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Original Research Article

Optimizing longifolene production in *Yarrowia lipolytica* via metabolic and protein engineering

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ABSTRACT

Longifolene ($C_{15}H_{24}$) is a tricyclic sesquiterpene widely utilized in the cosmetics and fragrances due to its versatile applications. Traditional extraction methods from plants suffer from low titer and lengthy production cycles, while chemical synthesis is hampered by the compound's complex structure, leading to high costs and insufficient market supply. This study aimed to develop a microbial cell factory for enhanced longifolene production. The strategy involved integrating longifolene synthase from *Pinus sylvestris* (*PsTPS*) into *Yarrowia lipolytica* and employing multiple metabolic engineering approaches. Initially, key genes in the mevalonate (MVA) pathway were overexpressed to enhance longifolene precursor availability for longifolene biosynthesis. Subsequently, protein engineering techniques were applied to optimize *PsTPS* (t*PsTPS*) for improved catalytic efficiency. Furthermore, co-expression of molecular chaperones was implemented to enhance the synthesis and secretion of *PsTPS*. The introduction of the isopentenol utilization pathway (IUP) further augmented the supply of C5 substrate. By optimizing the culture conditions, including a reduction in culture temperature, the efflux of longifolene was increased, and the dissolved oxygen levels were enhanced to promote the growth of the strain. These collective efforts resulted culminated in the engineered strain Z03 achieving a noteworthy production level of 34.67 mg/L of longifolene in shake flasks. This study not only demonstrates the feasibility of enhancing sesquiterpene production in *Y. lipolytica* but also highlights the potential of microbial platforms in meeting industrial demands for complex natural products.

1. Introduction

Longifolene, a tricyclic sesquiterpene compound, serves as the predominant constituent of heavy turpentine [1,2]. Known for its rosin fragrance, longifolene is widely utilized in spices formulations and plays a crucial role in the synthesis of isolongifolene and isolongifolol [3], alongside other fragrance products. Furthermore, its high net heat of combustion renders it valuable in the aerospace and new energy sectors, while its recognized anti-inflammatory, antibacterial, and antioxidant properties further enhance its industrial appeal [4–6]. The increasing market demand for longifolene underscores its significance in industrial applications.

Longifolene is currently obtained through plant extraction or chemical synthesis [7]. However, challenges such as prolonged extraction cycles and organic contamination hinder efficient plant-based production [8]. Chemical synthesis involves complex procedures that limit the attainment of high-purity longifolene [9]. Consequently, microbial synthesis emerges as a promising alternative for the production of longifolene.

Several studies have explored microbial synthesis strategies for longifolene using synthetic biology approaches in microorganisms. Such as, *Escherichia coli* has been engineered to produce longifolene, achieving titer of 2.64 mg/L in shake flasks [10]. Similarly, *Saccharomyces cerevisiae* has demonstrated production titer of 17.7 mg/L and 27.3 mg/L in shake flasks [11,12]. In addition, *Y. lipolytica* has been used to study the synthesis of longifolene; however, its titer is only about 6 mg/L, which needs to be further improved to meet the requirements of industrial production by optimizing metabolic pathways [13].

Y. lipolytica, recognized for its unique physiological properties and classified as Generally Recognized As Safe (GRAS) [14], exhibits superior flux of acetyl-CoA and the TCA cycle flux compared to traditional hosts such as *S. cerevisiae* and *E. coli* [15]. These metabolic advantages

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makes *Y. lipolytica* particularly suitable for terpenes synthesis, such as limonene [16], humulene [17], gibberellic acid [18], ginsenoside compound K [19], and astaxanthin [20]. Recently, advancements in synthetic biology tools have further facilitated the engineering of *Y. lipolytica* for the efficient expression of heterologous genes and the construction of complex biosynthetic pathways [21–23].

In this study, the microbial synthesis of longifolene was optimized using a systematic approach that integrates multiple metabolic engineering strategies: (1) overexpressing of key genes in the MVA pathway to enhance the supply of precursors; (2) selecting higher efficient *tPsTPS* enzymes predicted by SCANEER; (3) co-expressing molecular chaperones to improve the synthesis and secretion of heterologous protein; (4) introducing a two-step isopentenol utilization pathway to increase the availability of C5 substrates; and (5) optimizing the culture conditions to increase the efflux of longifolene and promote the growth of the strain (Fig. 1). The engineered *Y. lipolytica* strain exhibited significantly enhanced production of longifolene, demonstrating its potential as an optimized platform for industrial terpene production.

2. Material and methods

2.1. Strains and culture media

E. coli DH5α, utilized for plasmid construction was cultivated in LB medium (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl) at 37 °C. *Y. lipolytica* polf1 K6 served as the initial strain and cultured at 28 °C in YPD medium (2 % glucose, 2 % tryptone, and 1 % yeast extract).

2.2. Plasmids construction and strains development

All plasmids and primers for assembly used in this study are detailed in Tables S1 and S2. Primers were synthesized by Sangon Biotech (Shanghai). The longifolene synthase gene from *Pinus sylvestris* (*PsTPS*, GenBank ID: EF679332.2), the isopentenyl phosphate kinase gene from *Arabidopsis thaliana* (*AtIPK*, GenBank ID: AAN12957) and the choline kinase gene from *Saccharomyces cerevisiae* (*ScCK*, GenBank ID: AAA34499) were codon-optimized for expression in *Y. lipolytica* and synthesized by Sangon Biotech (Shanghai) (Table S3). Endogenous genes were cloned from the cDNA of *Y. lipolytica*. Plasmids were constructed using the ClonExpress Ultra One Step Cloning Kit (Vazyme). Yeast competent cells were transformed with the plasmids using the Frozen-EZ Yeast Transformation II™ kit (Zymo Research) following the manufacturer's instructions. Transformants were plated on SD-Leu or SD-Ura selection plates and incubated at 30 °C for 3 days. The engineered strains developed in this study are listed in Table 1.

2.3. Shake-flask culture and longifolene extraction

A single colony of the engineered *Y. lipolytica* strain was inoculated into a 50 mL conical flask containing 1 mL of YPD and cultured for 24 h at 28 °C with shaking at 140 rpm to start the seed culture stage. After cultivation for 24 h, the seed cultures were transferred into a 50 mL conical flask containing 5 mL of YPD achieving an initial optical density (OD) of 0.1 and cultivated for 120 h under the same conditions. At 24 h of cultivation, n-Dodecane (5 %, v/v) was introduced to facilitate the extraction of longifolene, continuing until the end of the culture period.

2.4. GC-MS analyses

The n-dodecane containing longifolene was harvested through centrifugation at 12,000 *g* for 2 min, and subsequently analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) (Agilent 7890B–7000C) according to the previous method [11]. The GC-MS analysis was conducted under the following conditions: the carrier gas was nitrogen and the flow rate was 1.0 mL/min. The initial oven temperature was held at 100 °C for 4 min and then increased at a rate of 30 °C/min to a final temperature of 250 °C and held for 1 min. An electron ionization detector with an ionization energy of 70 eV was used for the analysis. The fragmentation pattern of longifolene was first compared to the NIST library database. A longifolene standard (L873309, Macklin, Shanghai, China) was utilized for construct the standard curve and identify the substances.

2.5. Analyses for optical density and yeast biomass

The sample from the broth were diluted with culture medium and the OD was measured at 600 nm (OD_{600}) using a UV-1750 spectrophotometer (Shimadzu, Japan). The supernatant was subsequently removed

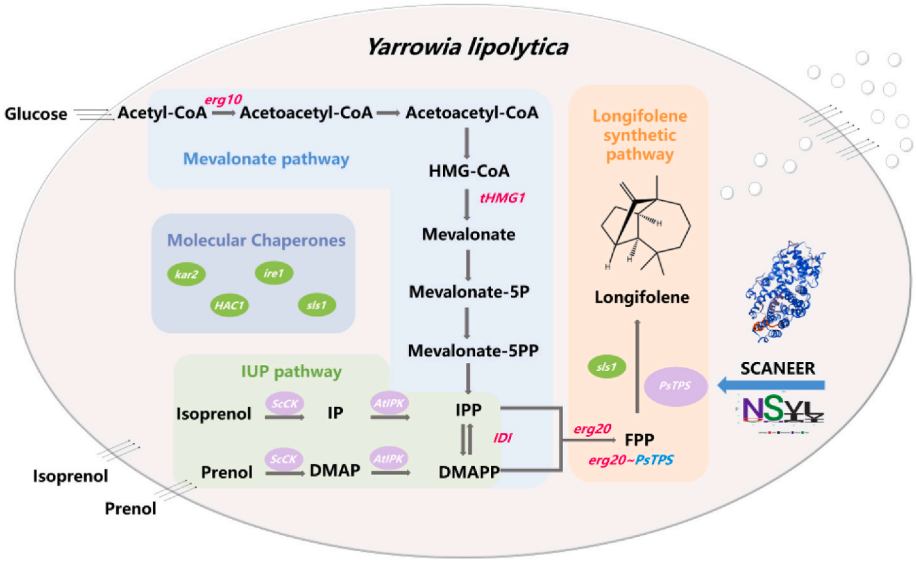


Fig. 1. Schematic diagram of longifolene synthesis in *Y. lipolytica*. *ERG10*, acetyl-CoA acetyltransferase; *tHMG1*, truncated HMG-CoA reductase; *IDI1*, isopentenyl-diphosphate δ-isomerase; *ERG20*, farnesyl diphosphate synthetase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallylpyrophosphate; FPP, farnesyl diphosphate; *PsTPS*, longifolene synthetase from *P. sylvestris*; $tPsTPS^{H157K}$, longifolene synthetase mutant; IP, isopentenyl phosphate; DMAP, dimethylallyl phosphate; *AtIPK*, isopentenyl phosphate kinase gene from *A. thaliana*; *ScCK*, choline kinase gene from *S. cerevisiae*; *HAC1*, endoplasmic reticulum-resident stress response regulatory protein; *KAR2*, the *Hsp90* molecular chaperone; *IRE1*, UPR sensor protein; *SLS1*, the endoplasmic reticulum-resident molecular chaperone.

Table 1
Y. lipolytica strains used in this study.

Strains	Host strains	Descriptions	Resources
Y. lipolytica po1f K6	–	*MATa, leu2-270, ura3-302, xpr2-322, axp1-2, Leu⁻, Ura⁻,* △ku70	This Lab
K6-1	*Y. lipolytica* po1f K6	pox3△:pPOX3-*IDI-ERG20*	This Study
K6-2	K6-1	pox2△:pPOX2-*tHMG1-ERG10*	This Study
F01P	*Y. lipolytica* po1f K6	pox5△:pPOX5-*PsTPS*	This Study
F02P	F01P	pox3△:pPOX3-*IDI-ERG20*	This Study
F03P	F02P	pox2△:pPOX2-*tHMG1-ERG10*	This Study
L01P	*Y. lipolytica* polf1 K6	pox5△:pPOX5TL-*PsTPS*, *Leu*$^+$	This Study
L02P	K6-1	pox5△:pPOX5TL-*PsTPS*, *Leu*$^+$	This Study
L03P	K6-2	pox5△:pPOX5TL-*PsTPS*, *Leu*$^+$	This Study
L04P	K6-2	pox5△:pPOX5TL-*ERG20-PsTPS*, *Leu*$^+$	This Study
L05P	K6-2	pox5△:pPOX5TL-*PsTPS* ~ *ERG20*, *Leu*$^+$	This Study
L06P	K6-2	pox5△:pPOX5TL-*ERG20~PsTPS*, *Leu*$^+$	This Study
L07P	K6-2	pox5△:pPOX5TL-*KAR2-PsTPS*, *Leu*$^+$	This Study
L08P	K6-2	pox5△:pPOX5TL-*IRE1-PsTPS*, *Leu*$^+$	This Study
L09P	K6-2	pox5△:pPOX5TL-*HAC1-PsTPS*, *Leu*$^+$	This Study
L10P	K6-2	pox5△:pPOX5TL-*SLS1-PsTPS*, *Leu*$^+$	This Study
Y124 N	K6-2	pox5△:pPOX5TL-*tPsTPS*^{Y124N}, *Leu*$^+$	This Study
N212S	K6-2	pox5△:pPOX5TL-*tPsTPS*^{N212S}, *Leu*$^+$	This Study
I329V	K6-2	pox5△:pPOX5TL-*tPsTPS*^{I329V}, *Leu*$^+$	This Study
I404L	K6-2	pox5△:pPOX5TL-*tPsTPS*^{I404L}, *Leu*$^+$	This Study
Y31D	K6-2	pox5△:pPOX5TL-*tPsTPS*^{Y31D}, *Leu*$^+$	This Study
M52L	K6-2	pox5△:pPOX5TL-*tPsTPS*^{M52L}, *Leu*$^+$	This Study
H157K	K6-2	pox5△:pPOX5TL-*tPsTPS*^{H157K}, *Leu*$^+$	This Study
Q342E	K6-2	pox5△:pPOX5TL-*tPsTPS*^{Q342E}, *Leu*$^+$	This Study
I365 M	K6-2	pox5△:pPOX5TL-*tPsTPS*^{I365M}, *Leu*$^+$	This Study
G487R	K6-2	pox5△:pPOX5TL-*tPsTPS*^{G487R}, *Leu*$^+$	This Study
Z01	K6-2	pox6△:pPOX6-*tPsTPS*^{H157K}-*SLS1*	This Study
Z02	Z01	rDNA△: prDNAU-*tPsTPS*^{H157K}-*tHMG1- ERG20*, *Ura*$^+$	This Study
Z03	Z02	rDNA△: prDNAL-*AtIPK-ScCK-tPsTPS*^{H157K}-*ERG20*, *Leu*$^+$	This Study

by centrifugation and the remaining biomass was freeze-dried using a vacuum freeze dryer to determine the dry cell weight (DCW) [24].

2.6. De novo prediction and sequence analysis of fusion proteins

Due to the absence of characterized structures for the fusion proteins (*PsTPS* ~ GGGGS*3~ ERG20, ERG20~GGGGS*3~*PsTPS*), AlphaFold2 was utilized for de novo prediction of their amino acid sequences. The *PsTPS* protein sequence was submitted to NCBI for homology sequence prediction to identify conserved amino acids.

2.7. Analysis of the coevolutionary conservation of the PsTPS

ClustalW was employed for multiple sequence alignments (Fig. S2). The conservation rate of amino acid residues was analyzed by WebLogo (https://weblogo.threeplusone.com/) (Fig. S3). Additionally, SCANEER analysis (https://github.com/SBIlab/SCANEER) was utilized to identify amino acids that underwent mutation during protein evolution.

2.8. Statistical analysis

Independent experiments were carried out in triplicates to ensure reproducibility. GraphPad Prism 9.01 software (GraphPad Prism) was used to data analysis. The results are presented as mean ± standard deviation (SD). Statistical analysis between groups were performed using t-tests following one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Expanding the mevalonate pathway to enhance longifolene synthesis

Y. lipolytica possesses an endogenous mevalonate (MVA) pathway that generates common terpenoid precursors. Previous studies have elucidated the biological pathways involved in the synthesis of longifolene [10]. Longifolene synthase from *P. sylvestris* has been identified as exhibiting the highest catalytic efficiency [12]. To enhance longifolene production, the codon-optimized *PsTPS* was integrated into strain *Y. lipolytica* polf1 K6 (△ku70) under the control of a strong promoter, resulting in strain L01P, which produced 3.28 ± 0.77 mg/L of longifolene (Fig. 2). In *Y. lipolytica*, the MVA pathway provides the direct precursor farnesyl pyrophosphate (FPP) for longifolene production [25]. The overexpression of *IDI* and *ERG20* within the MVA pathway has previously been shown to enhance zeaxanthin synthesis [26]. Subsequently, *IDI* and *ERG20* were overexpressed in strain *Y. lipolytica* polf1 K6 to obtain strain K6-1, which was further integrated with *PsTPS* to create strain L02P, achieving a longifolene titer of 4.33 ± 0.59 mg/L (Fig. 2A–C). Previous studies have demonstrated that increasing the expression of *tHMG1* and *ERG10* within the MVA pathway enhances β-carotene synthesis [27]. Further enhancement was achieved by overexpressing *tHMG1* and *ERG10* in K6-1, resulting in strain K6-2, which, upon integration with *PsTPS,* produced strain L03P with a longifolene production of 6.39 ± 0.80 mg/L, representing a 1.95-fold increase compared to the titer observed in strain L01P (Fig. 2A–C). These results demonstrate the feasibility of expanding the mevalonate pool to improve terpene synthesis. In addition, the concentration of n-dodecane added is critical, as it may influence the growth of *Y. lipolytica* (Fig. S1).

3.2. Enhancing longifolene production via fusion expression of ERG20 and PsTPS

FPP serves not only as a precursor for sesquiterpene production but also for the synthesis of squalene, ergosterol, and other essential compounds [28]. Internal cellular processes, including diffusion, degradation, and the conversion of FPP into by-products through competing pathways, can significantly impact the efficiency of target product synthesis [29]. To enhance sesquiterpene synthesis, one strategy involves fusing FPP synthase with sesquiterpene synthase to increase the flux of FPP into the sesquiterpene synthesis pathway [30]. This study aimed to enhance longifolene production by expressing two sequentially acting proteins, *ERG20* and *PsTPS*, as a fusion protein module with a linker region. The fusion of *ERG20* and *PsTPS* using linkers (GGGGS*3), resulted in a significant increase in longifolene production compared to separate expression cassettes, likely due to enhanced availability of FPP. Notably, the fusion of *PsTPS* at the C-terminus of *ERG20* via a linker led to a remarkable increase in longifolene production, reaching 14.11 ± 2.97 mg/L (Fig. 3A–B).

To investigate the structural basis of enhanced longifolene

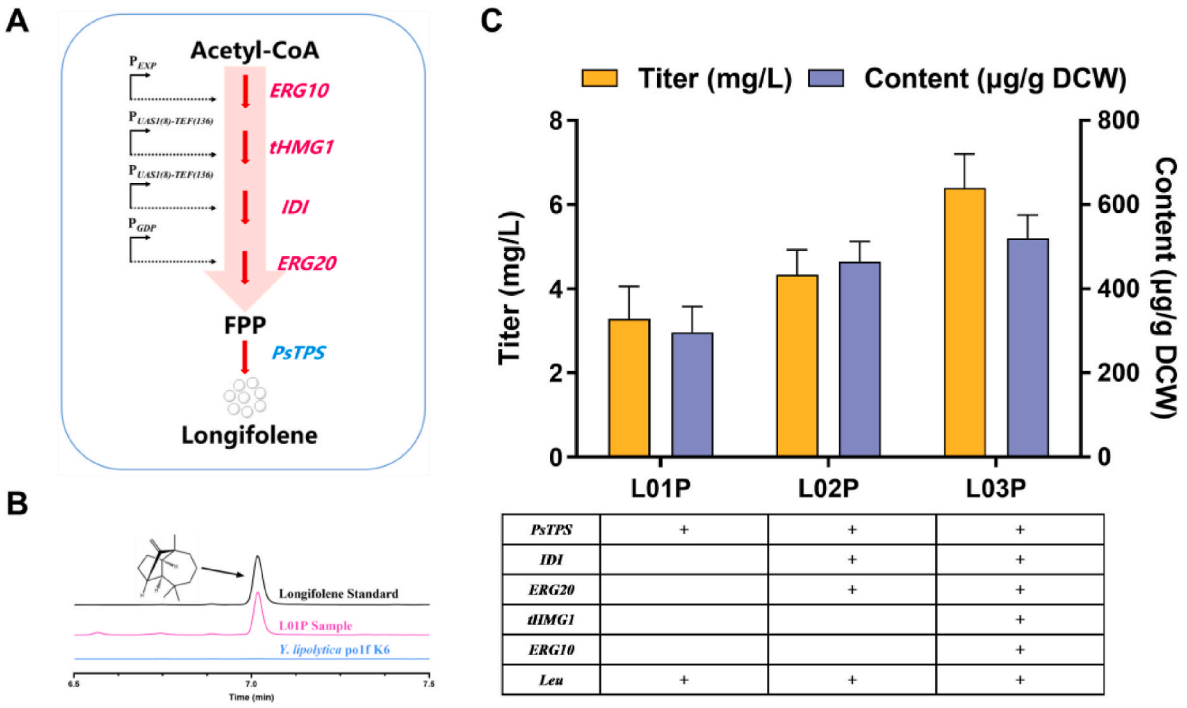


Fig. 2. Longifolene production in engineered strains utilizing the MVA pathway. (A) Schematic diagram of the MVA pathway leading to longifolene production; (B) GCMS chromatograms analysis results of the engineered strain L01P, the control strain *Y. lipolytica* polf K6 and longifolene ABA standard; (C) The longifolene produced by strains L01P, L02P and L03P. The growth of strains L01P, L02P and L03P. Longifolene production was assessed after 120 h of fermentation in 5 mL of YPD medium. The data are represented as mean ± standard deviation (n = 3).

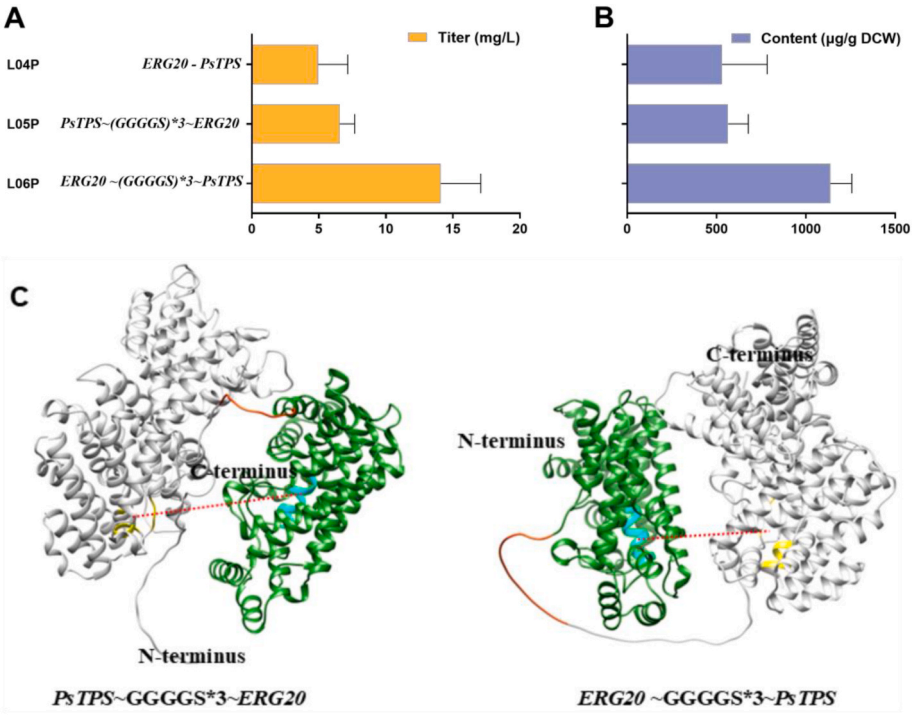


Fig. 3. Longifolene production in K6-2 strains overexpressing *PsTPS* and *ERG20* in different fused forms is presented. (A) Productivity of longifolene; (B) Content of longifolene; (C) Predicted 3D models of the fusion proteins *PsTPS*-GGGGS*3-*ERG20* and *ERG20*-GGGGS*3-*PsTPS*. The data are represented as mean ± standard deviation (n = 3).

production, the AlphaFold2-Multimer tool was employed to predict the three-dimensional structure of the fusion protein. The structural simulation revealed that the distance between the two active sites of *ERG20* and *PsTPS* in the *ERG20*~*PsTPS* configuration is shorter than that in the *PsTPS* ~ *ERG20* configuration (Fig. 3C). This proximity facilitates reduced spatial diffusion of intermediates, thereby enhancing their colocalization. This phenomenon mirrors observations made in the synthesis of miltiradiene and patchoulol [31,32].

3.3. Screening molecular chaperones for enhanced longifolene synthase activity

The low activity of longifolene synthase represents a significant bottleneck in its biosynthesis (Hassan et al., 2020). Despite the advantages of *Y. lipolytica* in synthesizing terpene compounds, the expression of heterologous proteins continues to face challenges [33], including mRNA transcription efficiency, protein folding accuracy, and stability. Protein misfolding can severely impact activity, as it relies on the specific three-dimensional structure [34]. In this context, molecular chaperone systems play a crucial role in maintaining protein functionality by facilitating proper folding and preventing the aggregation of misfolded proteins [35], thereby enhancing the quantity and quality of recombinant protein expression in *Y. lipolytica*. Paulina Korpys-Woźniak demonstrates that the overexpression of molecular chaperones Hac1p (encoded by *HAC1*), Sls1p (encoded by *SLS1*), and protein disulfide isomerase (encoded by *PDI1*) significantly enhances the expression and accumulation of heterologous genes [36].

In this study, *PsTPS* was co-overexpressed alongside the endoplasmic reticulum-resident stress response regulatory protein Hac1p, the endoplasmic reticulum-resident molecular chaperone Sls1p (a cofactor of KAR2), as well as the *Hsp90* molecular chaperone (encoded by *KAR2*) and Ire1p (the UPR sensor protein) involved in protein entry into the endoplasmic reticulum in strain K6-2. The effects of co-overexpression on longifolene accumulation were evaluated. Compared to the control strain L03, most molecular chaperones have a positive impact on longifolene titer. Notably, the endoplasmic reticulum-resident molecular chaperone *SLS1* significantly enhanced longifolene production, achieving a maximum of 9.86 mg/L and 828.72 μg/g (Fig. 4). As a molecular chaperone, *SLS1* plays a crucial role in facilitating the proper folding of proteins [35], thereby improving the folding efficiency of *PsTPS* in the endoplasmic reticulum and enhancing its expression. These findings underscore *SLS1* as the most effective molecular chaperone for longifolene synthesis.

3.4. Protein engineering of PsTPS to increase longifolene titer

The catalytic activity of longifolene synthase is essential for the conversion of FPP to longifolene. However, the X-ray crystal structure of longifolene synthase has yet to be documented, and its catalytic mechanism remains unclear. Currently, high-throughput detection of longifolene synthesis remains unfeasible, in contrast to the synthesis of compounds like astaxanthin, which can be achieved through irrational design methods such as error-prone PCR. In such cases, beneficial mutants can be easily distinguished and identified visually. Therefore, at this stage, it is crucial to rationally design PsTPS using predictive tools like SCANEER, which assists in identifying potential enzyme modifications that could enhance longifolene production [37]. While SCANEER helps guide the design process, future high-throughput methods will be necessary to identify and optimize beneficial mutants effectively. Consequently, this research employed statistical amino acid frequency analysis derived from multiple sequence alignment to modify the enzyme and enhance its catalytic performance.

Comparative sequence analysis of *PsTPS* revealed biased amino acid residues. Conserved residues identified from the multiple sequence alignment of *PsTPS* and 16 homologs were selected as potential mutation sites (Fig. S2). Four specific sites—Y124 N, N212S, I329V, and I404L—were targeted for site-directed mutation in *PsTPS* (Fig. S3). Compared to strain L03P, the Y124 N mutation exhibited a slight reduction in longifolene titer, whereas the N212S, I329V, and I404L mutations demonstrated a significant decrease, indicating these sites may be ineffective for mutation (Fig. 5A).

Using SCANEER, a novel enzyme activity prediction tool that analyzes the frequency of mutated amino acid pairs and provides feedback on mutated amino acid sites [38], amino acid sites with SCI scores exceeding 2 were identified for site-directed mutagenesis to generate mutant strains. SCANEER constructed a residue coevolution network by using a developed pipeline to predict mutational effects that may be associated with allosteric sites. Specifically, it collects homologs to be engineered and constructs a Multiple Sequence Alignment (MSA). Using the substitution correlation method based on McLachlan's approach, the tool calculates the covariation intensity between protein residues. The coevolution relationship between residues is then determined based on the MSA. This analysis enables the identification of key residues that could be targeted for mutagenesis to improve enzyme activity and pathway flux [39]. The production of longifolene and the dry weight ratio were increased in strains expressing the mutations Y31D, M52L, H157K, and I365 M. Among these mutations, the H157K and I365 M variants exhibited the most substantial increases in longifolene production, reaching 255 % and 175 % respectively (Fig. 5B–Table S4). Conversely, the mutants Q342E and G487R displayed a significant decrease in longifolene production, suggesting the importance of conserved amino acids in the enzyme's catalytic activity throughout evolution.

The predicted structure of longifolene synthase from *Picea abies* (Q675L0.1.A) was utilized as a template for homology modeling of the 3D structure of *PsTPS*, followed by the docking of the FPP substrate to *PsTPS*. Notably the mutation sites H157K and I365 M, which enhance the catalytic ability of *PsTPS,* are situated at a considerable distance from the binding pocket (Fig. S4). This observation suggests that distal residues within the protein sequence significantly influence the enzyme 's activity.

3.5. Integration of multiple strategies for enhanced longifolene synthesis

Increasing gene copy numbers to enhance gene expression can significantly improve metabolic pathway flux (Fig. 6A). *HMGR* serves as the primary rate-limiting enzyme in the MVA pathway, and augmenting its copy number has proven successful in optimizing pathway flux [40]. For instance, in studies on squalene production, increasing the copy number of the *tHMG1* gene from one to two copies resulted in a remarkable 16.8-fold increase in squalene production [41].

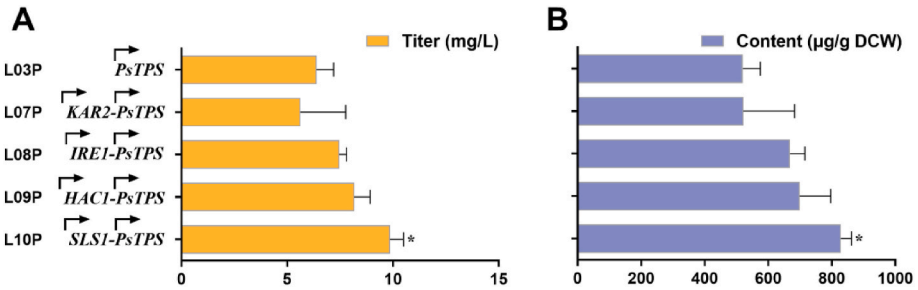


Fig. 4. Longifolene production in strains co-overexpressing molecular chaperones. (A) The longifolene titer by strains L03P, L07P, L08P, L09P and L10P; (B) The longifolene content by strains L03P, L07P, L08P, L09P and L10P. Longifolene production was evaluated after 120 h of fermentation in 5 mL of YPD medium. The data are represented as mean ± standard deviation (n = 3).

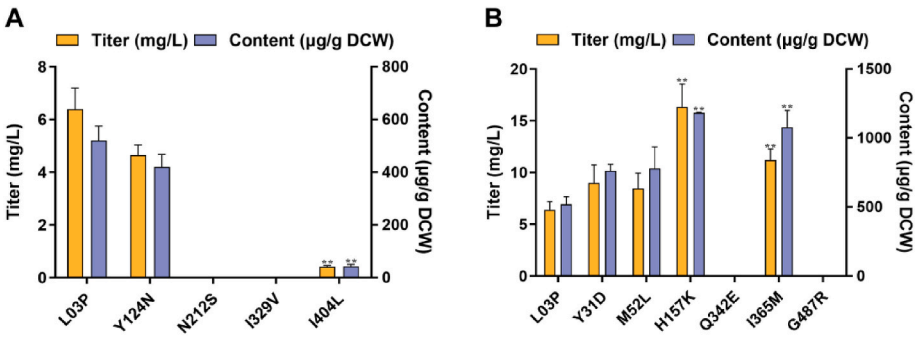
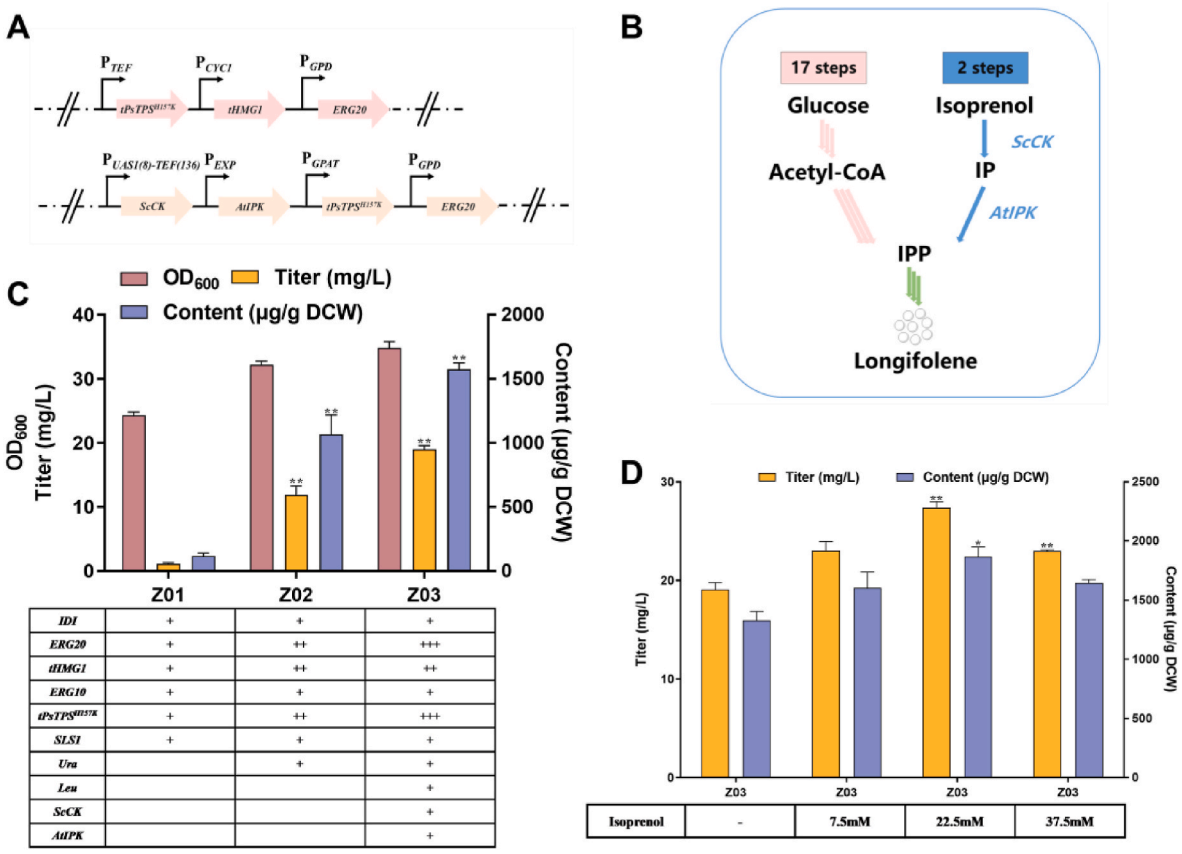


Fig. 5. Longifolene production in strains with protein engineering. (A) The longifolene produced in strains mutated by homology comparison; (B) The longifolene produced in strains mutated as predicted by SCANEER. Data reflect measurements taken after 120 h of fermentation in 5 mL of YPD medium. The data are represented as mean ± standard deviation (n = 3).



IDI	+	+	+
ERG20	+	++	+++
tHMG1	+	++	++
ERG10	+	+	+
tPsTPS^{H157K}	+	++	+++
SLS1	+	+	+
Ura		+	+
Leu			+
ScCK			+
AtIPK			+

Fig. 6. Longifolene production in engineered strains. (A) Different gene configuration of longifolene synthesis pathway; (B) Comparison of the IUP pathway and the native pathway; (C) The longifolene production and growth of strains Z01, Z02 and Z03; (D) The longifolene production of strain Z03 with different supplementary concentrations of isoprenol. Measurements were conducted after 120 h of fermentation in 5 mL of YPD medium. The data are represented as mean ± standard deviation (n = 3).

Optimizing metabolic pathways and fluxes is a crucial approach for enhancing the production of desired products in engineered strains. Terpenoid biosynthesis depends on isoprene as a key precursor, with the synthesis of its precursor, isopentenyl pyrophosphate (IPP), tightly regulated [42]. To overcome the limitations in endogenous metabolism affecting IPP synthesis, the isopentenol utilization pathway (IUP) was introduced to enhance IPP availability (Fig. 6B) [43].

Building upon the K6-2 strain, effective improvement strategies were implemented by elevating the copy number of MVA pathway genes, including *tHMGR1*, *ERG20*, and *PsTPS*, resulting in a significant increase in longifolene production (Fig. 6C). By incorporating genes associated with the IUP pathway into the engineered strain and exploring the effects of different supplementary concentrations of isoprenol on its growth and synthesis of longifolene, the optimal amount and time of isoprenol added was determined, and the titer was further improved. Our findings indicate that the addition of isoprenol at 0 h of culture significantly impairs the growth of *Y. lipolytica*, whereas the introduction of isoprenol at 24 h of culture has a little impact on its growth (Fig. 6S). Additionally, excessive isoprenol concentrations also inhibit growth, which is consistent with previous studies [44,45]. Consequently, we opted to introduce three isoprenol concentrations with a minimal impact on growth, 24 h post-culture, to investigate their effect on longifolene synthesis. The results revealed that the highest yield (27.38 mg/L) of longifolene was achieved with an isoprenol

concentration of 22.5 mM (Fig. 6D). While increasing isopentenol supplementation improved longifolene yield, further enhancements in intracellular precursor availability are required. Future work should focus on optimizing the endogenous MVA pathway to maximize precursor flux toward longifolene biosynthesis. Some strategies such as implementing dynamic regulatory systems to modulate precursor allocation based on cellular demand may help balance metabolic fluxes and improve production efficiency. Additionally, exploring heterologous expression of optimized MVA pathway genes or utilizing synthetic biology tools to create novel metabolic circuits may further enhance *Y. lipolytica*'s potential as a microbial cell factory for sesquiterpene biosynthesis. These strategies may provide a more comprehensive perspective on the challenges and future directions for optimizing longifolene production in *Y. lipolytica*.

3.6. Optimization of fermentation conditions to improve longifolene production

To enhance the yield of longifolene, we investigated the effects of culture temperature and dissolved oxygen on its production. During the fermentation process, minor alterations in conditions can result in significantly different outcomes [46]. Culturing at low temperatures can stimulate the synthesis and efflux of terpenes in yeast, thereby increasing terpene production [47]. Furthermore, for the aerobic organism *Y. lipolytica*, the concentration of dissolved oxygen is a critical factor influencing productivity during fermentation [48]. Enhancing oxygen supply is a crucial strategy for improving the efficiency of aerobic reactions and promoting cell growth [49]. To investigate whether low temperature and increased dissolved oxygen can enhance the production of longifolene in Z03, we selected two different temperatures (22 °C and 28 °C) and two types of shake flasks (baffled and non-baffled) for a cross-combination culture experiment.

Our findings indicate that fermentation at a lower temperature (22 °C) resulted in an increased yield and content of longifolene, despite the biomass being lower compared to fermentation at the standard temperature (28 °C) (Fig. 7). Previous research by Qin et al. explored the effects of low temperature (22 °C) on the production of β-caryophyllene and α-amino acids in *S. cerevisiae* [47]. Their study demonstrated that lower temperatures can enhance the synthesis and secretion of terpenoids in *S. cerevisiae*. These results underscore the significance of temperature as a critical factor in the production of longifolene, particularly highlighting that the optimal temperature can influence the activity of enzymes involved in the longifolene biosynthesis pathway.

To evaluate the effect of oxygen supply on longifolene production, Z03 was cultured using two types of shake flasks to facilitate longifolene production. The production of longifolene exhibited significant variation under different culture conditions. The highest production of longifolene was achieved in the baffled shake flask. *Y. lipolytica* is a strict aerobic microorganism, and an increase in oxygen availability enhances its growth. This indicates that augmenting the oxygen supply not only promotes cellular growth but also enhances the efficiency of the longifolene biosynthetic pathway in *Y. lipolytica*.

Ultimately, a longifolene-producing strain designated Z03 was successfully developed, producting 34.67 mg/L under optimized conditions, which represents a 5.78-fold increase compared to the titer reported by Zhang et al. (Table S5) [13]. In another study, Xia et al. achieved a longifolene yield of 1.25 g/L in *S. cerevisiae* through fermentation, which was significantly higher than the 27.3 mg/L yield observed in shake flasks [11]. Additionally, *Y. lipolytica* can also substantially enhance the yield of synthetic products optimized fermentation conditions [50]. Our strain also has the potential to achieve yields at the g/L level under fermentation conditions, indicating promising scalability for industrial production.

4. Conclusion

In this study, we have pioneered the development of a *Y. lipolytica* platform for optimized production of longifolene. This was achieved by reconstructing the endogenous MVA pathway and introducing the IUP pathway to increase the supply of precursors, along with improving enzyme catalytic activity through protein engineering. The engineered strain successfully produced 34.67 mg/L of longifolene in shake flasks, marking the highest titer of sesquiterpenes reported for *Y. lipolytica* to date. This achievement underscores the effectiveness of combining metabolic pathway optimization with protein engineering strategies to enhance terpenoid biosynthesis. The developed platform not only advances our understanding of *Y. lipolytica*'s potential as a robust microbial cell factory for sesquiterpene production but also sets a precedent for further optimizing biosynthetic pathways toward the bioproduction of valuable compounds.

CRediT authorship contribution statement

Yi-Tong Yao: Writing – original draft, Formal analysis, Data curation. **Xiao Zhang:** Investigation, Formal analysis. **Chen-Yu Wang:** Visualization, Investigation. **Yu-He Zhang:** Investigation. **Da-Wei Li:** Visualization. **Wei-Dong Yang:** Visualization. **Hong-Ye Li:** Writing – review & editing, Resources, Funding acquisition. **Li-Gong Zou:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

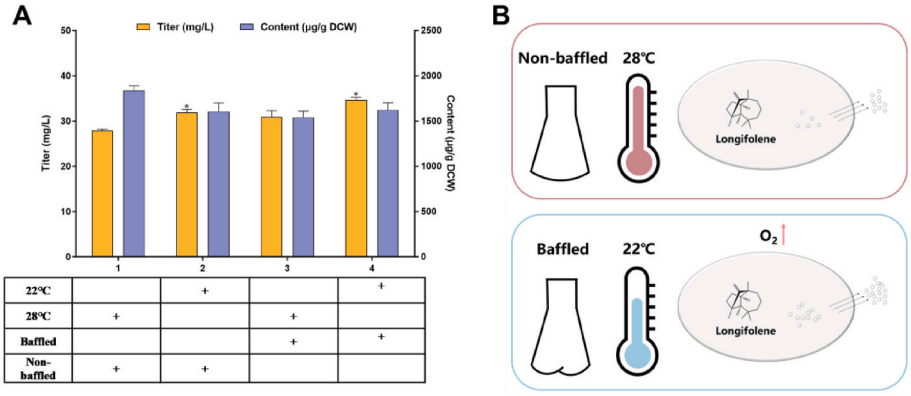


Fig. 7. Longifolene production in Z03 strains with different fermentation conditions. (A) The longifolene production; (B) Schematic diagram of the effect of temperature and dissolved oxygen on longifolene production. Measurements were conducted after 120 h of fermentation in 5 mL of YPD medium. The data are represented as mean ± standard deviation (n = 3).

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2025.01.004.

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