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Combinatorial metabolic engineering of *Yarrowia lipolytica* for high-level production of the plant-derived diterpenoid sclareol

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Abstract

Background Sclareol, a diterpene alcohol derived from *Salvia sclarea*, is primarily used in the synthesis of ambrox, an alternative to the expensive spice ambergris. However, commercial production of sclareol from plant extraction is costly and environmentally problematic, limiting its scalability. Recent advances in synthetic biology have enabled the construction of efficient cell factories for sclareol synthesis, offering a more sustainable solution.

Results In this study, we engineered *Yarrowia lipolytica* to produce sclareol by integrating genes encoding (13E)-8α-hydroxylabden-15-yl diphosphate synthase (LPPS) and sclareol synthase (SCS). Sclareol titers were further enhanced through the fusion of SsSCS and SsLPPS proteins, as well as multi-copy gene integration. To increase the precursor geranylgeranyl diphosphate (GGPP), we overexpressed various geranylgeranyl diphosphate synthases (GGS1), resulting in significant accumulation of GGPP. Additionally, optimization of the mevalonate pathway, coupled with the downregulation of lipid synthesis and upregulation of lipid degradation, directed more acetyl CoA towards sclareol production.

Conclusions In this study, we reprogrammed the metabolism of *Y. lipolytica* by combinatorial metabolic engineering with a sclareol titer of 2656.20 ± 91.30 mg/L in shake flasks. Our findings provide a viable strategy for utilizing *Y. lipolytica* as a microbial cell factory to produce sclareol.

Keywords Sclareol, GGPP, Diterpenoids, *Yarrowia lipolytica*, Synthetic biology

Background

Sclareol, a labdane diterpene alcohol, is extensively utilized in the food, cosmetic and pharmaceutical industries as an additive, flavor and fragrance due to its antimicrobial, bactericidal properties, and distinctive aroma. Additionally, it serves as a precursor for the synthesis of valuable downstream products, including sclareolide and ambrox, thereby holding significant industrial potential [1, 2]. Traditionally, sclareol is primarily sourced from the flowers and leaves *Salvia Sclarea*. However, conventional plant extraction methods face criticism for being environmentally damaging, cost-prohibitive, and inefficient [3]. In contrast, the rapid advancements in synthetic

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biology have positioned microbial biosynthesis of diterpene as a promising alternative, offering a more sustainable and efficient approach to traditional plant-based extraction [4, 5].

Yarrowia lipolytica is an emerging unconventional oleaginous yeast, as a safe yeast confirmed by the FDA. One of the primary advantages of *Y. lipolytica* over other microbial platform is its robust tricarboxylic acid (TCA) cycle, which efficiently generates key precursors such as acetyl-CoA, ATP, and various cofactors through the utilization of diverse carbon sources [6]. Furthermore, *Y. lipolytica* possesses a well-established endogenous mevalonate (MVA) pathway, enabling the production of significant quantities of isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) for terpene synthesis [7]. Therefore, *Y. lipolytica* is considered as an attractive and promising microbial chassis to produce high-value bioproducts, particularly terpenes [8]. In recent years, considerable efforts have been dedicated to the biosynthesis of various terpenoids using *Y. lipolytica*, including farnesene [9, 10], limonene [11, 12], squalene [13, 14], and β-carotene [15, 16]. However, research on the biosynthesis of diterpenes remains relatively limited, likely due to the limitation of geranylgeranyl pyrophosphate (GGPP) content, product toxicity, and other associated limitations.

The sclareol biosynthesis pathway in microorganisms involves two primary metabolic modules: the synthesis of GGPP from acetyl-CoA via the MVA pathway, and the conversion of GGPP to sclareol, catalyzed by LPPS and SCS. Recent advancements in metabolic engineering and synthetic biology have facilitated the successful biosynthesis of sclareol in engineered microbial cell factories through various strategies. For instance, Schalk et al.. reported the first successful biosynthesis of sclareol in *Escherichia coli*, achieving a yield of 1.5 g/L [17]. Cao et al. employed a global metabolic engineering approach in *Saccharomyces cerevisiae*, strengthening the MVA pathway and enzymatically modifying the ERG20 gene, resulting in a significant increase in sclareol production, reaching 11.4 g/L via fed-batch fermentation [18]. Furthermore, Sun et al. constructed a de novo sclareol biosynthetic pathway in *Y. lipolytica* and optimized the interaction between LPPS and SCS using a scaffold protein, enhancing the yield of sclareol up to 12.9 g/L in a 5-L bioreactor [19]. These pioneering efforts have made significant strides in improving sclareol biosynthesis. However, while these advancements represent substantial progress compared to previous reports, further optimization through more precise metabolic regulation holds the potential for even higher production yields.

In this study, we successfully achieved the production of sclareol in *Y. lipolytica* by introducing a heterologous biosynthesis pathway, integrating multiple copies of sclareol synthase, upregulating the mevalonate pathway, and enhancing intracellular acetyl-CoA synthesis (Fig. 1). This approach led to a substantial increase in sclareol yield, reaching 2656.20 ± 91.30 mg/L in shake flasks. These results underscore the significant potential of *Y. lipolytica* for the commercial biosynthesis of sclareol. Furthermore, this study highlights the effectiveness of precursor supply optimization and carbon flux remodeling in engineered strains, offering valuable insights for the microbial synthesis of other diterpenoids in *Y. lipolytica*.

Materials and methods

Strains and plasmids

The initial strain *Y. lipolytica* Po1f-tHEI and the related recombinant engineered strains are listed in Table 1. Construction and amplification of plasmids were completed using *Escherichia coli* DH5α (TransGen, Beijing). The primers used in this study were synthesized by Tsingke Biotech (Beijing) Co., Ltd. The plasmids and primers involved in this study are summarized in Tables S1 and S2, respectively.

DNA manipulation

The heterologous SsLPPS, SsSCS from *Salvia sclarea* [17, 20] and tPaGGPPS from *Phomopsis amygdali* [19] were codon-optimized and synthesized by Sangon Biotech (Shanghai) Co., Ltd. The endogenous genes used to overexpress were amplified from the Po1f genome. Gene elements like promoters and terminators (*pTEFin*, *pFBAin*, *php4d*, *xpr2t*, *Tsynth7t*) used in plasmid construction were amplified from conserved plasmids. The assembly methods of gene integration plasmids pINA1312, pINA1269 and target genomic loci knockout plasmids CRISPRyl-Cas9 vectors were illustrated in previous studies [21, 22]. Plasmids used in this study were constructed using *pEASY*®-Basic Seamless Cloning and Assembly Kit (TransGen, Beijing), and extracted using Plasmid Mini-Prep Kit (Generay, Shanghai). Details regarding plasmid construction and colony PCR identification can found in a previous study [23]. The sequences of the related genes are presented in Table S3.

Cultivation and medium

The *E. coli* DH5α used for vector construction was cultured at 37℃ in Luria-Bertani (LB) broth medium (25 g/L) supplemented with 50 μg/mL kanamycin or 100 μg/mL ampicillin. The engineered *Y. lipolytica* strains were cultured in Yeast Extract Peptone Dextrose (YPD) agar medium (10 g/L yeast extract, 20 g/L tryptone, and 20 g/L glucose) for obtaining yeast single colonies or in enrichment YPD liquid medium (20 g/L yeast extract, 40 g/L tryptone, and 60 g/L glucose) for cultivation of seed liquid and fermentation. Before fermentation,

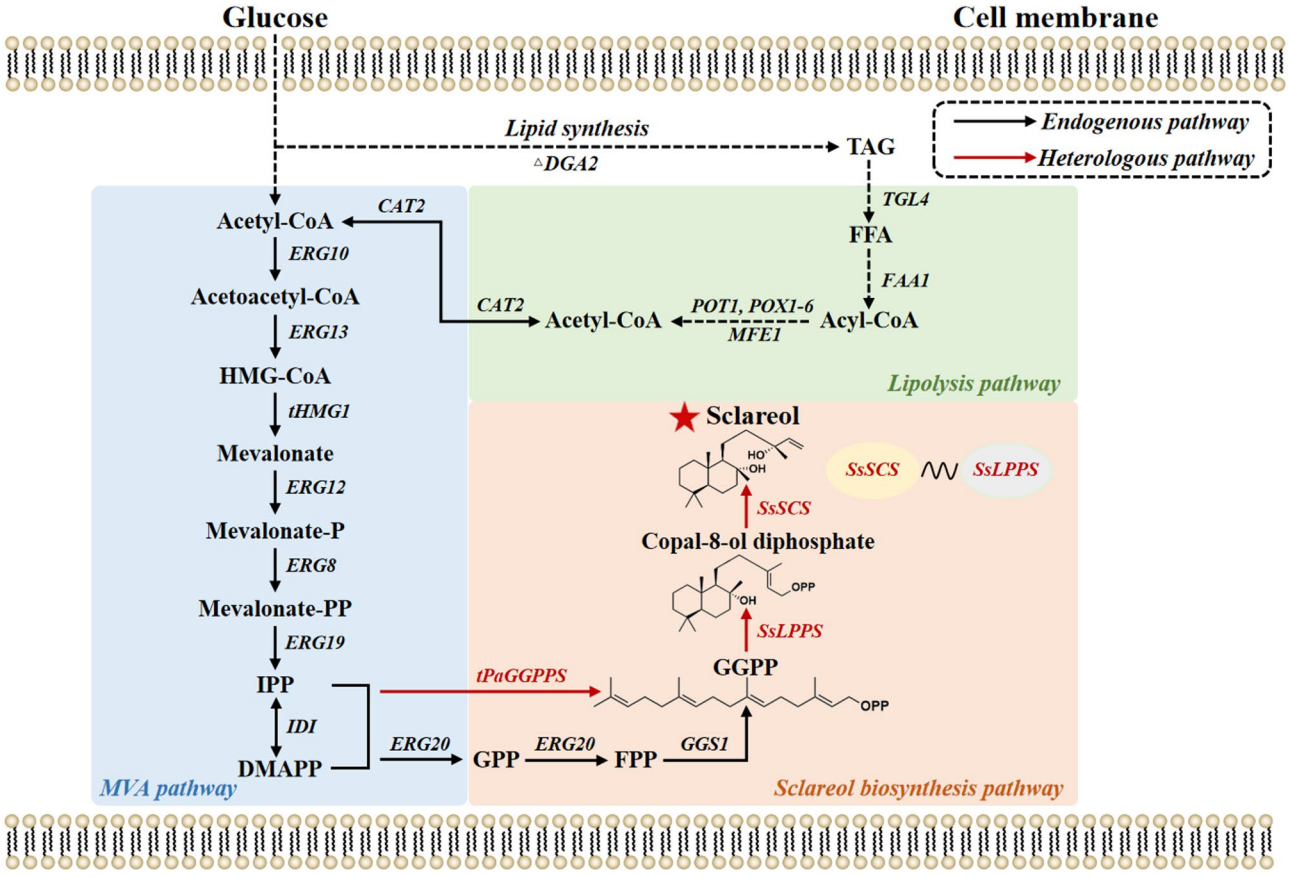

Fig. 1 Schematic diagram of biosynthetic pathways of sclareol in *Y. lipolytica*

several single colonies were cultured in 25 mL flask containing 5 mL YPD medium and grown at 30℃ and 220 rpm for 24 h as a seed culture liquid. The seed culture liquid was then transferred into a 250 mL flask containing 50 mL of YPD medium, with the initial fermentation solution OD_{600} controlled at 0.5. The fermentation culture condition is 30 ℃ and 220 rpm for 6 days, 10% of the fermentation volume of dodecane was added after 12 h of fermentation for product extraction.

Transformation and strain screening

The Frozen EZ Yeast Transformation II [ZYMO RESEARCH, USA] was used to perform linear and CRISPRyl-Cas9 plasmid transformation according to the method described in a previous study [24]. The transformed yeast was coated on the Yeast Nitrogen base (YNB) plate (6.7 g/L yeast nitrogen base, 10 g/L glucose, 2% agar) and cultivated at 30℃ for 2–4 days to obtain transformants. Then, the transformants were verified through colony PCR to confirm whether gene integration was successful. The transformants with successful gene integration were cultured on YPD plates containing 5-Fluoroorotic Acid (5-FOA) at least 36 h to recycle the URA3 selectable marker.

Quantification of biomass, glucose and metabolite concentrations

The quantification of biomass and residual glucose concentration during fermentation were performed as previously described [25]. After fermentation, the dodecane phase containing sclareol and by-product (GGOH) were collected by centrifugation at 7500 rpm for 8 min. The upper organic phase was diluted with dodecane to a suitable concentration within the range of standard curve and subsequently filtered through a 0.22 μm organic membrane filter. The samples processed by the above method were used for GC-MS analysis on a 7890–8975 C Network GC System (Agilent, USA) using an Agilent HP-5MS column (30 m × 250 μm × 0.25 μm). The GC-MS program was set as follows: the injector temperature was 250 °C and the initial oven temperature was 60 °C, then increase to 160 °C at a rate of 10 °C/min, hold for 1 min, and then increase to 280 °C at a rate of 40 °C/min with a hold for 4 min. The sample injection volume was 1μL, helium was used as carrier gas at a constant flow of 1 mL/min and the traffic splitting was 30 mL/min. The sclareol and GGOH standards (GC ≥ 98%) were purchased from Macklin and yuan ye Bio-Technology.

Table 1 Strains used in this study

Strains	Description	Source
Po1f-tHEI	Po1f-Δku70, URA3-, LEU2-, ΔIntC::*tHMG1*, ΔIntF::*IDI*, ΔScp2::*ERG20*	Prof. Xiaojun Ji from Nanjing Tech University
YAs0	Po1f-tHEI, pINA1312-L + S	This study
YAs1a	Po1f-tHEI, pINA1312-S-GGG-L	This study
YAs1b	Po1f-tHEI, pINA1312-S-GSG-L	This study
YAs1c	Po1f-tHEI, pINA1312-S-GGGS-L	This study
YAs1d	Po1f-tHEI, pINA1312-S-GSTSSG-L	This study
YAs2	Po1f-tHEI-D069::FBAin-SL	This study
YAs3	Po1f-tHEI, pINA1269-tPaGGPPS-FBAin-SL	This study
YAs4	YAs3-D069::FBAin-SL	This study
YAs5	YAs3-D069::GPD-SL	This study
YAs6	YAs3-D069::TEFin-SL	This study
YAs7	YAs3-D069::TEFxo-SL-E153::VPRHX	This study
YAs8	YAs6-D17::ERG19	This study
YAs9	YAs6-D17::ERG13	This study
YAs10	YAs6-D17::ERG12	This study
YAs11	YAs6-D17::ERG8	This study
YAs12	YAs6-D17::ERG19-ERG13	This study
YAs13	YAs6-E153::ERG12-ERG8	This study
YAs14	YAs12-E153::ERG12-ERG8	This study
YAs15	YAs14-EXG2::TGL4	This study
YAs16	YAs14-EXG2::FAA1	This study
YAs17	YAs14-EXG2::POX2	This study
YAs18	YAs14-EXG2::MFE1	This study
YAs19	YAs14-EXG2::POT1	This study
YAs20	YAs14-EXG2::CAT2	This study
YAs22	YAs20-ΔDGA2	This study
YAs23	YAs22-AXP::SL	This study
YAs24	YAs23-F262::SL	This study

Results

Construction of the sclareol biosynthetic pathway in *Y. lipolytica*

The native *Y. lipolytica* is capable of synthesizing the sclareol precursor GGPP via an endogenous metabolic pathway. In this study, the chassis strain po1f-tHEI has been engineered to enhance the expression of key endogenous enzymes within the MVA pathway, including 3-hydroxy-3-methylglutaryl coenzyme A reductase (tHMG1), isopentenyl diphosphate delta-isomerase (IDI), and farnesyl diphosphate synthase (ERG20), thereby promoting the accumulation of GGPP. Subsequently, codon-optimized sclareol biosynthetic genes from *Salvia sclarea* (SsLPPS and SsSCS) were integrated into *Y. lipolytica* Po1f-tHEI using the plasmid pINA1312, resulting in the strain YAs0. After 6 days of fermentation, samples were analyzed by GC-MS and compared to a standard, yielding 3.56 mg/L of sclareol (Fig. 2 and Fig. S1).

Moreover, fusion proteins linked by short flexible peptides have been shown to enhance the probability of enzyme collision with intermediate complexes, thereby improving the catalytic efficiency of substrate utilization [26]. In this study, we explored the effects of different flexible linkers by fusing SsSCS and SsLPPS, obtaining strains YAs1a-d (Fig. 2). The fusion of the two enzymes with various linkers aimed to investigate the impact of linker flexibility on protein function and sclareol yield. As expected, strain YAs1a expressing the fused module SsSCS-GGG-SsLPPS (SL) produced 10.47 ± 0.37 mg/L sclareol, which was 2.94 times higher than that of strain YAs0. A similar effect was observed with the GSG linker, yielding 9.87 ± 0.40 mg/L of sclareol. However, as the linker length increased, the yield of sclareol declined sharply, with no sclareol detectable in strain YAs1d, which expressed the fused module SsSCS-GSTSSG-SsLPPS. These results

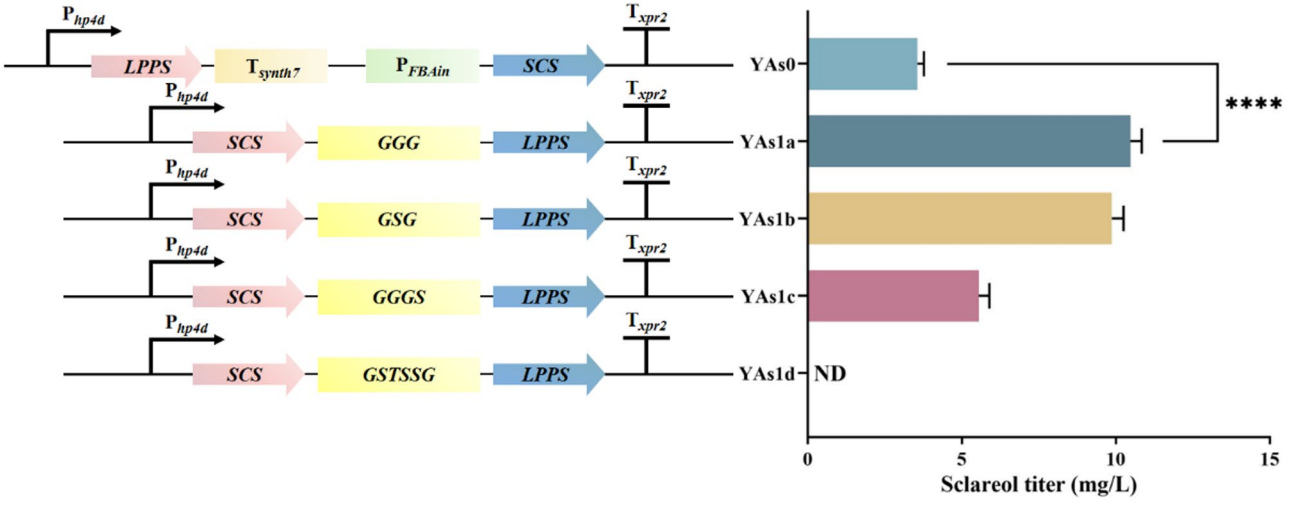

Fig. 2 The sclareol production of strains expressing the different fused module (ND: not detected). YAs0: Co-expression of SsSCS and SsLPPS. YAs1a–d: Fusion of SsSCS and SsLPPS using four flexible linkers: GGG, GSG, GGGS, GSTSSG. Bars and error bars represent the mean and standard deviation of three biological replicates. ****$p < 0.0001$

suggest that the length of the linker plays a crucial role in the expression and stability of fusion proteins, with longer linkers potentially leading to protein degradation, which adversely affects product yield [27]. Furthermore, Wei et al.. reported improved miltiradiene production in *S. cerevisiae* using a GSTSSG linker [27], while Cao et al.. increased the yield of sclareol in *S. cerevisiae* using the fused module SsSCS-GGGS-SsLPPS [18]. In contrast, Ye et al.. used the GGGGS linker to construct a fusion protein of ERG20 and LsLTC2, resulting in significantly enhanced β-elemene production in *Ogataea polymorpha* [28]. These discrepancies in the literature may be attributed to differences in the enzymes, target products, and host organisms used in each study, underscoring the complexity of optimizing fusion protein systems for specific biosynthetic pathways.

Redirecting metabolic fluxes to improve sclareol biosynthesis

To obtain a stable strain suitable for subsequent transformation, the SL was integrated into the *Y. lipolytica* Po1f-tHEI genome using the CRISPRyl-Cas9 system. However, sclareol production was undetectable in the strain YAs2 (Fig. 3B). We hypothesized that this failure could be attributed to insufficient accumulation of the endogenous GGPP and inadequate expression of sclareol synthase. Consequently, the endogenous geranylgeranyl pyrophosphate synthase, GGS1, was overexpressed to enhance the GGPP pool [29, 30], with two directed evolution mutants of GGS1 introduced to assess their effects [31], obtaining strains YAs2a-c. The GGPP content was analyzed through its dephosphorylated derivative, geranylgeraniol (GGOH) [26]. The findings revealed that strain YAs2a produced only 32.32 ± 2.68 mg/L GGOH and 3.24 ± 0.26 mg/L sclareol, suggesting that the activity of endogenous GGS1 was insufficient to direct the farnesyl pyrophosphate (FPP) flux toward sclareol biosynthesis. No significant improvement was observed in the other two strains (Fig. 3B). To screen for an efficient GGPP synthase, we compared three exogenous GGPPS genes with distinct enzymatic functions: XdGGPPS from *Xanthophyllomyces dendrorhous* [15], which generates GGPP by catalyzing the condensation of FPP and IPP; SaGGPPS from *Sulfolobus acidocaldarius* [32], and tPaGGPPS from *Phomopsis amygdali* [33], which catalyze the direct condensation of IPP and DMAPP to form GGPP. Among these, tPaGGPPS exhibited the highest efficacy, leading to a GGOH titer of 999.69 ± 67.12 mg/L and a sclareol titer of 29.83 ± 6.57 mg/L (Fig. 3B). This improvement is likely due to the highly efficient catalytic activity of tPaGGPPS, as well as the shorter GGPP synthesis pathway, which minimized metabolic shunting [34]. The tPaGGPPS and SL were co-expressed using pINA1269 to construct strain YAs3 for further investigations.

To further enhance the flux of GGPP toward sclareol biosynthesis, we sought to outcompete the endogenous phosphatases by integrating an additional copy of SL into the D069 locus of strain YAs3. Simultaneously, we employed different promoters, including GPD, TEFin, and TEFxo to drive the expression of the SL gene, with the aim of optimizing sclareol production. Among these, the TEFin promoter resulted in the highest sclareol titer of 342.68 ± 14.54 mg/L, accompanied by a GGOH concentration of 576.31 ± 17.98 mg/L (Fig. 3D). In addition to monitoring sclareol production, we evaluated the growth performance and glucose consumption of the various strains over a 6-day fermentation period in YPD medium, focusing specifically on strain YAs3-7. Compared to the control strain YAs3 and the other variants, strain YAs6 exhibited the highest sclareol titer, displayed a modest growth impairment during the first 72 h of fermentation (Fig. 3E). In contrast, the glucose consumption rate in strain YAs6 was notably higher than in the other strains (Fig. 3F).

Enhancement of the MVA pathway improves sclareol production

The endogenous MVA pathway is crucial for terpene biosynthesis in eukaryotic systems (Fig. 4A). A potential limitation in the MVA pathway becomes evident as product titers increase, leading to a rise in metabolic burden. Given that the genes tHMG1, IDI and ERG20 had already been overexpressed in the initial strain, we further explored the integration of additional key enzymes involved in the MVA pathway. These enzymes-mevalonate diphosphate decarboxylase (ERG19), hydroxymethylglutaryl-CoA synthase (ERG13), mevalonate kinase (ERG12) and phosphomevalonate kinase (ERG8)-were incorporated into the D17 locus of strain Yas6 to enhance the MVA pool and mitigate potential MVA depletion. As shown in Fig. 4B, sclareol production was significantly improved across all four engineered strains. Specifically, strain Yas8 produced 392.61 ± 10.59 mg/L of sclareol (a 14.57% increase), strain Yas9 yielded 423.26 mg/L (23.51% increase), strain Yas10 achieved 470.37 ± 26.23 mg/L (37.26% increase), and strain Yas11 generated 389.90 ± 11.78 mg/L (13.78% increase), all relative to the control strain. Our experiments demonstrated that most of the MVA pathway key enzymes were beneficial for the increase of GGPP and sclareol production, which can be attributed to product specificity.

To investigate the synergistic effect of the co-expression of key enzymes in the MVA pathway, we co-expressed ERG19 and ERG13, as well as ERG12 and ERG8, to generate YAs12 and YAs13, respectively. The sclareol titer in strain Yas13 increased to 549.67 ± 68.50 mg/L, representing a 60.40% enhancement. However, the co-expression of ERG19 and ERG13 resulted in a similar

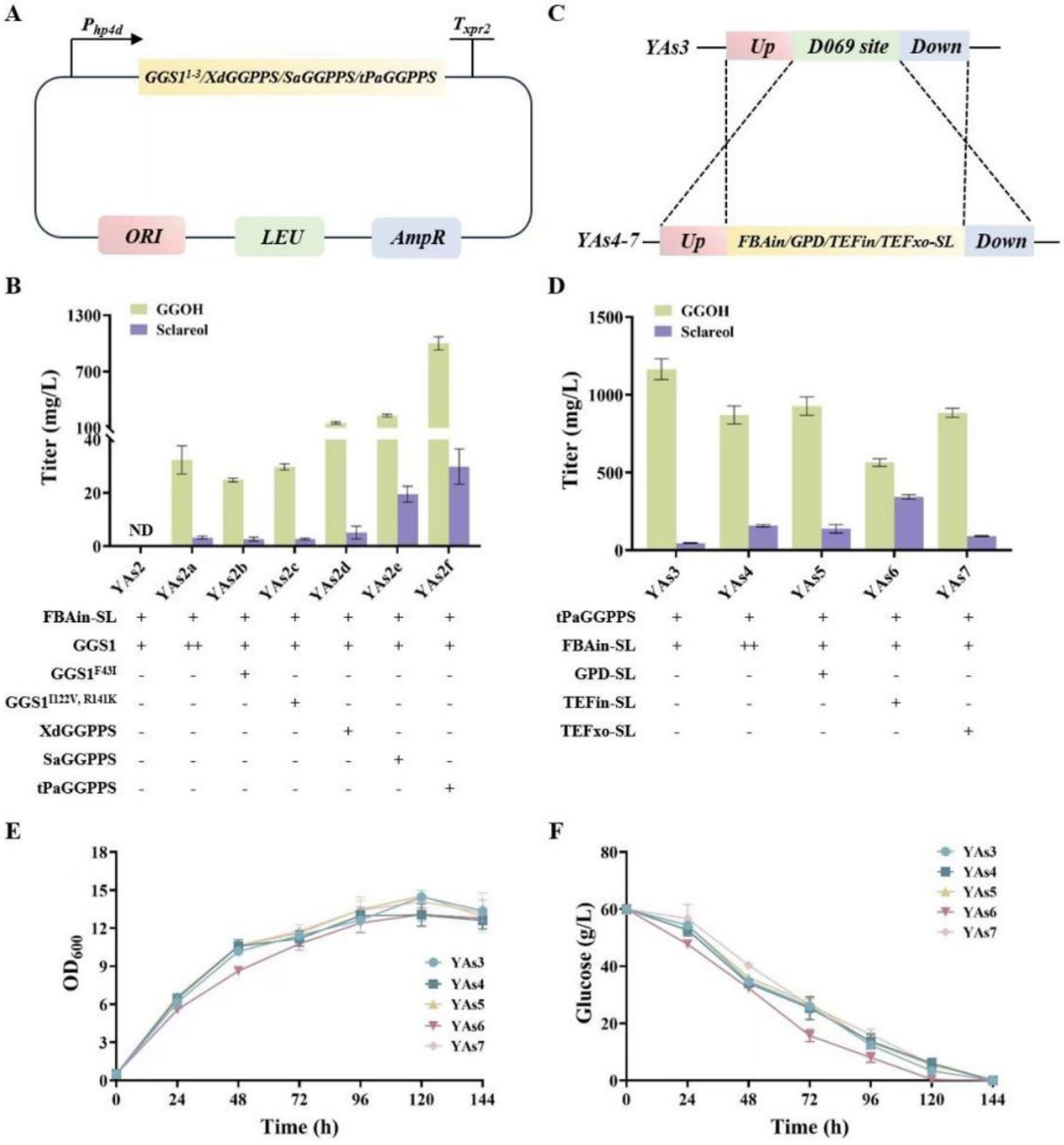

Fig. 3 Enhancement of sclareol synthesis via screening of GGPP synthase and promoters. (**A**) Schematic diagram of pINA1269 plasmid expressing GGPPS from different sources. (**B**) Screening for GGPPS genes to increase GGPP content. (**C**) The SL genes initiated by pFBAin, pGPD, pTEFin and pTEFxo at the D069 site. (**D**) Comparison of sclareol production in different promoters express SL. (**E**) The cell density (OD_{600}) curves of strain YAs3-7 during 6-days-long fermentation. (**F**) Glucose consumption curves of strain YAs3-7 during 6-days-long fermentation. Bars and error bars represent the mean and standard deviation of three biological replicates

outcome to the overexpression of ERG13 alone, suggesting a potential bottleneck in the pathway. This outcome may be attributed to the excessive accumulation of intermediate metabolite MVA [35], which may not be efficiently converted into downstream products. To further test this hypothesis, we integrated ERG12 and ERG8 into strain YAs12 to generate YAs14. As a result, the sclareol titer significantly increased from 445.93 ± 5.06 to 858.11 ± 17.17 mg/L. In parallel, strain YAs14 produced 1610.26 ± 16.76 mg/L of GGOH, which was 185.44%

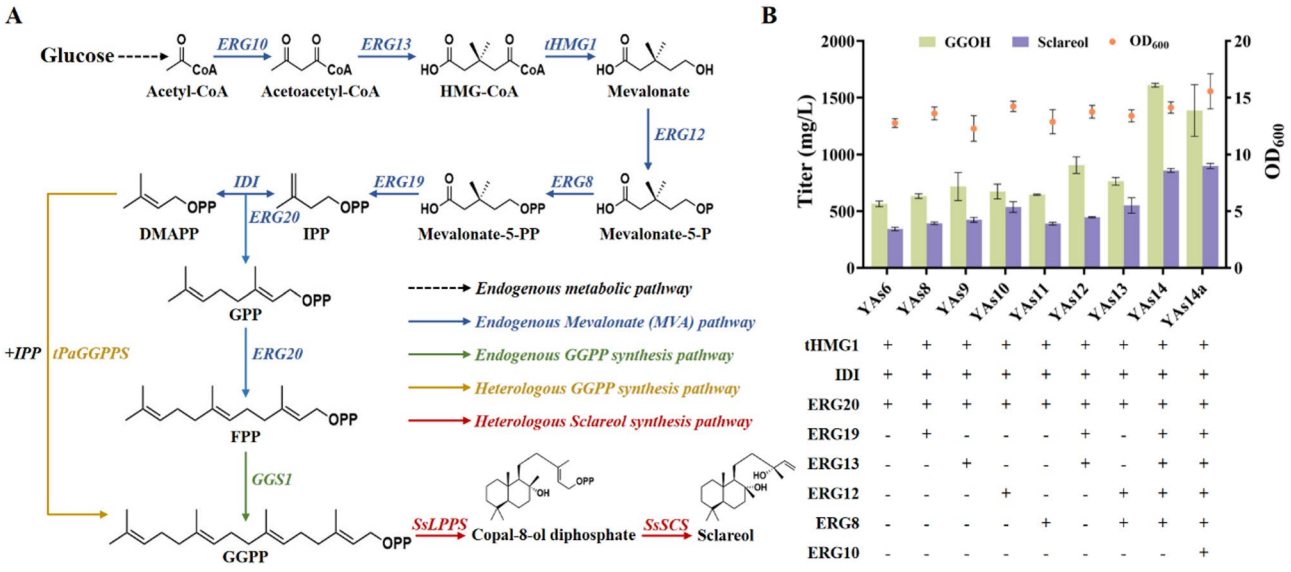

Fig. 4 Enhancement of the MVA pathway in *Y. lipolytica*. (**A**) Schematic representation of the sclareol biosynthetic pathway starting from glucose. (**B**) Titers of GGOH and sclareol in engineered strains overexpressing the genes of the MVA pathway after 6-days-long fermentation. Bars and error bars represent the mean and standard deviation, respectively, of biological triplicates

higher compared to YAs6 (Fig. 4B). This experiment highlights the successful balancing and enhancement of the MVA pathway, leading to strains with significantly higher titers of both sclareol and GGOH. These findings suggest that expanding the MVA pool is a promising strategy for improving terpenoid biosynthesis. Subsequently, the endogenous ERG10 gene [18], coding for acetoacetyl-CoA thiolase, was selected for overexpression in YAs14 using the vector pINA1312 to generate strain YAs14a. Contrary to expectations, no significant increase in sclareol production was observed in YAs14a (Fig. 4B). This lack of improvement suggests that the native ERG10 is already sufficiently active in converting acetyl-CoA to acetoacetyl-CoA, allowing efficient entry into the MVA pathway. Consistent with these results, previous studies investigating the overexpression of ERG10 have reported either marginal improvements or even slight reductions in production yields, further supporting the notion that additional overexpression of ERG10 may not provide a substantial benefit in this context [13, 36].

In short, these results likely uncover a previously unrecognized bottleneck in the biosynthesis of diterpenes in *Y. lipolytica*, demonstrating that ERG19, ERG13, ERG12 and ERG8 contribute distinctively to the MVA pathway, in addition to the well-established tHMG1. Moreover, the strain YAs14 which co-expresses ERG19, ERG13, ERG12 and ERG8, exhibited a significant enhancement in the titers of both sclareol and GGOH compared to other strains. This result suggests that overexpression of individual enzymes can lead to excessive accumulation of intermediates within the MVA pathway, which may impede optimal product synthesis. Consequently, it underscores the importance of balancing and optimizing the metabolic flus throughout MVA pathway. while earlier works established the foundational role of tHMG1 in MVA pathway, our study advanced the field by demonstrating that holistic pathway balancing — rather than single-enzyme amplification — was critical for high-yield diterpenoid synthesis in *Y. lipolytica*. This observation aligns with similar findings in other studies on terpene biosynthesis, including those focused on trans-nerolidol [37], patchoulol [38], α-humulene [39], β-carotene [40], where metabolic balancing was also found to be critical for efficient product yields.

Enhancing supply of acetyl-CoA and increasing the copy number of sclareol synthase for further sclareol production

Y. lipolytica, an oleaginous yeast, is capable of accumulating lipids that constitute 30-50% of its dry cell weight [41]. Acetyl-CoA serves as a central precursor for both lipid and terpene biosynthesis in this organism. Therefore, strategies aimed at attenuating lipid biosynthesis while enhancing lipid degradation to redirect acetyl-CoA flux towards terpene production may effectively increase sclareol yield (Fig. 5A). The degradation of triacylglycerides (TAG) [42], the conversion of acyl-CoA to free fatty acid (FFA) [43], and the β-oxidation pathway all contribute to the generation of acetyl-CoA. In preoxisomal β-oxidation, acetyl-CoA is produced through a four-step enzymatic cycle involving acyl-CoA oxidase (POX2) [44], multifunctional enzyme (MFE1) [45] and 3-ketoacyl-CoA thiolase (POT1) [42]. Ultimately, the acetyl-CoA generated is transported into the cytoplasm with the assistance of peroxisomal carnitine acetyltransferase (CAT2) [37].

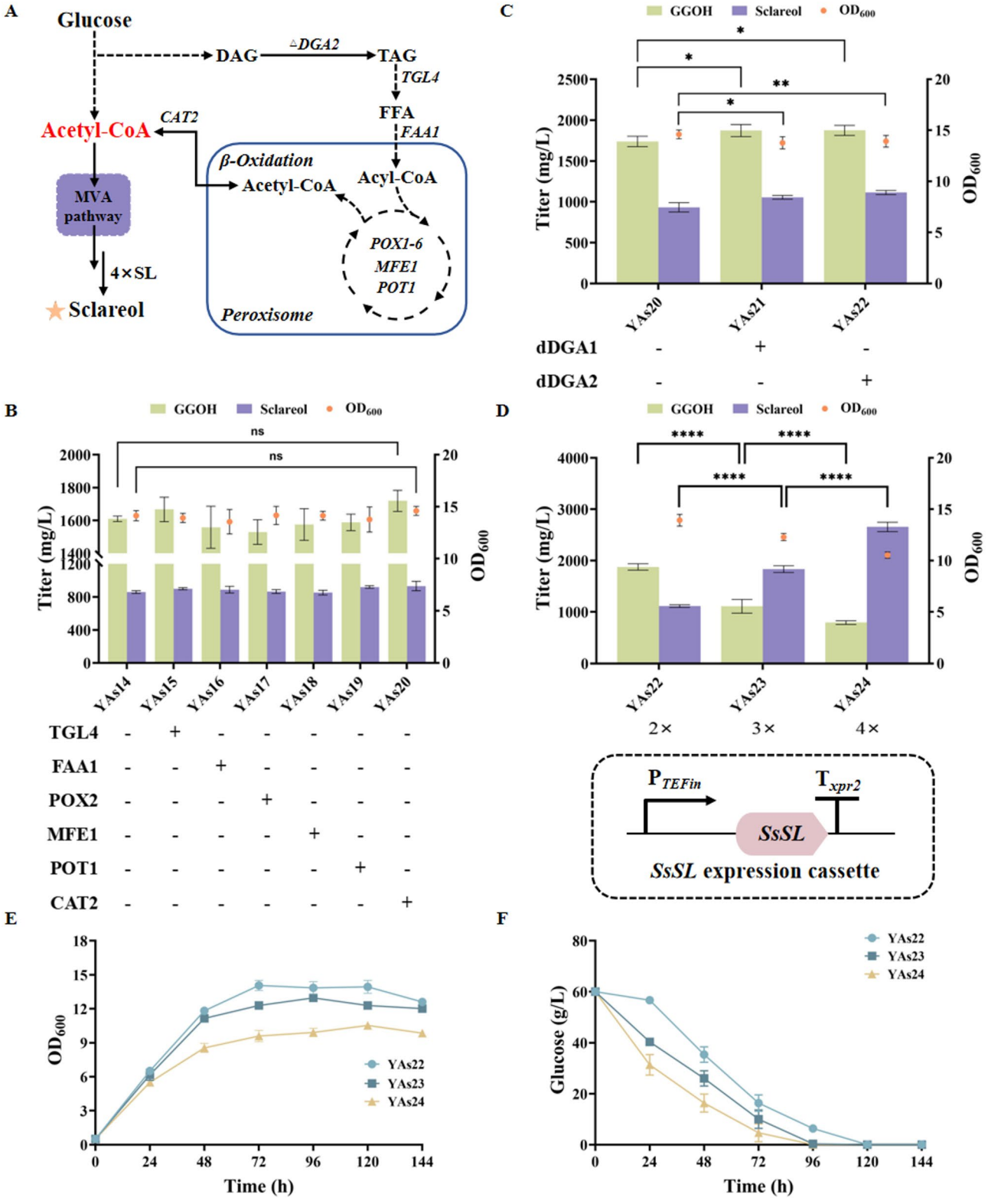

Fig. 5 Enhancing supply of acetyl-CoA and increasing the copy number of sclareol synthase for further sclareol production (**A**) Schematic diagram for increasing acetyl-CoA supply. (**B**) Titers and OD_{600} of strains which separately overexpressed lipolytic pathway genes. (**C**) Titers and OD_{600} of strains which separately deleted the lipid synthesis pathway genes. (**D**) Effects of increasing SsSL copy numbers on the titers of sclareol and GGOH. (**E**) The OD_{600} curves of strain YAs22-24 during 6-days-long fermentation. (**F**) Glucose consumption curves of strain YAs22-24 during 6-days-long fermentation. Bars and error bars represent the mean and standard deviation of three biological replicates. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

These enzymes were subsequently engineered in strain YAs14 to assess the potential for enhanced acetyl-CoA flux and its impact on terpene production.

As shown in Fig. 5B, strains YAs15-19 overexpressing the endogenous genes TGL4, FAA1, POX2, MFE1 and POT1 showed a modest variation in sclareol production, with titers of 899.14 ± 13.51 mg/L, 887.26 ± 40.44 mg/L, 864.46 ± 23.56 mg/L, 851.78 ± 28.72 mg/L and 919.83 ± 15.12 mg/L, respectively. In contrast, the strain YAs20 overexpressing CAT2 exhibited the highest sclareol yield of 931.30 ± 56.98 mg/L, representing an 8.53% increase compared to the control strain YAs14. However, there was no significant difference (Fig. 5B). Furthermore, diacylglycerol acyltransferases (DGA1 and DGA2) are pivotal in lipid synthesis [46]. Inhibition of TAG synthesis by knocking out DGA1 and DGA2 has been shown to increase the production of various metabolites, including β-farnesene [43], polydatin [22], scutellarin [47] etc. Therefore, DGA1 and DGA2 were deleted in strain YAs20, resulting in the generation of YAs21 and YAs22, respectively. Shake-flask fermentation results demonstrated that the deletion of DGA1 or DGA2 significantly enhanced sclareol production, achieving titers of 1054.53 ± 23.01 mg/L (YAs21) and 1113.17 ± 25.69 mg/L (YAs22), respectively. Notably, both strains accumulated nearly 1900 mg/L of sufficient GGOH pool under normal growth conditions (Fig. 5C).

By enhancing the MVA pathway and the supply of acetyl-CoA, a substantial accumulation of the by-product GGOH was observed in strain YAs22, indicating that the supply of sclareol precursor GGPP was adequate and sclareol biosynthesis may be hindered by the incomplete conversion of GGPP to sclareol. Consequently, we sought to further enhance the conversion of GGPP to sclareol by increasing the expression of SsSL gene. This strategy led to a significant increase in sclareol production, reaching 1833.67 ± 64.89 mg/L in strain YAs23, resulted in a 1.65-fold improvement compared to strain YAs22. Subsequently, to explore the effect of further increasing the copy number, an additional copy of SsSL was integrated into strain YAs23. The resulting strain YAs24, which harbored four additional SsSL copies, produced 2656.20 ± 91.30 mg/L of sclareol, corresponding to a 2.39-fold increase over YAs22 (Fig. 5D). Notably, the GGOH concentration in strain YAs24 significantly decreased to 797.17 ± 35.59 mg/L, accompanied by a slower growth rate (Fig. 5D and E). These findings suggest that the concentration of GGPP or GGOH may serve as an indicator of the strain's growth status. When considering future metabolic engineering modifications to strain YAs24, it will be essential to focus on optimizing GGPP accumulation rather than further increasing the expression of SsSL. Overexpression of SsSL may induce a metabolic burden, thereby disrupting the internal metabolic balance of the strain.

Discussion

While previous efforts in *E. coli* and *S. cerevisiae* have laid the groundwork for sclareol production, our study introduces several transformative modifications that leverage the unique physiological and metabolic characteristics of *Y. lipolytica*. Unlike *E. coli*, which lacks a native MVA pathway and requires extensive modifications for terpene production [17], *Y. lipolytica* inherently possesses a robust MVA pathway and an efficient acetyl-CoA supply for diterpene synthesis [7]. In comparison to *S. cerevisiae*, which achieved 11.4 g/L of sclareol via fed-batch fermentation [18], the strain (YAs24) produced 2656.20 ± 91.30 mg/L in shake flasks without process optimization, demonstrating its inherent scalability potential. Previous studies in *Y. lipolytica* used scaffold proteins to enhance enzyme colocalization for sclareol synthesis [19]. However, our work used flexible short peptide linkers (GGG or GSG) to directly fuse SsSCS and SsLPPS, achieving a 2.94-fold increase in sclareol titer (Fig. 2). This approach avoids the potential metabolic burden of oversized scaffold protein expression and simplifies genetic manipulation. Notably, linker length critically impacted enzyme stability, with longer linkers (e.g., GSTSSG) potentially leading to protein degradation (YAs1d), which adversely affects product titer. These findings underscore the precision required in fusion enzyme design and highlight a trade-off between flexibility and stability. Moreover, while previous studies on the MVA pathway have focused on the rate-limiting gene tHMG1 [19, 30], our work systematically optimized the MVA pathway in *Y. lipolytica* by co-expressing ERG19, ERG13, ERG12, and ERG8 to balance the flux, avoiding intermediate accumulation and increasing sclareol production by 1.5-fold (Fig. 4A&B). This approach mirrors similar strategies used in *S. cerevisiae* for sesquiterpene production, demonstrating its broader applicability [36]. Notably, our work is the first to apply this strategy to diterpene biosynthesis in *Y. lipolytica*.

In contrast to previous efforts that focused solely on enhancing precursor GGPP supply, we implemented a dual strategy by downregulating lipid synthesis (ΔDGA1/ΔDGA2) and upregulating lipid degradation (CAT2 overexpression) to redirect acetyl-CoA flux towards sclareol biosynthesis (Fig. 5A). This metabolic reprogramming resulted in a 29.72% increase in sclareol titer (Fig. 5B&C). While plasmid-based expression systems are commonly used in *S. cerevisiae* [18] and *E. coil* [17], they are prone to genetic instability and antibiotic dependency. We addressed these limitations by integrating multiple copies of the SsSL fusion gene into the genome under the strong constitutive promoter TEFin. This strategy minimized

the risk of plasmid loss in industrial-scale fermentation, achieving a sclareol titer of 2656.20 ± 91.30 mg/L. However, there are limitations to this study. The integration of multiple gene copies led to a reduced growth rate of the engineered strain, and the potential inhibitory effects of high sclareol concentrations on cellular activity remain unexplored. Further studies are need to investigate product toxicity. Additionally, enhancing strain tolerance through adaptive evolution or targeted genetic modifications could help alleviate growth impairments under high-productivity conditions. Finally, while the shake-flask experiments showed promising results, scaling up to bioreactor systems is essential for validate industrial applicability, particularly in terms of oxygen transfer, pH control and long-term culture stability.

In summary, our study presents a combinatorial metabolic engineering framework tailored specifically for *Y. lipolytica*, incorporating enzyme fusion, lipid metabolism regulation, and multi-copy genome editing. The genetic and metabolic engineering strategies we employed offer a systematic and innovative approach to diterpenoid biosynthesis in *Y. lipolytica*, overcoming critical challenges encountered in previous microbial platforms. These advancements address key limitations from prior research, such as precursor competition and enzyme instability.

Conclusion

This study successfully engineered a *Y. lipolytica* strain capable of producing a sclareol titer of 2656.20 ± 91.30 mg/L. The strategies employed included the introduction of a heterologous biosynthesis pathway, enhancement of precursors supply, upregulation of the mevalonate pathway, optimization of intracellular acetyl-CoA synthesis, and integration of multiple copies of sclareol synthase. This work provides a valuable framework for the efficient production of sclareol and other diterpenes in *Y. lipolytica*, presenting a promising approach to accelerate the industrial-scale production of sclareol.

Supplementary Information

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Supplementary Material 1

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Author contributions

B.C.Y, Y.Z and J.C designed the research. J.C and L.Z.H performed the experiments. J.C analyzed the data and wrote the manuscript. Y.Z and J.C revised the manuscript. B.C.Y provided funding support. All authors read and approved the final manuscript.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

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