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Engineered Microbial Consortium for *De Novo* Production of Sclareolide

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ABSTRACT: Sclareolide, a natural product with bioactive and fragrant properties, is not only utilized in the food, healthcare, and cosmetics industries but also serves as a precursor for the production of ambroxide and some bioactive compounds. Currently, there are three primary methods for producing sclareolide: direct extraction from plants, chemical synthesis using sclareol as a precursor, and the biotransformation of sclareol. Here, we established a platform for producing sclareolide through a modular coculture system with *Saccharomyces cerevisiae* and *Cryptococcus albidus* ATCC 20918. *S. cerevisiae* was engineered for *de novo* sclareol biosynthesis from glucose, while *C. albidus* enabled the production of sclareolide via sclareol biotransformation. To enhance the supply of sclareol, a recombinant yeast strain was constructed through metabolic engineering to produce 536.2 mg/L of sclareol. Further improvement of the coculture system for sclareolide production was achieved by incorporating Triton X-100 facilitated intermediate permeability, inoculation proportion adjustment, and culture temperature optimization. These refinements culminated in a sclareolide yield of 626.3 mg/L. This study presents a novel streamlined and efficient approach for sclareolide preparation, showcasing the potential of the microbial consortium in sustainable bioproduction.

KEYWORDS: *sclareolide, sclareol, cocultivation, microbial consortium, Saccharomyces cerevisiae, Cryptococcus albidus*

1. INTRODUCTION

Sclareolide is a sesquiterpene lactone naturally isolated from arnica and other plants, with robust antifungal, antitumor, and antiviral activities.[1] Sclareolide is not only used as raw material in various industrial sectors of fragrance,[2] botanical pesticides,[3] and medicinal reagents,[4] but also applied as a flavoring agent in tobacco and foods.[5] In addition to these applications, sclareolide also serves as a precursor for ambroxide production. Ambergris is a rare intestinal secretion from sperm whales and has been used as a natural fragrance and medicine for a long history.[6] As the main odor constitute of ambergris, ambroxide is now a highly regarded substitute for natural ambergris in the perfume industry.[7] Currently, ambroxide is mainly produced from plant-derived sclareol via chemical synthesis, involving three stages: side chain oxidation of sclareol alcohol to sclareolide, reduction of sclareolide to ambradiol, and dehydration and cyclization of ambradiol to ambroxide (Figure S1).[8] However, challenges such as clean extraction of sclareol from *Salvia sclarea*, the residues of metal hydride reduction reagents, and the low yield of the chemical route render this manufacturing technology economically inefficient and environmentally detrimental.[9,10]

At present, sclareolide can be prepared by either chemical synthesis[11] or plant extraction.[12] Most chemical synthesis approaches are based on the oxidation of sclareol using potassium permanganate, but the chemical reagent residues and poor stereoselectivity have greatly limited its application. Although sclareolide is naturally present in some plants, large-scale extraction of plant-derived sclareolide is impractical due to its extremely low content and complicated purification processes.[13] Thus, both academia and industries are increasingly drawn to pioneering biosynthetic/biotransformation methods, aiming to revolutionize the industrial production of sclareolide.

Inspired by the ubiquitous natural microbial communities, artificial microbial communities have been emerging for enhancement of biomanufacturing.[14] In coculture communities, microbes interact with each other through signaling and/or defense molecules. Microbial coculture strategies are increasingly applied in biotechnology, ecology, and other fields.[15] Despite notable achievements in metabolic engineering and strain improvement, challenges persist in using single microorganisms to synthesize various value-added chemicals, particularly in the biosynthesis of complex natural products in the context of unresolved or complicated metabolic pathways.[16] Modular coculture engineering offers a solution by allowing the rational design of an entire metabolic pathway distributed in different organism modules. For instance, artificial microbial communities have been designed to produce valuable natural products, such as the scaffold molecules of paclitaxel and tanshinone,[17] flavonoids,[18] salidroside,[19] and ambradiol.[20,21]

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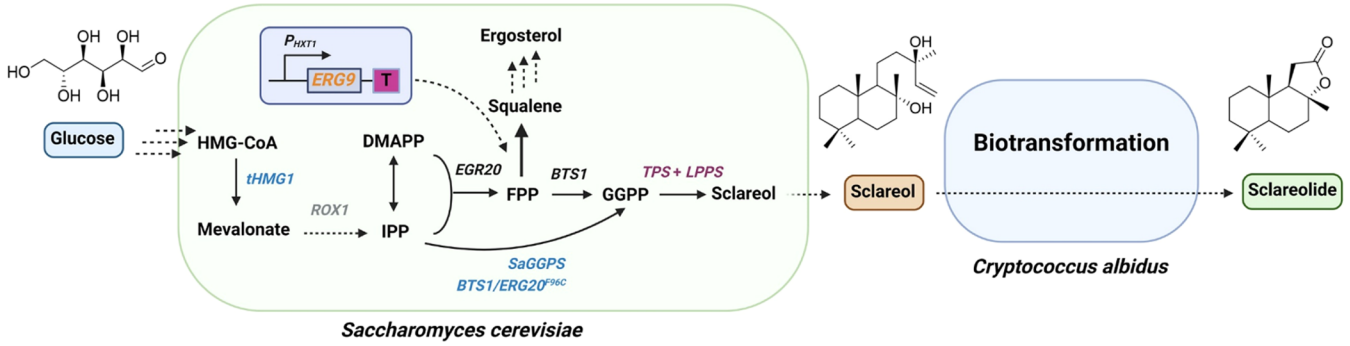

Figure 1. Schematic overview of the engineered microbial consortium for sclareolide production. *S. cerevisiae* was engineered for *de novo* sclareol production from glucose, while *C. albidus* enabled the production of sclareolide via sclareol biotransformation. Solid-line arrows indicate one-step conversions, while dotted-line arrows represent multiple steps. Blue fonts, overexpression of endogenous genes; purple fonts, overexpression of exogenous genes; orange fonts, gene restraint; gray fonts, gene inactivation. tHMG1, the catalytic domain of 3-hydroxy-3-methylglutaryl-CoA reductase; ROX1, a transcriptional regulator of sterol biosynthesis; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; BTS1, farnesyltransferase; ERG20^{F96C}, the F96C mutant of farnesyl diphosphate synthase; SaGGPS, geranylgeranyl diphosphate synthase; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; ERG9, squalene synthetase; LPPS, LDPP synthase from *S. sclarea*; TPS, sclareol synthase from *S. sclarea*.

Thus, harnessing the innate ability of natural isoprene related products to traverse cell membranes for signal transmission, sustainable and green production of sclareolide from simple carbon sources becomes a tangible prospect through coculture engineering. The biosynthetic pathway of sclareolide can be compartmentalized into two modules: the sclareol synthesis module and the sclareolide formation module. Previous studies have functionally characterized the genes responsible for sclareol biosynthesis in *S. sclarea*, leading to the reconstruction of sclareol biosynthetic pathway in *Escherichia coli*.[22] *Saccharomyces cerevisiae* has been widely recognized as a superior chassis for diterpene compounds production. While numerous strains can transform sclareol, the majority of these strains can merely modify the main ring by hydroxylation rather than the branched chain rearrangement.[23] Only a limited number of strains are capable of modifying sclareol at the branched chain to form ambradiol or sclareolide; for instance, *Cryptococcus albidus* ATCC 20918 and *Filobasidium magnum* JD1025 can convert sclareol to sclareolide.[24,25] Notably, the *C. albidus* strain has garnered significant attention for its excellent sclareol conversion capacity.[24] Given the lack of knowledge regarding the biocatalytic mechanisms for this valuable bioconversion, microbial coculture engineering could bypass the obstacle to achieve green biosynthesis of sclareolide.

In this study, we devised a modular coculture system for sclareolide production within an artificial microbial consortium. Initially, we engineered a recombinant strain of *S. cerevisiae* to produce sclareol, serving as the upstream substrate supply module. Concurrently, strain *C. albidus* ATCC 20918 was applied as the downstream biotransformation module, facilitating the conversion of sclareol to sclareolide. By integrating the two modules, *de novo* synthesis of sclareolide was accomplished by the coculture system utilizing a simple carbon source. Subsequently, the production of sclareolide by the microbial consortium was optimized by cell permeabilization, adjusting coculture temperature, and inoculation ratio, resulting in a titer of 626.3 mg/L. This is a new strategic scheme for the biosynthesis of sclareolide, laying a robust foundation for sustainable biomanufacturing of sclareolide.

2. MATERIALS AND METHODS

2.1. Strains, Media, and Reagents. *E. coli* DH5α was used for plasmid construction and amplification. All recombinant *E. coli* cells were cultured in Luria broth (LB) medium supplemented with 100 μg/mL ampicillin when necessary. All recombinant *S. cerevisiae* strains used in this study were derivatives of strain *S. cerevisiae* INVSc1, as listed in Table S1. *S. cerevisiae* strains were cultured at 30 °C in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) or synthetic dextrose (SD) minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.2% amino acid mix without the selected amino acid). *C. albidus* ATCC 20918 was cultured at 25 °C in a YPD medium. For batch fermentations, including mono- and coculture systems, Y1 medium (0.1% NH_4Cl, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.1% yeast extract, and 2.5% glucose) was applied. PrimeSTAR Max DNA Polymerase, restriction endonucleases, and T4 DNA ligase were purchased from Takara (Dalian, China). Phanta Super-Fidelity DNA Polymerase and ClonExpress Ultra One Step Cloning Kit were bought from Vazyme Biotech Co., Ltd. (Nanjing, China). Antibiotics and amino acids were acquired from Sangon Biotech (Shanghai, China). Sclareol and sclareolide (98% purity) were purchased from Sigma-Aldrich (Darmstadt, Germany).

2.2. Construction of Plasmids and Strains. All primers used in this study (Table S2) were synthesized by Tsingke Biotechnology Co., Ltd. (Qingdao, China). Codon-optimized genes, including diterpene synthases genes *LPPS* (GenBank accession number: JN133923) and *TPS* (JN133922) from *S. sclarea*, and geranylgeranyl diphosphate synthase gene *SaGGPS* (WP_011277020) from *Sulfolobus acidocaldarius*, were codon-optimized for *S. cerevisiae* and synthesized by BGI Genomics Co., Ltd. (Qingdao, China). The DNA sequences of the synthesized genes *TPS*, *LPPS*, and *SaGGPS* are listed in Table S3. The endogenous genes were amplified by PCR with the genomic DNA of *S. cerevisiae* BY4741 as a template. The pRS415 plasmid was used for constructing expression vectors. The plasmids utilized in this study are given in Table S4. Genome editing was performed using CRISPR/Cas9 system.[26] The donor DNAs for gene deletion and integration were prepared by fusion PCR reactions. The plasmids or donor DNAs were transformed into *S. cerevisiae* cells using the LiAc/SS carrier DNA/PEG method.[27]

2.3. Cultivation Conditions. For monoculture of engineered *S. cerevisiae* strains, a single colony of the engineered yeast strain was inoculated into 3 mL of YPD medium and cultured overnight at 30 °C, 220 rpm to prepare the seed cultures. Subsequently, 2% (v/v) of the seed cultures were inoculated into 30 mL of Y1 medium with an initial optical density (OD_{600}) of 0.1 and then cultured at 30 °C, 220 rpm for 72 h. To facilitate in situ product extraction, a biphasic

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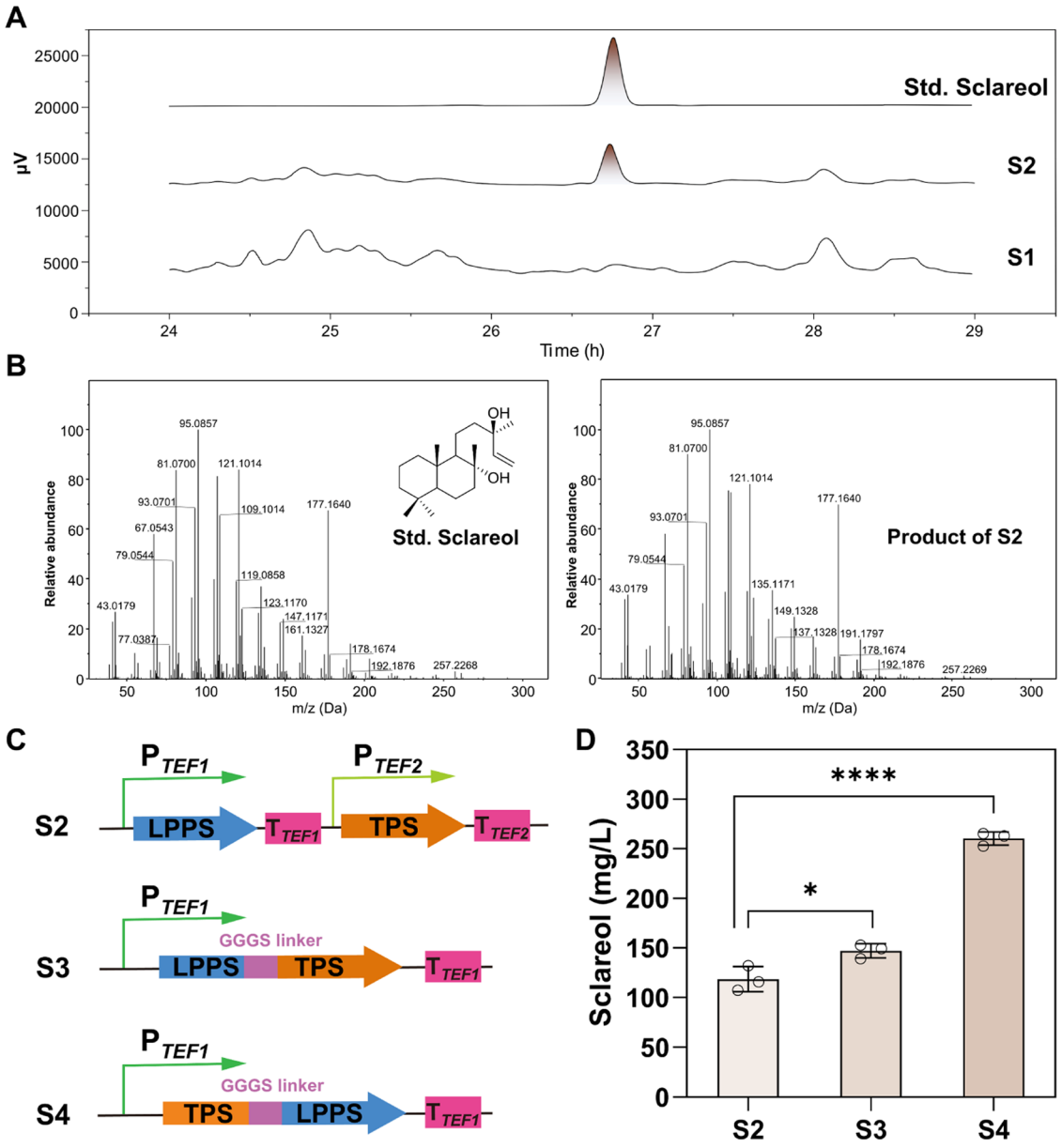

Figure 2. Construction of the biosynthetic pathway of sclareol in *S. cerevisiae*. (A) GC analysis of sclareol standard and the extracts of strains S1 and S2. (B) Mass spectra of sclareol standard and the corresponding compound produced by strain S2. (C) Schematic diagram of LPPS and TPS gene expression in strains S2, S3, and S4. P_{TEF1}, TEF1 promoter; T_{TEF1}, TEF1 terminator; P_{TEF2}, TEF2 promoter; T_{TEF2}, TEF2 terminator. (D) Sclareol production of different combinations of *TPS* and *LPPS* in shake flasks by biphasic fermentation for 72 h at 220 rpm, 30 °C. Triplicated measurements were conducted for each strain. The bars and circles represent the average and individual values of sclareol production. Statistical analyses using a one-sided *t*-test were performed to compare the sclareol concentration obtained by strains expressing fused TPS and LPPS against strain S2. $^{*}P < 0.05$, $^{****}P < 0.0001$.

fermentation was applied by adding 10% (v/v) *n*-dodecane to the culture after 24 h.

For the coculture of *S. cerevisiae* and *C. albidus*, the seed preparation procedure of engineered *S. cerevisiae* was the same as that of monocultures. A single colony of *C. albidus* ATCC 20918 was inoculated into a culture tube containing 3 mL of YPD medium and cultured at 25 °C and 180 rpm for 48 h to prepare the seed cultures. Subsequently, *S. cerevisiae* and *C. albidus* cells were centrifuged and collected at 5000*g* for 5 min and then cotransferred to Y1 medium with a respective initial OD_{600} of 0.1. The coculture was conducted at 25 °C and 180 rpm for 144 h. The glucose of the fermentation broth was measured each 12 h using a SBA-40E biosensor analyzer (Shandong, China). When the glucose level dropped below 5 g/L, supplementary glucose was added to maintain the concentration to 5 g/L. To investigate the impacts of the inoculation ratio and cultivation temperature, one parameter was modified while keeping the other factor constant. Cell optical density (OD_{600}) was measured at the wavelength of 600 nm, and dry cell weight (DCW) measurements were performed at 105 °C until a constant weight was achieved. All experiments were conducted three times, and all of the results were shown as means ± standard deviations.

2.4. Extraction and Metabolites Analysis. The resulting sclareol comprised extracellular and intracellular fractions. To determine the extracellular sclareol titer, the supernatant of the biphasic fermentation was collected by centrifugation at 5000*g* for 5 min. The *n*-dodecane phase was diluted 10-fold in ethyl acetate, while the aqueous phase was extracted with an equal volume of ethyl acetate. Subsequently, rotary evaporation of organic extracts was applied, and the residues were redissolved using ethyl acetate. To determine intracellular sclareol titer, the collected cell pellets were

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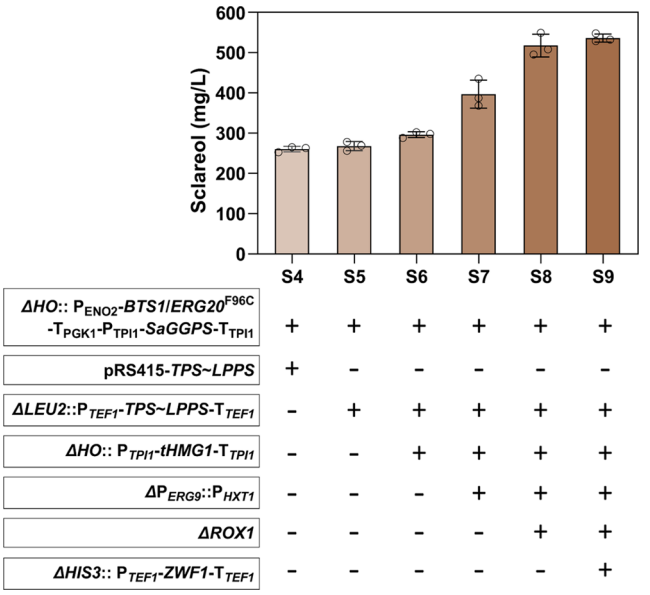

Figure 3. Engineering *S. cerevisiae* strains for optimization of sclareol production. Starting with the parental strain S1 (*S. cerevisiae* INVSc1, Δ*HO*:: P_{ENO2}-BTS1/ERG20^{F96C}-T_{PGK1}-P_{TPI1}-SaGGPS-T_{TPI1}), a series of modifications were implemented to generate recombinant strains S4−S9. The sclareol production of these strains was performed in shake flasks by biphasic fermentation for 72 h at 220 rpm, 30 °C. Triplicated measurements were conducted for each strain. The bars and circles respectively represent the average and individual values of sclareol production.

washed twice with distilled water and then disrupted at 75 Hz for 5 min in a high-throughput tissue grinder SCIENTZ-48 (Ningbo Xinzhi Biotechnology Co., Ltd., China), followed by extraction with 500 μL of ethyl acetate.

Quantification of sclareol was performed using gas chromatography (GC) (7890B; Agilent) equipped with a flame ionization detector and SH-Rxi-5Sil MS column (30 m × 0.25 mm × 0.25 μm; Shimadzu, Japan). The GC temperature program was as follows: the initial temperature was set as 50 °C for 2 min, ramped to 150 °C at a rate of 20 °C/min, and then increased to 300 °C at a rate of 10 °C/min. The inlet temperature was set to 300 °C. The injection volume was 1 μL. The flow rate of the carrier gas (nitrogen) was maintained at 1.0 mL/min. The extraction and analytical method of sclareolide was the same as that of sclareol. The quantification of sclareol and sclareolide in fermentation samples was performed according to the corresponding standard curves (Figure S2).

Gas chromatography−mass spectrometry (GC-MS) analysis was performed on an Agilent Thermo 01193K gas chromatograph equipped with a DB-5MS column (30 m × 0.25 mm × 0.25 μm). The GC-MS temperature program was the same as that of the GC analysis. The temperatures of the injection port, mass transfer tubing, and ion source were maintained at 300, 300, and 250 °C, respectively. The injection volume was 1 μL. The flow rate of the carrier gas (helium) was set at 1.0 mL/min, and the data was acquired in the electron impact mode (EI) with full scan range of 31−450 *m*/*z*. Final quantification was performed using Xcalibur software.

3. RESULTS AND DISCUSSION

3.1. Coculture Design for *De Novo* Production of Sclareolide. Compartmentalizing target biological processes into different strains allows for leveraging the unique properties and functions of involved microbes.[17] In the context of sclareolide bioproduction, the upstream sclareol biosynthetic pathway has been well understood. However, the downstream pathway for sclareolide biosynthesis in plants, as well as its biotransformation in *C. albidus* ATCC 20918 and other functionally similar strains, remain elusive. Thus, for *de novo* production of sclareolide, an engineered microbial consortium was designed as shown in Figure 1. In this coculture system, *S. cerevisiae* is genetically modified to produce sclareol, serving as the upstream substrate supply module. Meanwhile, strain ATCC 20918 serves as the downstream biotransformation module, facilitating the production of sclareolide from sclareol. By integrating the two modules, *de novo* synthesis of sclareolide is expected to be accomplished by the coculture system from a simple carbon source.

3.2. Construction of the Sclareol Biosynthetic Pathway in *S. cerevisiae*. Sclareol is synthesized through a two-step reaction catalyzed by two diterpene synthases in *S. sclarea*,[22,28] using geranylgeranyl diphosphate (GGPP) as a precursor. Initially, (13*E*)-8α-hydroxylabden-15-yl diphosphate (LDPP) synthase (LPPS), a type II diterpene synthase, catalyzes the protonation-initiated cyclization of GGPP to LDPP. Subsequently, LDPP is converted to sclareol by TPS, which is a type I diterpene synthase. The identification of the two-step pathway has enabled the heterologous biosynthesis of sclareol.[22,29] The natural mevalonate (MVA) pathway for GGPP production renders *S. cerevisiae* an ideal platform for diterpene production. The optimization of GGPP accumulation was demonstrated to be critical for the production of downstream products.[18] The effective strategy for improving GGPP accumulation involves the overexpression of geranylgeranyl diphosphate synthase (SaGGPS) from *S. acidocaldarius*, as well as a fusion protein of native farnesyltransferase (BTS1) and farnesyl diphosphate synthase (ERG20^{F96C}).[19,30,31]

To construct a host strain for efficient GGPP production, *BTS1-ERG20*^{F96C} and *SaGGPS* were integrated into the HO locus, resulting in the S1 stain. Then, *TPS* and *LPPS* were cloned into the pRS415 plasmid to construct the sclareol biosynthetic pathway, giving rise to strain S2. As expected, a new product was produced by strain S2 when compared to S1 (Figure 2A), which was identified as sclareol by GC and GC-MS with authentic sclareol as the reference (Figure 2B). In order to maintain inherent activity of individual proteins and enhance the catalytic capacities in cascade reactions, the strategy of designing fusion proteins with a linker region has widely been employed.[32] Accordingly, we fused the two diterpene synthases with the flexible linker "GGGS" and expressed the fusion proteins (Figure 2C). The yield of sclareol was improved through either LPPS-TPS fusion (strain S3) or TPS-LPPS fusion (strain S4), with TPS-LPPS fusion demonstrating prominent advantages over LPPS-TPS fusion. Notably, strain S4 achieved a sclareol titer of 260.3 mg/L, representing a 2.2-fold increase compared to S2 that harbors separate enzymes (Figure 2D).

3.3. Pathway Optimization for Improvement of Sclareol Production. It is necessary to further increase the production of sclareol in order to facilitate an adequate substrate supply for the subsequent bioconversion stage. Thus, in pursuit of stable expression of *TPS* and *LPPS*, the cassette of TPS-LPPS fusion protein was integrated into the genome of strain S1, resulting in strain S5. However, this integration did not result in a discernible increase in sclareol yield compared with strain S4. *HMG1* encoded 3-hydroxy-3-methylglutaryl-CoA reductase has been identified as the rate-limiting enzyme in the MVA pathway, and overexpression of *tHMG1* (encoding the catalytic domain of HMG1) could effectively enhance MVA metabolic flux.[33] Hence, we integrated the *tHMG1*

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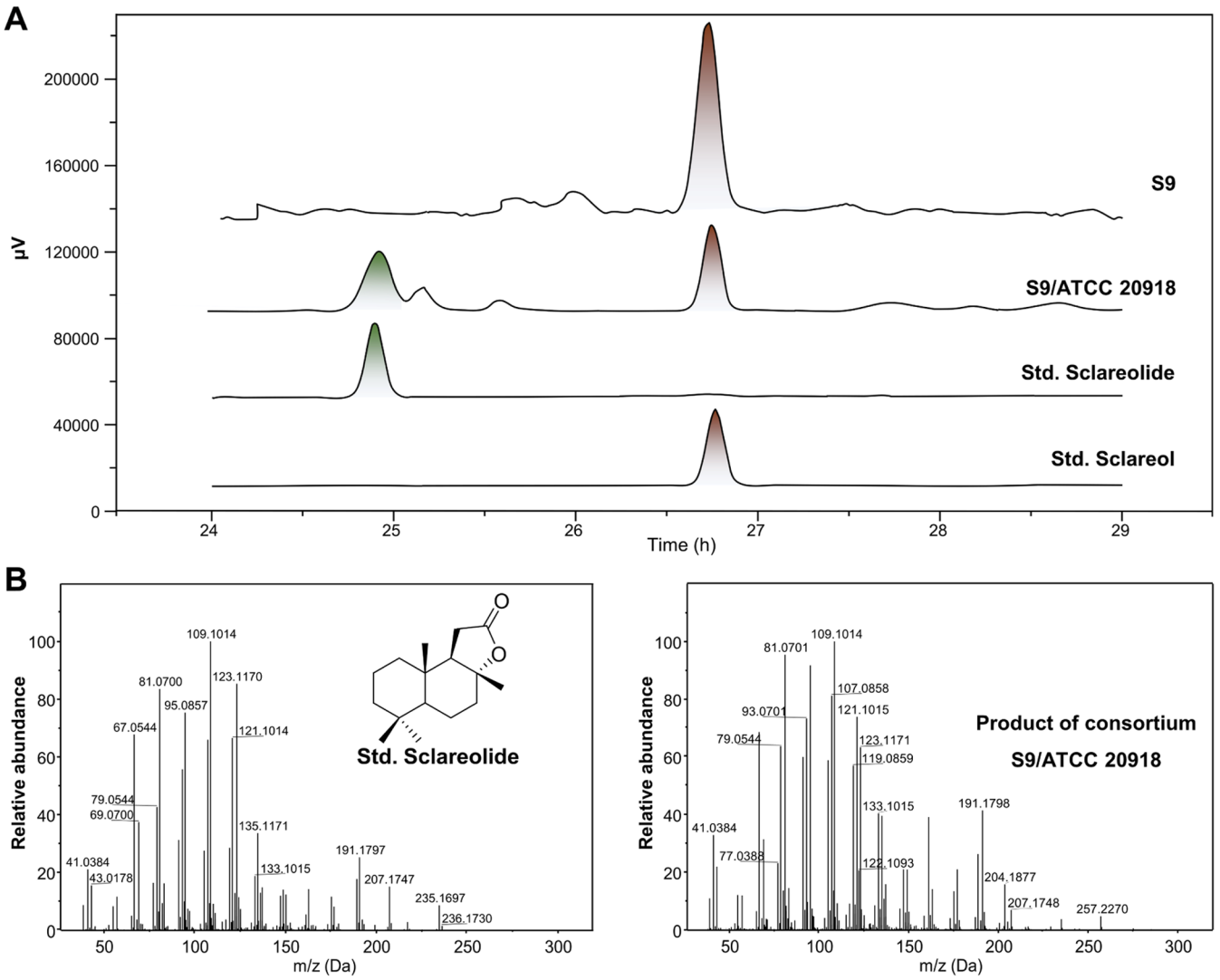

Figure 4. Establishment of a sclareolide production system through *S. cerevisiae-C. albidus* coculture engineering. (A) GC analysis of standard sclareolide, sclareol, and the extracts of strains S9, and of the coculture of S9/ATCC 20918. (B) GC-MS spectra of sclareolide standard and the corresponding compound produced by the coculture of S9/ATCC 20918.

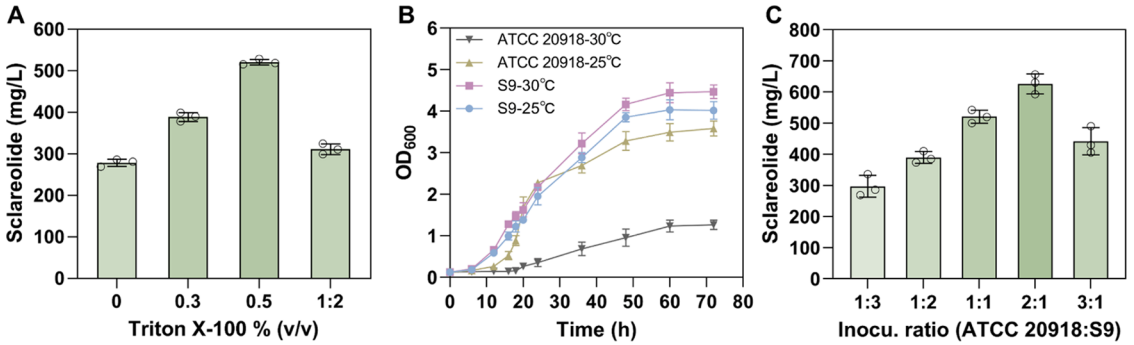

Figure 5. Optimization of sclareolide production. (A) Optimization of sclareolide production by adding Triton X-100 in different proportions. (B) Growth curves of *S. cerevisiae* S9 and *C. albidus* ATCC 20918 at 25 and 30 °C. (C) Optimization of sclareolide production by tuning inoculation ratios. All data represent the averages of three independent biological replicates. The error bars depict standard deviations, and gray cycles indicate the individual values.

expressing cassette into another HO locus, generating strain S6, which elevated the sclareol titer to 296.3 mg/L (Figure 3).

Squalene synthase (ERG9) catalyzes the conversion of FPP to squalene during triterpenoid and ergosterol biosynthesis,[34] thereby competitively consuming FPP and diminishing GGPP accumulation. To mitigate this effect, we replaced the native promoter of *ERG9* with the *HXT1* promoter for the downregulation of the squalene branching pathway, giving rise to strain S7. This modification led to a sclareol titer of 396.9 mg/L, which was 27.2% higher than that of strain S6.

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Transcriptional regulators have been shown to significantly influence isoprenoid production.[29] Notably, ROX1 acts as a global transcriptional repressor of MVA pathway, and the knockout of *ROX1* was reported to significantly improve the precursor supply for isoprenoids.[35] Therefore, we knocked out *ROX1* in strain S7, generating strain S8. A further increased yield of sclareol was achieved by strain S8, which was 517.6 mg/L, and 30.4% higher than that of strain S7. In addition, to bolster NADPH generation—an effective strategy to improve terpenoid production,[36] we sought to overexpress glucose-6-phosphate dehydrogenase (ZWF1) to enhance the NADPH level and hence terpenoid production.[37] Experimentally, we integrated the *ZWF1* expressing cassette into the *HIS3* locus of strain S8. The resulting strain S9 produced 536.2 mg/L of sclareol (Figure 3), representing a modest increase of 3.6% compared to S8. This limited enhancement might be due to the complexity of intracellular metabolic processes, which could affect the overall yield. Despite the outcome against the anticipation, strain S9 showed a remarkable 4.5-fold increase over its parental strain S2.

3.4. Modular Coculture for Sclareolide Biosynthesis. Several strains have demonstrated the capability to convert sclareol into sclareolide, with *C. albidus* ATCC 20918 exhibiting notable conversion efficiency.[25] Consequently, we selected this strain as the downstream module for the production of sclareolide from yeast-produced sclareol (Figure 1). Combining the sclareol-producing strain S9 and sclareol conversion strain ATCC 20918, the modular coculture system was established, and successful production of sclareolide was confirmed by GC (Figure 4A) and GC-MS (Figure 4B) analyses. However, the sclareolide titer reached only 278.4 mg/L (Figure 5A). Of note, up to 360.7 mg/L sclareol persisted intracellularly in *S. cerevisiae*, with an additional 56.3 mg/L of sclareol detected in the coculture fermentation broth (Figure S3). This result suggested that the inefficient transmembrane transport of strain S9 might hinder sclareol conversion by strain ATCC 20918 in the coculture system. Previous studies have indicated that the secretion of terpenoids by *S. cerevisiae* could be enhanced by overexpressing transporters, incorporating hydrophobic solvents, extractants, surfactants, or cyclic oligosaccharides.[38] The permeabilization of *S. cerevisiae* cells could be achieved by treatment with nonionic surfactant Triton X-100.[39] Therefore, we optimized the concentration of Triton X-100 to promote the substance transfer and solubility within the coculture system. As a result, the addition of 0.5% Triton X-100 led to a sclareolide yield of 520.5 mg/L (Figure 5A), 2.87-fold higher compared to the nonsurfactant condition. The residual sclareol in the broth was reduced to 10.7 mg/L (Figure S3). Conversely, when the concentration of Triton X-100 was further increased to 1%, sclareolide production sharply declined by 67.34% to 311.1 mg/L. Despite limited knowledge on the impact of Triton X-100 on *C. albidus* ATCC 20918, we hypothesized that its addition to the coculture system might facilitate the transport of sclareol between the two strains and enhance the exocytosis of sclareolide by strain ATCC 20918. However, membrane damage or even cell lysis should also be taken into consideration when an excessive amount of Triton X-100 was applied. Additionally, sclareol or sclareolide may also stick to the cell wall of yeasts, and the addition of Triton X-100 may facilitate their wash-off from the yeast cells.

Improving the composition and behavioral control of microbial coculture systems has consistently been a primary objective in the field of microbial cocultivation. The objective is to attain stable productivity under optimal conditions. Throughout the microbial cocultivation process, the interactions between microbial populations are highly dynamic, necessitating measures to harmonize growth and metabolism, such as optimization of fermentation conditions.[40] Given the disparate optimal growth temperature of *S. cerevisiae* and *C. albidus*, we first assessed the growth curves of the two strains at 25 and 30 °C, respectively. As shown in Figure 5B, the growth rate of *C. albidus* was slow at 30 °C, while *S. cerevisiae* exhibited consistent growth under both conditions. Therefore, 25 °C was selected as the optimum temperature for cocultivation. Subsequently, we optimized the initial inoculation ratio of the two strains. Cocultivation of strains ATCC 20918 and S9 was conducted with varying inoculation proportions. The highest sclareolide yield (626.3 mg/L) was achieved when the initial inoculation ratio was 2:1, representing a 2.1-fold increase compared to the initial inoculation ratio of 1:3 (Figure 5C). These results highlight the significance of maintaining a balanced proportion among different microbial modules for achieving efficient sclareolide production through coculture fermentation.

In summary, we devised and optimized *de novo* production of sclareolide via modular coculture engineering. However, despite the successful flask-scale fermentation, large-scale implementation would require further optimization, particularly in terms of maintaining the balance between the two strains during fermentation, controlling environmental conditions, and ensuring a consistent product yield. Recently, a coculture approach was reported to achieve the *de novo* production of up to 644.2 mg/L of ambradiol—the direct precursor of ambroxide,[20] which is comparable to the sclareolide yield in this study. Moreover, a *S. cerevisiae* strain was engineered to achieve high-level production of sclareol up to 11.4 g/L.[29] Given the superior biotransformation capacity of *C. albidus* ATCC 20918 as described by Farbood et al.,[24] we believe that the coculture system of engineered *S. cerevisiae* and strain ATCC 20918 holds significant potential for further optimization to achieve higher sclareolide yield.

■ ASSOCIATED CONTENT

Data Availability Statement

The authors declare that all data supporting the findings of this study are available within the article, Supporting Information, or from the corresponding author upon reasonable request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c05506.

Strains, primers, nucleotide sequences of codon-optimized genes, and plasmids used in this study (Tables S1−S4); chemical synthetic route of ambroxide from sclareol (Figure S1); standard curves of the sclareol and sclareolide for GC analysis (Figure S2); intracellular sclareol in S9 and sclareol in coculture broth under 0 and 0.5% Triton-100 treatment (Figure S3) (PDF)

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Author Contributions

D.T., L.D., K.L., and S.L. conceived the concept and designed the experiments. D.T. performed the experiments and analyzed the data. X.Z., Y.Z., C.Z., C.C., and Y.C. provided technical assistance and materials. D.T. wrote the original draft. S.L. and K.L. reviewed and edited the manuscript. All authors listed read and approved the manuscript.

Notes

The authors declare no competing financial interest.

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