Synthetic and Systems Biotechnology 10 (2025) 120–126

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

Development of a green *Komagataella phaffii* cell factory for sustainable production of plant-derived sesquiterpene (–)-α-bisabolol

Jintao Cheng [a,**,1], Zhongji Pu [a,1], Jiali Chen [a,c], Dingfeng Chen [a,d], Baoxian Li [a,c], Zhengshun Wen [a,d], Yuanxiang Jin [a,c], Yanlai Yao [a], Kan Shao [e], Xiaosong Gu [f,***], Guiling Yang [a,b,*]

[a] *Xianghu Laboratory, Hangzhou, 310027, China*
[b] *State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products, Laboratory (Hangzhou) for Risk Assessment of Agricultural Products of Ministry of Agriculture, Institute of Agro-product Safety and Nutrition, Zhejiang Academy of Agricultural Sciences, Hangzhou, 310021, Zhejiang, China*
[c] *College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou, 310032, China*
[d] *School of Food and Pharmacy, Zhejiang Ocean University, Zhoushan, 316022, China*
[e] *Department of Environmental and Occupational Health, School of Public Health, Indiana University, Bloomington, IN, 47405, USA*
[f] *Hubei Province Key Lab Yeast Function, Yichang, 443003, China*

ARTICLE INFO

Keywords:
(–)-α-Bisabolol
Komagataella phaffii
Enzyme engineering
Microbial cell factory
Metabolic engineering

ABSTRACT

(–)-α-Bisabolol is a plant-derived sesquiterpene derived from *Eremanthus erythropappus,* which can be used as a raw material in cosmetics and has anti-inflammatory function. In this study, we designed six mutation sites of the (–)-α-bisabolol synthase BOS using the plmDCA algorithm. Among these, the F324Y mutation demonstrated exceptional performance, increasing the product yield by 73 %. We constructed a *de novo* (–)-α-bisabolol biosynthesis pathways through systematic synthetic biology strategies, including the enzyme design of BOS, selection of different linkers in fusion expression, and optimization of the mevalonate pathway, weakening the branching metabolic flow and multi-copy strategies, the yield of (–)-α-bisabolol was significantly increased, which was nearly 35-fold higher than that of the original strain (2.03 mg/L). The engineered strain was capable of producing 69.7 mg/L in shake flasks. To the best of our knowledge, this is the first report on the biosynthesis of (–)-α-bisabolol in *Komagataella phaffii*, implying this is a robust cell factory for sustainable production of other terpenoids.

1. Introduction

Sesquiterpenes are mainly derived from plants, particularly aromatic plants, herbaceous plants, and resins. Sesquiterpenes and their derivatives have a variety of uses, including spices, cosmetic products, food additives, and biopesticides [1,2]. (–)-α-Bisabolol is a type of sesquiterpene obtained by extracting essential oils from *Eremanthus erythropappus* [3]. (–)-α-Bisabolol as a cosmetic raw material, has analgesic, anti-inflammatory, and antibacterial properties [3,4]. (–)-α-Bisabolol was an important scientific research topic because of its diverse biological activities and potential applications [5]. Extracting (–)-α-bisabolol from plants is time-consuming, laborious, and has ineffective. Therefore, more efficient methods for (–)-α-bisabolol production are urgently needed.

With the advancements in synthetic biology, various microbial cell factories have been employed for the production of (–)-α-bisabolol [3,4,6,7]. For the sustainable production of (–)-α-bisabolol, *Escherichia coli* was used to express the key enzymes associated with the valeric acid pathway and (–)-α-bisabolol synthase (BOS) responsible for the synthesis of (–)-α-bisabolol. Researchers have identified a new (–)-α-bisabolol synthase BOS (CcBOS) from *Cynara cardunculus* var. *Scolymus*, which can efficiently increase the production of

Peer review under responsibility of KeAi Communications Co., Ltd.
* Corresponding author. Xianghu Laboratory, Hangzhou, 310027, China.
** Corresponding author.
*** Corresponding author.
E-mail addresses: jintaocheng@zju.edu.cn (J. Cheng), xiaosonggu@yeah.net (X. Gu), guilingchina2008@163.com (G. Yang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.synbio.2024.09.006
Received 20 July 2024; Received in revised form 18 August 2024; Accepted 12 September 2024
Available online 17 September 2024

(–)-α-bisabolol [3]. Considering that *E. coli* is easily infected by phages during fermentation, some researchers used yeast cell factories to produce (–)-α-bisabolol. By introducing the heterologous (–)-α-bisabolol synthase MrBOS (*Matricaria recutita*), researchers constructed a *de novo* biosynthetic (–)-α-bisabolol recombinant strain in *Saccharomyces cerevisiae*. Subsequently, in a 5 L bioreactor, the yield of (–)-α-bisabolol reached 7.02 g/L [4]. In addition, *Yarrowia lipolytica* has also been used to produce (–)-α-bisabolol [6].

Komagataella phaffii is a yeast model organism widely used in biotechnology [8]. It is often used as a protein expression system, particularly for the production of eukaryotic proteins which are difficult to express in bacteria or that require complex post-translational modifications [9,10]. While the primary application of *K. phaffii* is for protein production, an appropriate bioengineering strategy can exploit its potential for the synthesis of small molecules, especially in the field of biocatalysis and biotransformation [11–13]. With advances in synthetic biology and metabolic engineering, there are increasing examples of the production of specific small-molecule compounds using *K. phaffii* [14–17]. To the best of our knowledge, no reports on the production of (–)-α-bisabolol in *K. phaffii* have been published.

In this study, *de novo* production of (–)-α-bisabolol in *K. phaffii* was investigated using a systematic synthetic biology strategy (Fig. 1). These included the screening for the optimal (–)-α-bisabolol synthase, modification of (–)-α-bisabolol synthase, optimization of combinatorial expression, enhancement of metabolic flux in the mevalonate (MVA) pathway, reduction in metabolic flux to branching pathway, and multicopy strategies. Eventually, the (–)-α-bisabolol titer reached 69.7 mg/L at shaker level. This study broadens the application of *K. phaffii* as cell factory for production of natural products.

2. Materials and methods

2.1. Strain and growth conditions

E. coli DH5α was used as a cloning host. Lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was used for the culture of *E. coli*. YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) was used for the culture of *K. phaffii*. *K. phaffii* strains were grown at 30 °C for 220 rpm. Yeast cultures were supplemented with Zeocin (100 mg/L), G418 (200 mg/L), or Hygromycin B (200 mg/L) to maintain stability of recombinant plasmids.

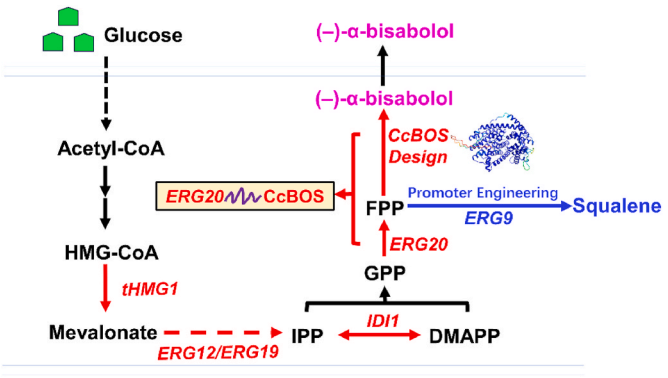

Fig. 1. Schematic diagram of (–)-α-bisabolo biosynthesis by *K. phaffii*. Red indicates that it needs to be strengthened, and blue indicates that it needs to be weakened. HMG-CoA, 3-Hydroxy-3-methylglutaryl-CoA; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate. *ERG20*, farnesyl diphosphate synthase. IPP, isopentenyl pyrophosphate. *IDI1*, isopentenyl diphosphate isomerase 1. *tHMG1*, truncated HMG-CoA reductase; *ERG12*, mevalonate kinase; *ERG19* mevalonate pyrophosphate decarboxylase.

2.2. Plasmid construction

All plasmids and primers used in this study are summarized in Table S1 and Table S2. The synthesis of heterologous genes was carried out by Nanjing GenScript Biotech Co., Ltd. The genes for three heterologous (–)-α-bisabolol synthases require codon optimization (MrBOS, GenBank AIG92846.1; AaBOS, GenBank PWA60940.1; CcBOS, GenBank XP_024994640.1). The sequences of these synthetic genes are shown in Table S3. The other key genes in this study were derived from *K. phaffii*, isopentenyl diphosphate isomerase *(IDI1)*, truncated HMG-CoA reductase *(tHMG1)*, Mevalonate kinase (*ERG12)*, and Mevalonate pyrophosphate decarboxylase (*ERG19)*. All plasmids in this study were constructed primarily by restriction digest, ligation and seamless cloning. Referring to the previous report on the *K. phaffii* CRISPR/Cas9 gene editing system, we first constructed the plasmid pGAP-Cas9 and transferred it to *K. phaffii* to obtain the host GS115-Gas9 [11]. Subsequently, the sgRNA plasmid and expression plasmid were constructed.

For the expression plasmid, the initial plasmid mainly contains a yeast promoter, a yeast terminator, and a pUC19 core part combined to form a single gene expression vector pUC19-P_{TEF1}-T_{AOX1} and a multigene expression vector pUC19-P_{TEF1}-T_{AOX1}-P_{GAP}-T_{0547t}. In order to construct plasmids that can be integrated into different loci on the genome (IntegX-donor), homology arms of about 500–1000 bp at specific loci were inserted upstream and downstream of vector pUC19-PTEF1-TAOX1, which can be obtained by homologous recombination construction, and finally we constructed the departure plasmids IntegX-donor with different integration sites [11,12], details were shown in Table S4.

For the construction of the sgRNA plasmid, the departure plasmid mainly contains the *K. phaffii* replicon, the expression frame of the resistance gene, and the expression frame of the expression of the gRNA [17]. To construct plasmids with different resistances, three plasmids were constructed, namely hygromycin, G418, and geneticin. Recombinant plasmids HZP-IntegX (zeocin), HHP-IntegX (hygromycin), HZP-IntegX (G418) with different corresponding sites were designed, the Benchling CRISPR tool (https://benchling.com/crispr/) can be employed for designing gRNAs.

2.3. Strain construction

The genes related to the (–)-α-bisabolol biosynthesis pathway were sequentially integrated into the *K. phaffii* chromosome by CRISPR/Cas9 method. The fragment containing the target gene expression cassettes and the corresponding gRNA were simultaneously transformed into *K. phaffii* to obtain the corresponding recombinant strain. gRNA plasmids can be lost by passaging. *K. phaffii* transformation was carried out by electroconversion method [15]. In this study, all constructed recombinant strains are listed in Table 1 and the corresponding integration sites are listed in Table S4.

2.4. Enzyme engineering

We utilized an in-house enzyme mining and design platform, which comprehensively considers one-dimensional sequence analysis, two-dimensional coevolution information, three-dimensional enzyme-substrate complex structures, and four-dimensional molecular dynamics trajectories. By integrating deep learning prediction models, this platform provides robust computational support for enzyme research and application.

First, we searched the UniRef100 database and obtained 5601 homologous sequences [18], constructing a multiple sequence alignment (MSA) using Muscle V5 [19]. Subsequently, we analyzed the top 20 coevolving residue pairs using plmDCA. We then examined the occurrence frequency of the 20 amino acids at these sites to guide mutation design. Additionally, we used CAVER3.0 to analyze the enzyme channels and identify potential bottleneck residues for further mutation

Table 1
Strains used in this study.

Strain	Genotype	Source
BOS0	*K. phaffii* GS115-HIS4:Cas9	This study
BOS1	BOS0-Int1:TEF1p-AaBOS-AOX1t	This study
BOS2	BOS0-Int1:TEF1p-CcBOS-AOX1t	This study
BOS3	BOS0-Int1:TEF1p-MrBOS-AOX1t	This study
BOS4	BOS0-Int1:TEF1p-CcBOS(L57E)-AOX1t	This study
BOS5	BOS0-Int1:TEF1p-CcBOS(V381S)-AOX1t	This study
BOS6	BOS0-Int1:TEF1p-CcBOS(T323I)-AOX1t	This study
BOS7	BOS0-Int1:TEF1p-CcBOS(F324Y)-AOX1t	This study
BOS8	BOS0-Int1:TEF1p-CcBOS(L403A)-AOX1t	This study
BOS9	BOS0-Int1:TEF1p-CcBOS(T421S)-AOX1t	This study
BOS10	BOS0-Int1:TEF1p-CcBOS(T421S)-AOX1t-GPAp-ERG20-CYCt1	This study
BOS11	BOS0-Int1:TEF1p-ERG20-GGGGS-CcBOS(T421S)-AOX1t	This study
BOS12	BOS0-Int1:TEF1p-ERG20-(PT)4 P-CcBOS(T421S)-AOX1t	This study
BOS13	BOS0-Int1:TEF1p-ERG20-(PA)5-CcBOS(T421S)-AOX1t	This study
BOS14	BOS13-Int2:TEF1p-IDI1-AOX1t	This study
BOS15	BOS13-Int2:TEF1p-IDI1-AOX1t-GPAp-tHMG1-CYCt1	This study
BOS16	BOS15-Int3:TEF1p-ERG12-AOX1t	This study
BOS17	BOS15-Int3:TEF1p-ERG12-AOX1t-GPAp-ERG19-CYCt1	This study
BOS18	BOS17-ΔERGp (−50bp)-L	This study
BOS19	BOS17-ΔERGp (−100bp)-L	This study
BOS20	BOS17-ΔERGp (−100bp)-R	This study
BOS21	BOS19-Int4:TEF1p-ERG20-(PA)5-CcBOS(T421S)-AOX1t	This study
BOS22	BOS21-Int5:TEF1p-ERG20-(PA)5-CcBOS(T421S)-AOX1t	This study
BOS23	BOS22-Int6:TEF1p-ERG20-(PA)5-CcBOS(T421S)-AOX1t	This study

design [20].

2.5. Fermentation conditions

We inoculated a single colony strain of the recombinant strain into tubes containing 5 mL of YPD medium, incubated at 30 °C and 220 rpm for 24 h, and then transferred to 50 mL of YPD at 1 % inoculum incubate in 250 mL shake flasks for 72 h. 10 % *n*-dodecane was added after 24 h of incubation.

2.6. Detection method of (–)-α-bisabolol

After the fermentation, the 5000 g culture broth was centrifuged for 10 min to obtain *n*-dodecane containing (–)-α-bisabolol in the upper layer of the fermentation broth. Samples need to be diluted 10–1000 times. *n*-dodecane was appropriately diluted and then detected by GC-MS using the gas chromatography method: gas chromatography (GC; GCMS-TQ8050 NX; SHIMADZU) equipped with a flame ionization detector and Rtx-5MS column (30 m × 0.25 mm × 0.25 μm; SHIMADZU). Helium was used as the carrier gas and the flow rate was 1 mL/min. The ejector and flame ionization detector were maintained at 240 and 250 °C, respectively. The oven temperature was maintained at 60 °C for 2 min, followed by an increase to 200 °C at a rate of 4 °C/min, then an increase to 300 °C at a rate of 10 °C/min and maintained at 300 °C for 5 min.

3. Results

3.1. Construction of a recombinant K. phaffii strain for producing (–)-α-bisabolol

Due to the presence of genes associated with the mevalonate pathway within *K. phaffii*, a recombinant strain was constructed for the biosynthesis of (–)-α-bisabolol by introducing the (–)-α-bisabolol synthase expression cassette into *K. phaffii*. To evaluate the effects of different sources of (–)-α-bisabolol synthase (BOS), three distinct BOSs (AaBOS, from *Artemisia annua*; CcBOS, from *C. cardunculus* var. *scolymus*; and MrBOS, from *M. recutita*) were selected for test [3,4], and three recombinant strains (BOS1, BOS2, and BOS3) were constructed correspondingly. The retention time of (–)-α-bisabolol produced by *K. phaffii* strains was consistent with that of the (–)-α-bisabolol standard (Fig. 2A), the mass spectrometry detection is shown in Fig. S1. The outcomes of the experiment are illustrated in Fig. 2B, and the recombinant strain BOS2 produced the highest amount of (–)-α-bisabolol, 2.03 mg/L. This finding suggests that the *C. cardunculus* var. *scolymus*-derived (–)-α-bisabolol synthase (CcBOS) is more suitable for (–)-α-bisabolol production in *K. phaffii*.

3.2. Design of (–)-α-bisabolol synthase CcBOS

Enzyme engineering has been a key strategy to improve enzyme activity [21,22]. The coevolving residue pair analysis revealed that Leu57-Lys247, Val318-Tyr549, Phe282-Thr323, Phe324-Thr337, and Ser395-Thr421 are not the most frequently occurring combinations (Fig. 3A). We designed the following mutations based on an amino acid frequency analysis (Fig. 3B and Fig. S2): For Leu57-Lys247, the EK combination had the second-highest frequency (0.091), leading to the design of mutation L57E. For Val318-Tyr549, the SY combination had the highest frequency (0.300), leading to the design of mutation V318S. For Phe282-Thr323, the FI combination had the highest frequency (0.370), leading to the design of mutation T323I. For Phe324-Thr337, the YT combination had the highest frequency (0.732), leading to the design of mutation F324Y. For Ser395-Thr421, the SS combination had the second-highest frequency (0.154), leading to the design of mutation T421S. Using CAVER3.0, we identified L403 as a bottleneck residue in the enzyme channel, leading to the design of mutation L403A (Fig. S2).

Experimental results showed that the L57E and F324Y mutants exhibited increased enzyme activity, with yields increasing by 45.7 % and 73 % (Fig. 3C), respectively. Structural analysis revealed that the L57E mutation resulted in the formation of a salt bridge interaction between E57 and L247, while the F324Y mutation led to the formation of a hydrogen bond interaction between Y324 and T337 (Fig. S3). These stronger non-covalent interactions likely stabilized the protein conformation, enhancing the enzyme's catalytic efficiency.

3.3. Expression of fusion proteins increased the yield of (–)-α-bisabolol

Fusion protein expression can improve the catalytic efficiency and thus increase the product yield.[22] In this study, three different linkers (GGGGS, (PT)4 P, (PA)5) were chosen to fuse farnesyl diphosphate synthase (ERG20) with CcBOS, and the constructed expression cassettes were transformed into *K. phaffii* to obtain three identical recombinant strains BOS11 (ERG20-GGGGS-CcBOS(F324Y)), BOS12 (ERG20-(PT)4 P-CcBOS(F324Y)), BOS13 (ERG20-(PA)5-CcBOS(F324Y)). At the same time, as shown in Fig. 4, we constructed the control strain BOS10, which overexpressed ERG20 and CcBOS without fusion.

The recombinant strain BOS10, which expresses ERG20 and CcBOS simultaneously, can further increase the yield of (–)-α-bisabolol (Fig. 4). The yield of (–)-α-bisabolol in three recombinant strains (BOS11, BOS12 and BOS13) with different linkers were higher than that of the recombinant strain BOS10, and the yield of the recombinant strain BOS13 increased the most significantly, with a yield of 8.58 mg/L. Compared to the previous starting strain BOS7, the yield of (–)-α-bisabolol in BOS13 was increased by 143 %.

3.4. Overexpression of key genes in the mevalonate pathway further increased the yield of (–)-α-bisabolol

The mevalonate (MVA) pathway is an important step in the biosynthesis of sesquiterpene products [16]. The accumulation of intermediate products in the mevalonate pathway contributes to the high yield of downstream products. In the MVA pathway, *IDI1*, *tHMG1*, *ERG12*, and *ERG19* are key genes, and their high expression contributes to the improvement of metabolic flux. Here, *IDI1*, *tHMG1*, *ERG12*, and *ERG19* were overexpressed to increase precursor supplement, respectively. Recombinant strains (BOS14, BOS15, BOS16, BOS17) were

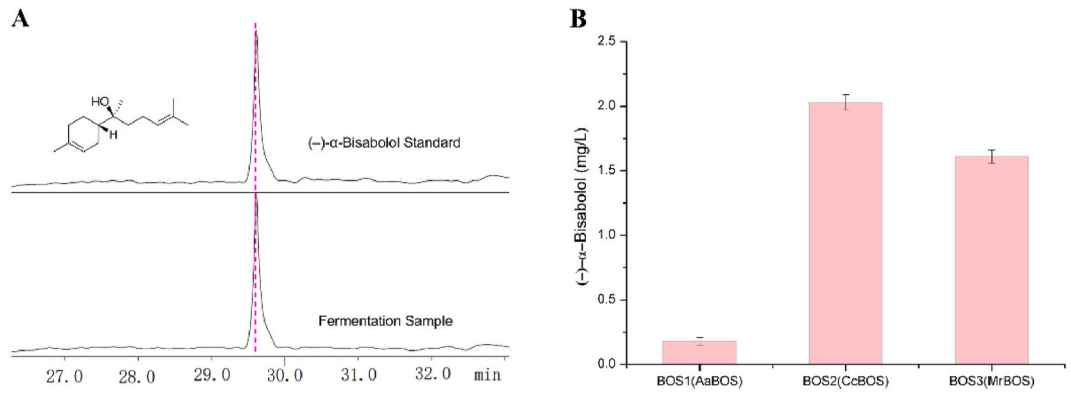

Fig. 2. Construction of a recombinant *K. phaffii* strain for producing (–)-α-bisabolol. (A) Ion chromatography for detection of (–)-α-bisabolol standard and fermentation broths of *K. phaffii* expressing CcBOS. (B) The yield of (–)-α-bisabolol in three different sources of (–)-α-bisabolol synthase (AaBOS, CcBOS, MrBOS) overexpression in *K. phaffii*, respectively. The values of product are the average of three biological replicates.

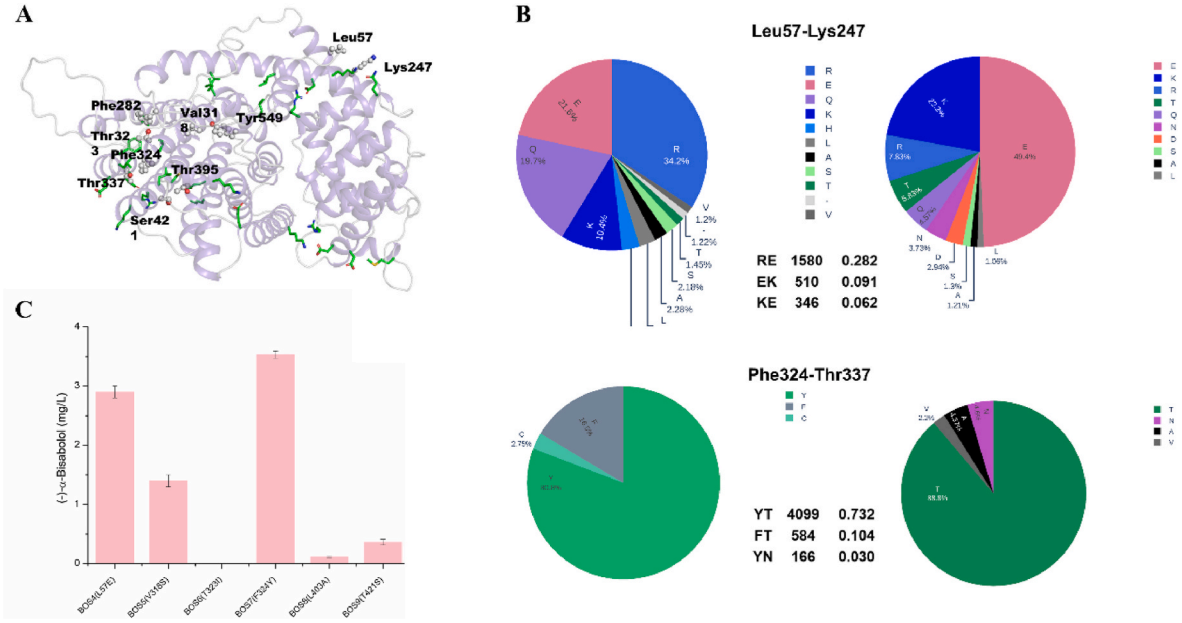

Fig. 3. Computational Design of BOS Enzyme. A: Coevolving residue pairs predicted by plmDCA. B: Analysis of amino acid combination frequencies for key coevolving residue pairs. C: Assay of (–)-α-bisabolol yield in different mutants.

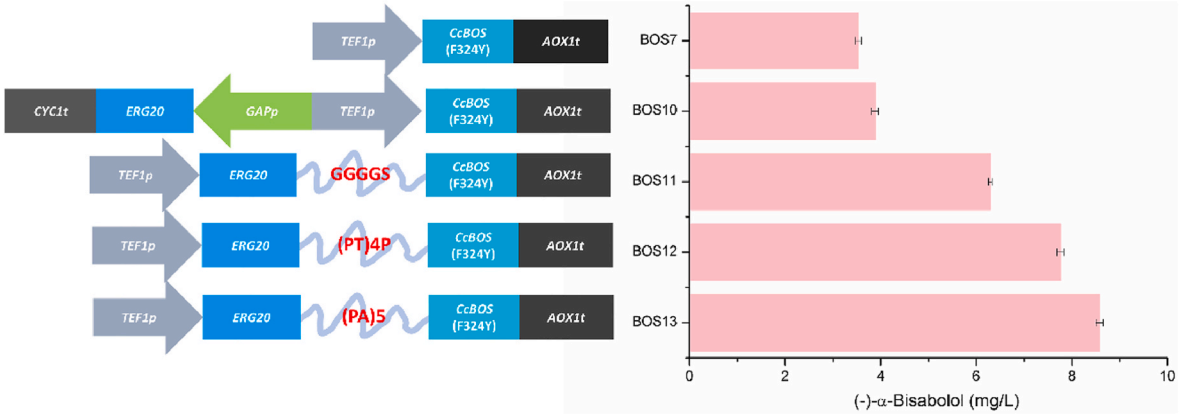

Fig. 4. Recombinant strains containing three different linkers (ERG20-LinkerX-CcBOS, GGGGS, (PT)4P, (PA)5) were constructed to compare their effects on (–)-α-bisabolol production. (–)-α-bisabololin the fermentation broth of the recombinant strain was detected by GC-MS. The values of product are the average of three biological replicates.

obtained by sequentially integrating these genes into *K. phaffii* BOS13 mentioned above.

As shown in Fig. 5, (–)-α-bisabolol was further improved by increasing the expression of genes related to the MVA pathway. Among them, the yield in the recombinant strain BOS17 reached 16.6 mg/L. This suggests that enhancing the metabolic flux of the MVA pathway does help increase the production of (–)-α-bisabolol.

3.5. The deletion of the ERG9 promoter weakened the metabolic flow of the branch pathway

In the sesquiterpene product biosynthesis process, ERG9 is the squalene synthase in yeast, which converts farnesyl diphosphate (FPP) to squalene, and it is the first enzyme in the triterpene and ergosterol biosynthesis branch. High expression of ERG9 would consume a large amount of FPP, thereby reducing the supply of precursors for GGPP production and ultimately reducing the generation of sesquiterpenoids. Given that ERG9 is essential for ergosterol biosynthesis, yeast cannot survive properly without it. Therefore, many researchers have tried to reduce the expression of the original promoter P_{ERG9}.

In the biosynthetic pathway of (–)-α-bisabolol, decreasing the metabolic flux of the branch pathway is helpful to increase the production of (–)-α-bisabolol. Here, we tried to delete the original promoter to reduce the expression of ERG9. The promoters were subtracted in turn, as shown in Fig. 6. The results showed that truncation of the promoter could reduce the expression of ERG9, resulting in higher production of (–)-α-bisabolol. As expected, the yield of (–)-α-bisabolol in the recombinant strain BOS19 (29.4 mg/L) was higher than that of the recombinant strain BOS17(16.6 mg/L), owning to a lower promoter activity. However, there was a significant fall in BOS20 (20.3 mg/L) compared with BOS18 (24.9 mg/L), with a same-length promotor truncation. We speculated that it was distinct in the function of different regions on the promoter, and overinhibition of *ERG9* transcription had harmful effects for (–)-α-bisabolol biosynthesis.

3.6. The multi-copy strategy further increased the yield of (–)-α-bisabolol

To further increase the yield of (–)-α-bisabolol, a multi-copy integration strategy was used to optimize the copy number of the fusion protein (ERG20-(PA)5-CcBOS(F324Y)). As shown in Fig. 7, the production of (–)-α-bisabolol increased as the copy number of the fusion protein accumulated. When the copy number of the fusion protein was three, the yield of (–)-α-bisabolol in the recombinant strain BOS22 reached 69.7 mg/L, which was 30-fold higher than that of the initial strain BOS2.

4. Discussion

(–)-α-Bisabolol is an important functional ingredient in cosmetic products of plant origin [1]. The extraction of (–)-α-bisabolol from plants is a traditional method that is complex and costly [2]. With the rapid development of synthetic biology and gene editing technology, (–)-α-bisabolol can be biosynthesized in microbial cell factories [3,4]. In this study, we used *K. phaffii* to biosynthesize (–)-α-bisabolol for the first time.

During the biosynthesis of (–)-α-bisabolol we expressed three different sources of BOSs, and the results indicated that BOSs from different sources significantly influenced the production of (–)-α-bisabolol. The CcBOS showed the highest efficiency, which is consistent with previous reports [6]. In order to further enhance the initial production of (–)-α-bisabolol, other novel BOSs could also be explored in the future.

Moreover, in order to improve the catalytic activity of CcBOS, we designed different mutants of CcBOS with assistance from deep learning prediction models, and the experimental results showed that two of the six mutants further increased the yield of (–)-α-bisabolol. The best-performing mutant produced a 40 % increase in the yield of (–)-α-bisabolol. The plmDCA (Pseudolikelihood Maximization Direct Coupling Analysis) algorithm is a method used to infer direct residue-residue interactions within protein structures by analyzing multiple

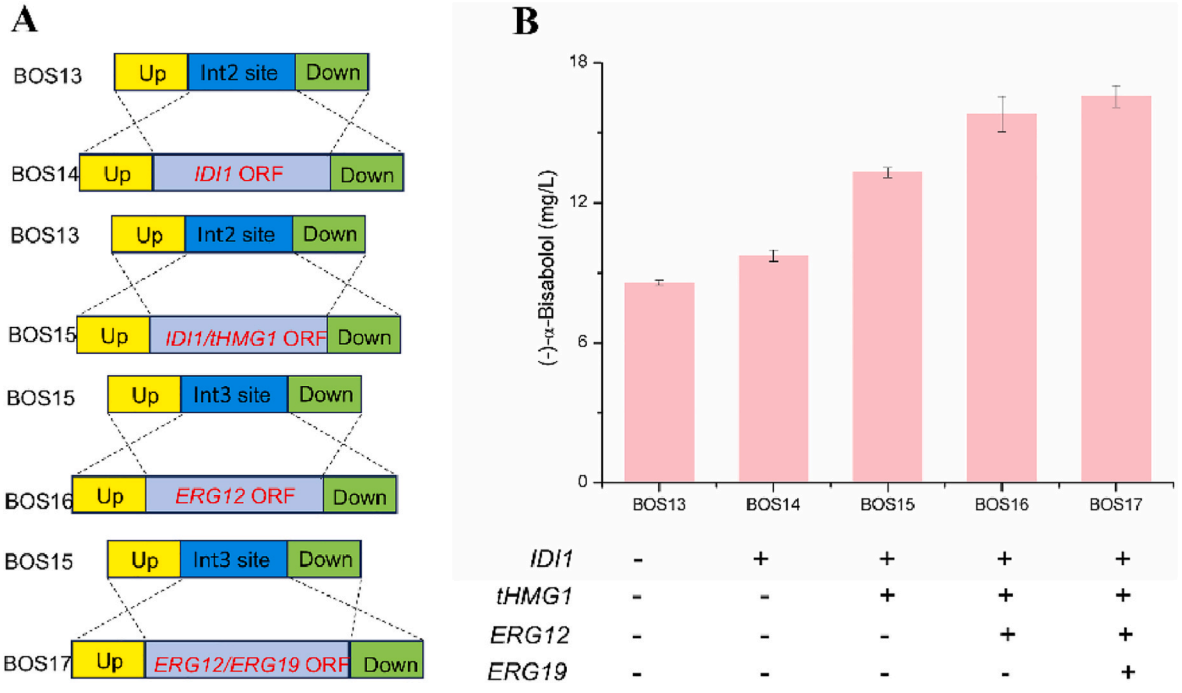

Fig. 5. Enhances the MVA pathway and thereby increases the production of (–)-α-bisabolol. (A) Expression cassettes containing different genes (*IDI1*, *tHMG1*, *ERG12* and *ERG19*) are inserted into specific loci on the genome of *K. phaffii*. (B) (–)-α-bisabolol titer of strain BOS13 (with integrated *IDI1*, *tHMG1*, *ERG12*, and *ERG19*) after 72 h of fermentation. "+" indicates that the expression cassette had been integrated into the genome of the target strain. "–" indicates that the gene had not been integrated into the expression cassette of the target strain. The values of product are the average of three biological replicates.

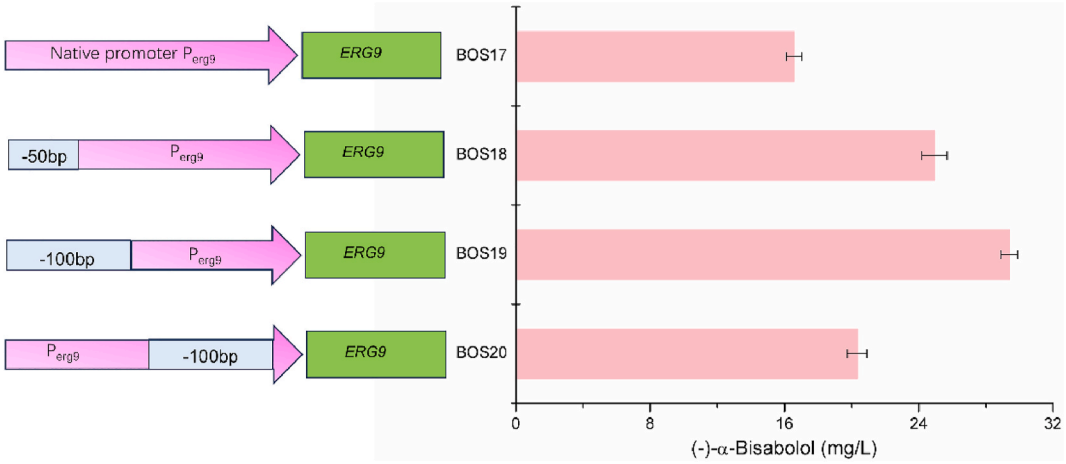

Fig. 6. Effect of promoter truncation on (–)-α-bisabolol production. The ERG9 promoter of the host itself is truncated to varying degrees, which are 50 bp, 100 bp, from 5 to 3 ends, and 100 b truncation is also designed, from 3 to 5 ends. The values of product are the average of three biological replicates.

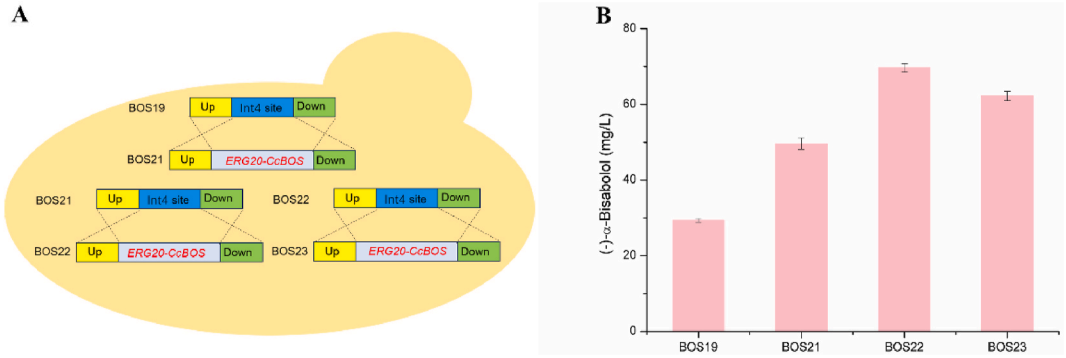

Fig. 7. Effect of multiple copies on (–)-α-bisabolol production. (A) Expression cassettes (ERG20-(PA)5-CcBOS(F324Y))are inserted into specific loci on the genome of *K. phaffii*. (B) (–)-α-bisabolol titer of strains. Two multi-copy, three multi-copy, four multi-copy recombinant strains were designed to compare the yield of (–)-α-bisabolol, respectively. The values of product are the average of three biological replicates.

sequence alignments (MSAs). It works by maximizing the likelihood of observed residue pairs under a global probability model, effectively disentangling direct couplings from indirect ones, thereby improving the accuracy of contact predictions. This algorithm has demonstrated higher precision compared to other DCA methods. The F324Y mutation was particularly effective because it introduced a hydroxyl group that forms a stabilizing hydrogen bond, as shown in the structure (Fig. S3). This interaction likely enhances the local stability and function, making it more impactful compared to other mutations. Fusion expression is another effective way to improve catalytic efficiency. In this study, we explored the effect of different linkers between *ERG20* and *CcBOS* on (–)-α-bisabolol production. The results showed that different linkers did further raise the yield of (–)-α-bisabolol, suggesting that the fusion of the two proteins is likely to improve the catalytic efficiency.

The mevalonate pathway is one of the important intermediate pathways for the synthesis of terpenoids, and enhancing the metabolic flux is helpful to improve the yield of the target product [16]. The overexpression of the four genes (*IDI1*, *tHMGR*, *ERG12*, *ERG19*) did increase the metabolic flux, which would enhance precursor supply for (–)-α-bisabolol synthesis. There are competing branching pathways in the biosynthesis of α-bisabolol. When α-bisabolol is synthesized with FPP as substrate, squalene synthesized with the same substrate will affect the yield of α-bisabolol. However, ergosterol, a downstream product of squalene, is an important component of membrane structure, and direct knockdown of the squalene synthesis gene *ERG9* will affect cell growth, and the dynamic expression of the *ERG9* gene is usually down-regulated by replacing the original promoter with other promoters. In order to reduce the effect of branched metabolic flux, we tried to downregulate the expression of *ERG9* using a promoter truncation strategy, and we designed three different promoters to replace the original promoter. The best result was to truncate the promoter P_{ERG9} by 100 bp from the 5th to the 3rd end, and the recombinant strain BOS19 had the highest yield, with a yield of 29.4 mg/L, which was higher than the reference strain BOS17 (16.6 mg/L). The yields of α-bisabolol in the other two recombinant strains (BOS18 and BOS20) were also significantly higher than those of the reference strain BOS17. This suggests that the truncation of the promoter P_{ERG9} may reduce the expression of ERG9, by reducing the metabolic flux of the branch subsequently, and ultimately enhancing the metabolic flux for (–)-α-bisabolol. This provides a good reference for the subsequent production of other terpenoids.

5. Conclusions

In summary, for the first time, we used the *K. phaffii* cell factory to produce (–)-α-bisabolol sustainably. With the low initial production (2.03 mg/L), we finally achieved a yield of 69.7 mg/L through systematic synthetic biology strategies, including the screening and protein engineering of (–)-α-bisabolol synthase, optimization of fusion protein linker, regulation of metabolic pathways with weakening branch pathways, and multiple copies. Compared with the starting strain BOS2, the yield of (–)-α-bisabolol in the recombinant strain BOS23 increased by approximately 35-fold. Our study shows that these strategies are very effective and lays the foundation for the subsequent biosynthesis of

other terpenoids in *K. phaffii*.

Competing interests

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Jintao Cheng: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Zhongji Pu:** Software, Investigation. **Jiali Chen:** Investigation. **Dingfeng Chen:** Investigation. **Baoxian Li:** Investigation. **Zhengshun Wen:** Investigation. **Yuanxiang Jin:** Investigation. **Yanlai Yao:** Investigation. **Kan Shao:** Investigation. **Xiaosong Gu:** Writing – review & editing, Investigation. **Guiling Yang:** Writing – review & editing, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the key scientific research and development project of Zhejiang Province (2024SSYS0103), the Start-up funds of Xianghu Laboratory (2023C4S02002). This work was supported by the National Natural Science Foundation of China (32301255). Thanks to the technical support provided by the public instrument platform of Xianghu Laboratory.

Abbreviations

HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
DMAPP	Dimethylallyl pyrophosphate
GPP	Geranyl diphosphate
FPP	Farnesyl diphosphate
ERG20	Farnesyl diphosphate synthase
IPP	Isopentenyl pyrophosphate
IDI1	Isopentenyl diphosphate isomerase 1
tHMG1	Truncated HMG-CoA reductase
ERG12	Mevalonate kinase
ERG19	Mevalonate pyrophosphate decarboxylase

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.09.006.

References

[1] Chen T, Liu Y, Ma B, Sun B, Pan Y, Ou Y, Yu H, She Z, Long Y. Anti-inflammatory sesquiterpenes from fruiting bodies of *Schizophyllum commune*. J Agric Food Chem 2024;72(10):5416–27. https://doi.org/10.1021/acs.jafc.3c08313.

[2] Gong DY, Chen XY, Guo SX, Wang BC, Li B. Recent advances and new insights in biosynthesis of dendrobine and sesquiterpenes. Appl Microbiol Biotechnol 2021;105(18):6597–606. https://doi.org/10.1007/s00253-021-11534-1.

[3] Lim HS, Kim SK, Woo SG, Kim TH, Yeom SJ, Yong W, Ko YJ, Kim SJ, Lee SG, Lee DH. (-)-α-Bisabolol production in engineered *Escherichia coli* expressing a novel (-)-α-Bisabolol synthase from the Globe Artichoke *Cynara cardunculus* var. *Scolymus*. J Agric Food Chem 2021;69(30):8492–503. https://doi.org/10.1021/acs.jafc.1c02759.

[4] Jiang Y, Xia L, Gao S, Li N, Yu S, Zhou J. Engineering *Saccharomyces cerevisiae* for enhanced (-)-α-bisabolol production. Synth Syst Biotechnol 2023;8(2):187–95. https://doi.org/10.1016/j.synbio.2023.01.004.

[5] Ramazani E, Akaberi M, Emami SA, Tayarani-Najaran Z. Pharmacological and biological effects of alpha-bisabolol: an updated review of the molecular mechanisms. Life Sci 2022;304:120728. https://doi.org/10.1016/j.lfs.2022.120728.

[6] Li YW, Yang CL, Shen Q, Peng QQ, Guo Q, Nie ZK, Sun XM, Shi TQ, Ji XJ, Huang H. YALIcloneNHEJ: an efficient modular cloning toolkit for NHEJ integration of multigene pathway and terpenoid production in *Yarrowia lipolytica*. Front Bioeng Biotechnol 2022;9:816980. https://doi.org/10.3389/fbioe.2021.816980.

[7] Kim TY, Park H, Kim SK, Kim SJ, Park YC. Production of (-)-α-bisabolol in metabolically engineered *Saccharomyces cerevisiae*. J Biotechnol 2021;340:13–21. https://doi.org/10.1016/j.jbiotec.2021.08.008.

[8] Karbalaei M, Rezaee SA, Farsiani H. *Pichia pastoris*: a highly successful expression system for optimal synthesis of heterologous proteins. J Cell Physiol 2020;235(9):5867–81. https://doi.org/10.1002/jcp.29583.

[9] Mastropietro G, Aw R, Polizzi KM. Expression of proteins in *Pichia pastoris*. Methods Enzymol 2021;660:53–80. https://doi.org/10.1016/bs.mie.2021.07.004.

[10] Schwob M, Kugler V, Wagner R. Cloning and overexpressing membrane proteins using *Pichia pastoris* (*Komagataella phaffii*). Curr Protoc 2023;3(11):e936. https://doi.org/10.1002/cpz1.936.

[11] Gao J, Jiang L, Lian J. Development of synthetic biology tools to engineer *Pichia pastoris* as a chassis for the production of natural products. Synth Syst Biotechnol 2021;6(2):110–9. https://doi.org/10.1016/j.synbio.2021.04.005.

[12] Gao J, Ye C, Cheng J, Jiang L, Yuan X, Lian J. Enhancing homologous recombination efficiency in *Pichia pastoris* for multiplex genome integration using short homology arms. ACS Synth Biol 2022;11(2):547–53. https://doi.org/10.1021/acssynbio.1c00366.

[13] Cen YK, Wang Q, Gong H, Xue YP, Zheng YG. Expression of L-phosphinothricin synthesis enzymes in *Pichia pastoris* for synthesis of L-phosphinothricin. Biotechnol Lett 2022;44(4):561–70. https://doi.org/10.1007/s10529-022-03239-w.

[14] Cheng J, Zuo Y, Liu G, Li D, Gao J, Xiao F, Huang L, Xu Z, Lian J. Development of a *Pichia pastoris* cell factory for efficient production of germacrene A: a precursor of β-elemene. Bioresour Bioprocess 2023;10(1):38. https://doi.org/10.1186/s40643-023-00657-0.

[15] Cai P, Duan X, Wu X, Gao L, Ye M, Zhou YJ. Recombination machinery engineering facilitates metabolic engineering of the industrial yeast *Pichia pastoris*. Nucleic Acids Res 2021;49(13):7791–805. https://doi.org/10.1093/nar/gkab535.

[16] Zuo Y, Xiao F, Gao J, Ye C, Jiang L, Dong C, Lian J. Establishing *Komagataella phaffii* as a cell factory for efficient production of sesquiterpenoid α-Santalene. J Agric Food Chem 2022;70(26):8024–31. https://doi.org/10.1021/acs.jafc.2c02353.

[17] Gao J, Zuo Y, Xiao F, Wang Y, Li D, Xu J, Ye C, Feng L, Jiang L, Liu T, Gao D, Ma B, Huang L, Xu Z, Lian J. Biosynthesis of catharanthine in engineered *Pichia pastoris*. Nat Synth 2023;2:231–42. https://doi.org/10.1038/s44160-022-00205-2.

[18] Suzek BE, Wang Y, Huang H, McGarvey PB, Wu CH, Consortium UniProt. UniRef clusters: a comprehensive and scalable alternative for improving sequence similarity searches. Bioinformatics 2015;31(6):926–32. https://doi.org/10.1093/bioinformatics/btu739.

[19] Edgar RC. Muscle5: high-accuracy alignment ensembles enable unbiased assessments of sequence homology and phylogeny. Nat Commun 2022;13(1):6968. https://doi.org/10.1038/s41467-022-34630-w.

[20] Chovancova E, Pavelka A, Benes P, Strnad O, Brezovsky J, Kozlikova B, Gora A, Sustr V, Klvana M, Medek P, Biedermannova L, Sochor J, Damborsky J. Caver 3.0: a tool for the analysis of transport pathways in dynamic protein structures. PLoS Comput Biol 2012;8(10):e1002708. https://doi.org/10.1371/journal.pcbi.1002708.

[21] Abdul Manas NH, Md Illias R, Mahadi NM. Strategy in manipulating transglycosylation activity of glycosyl hydrolase for oligosaccharide production. Crit Rev Biotechnol 2018;38(2):272–93. https://doi.org/10.1080/07388551.2017.1339664.

[22] Nie S, Wang S, Chen R, Ge M, Yan X, Qiao J. Catalytic Mechanism and heterologous biosynthesis application of sesquiterpene synthases. J Agric Food Chem 2024;72(13):6871–88. https://doi.org/10.1021/acs.jafc.4c00030.