]. In this study, *LIS* and *CrGPPS* genes were fused with a flexible GGGS linker and transformed into the best performing strain, G9 (Fig. 4). Both strains G11 (expressing a hybrid enzyme where *CrGPPS* fused through its C-terminus to *LIS*; *CrGPPS-LIS*) and G12 (expressing a hybrid enzyme where *LIS* fused through its C-terminus to *CrGPPS*;

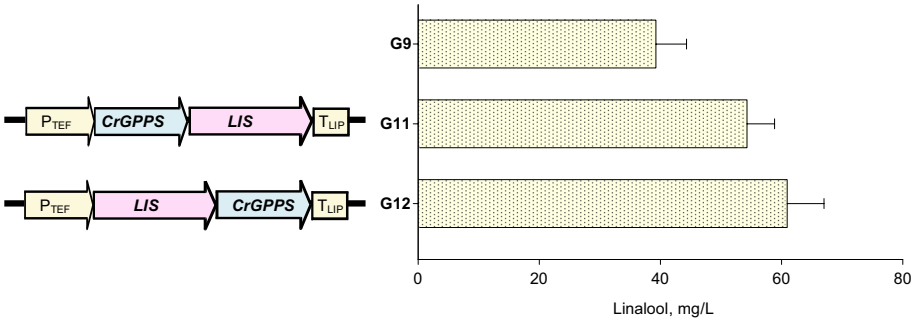


Fig. 4 The linalool production of strains G11 and G12 expressing the fusion proteins *CrGPPS-LIS or LIS-CrGPPS*, respectively and G9 control parent strain

LIS-CrGPPS) demonstrated enhancing linalool production compared with the parental G9 strain (Fig. 4). The best performing strain, G12, accumulated 60.9 mg/L of linalool in flask culture at the 5th day of cultivation.

Time Course Flask Cultivation

Linalool production by the final engineered strain G12 was evaluated in flasks for 7 days of dual phase cultivation in rich YP medium with 90 g/L sucrose. IPM was added at the third day of cultivation. The biomass (DCW, g/L) accumulated linearly from the beginning of cultivation to 144 h (Fig. 5). Linalool production was evaluated starting from 72 h during "dual-phase" cultivation. After 7 days, the linalool titer of strain G12 reached 109.6 mg/L while biomass continued to increase linearly.

Sugar consumption during cultivation was also evaluated. Like all descendants of the parental VKPM Y-4620 strain, G12 can utilize sucrose as a single carbon source due to the overexpression of *S. cerevisiae ScSUC2* and *Y. lipolytica HXK1* genes [26]. Invertase (Suc2) is secreted to the culture medium where sucrose is hydrolyzed to glucose and fructose, which is then taken up by cells [36]. Hexokinase was also demonstrated to have an affinity to fructose, allowing its assimilation into glycolysis [37].

Sucrose was totally hydrolyzed by 48 h (Supplementary Fig. S3). Glucose concentration at 24 h was 9.7 g/L and was consumed totally by 72 h. However, fructose content remained at 16.8–36.7 g/L for 72 h and was consumed only by 168 h. We observed the same pattern of sugar consumption in the control strains G2 and G9 (Supplementary Fig. S4, S5) with a noticeable delay in fructose uptake. This may be due to delayed transport and the inefficient operation of hexokinase (HXK1) in *Y. lipolytica* [38].

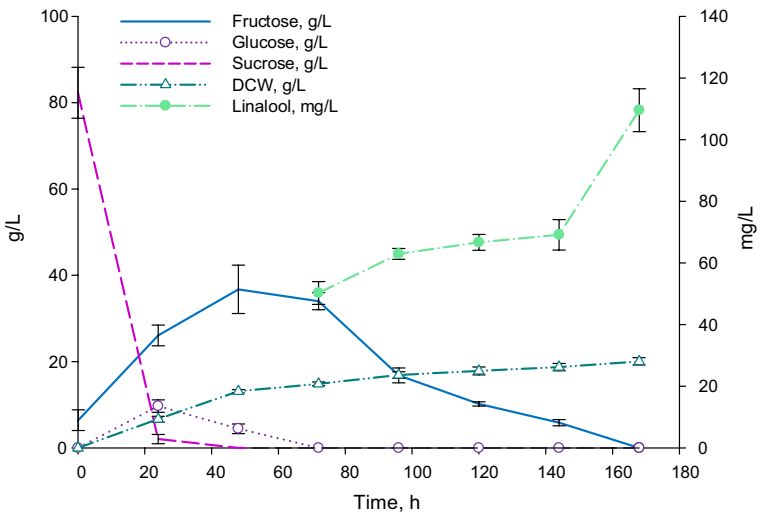


Fig. 5 Time course flask cultivation of G12 strain. Concentrations of linalool (light green dot dash line), dry cell weight (DCW) (green two-dot dash line), sucrose (pink dashed lines), glucose (purple dotted line with circles), and fructose (blue line)

Conclusions

Our linalool-producing strain was engineered by increasing the flux towards MVA precursors, expressing two types of GPP synthase, introducing three copies of a linalool synthase gene, and disrupting *DGK1*. The base strain with 2 copies of GPP synthase (*ERG20*$^{F88W-N119W}$) and 3 copies of linalool synthases (*LIS*) accumulated 12.6 mg/L of linalool. Introduction of just one gene copy encoding *C. roseus* GPP synthase (*CrGPPS*) increased linalool production by 2.5 times resulting in 31.6 mg/L of linalool being produced. Disrupting *DGK1* further increased accumulation of linalool. Finally, the fusion of two key enzymes (linalool synthase and geranyl diphosphate synthase) yielded a recombinant strain producing 109.6 mg/L of linalool at 168 h of cultivation, which exceeds previous results obtained in *Y. lipolytica* [12]. Thus, increasing the GPP precursor pool is critical for linalool synthesis.

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Author Contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Maria O. Taratynova, Iuliia M. Fedyaeva, Dmitry A. Dementev, and Evgeniya Y. Yuzbasheva. The first draft of the manuscript was written by Maria O. Taratynova and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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