

RESEARCH

Open Access



Fermentative production and direct extraction of (–)- α -bisabolol in metabolically engineered *Escherichia coli*

Gui Hwan Han^{1†}, Seong Keun Kim^{1,2†}, Paul Kyung-Seok Yoon^{1,3}, Younghwan Kang¹, Byoung Su Kim⁴, Yaoyao Fu¹, Bong Hyun Sung⁵, Heung Chae Jung¹, Dae-Hee Lee^{1,2*}, Seon-Won Kim^{6*} and Seung-Goo Lee^{1,2*}

Abstract

Background: (–)- α -Bisabolol, also known as levomenol, is an unsaturated sesquiterpene alcohol that has mainly been used in pharmaceutical and cosmetic products due to its anti-inflammatory and skin-soothing properties. (–)- α -Bisabolol is currently manufactured mainly by steam-distillation of the essential oils extracted from the Brazilian candeia tree that is under threat because its natural habitat is constantly shrinking. Therefore, microbial production of (–)- α -bisabolol plays a key role in the development of its sustainable production from renewable feedstock.

Results: Here, we created an *Escherichia coli* strain producing (–)- α -bisabolol at high titer and developed an in situ extraction method of (–)- α -bisabolol, using natural vegetable oils. We expressed a recently identified (–)- α -bisabolol synthase isolated from German chamomile (*Matricaria recutita*) (titer: 3 mg/L), converted the acetyl-CoA to mevalonate, using the biosynthetic mevalonate pathway (12.8 mg/L), and overexpressed farnesyl diphosphate synthase to efficiently supply the (–)- α -bisabolol precursor farnesyl diphosphate. Combinatorial expression of the exogenous mevalonate pathway and farnesyl diphosphate synthase enabled a dramatic increase in (–)- α -bisabolol production in the shake flask culture (80 mg/L) and 5 L bioreactor culture (342 mg/L) of engineered *E. coli* harboring (–)- α -bisabolol synthase. Fed-batch fermentation using a 50 L fermenter was conducted after optimizing culture conditions, resulting in efficient (–)- α -bisabolol production with a titer of 9.1 g/L. Moreover, a green, downstream extraction process using vegetable oils was developed for in situ extraction of (–)- α -bisabolol during fermentation and showed high yield recovery (>98%).

Conclusions: The engineered *E. coli* strains and economically viable extraction process developed in this study will serve as promising platforms for further development of microbial production of (–)- α -bisabolol at large scale.

Keywords: (–)- α -Bisabolol, (–)- α -Bisabolol synthase, Mevalonate pathway, Farnesyl diphosphate synthase, In situ extraction, Vegetable oils, *Escherichia coli*

Background

Terpenoids, also known as isoprenoids, are one of the largest and most diverse classes of naturally occurring products [1]. In animals, terpenoids play crucial roles as

membrane constituents (e.g., cholesterol) and components of the respiratory electron transport chain (e.g., ubiquinone) [2], whereas in microbes and plants, they are found as secondary metabolites that have been used as pharmaceuticals (e.g., paclitaxel and artemisinin), flavors, and fragrances (e.g., menthol and patchouli) [3]. Terpenoids, including monoterpenes, sesquiterpenes, and diterpenes, are synthesized by terpene synthases (TPs) with universal five-carbon building units, isopentenyl diphosphate (IPP) and its isomer dimethyl allyldiphosphate (DMAPP). IPP and DMAPP are synthesized in

*Correspondence: dhlee@kribb.re.kr; swkim@gnu.ac.kr; sglee@kribb.re.kr

†Gui Hwan Han and Seong Keun Kim contributed equally to this work

¹ Synthetic Biology and Bioengineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Republic of Korea

⁶ Division of Applied Life Science (BK21 Plus), PMBBRC, Gyeongsang National University, Jinju 52828, Republic of Korea

Full list of author information is available at the end of the article

two different pathways: the mevalonate (MVA) pathway [4], and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [5] (Fig. 1a). In general, Gram-negative bacteria, including *Escherichia coli*, employ the MEP pathway, whereas eukaryotes, archaea, humans, and Gram-positive cocci use the MVA pathway [6]. Prenyltransferases assemble the IPP and DMAPP units to produce the prenyl diphosphates, which are terpenoid precursors, including geranyl diphosphate (GPP, C₁₀), farnesyl diphosphate (FPP, C₁₅), and geranyl geranyl diphosphate (GGPP, C₂₀). These prenyl diphosphate molecules are converted into a large variety of terpenoids by TPSs [7, 8].

(-)- α -Bisabolol is an unsaturated sesquiterpene alcohol that occurs naturally in the Brazilian candeia tree (*Eremanthus erythropappus*) and in medicinal herbs such as German chamomile (*Matricaria recutita*) [9, 10]. It has been shown to have pharmaceutical functions (e.g., antibacterial, antiseptic, and anti-inflammatory activities), and skin-soothing and -moisturizing properties [11–13]. Owing to the low toxicity associated with (-)- α -bisabolol, the Food and Drug Administration (FDA) has granted it the Generally Regarded as Safe (GRAS) status that has promoted its use as an active ingredient in several commercial products [14]. At present, natural (-)- α -bisabolol is primarily manufactured through the steam-distillation of candeia essential oils extracted from the Brazilian candeia tree, which has raised environmental and bio-conservation issues in recent years [10]. (-)- α -Bisabolol can also be chemically synthesized; however, chemical synthesis requires an additional economically unviable purification step because of the formation of other diastereomers ((+)- α -bisabolol and (\pm)-*epi*- α -bisabolol) and undesirable byproducts [15]. Therefore, a sustainable supply of nature-identical (-)- α -bisabolol is essential to specialty chemical industries. Recently, a (-)- α -bisabolol synthase capable of synthesizing enantio-selective (-)- α -bisabolol as a single terpenoid product has been identified in German chamomile. (-)- α -bisabolol synthase was expressed in *Saccharomyces cerevisiae* for the production of (-)- α -bisabolol and had a yield of 8 mg/L after 4 days of cultivation [15]. This previous study has paved the way for the biological production of enantioselective (-)- α -bisabolol in engineered microorganisms, but the productivity is very low for industrial production of (-)- α -bisabolol.

The aim of this study was to produce (-)- α -bisabolol by large-scale fermentation of *E. coli*, which has been widely used for various industrial applications, and to develop an economically viable direct (-)- α -bisabolol extraction process during fermentation. To achieve this, we created (-)- α -bisabolol-producing *E. coli* in three steps: (1) We introduced the German chamomile (-)- α -bisabolol synthase gene (*MrBBS*) into *E. coli* convert endogenous

FPP to (-)- α -bisabolol; (2) We engineered the exogenous MVA pathway to increase IPP and DMAPP pool; and (3) We overexpressed an *ispA* gene encoding an FPP synthase that efficiently provides the (-)- α -bisabolol precursor (FPP) from IPP and DMAPP (Fig. 1b).

Methods

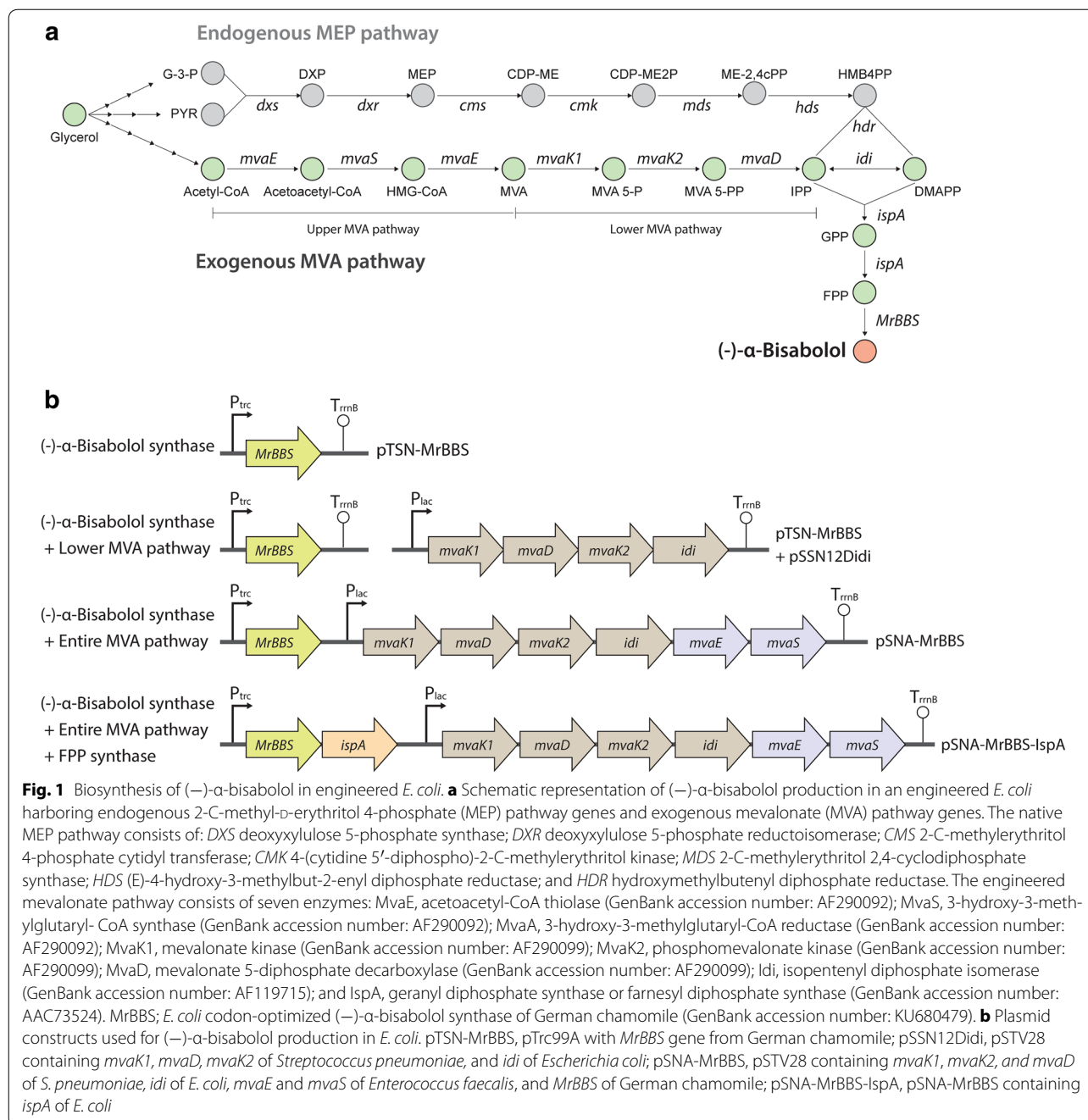
Bacterial strains, media, and culture conditions

Strains used in this study are listed in Table 1. *E. coli* DH5 α was used for cloning experiments and (-)- α -bisabolol production. BL21(DE3) and MG1655 strains were used to compare effects of different host strains on the production and toxicity of (-)- α -bisabolol. *E. coli* strains were grown in Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) for cloning experiments and (-)- α -bisabolol production. To produce (-)- α -bisabolol in *E. coli*, we used various media at 30 °C and 200 rpm; terrific broth (TB) medium (12 g/L enzymatic casein digest, 24 g/L yeast extract, 9.4 g/L K₂HPO₄, and 2.2 g/L KH₂PO₄), 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl), and M9 minimal medium (6.78 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, 0.241 g/L MgSO₄, 0.0111 g/L CaCl₂, and 0.1 g/L thiamine). M9 minimal medium was supplemented with 4 g/L of glucose. Ampicillin (100 μ g/mL), chloramphenicol (34 μ g/mL), or isopropyl β -D-1-thiogalactopyranoside (IPTG) as required.

n-Dodecane was used as an overlay to prevent the loss of volatiles. Furthermore, *n*-dodecane was used to solubilize and extract (-)- α -bisabolol, which is toxic to *E. coli* cell growth in high concentrations, from the culture media during cultivation. MVA was prepared from mevalonolactone (Sigma Aldrich) as previously described [16]. Cell growth was monitored by measuring the optical density at a wavelength of 600 nm (OD₆₀₀) with a spectrophotometer (Ultrospec 8000, GE Healthcare, Uppsala, Sweden). The inhibitory effect of (-)- α -bisabolol on the growth of *E. coli* strains was investigated in LB liquid medium supplemented with various concentrations of (-)- α -bisabolol. Diluted samples from cultures during the stationary phase were plated on LB solid medium and the colony-forming units (CFUs) were determined after overnight incubation at 30 °C.

Plasmid construction

Plasmids and polymerase chain reaction (PCR) primers used in this study are listed in Table 1. Common procedures, including genomic DNA preparation, restriction digestions, transformations, and other standard molecular biological techniques were performed as previously described [17]. All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich,



USA). PCR was performed following the manufacturer's instructions, using a high fidelity KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan). Plasmid preparation and gel extraction kits were obtained from Promega (Madison, USA) and oligonucleotides were synthesized by Bioneer (Daejeon, Korea). The *MrBBS* gene (GenBank accession number: KJ020282) was optimized to *E. coli* codons and synthesized by Bioneer (Additional file 1: Figure S1). We deposited the nucleotide sequence data of *E. coli*

codon-optimized *MrBBS* in the GenBank database (accession number: KU680479). The synthesized *MrBBS* was amplified by PCR with Bis-IF and Bis-IR primers (Table 1) and the plasmid backbone was amplified with the Bis-VF and Bis-VR primers from pTrc99A (GE Healthcare). The two PCR-amplicons were assembled via the Gibson Assembly Method [18] using Gibson Assembly Master Mix (New England Biolabs), to produce the pTSN-MrBBS plasmid. The entire polycistronic MVA pathway genes

Table 1 List of strains, plasmids, and primers used in this study

Name	Description	Refs
Strains		
DH5 α	F^- , $\Phi 80lacZ-\Delta M15-f(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk-, mk+)$ <i>phoA supE44 thi-1 gyrA96 relA1</i>	Enzymomics
MG1655	F^- , λ^- , <i>ilvG^-</i> , <i>rfb-50</i> , <i>rph1</i>	ATCC 700926
BL21(DE3)	BL21 F^- , <i>dcm ompT hsdS (rB- mB-)</i> <i>gal</i> λ (DE3)	Enzymomics
Plasmids		
pSSN12Didi	pSTV28 containing <i>mvaK1</i> , <i>mvaD</i> , <i>mvaK2</i> of <i>Streptococcus pneumoniae</i> , and <i>idi</i> of <i>E. coli</i>	[19]
pPROLar.A	$P_{lac/ara-1}$ expression vector, Kan ^r , p15A ori	Clontech
pTrc99A	P_{trc} expression vector, Amp ^r , <i>lacI^q</i> , pBR322 ori	GE Healthcare
pTSN-MrBBS	pTrc99A containing <i>MrBBS</i> of <i>Matricaria recutita</i>	This study
pPR-IspA	pPROLar.A containing <i>ispA</i> of <i>E. coli</i>	This study
pTSN-MrBBS-IspA	pTSN-MrBBS containing <i>ispA</i> of <i>E. coli</i>	This study
pSNA	pSTV28 containing <i>mvaK1</i> , <i>mvaK2</i> , and <i>mvaD</i> of <i>S. pneumoniae</i> , <i>idi</i> of <i>E. coli</i> , and <i>mvaE</i> and <i>mvaS</i> , of <i>Enterococcus faecalis</i>	[19]
pSNA-MrBBS	pSNA containing <i>MrBBS</i> of <i>M. recutita</i>	This study
pSNA-MrBBS-IspA	pSNA-MrBBS containing <i>ispA</i> of <i>E. coli</i>	This study
Primers ^a		
Bis-IF	<u>aggttaaacctgagcacactgagcgtcag</u>	This study
Bis-IR	<u>cgactctagattagactatcatcggatgta</u>	This study
Bis-VF	<u>gatagtctaacttagagctgacctgcaggc</u>	This study
Bis-VR	<u>gtgtgctcatggttaacctcctgtgtgaaattgttatc</u>	This study
IspAI-F	<u>ggtaccccatatggactttcgcagcaact</u>	This study
IspAI-R	<u>tgctctagattatttatacgctggatga</u>	This study
IspAV-F	<u>taataaataatctagaggcatcaataaaaa</u>	This study
IspAV-R	<u>gaaagtccataggggtacctttctcctct</u>	This study
IspAop-IF	<u>acatccgatgatagctctaattattcattaagaggagaaaag</u>	This study
IspAop-IR	<u>tgcatgctgcaggctgacttagattatttattacgctg</u>	This study

Overlap region for Gibson Assembly is underlined

^a Primer sequences are indicated in the 5'-3' direction

were inserted into pTSN-MrBBS through *XbaI* restriction site digestion of both the pTSN-MrBBS and the pSNA plasmids containing all the mevalonate pathway genes [19], followed by ligation with T4 DNA ligase. The resultant plasmid was designated pSNA-MrBBS. To overexpress FPP synthase, the *ispA* gene (GenBank accession number: AAC73524) was PCR-amplified from *E. coli* MG1655 genomic DNA, using IspAI-F and IspAI-R primers. To avoid proofreading errors introduced during PCR, the amplified *ispA* gene was first inserted into the *XbaI* restriction site of pTSN-MrBBS (instead of pSNA-MrBBS). The pTSN-MrBBS-IspA and pSNA plasmids were digested with *XbaI* followed by ligation with T4 DNA ligase to create pSNA-MrBBS-IspA. The direction of the MVA pathway genes was verified by Sanger sequencing.

Batch and fed-batch fermentation

To prepare the pre-culture, the engineered *E. coli* was cultured in 100 mL of TB medium at 30 °C and 180 rpm. The batch fermentation was performed by inoculating

1% (v/v) of pre-culture into 400 mL TB medium supplemented with 10 g/L glycerol and overlaid with 80 mL *n*-dodecane in a 1 L fermenter. The cultures were incubated at 30 °C and air-aerated with a flow rate of 1 volume of air per volume of medium per min (vvm), the agitation speed remained fixed at 200 rpm in all experiments. Glycerol was used as a carbon source at a concentration of 10 g/L. The pH was balanced to pH 7.0 by the addition of 1 M HCl and 25% NH₄OH solutions. *n*-Dodecane was overlaid up to 20% (v/v) of culture volume to continuously extract (–)- α -bisabolol from the culture broth during fermentation. The fed-batch fermentation began as a batch operation and was switched to the fed-batch mode at 24 h, when OD₆₀₀ reached 3.0. At the beginning of fed-batch fermentation, TB medium was supplemented with 10 g/L glycerol, 0.1% (v/v) trace element solution (27 g/L FeCl₃·6H₂O, 2 g/L ZnCl₂·4H₂O, 2 g/L CoCl₂·6H₂O, 2 g/L Na₂MoO₄·2H₂O, 1 g/L CaCl₂·2H₂O, 1.3 g/L CuCl₂·6H₂O, and 0.5 g/L H₃BO₃) and 1% (v/v) vitamin solution (Sigma Aldrich, Cat. No. M6895). Glycerol was then fed at a rate

of 0.01 L/h and 10% (v/v) canola oil (instead of 20% (v/v) *n*-dodecane) was used to overlay 30 L TB medium in a 50 L fermenter.

(–)- α -Bisabolol extraction with *n*-dodecane and vegetable oils

Extraction of (–)- α -bisabolol was performed using four common vegetable oils: canola, olive, corn, and soybean oil. A total of 0.1 g of (–)- α -bisabolol was added to 10 mL of sterile TB medium in a glass vial and 1 mL of

(Agilent, Santa Clara, USA). The quantification of (–)- α -bisabolol was performed using GC equipped with a flame ionization detector (FID) with a HP-5 column (30 m \times 0.320 mm \times 0.25 μ m) at a flow rate of 1 mL/min. The starting temperature of the oven was 60 °C for 2 min, increased at 5 °C/min to 200 °C, held at 200 °C for 2 min, increased by 50 °C/min to 300 °C, and held at 300 °C for 5 min. Helium was used as the carrier gas with an inlet pressure of 5.58 psi. The (–)- α -bisabolol concentration produced by engineered *E. coli* was determined as follows:

$$(-)\text{-}\alpha\text{-bisabolol [mg/L]} = \frac{[(+)\text{-}\alpha\text{-bisabolol in } n\text{-dodecane phase}] \times [\text{volume of } n\text{-dodecane phase}]}{[\text{volume of medium phase}]}$$

individual vegetable oil was overlaid to the solution. After gentle stirring of the mixture, it was incubated at 25 °C for 1 h to separate the water-based TB medium and oil phase. The aqueous layer was removed and the organic layer was transferred to a new vial for gas chromatography (GC) analysis of (–)- α -bisabolol. The recovery yield of each vegetable oil was compared with that of *n*-dodecane. To determine the recovery efficiency of *n*-dodecane from cell pellets and TB medium, *E. coli* DH5 α cells transformed with the pSNA-MrBBS-IspA plasmid were cultivated in TB medium in the presence of 20% (v/v) *n*-dodecane at 30 °C at 180 rpm for 48 h. After centrifugation of the culture at 14,000 rpm for 10 min, the pellet, supernatant, and organic layers of *n*-dodecane were fractionated. To determine the recovery yield of *n*-dodecane, the organic layer was directly subjected to GC analysis of (–)- α -bisabolol. The isolated supernatant was mixed with 20% (v/v) *n*-dodecane, incubated for 30 min, and centrifuged at 14,000 rpm for 10 min. The organic layer was isolated and used for GC analysis of (–)- α -bisabolol. The cell pellet was first disrupted by sonication and the lysate was used for the extraction and quantification of (–)- α -bisabolol through the process described above.

(–)- α -Bisabolol identification and quantification

To analyze (–)- α -bisabolol production, the culture broth was centrifuged at 13,000 rpm for 10 min to separate the overlaid *n*-dodecane phase. The recovered *n*-dodecane-phase was subsequently analyzed for (–)- α -bisabolol by GC and GC-mass spectrometry (GC-MS). The identification of (–)- α -bisabolol was conducted by GC-MS (5977A MSD) with a HP-5MS column (30 m \times 0.250 mm \times 0.25 μ m, Agilent, Santa Clara, USA). The column flow was maintained at 1 mL/min. The oven temperature was initially held at 60 °C for 2 min, increased by 5 °C/min to 300 °C, and held at 300 °C for 10 min. (–)- α -Bisabolol (Cat. No. 23089-26-1, Sigma Aldrich) was used as the standard, and was confirmed through the GC-MS software, Mass Hunter

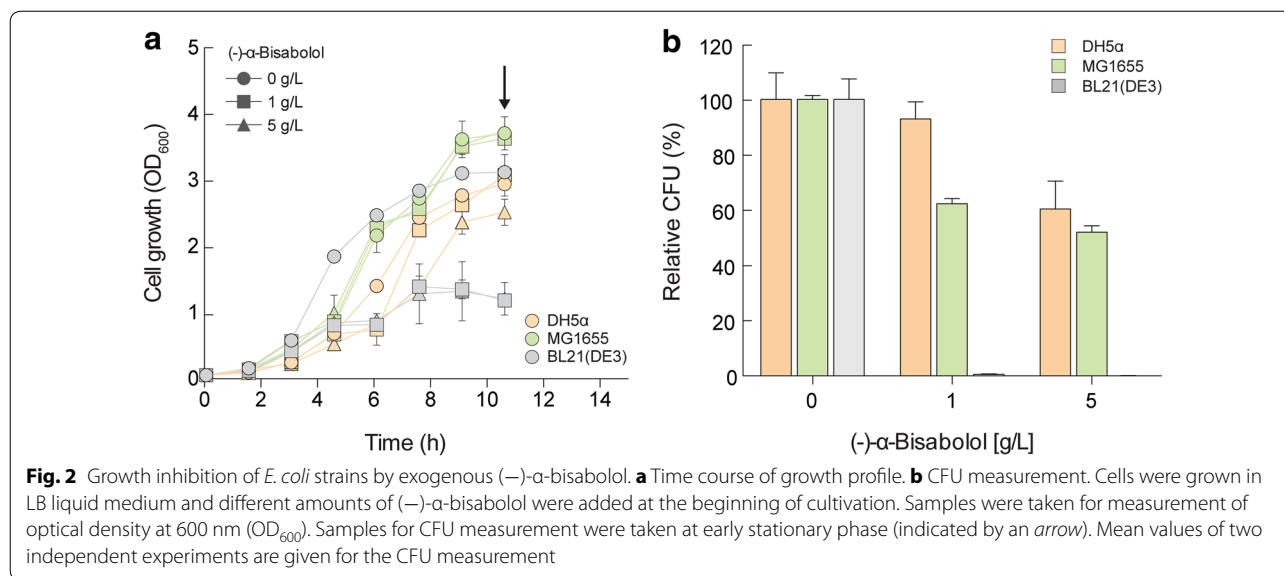
Analysis of glycerol consumption and byproduct formation

The culture broth was centrifuged and the supernatant was used to quantify glycerol consumption and byproduct formation. Glycerol, acetate, and mevalonate concentrations were determined by high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) with a refractive index detector at 454 nm using Aminex HPX-87H column (1300 mm \times 7.8 mm, Bio-Rad, Hercules, USA). Sulfuric acid (0.4 mM) was used as the mobile phase at a flow rate of 0.3 mL/min at 50 °C.

Results and discussion

Suitability of *E. coli* DH5 α as a host for (–)- α -bisabolol production

(–)- α -Bisabolol is a naturally occurring sesquiterpene alcohol that exhibits antibacterial activity [13]. This property would be a hurdle for the use of *E. coli* as a terpenoid production host [20]. Therefore, we examined the suitability of three different *E. coli* strains [DH5 α , MG1655, and BL21(DE3)] for (–)- α -bisabolol production by conducting growth assays in LB in the presence of various concentrations of (–)- α -bisabolol (Fig. 2). After 3 h of cultivation (mid-log phase), the growth of BL21(DE3) significantly decreased by 1 and 5 g/L (–)- α -bisabolol (Fig. 2a). The growth of MG1655 was not affected by (–)- α -bisabolol at concentrations of 1 or 5 g/L (Fig. 2a). The growth of DH5 α slightly decreased at (–)- α -bisabolol concentration of 5 g/L, but growth was not affected by 1 g/L (–)- α -bisabolol (Fig. 2a). This result was corroborated with CFU measurements for each culture. As expected, DH5 α showed the best tolerance to (–)- α -bisabolol whereas BL21(DE3) formed no colonies on the solid LB medium (Fig. 2b). These results are consistent with a previous study showing that of five different B- and K-type *E. coli* strains (MG1655, DH5 α , S17-1, XL1-Blue, and BL21), DH5 α is the best producer of the β -carotene (C40) terpenoid [21]. In addition, *E. coli* DH5 α has been used for production of retinol [22], farnesol [23], and protoilludene [23]. We concluded that



E. coli DH5 α is suitable as a host for (–)- α -bisabolol production and it was used for all subsequent experiments.

(–)- α -Bisabolol production in *E. coli* DH5 α expressing the *MrBBS* gene

To implement the *de novo* production of (–)- α -bisabolol in *E. coli* DH5 α , the gene of *MrBBS*, encoding the (–)- α -bisabolol synthase, was codon-optimized for *E. coli* and introduced via the pTSN-*MrBBS* plasmid (Fig. 1b). The resulting transformant cells were cultured in LB medium overlaid with 20% (v/v) *n*-dodecane for 48 h. Two-phase cultures have been successfully used to extract toxic, water-immiscible or volatile products [24–26] and *n*-dodecane was successfully used for extraction of (–)- α -bisabolol from the culture broth of *S. cerevisiae* [15]. As expected, in the present study, (–)- α -bisabolol was extracted into the *n*-dodecane phase in the two-phase *E. coli* culture. As the (–)- α -bisabolol was recovered in *n*-dodecane without significant residual amounts left in the cells (Additional file 1: Figure S2), (–)- α -bisabolol purification in a later step will be facilitated. The *n*-dodecane phase was used for GC analysis to measure (–)- α -bisabolol concentrations. There was a peak at 25.6 min in the *n*-dodecane phase sample of *E. coli* DH5 α with pTSN-*MrBBS*, corresponding to the standard (–)- α -bisabolol compound dissolved in *n*-dodecane (Fig. 3a). Mass spectrometry confirmed that the peak at 25.6 min was (–)- α -bisabolol (Fig. 3b, c) and a maximum concentration of 3 mg/L (–)- α -bisabolol was produced by the endogenous MEP pathway and exogenous *MrBBS* enzyme in *E. coli* DH5 α . The peak was not observed in two-phase cultures of DH5 α cells containing an empty plasmid as a control experiment (Fig. 3a). The (–)- α -bisabolol synthase gene, *MrBBS*, used in

this study was recently isolated from German chamomile and, unlike other TSPs, exclusively synthesizes (–)- α -bisabolol as a single terpene product [15]. Several TSPs producing α -bisabolol as a single major product have been previously cloned and biochemically characterized. However, these enzymes usually produce undesirable isomers with different structures or α -bisabolol of unknown stereochemistry [27–29]. The *MrBBS* enzyme has shown unique catalytic features including the formation of a single enantiopure (–)- α -bisabolol indicating the possibility of a biotechnological application to natural (–)- α -bisabolol production in *S. cerevisiae* [15]. Here, we have expressed the codon-optimized synthetic *MrBBS* in *E. coli* and successfully produced (–)- α -bisabolol (titer: 3 mg/L). A similar result has been observed when attempting to express other plant terpene synthases in *E. coli* [30, 31].

(–)- α -Bisabolol production in *E. coli* DH5 α expressing *MrBBS* and MVA pathway genes

Terpenoid production in microbes is mainly limited by the flux from intermediates of central metabolism (acetyl-CoA for MVA pathway or pyruvate and glyceraldehyde-3-phosphate for MEP pathway) to the substrates of TPSs (GPP, FPP, or GGPP) [32]. The introduction and engineering of the biosynthetic MVA pathway in *E. coli* have improved the production of terpenoids such as amorphadiene [32], carotenoids [19, 21], coenzyme Q10 [33], and farnesol [30] by efficiently supplying IPP and DMAPP. Based on these studies, we exploited the biosynthetic MVA pathway for the biosynthesis of (–)- α -bisabolol in *E. coli*. To increase carbon flux towards TPS substrates, a plasmid (pSSN12Didi) containing the lower MVA pathway

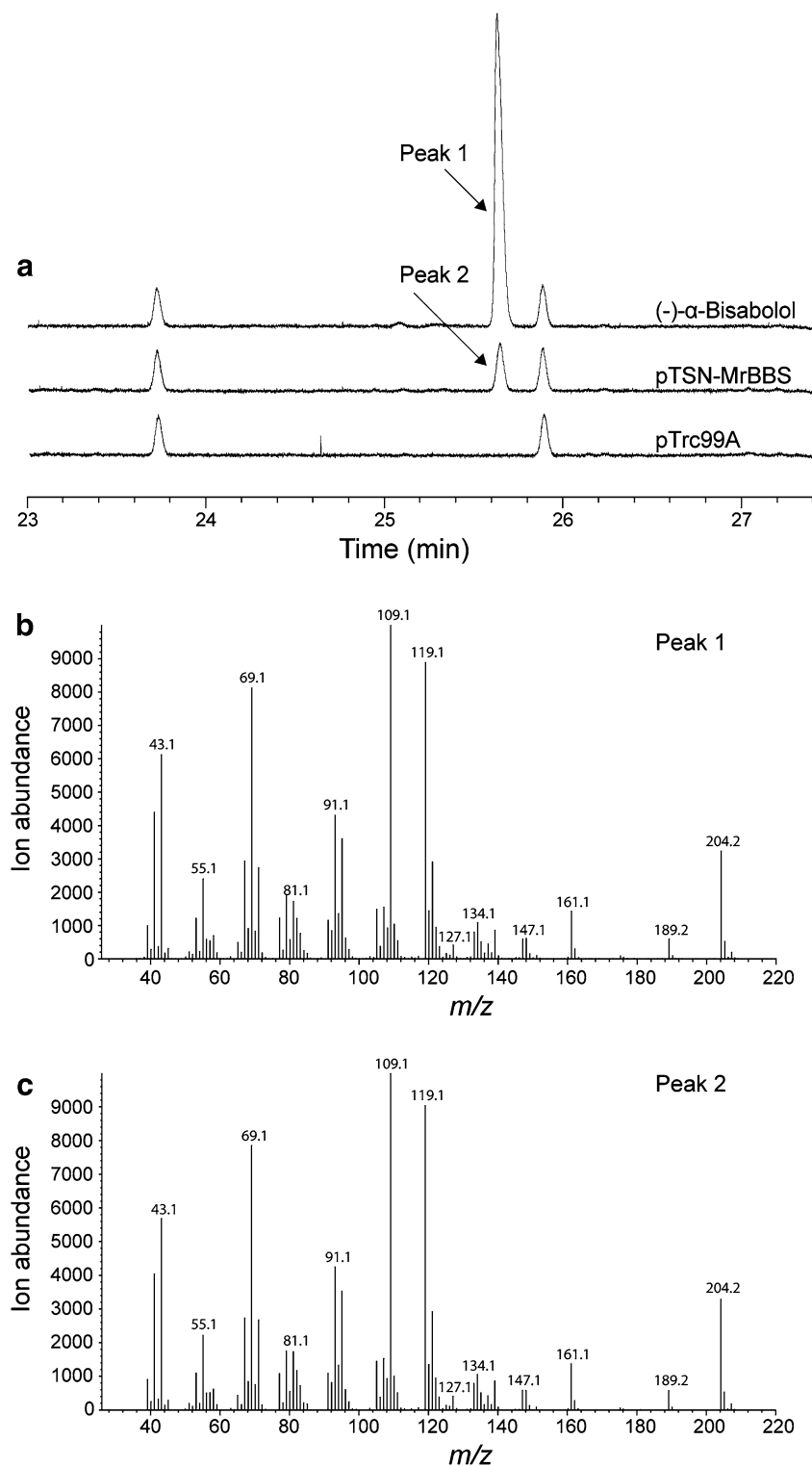
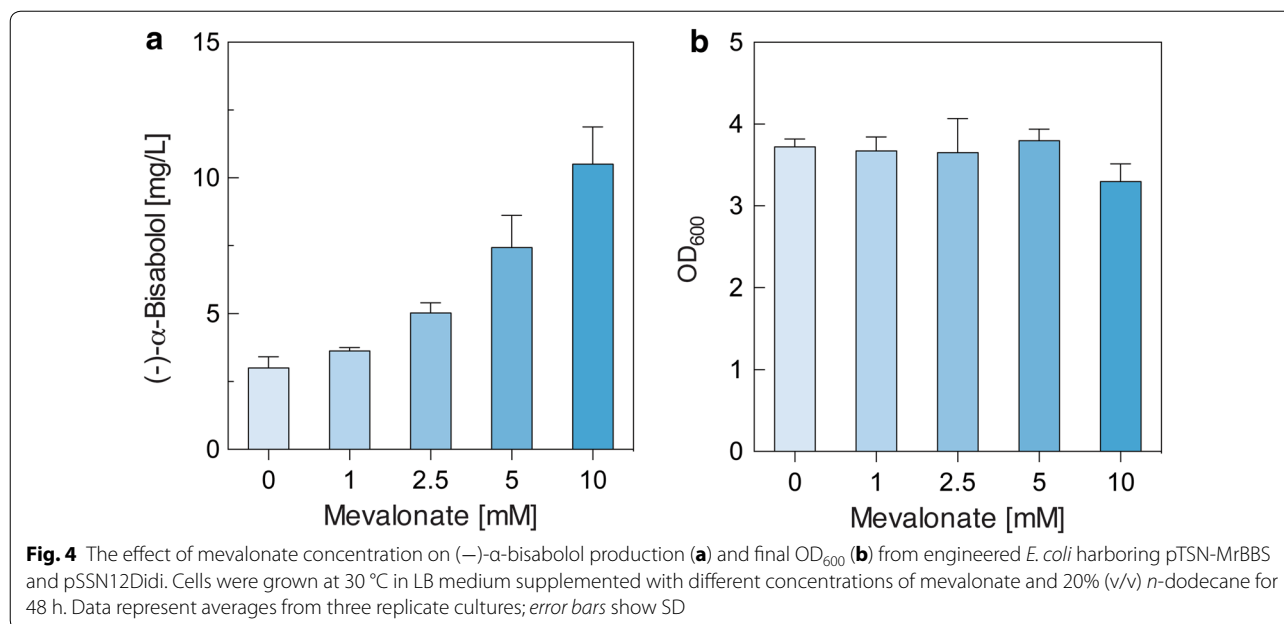


Fig. 3 GC and GC-MS analysis of *n*-dodecane extracts from *E. coli* expressing the *MrBBS* gene. **a** Total ion chromatograms of an authentic (-)- α -bisabolol standard (100 mg/L) and the *n*-dodecane extracts from the *E. coli* expressing MrBBS (pTSN-MrBBS) or containing an empty vector (pTrc99A). **b** Mass spectra of (-)- α -bisabolol standard. **c** Mass spectra of (-)- α -bisabolol produced in *E. coli* cells harboring the plasmid pTSN-MrBBS. Each cell was cultivated in LB medium overlaid with 20% (v/v) *n*-dodecane at 30 °C for 48 h

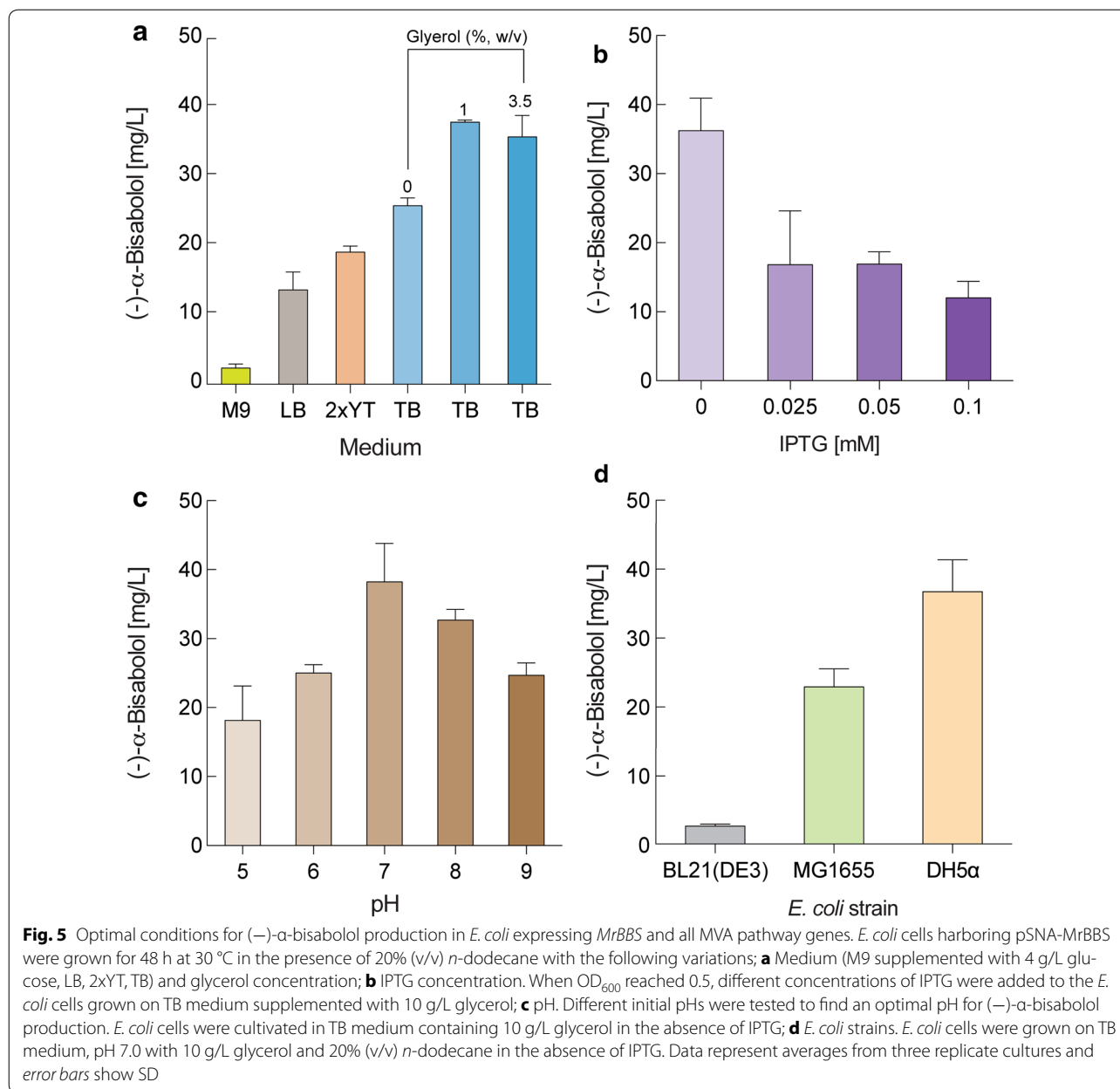


genes (Fig. 1b), leading from MVA to IPP and DMAPP [30], was introduced into *E. coli* DH5 α expressing the *MrBBS*. MVA is an exogenous substrate for *E. coli*, so supplemented MVA can only be converted to IPP and DMAPP by the engineered MVA lower pathway in *E. coli*. Different concentrations of MVA, ranging from 0 to 10 mM, were examined to determine whether MVA availability limits (–)- α -bisabolol production in LB medium. (–)- α -bisabolol production increased as the concentration of the supplied MVA increased (Fig. 4a) and cell growth was not significantly inhibited (Fig. 4b). With the addition of 10 mM MVA, (–)- α -bisabolol production was 10.5 mg/L, approximately 3.5-fold higher than that obtained without the addition of MVA (3 mg/L, Fig. 4a). Expression of the lower MVA pathway genes in *E. coli* improved (–)- α -bisabolol production with the addition of MVA. However, MVA is not an economically viable substrate. To achieve (–)- α -bisabolol production from cost-effective renewable resources such as glycerol, a major byproduct of the biodiesel industry, we constructed a plasmid pSNA-MrBBS (Fig. 1b). pSNA-MrBBS harbors the complete biosynthetic MVA pathway genes, including the *MrBBS* gene, and enables production of IPP and DMAPP in the absence of MVA. A total of 12.8 mg/L of (–)- α -bisabolol was produced from glycerol in *E. coli* DH5 α with pSNA-MrBBS grown in LB (Fig. 5a). This is a slightly higher concentration than that produced in DH5 α containing only *MrBBS* and lower MVA pathway genes with the addition of 10 mM MVA (10.5 mg/L). Overall, the expression of biosynthetic MVA pathway

genes significantly improved (–)- α -bisabolol production in *E. coli*.

Optimization of (–)- α -bisabolol production in *E. coli* DH5 α expressing *MrBBS* and entire MVA pathway genes

IPP and DMAPP, precursors of FPP, are essential metabolites in *E. coli* that are used for tRNA prenylation, synthesis of quinone and dolichol for respiration, and cell wall biosynthesis [31]. Thus, the metabolic balance of IPP and DMAPP between endogenous essential metabolism and exogenous (–)- α -bisabolol production plays a crucial role in improving production of (–)- α -bisabolol and alleviation of growth inhibition. To determine the optimal IPP and DMAPP pool balancing conditions in the engineered *E. coli* harboring the entire MVA pathway and *MrBBS* genes (pSNA-MrBBS), we examined if cultivation media and glycerol availability limit (–)- α -bisabolol production. To do this we tested different media and concentrations of glycerol ranging from 0 to 3.5% (w/v) (Fig. 5a). *E. coli* DH5 α containing the pSNA-MrBBS produced the highest concentration of (–)- α -bisabolol (24.2 mg/L) in TB medium without the addition of an additional carbon source (Fig. 5a). (–)- α -Bisabolol production was dependent on the amount of provided glycerol in the TB medium. With 1% (w/v) glycerol, (–)- α -bisabolol concentration was 38.3 mg/L, approximately 1.4-fold higher than the concentration of (–)- α -bisabolol obtained without the inclusion of glycerol. However, 3.5% (w/v) of glycerol concentration did not show further improvement of (–)- α -bisabolol production (Fig. 5a). Because cell growth of *E. coli* DH5 α varied in different culture media, the



differences in (-)-α-bisabolol production are partly due to differences in growth (Figure S3A).

Plasmid pSNA-MrBBS contained IPTG-inducible *lac* and *trc* promoters for expression of the complete MVA pathway genes and *MrBBS* gene, respectively (Fig. 1b). To ascertain the optimal inducer concentration for (-)-α-bisabolol production in *E. coli* DH5α with pSNA-MrBBS, induction was conducted with varying concentrations of IPTG ranging from 0 to 0.1 mM. Interestingly, leaky expression of the *MrBBS* and MVA pathway genes in the absence of IPTG showed the highest production of (-)-α-bisabolol (38.9 mg/L). At all other IPTG

concentrations (-)-α-bisabolol production decreased as the IPTG concentration increased (Fig. 5b) whereas the growth of *E. coli* DH5α cells is similar regardless of IPTG concentration (Additional file 1: Figure S3B). This result is compatible with a previous report [31] that showed lycopene production in *E. coli* expressing lower MVA pathway genes regulated by the *trc* promoter decreased under all IPTG-induced conditions. In addition, constitutive promoters controlling the expression of MVA pathway genes showed much lower production of amorphadiene than that from IPTG-inducible *lacUV5* and *trc* promoters [34]. High expression of the

MVA pathway and *MrBBS* genes under IPTG induced conditions could cause a deficiency of FPP for essential cellular metabolism. Furthermore, the accumulation of toxic intermediates of the MVA pathway, including IPP and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) [32, 35], could cause growth inhibition along with a decrease of (–)- α -bisabolol production. In addition, we investigated the effects of initial pH of the cultivation of *E. coli* DH5 α with pSNA-MrBBS on the production of (–)- α -bisabolol. The optimal pH for (–)- α -bisabolol production was pH 7 (38.2 mg/L, Fig. 5c). To determine the ideal *E. coli* strains for (–)- α -bisabolol production under the optimized culture conditions, we individually introduced the plasmid pSNA-MrBBS into three different *E. coli* strains (DH5 α , MG1655, or BL21(DE3)) and measured the (–)- α -bisabolol concentration produced from each. *E. coli* DH5 α cells produced the highest amount of (–)- α -bisabolol, and BL21(DE3) showed an approximate 13-fold decrease in (–)- α -bisabolol production compared to DH5 α (38.4 mg/L, Fig. 5d). We also measured cell growth during examination of pH effects on (–)- α -bisabolol production in *E. coli* DH5 α expressing *MrBBS* and entire MVA pathway genes. Although cell growth slightly decreased at a pH 5 or 6, it was similar at all other pHs (Additional file 1: Figure S3C).

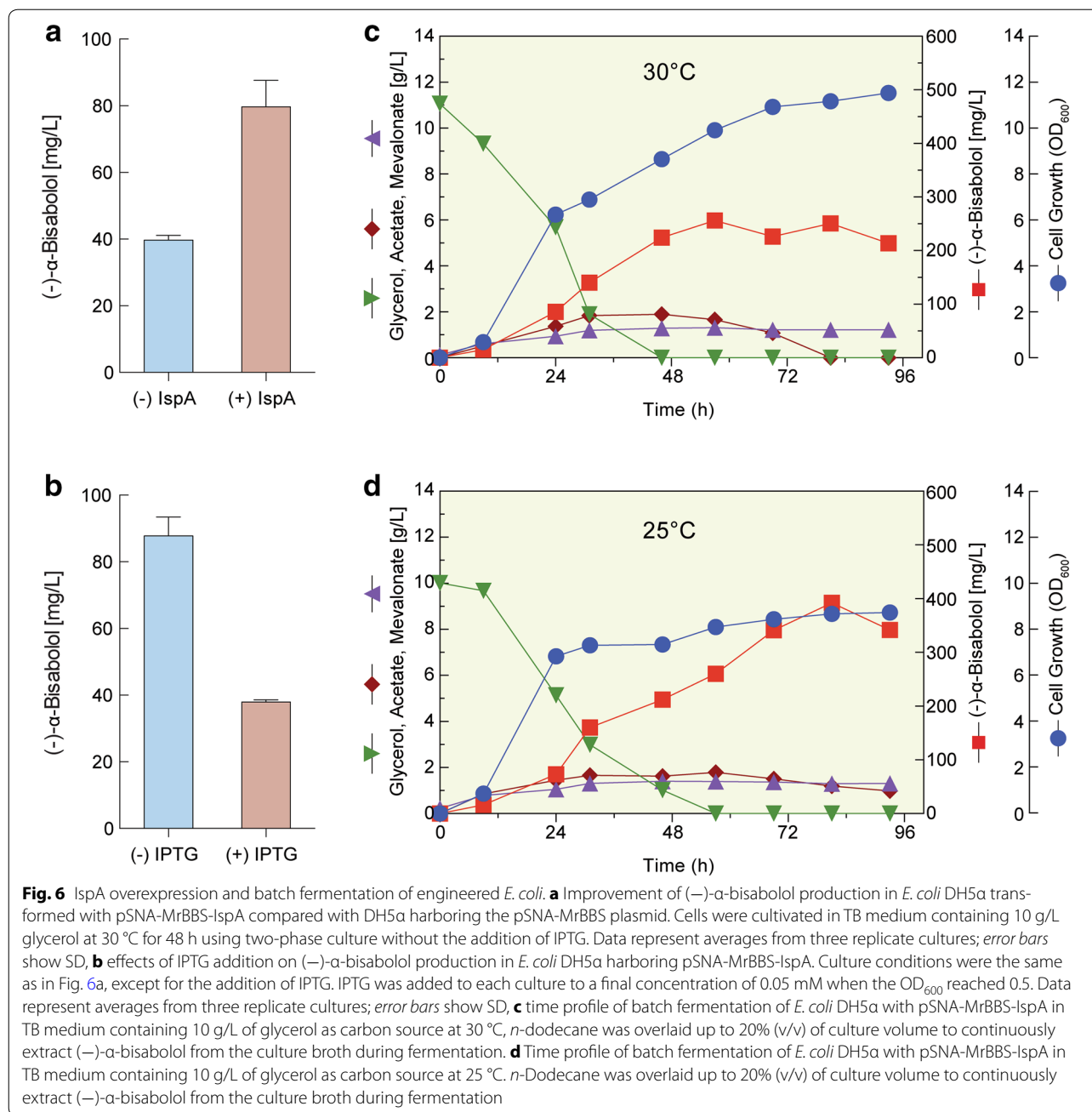
Effect of the overexpression of FPP synthase on (–)- α -bisabolol production

(–)- α -Bisabolol is produced from dephosphorylation of the prenyl diphosphate precursor FPP. Therefore, synthesis of FPP from IPP and DMAPP by FPP synthase, encoded by the *ispA* gene, plays a key role in the improved production of (–)- α -bisabolol. Furthermore, high concentrations of prenyl diphosphates, such as FPP, are toxic to *E. coli* cell growth [36]. FPPs need to be efficiently converted into downstream products by TPSs to reduce its toxicity. To increase the synthesis of the cellular terpenoid biosynthesis intermediate FPP, and (–)- α -bisabolol production, overexpression of an FPP synthase, encoded by the *ispA* gene, was performed. The *ispA* gene of *E. coli* was inserted downstream of the *MrBBS* gene in the plasmid pSNA-MrBBS, resulting in the pSNA-MrBBS-*IspA* plasmid (Fig. 1b). This plasmid was transformed into *E. coli* DH5 α and cultivated in TB medium containing 1% (w/v) glycerol at 30 °C for 48 h using a two-phase culture without the addition of IPTG. Overexpression of the *ispA* gene improved (–)- α -bisabolol production compared with pSNA-MrBBS alone. After 48 h, a concentration of 79.7 mg/L of (–)- α -bisabolol was obtained in a culture of DH5 α with pSNA-MrBBS-*IspA*. This was twofold higher than the 39.7 mg/L of (–)- α -bisabolol produced by DH5 α with pSNA-MrBBS alone (Fig. 6a), without significant difference in cell growth (Additional file 1: Figure S3D).

This suggested that sufficient supply of FPP is important to improve (–)- α -bisabolol production in *E. coli*. While overexpressing the *ispA* gene, 0.05 mM IPTG at OD₆₀₀ ~0.5 was added to induce expression of the MVA pathway and *MrBBS* genes. Similar to the results without *ispA* overexpression, (–)- α -bisabolol production decreased from 87.8 mg/L (no IPTG) to 40 mg/L (–)- α -bisabolol (0.05 mM IPTG, Fig. 6b) with no significant difference in cell growth (Additional file 1: Figure S3D). To monitor the time-course of (–)- α -bisabolol production, batch fermentation of *E. coli* DH5 α harboring the pSNA-MrBBS-*IspA* plasmid, using a 1 L fermenter, was conducted at 30 °C in the absence of IPTG for 93 h (Fig. 6c). (–)- α -bisabolol production of 214 mg/L was achieved and cell growth reached an OD₆₀₀ value of 11.5 after 93 h. MVA and acetate were the main byproducts, although the acetate was consumed after glycerol depletion at 48 h. The solubility of heterologous enzymes increases at lower growth temperatures because the transcription rate is slower, leading to better protein folding in *E. coli* [37, 38]. In addition, improved solubility of metabolic pathway enzymes at low temperature enhances the production yield [39]. Therefore, we performed the batch fermentation at 25 °C under the same conditions. Indeed, (–)- α -bisabolol production was 342 mg/L after fermentation for 93 h, approximately 1.6-fold higher than that obtained at 30 °C, and cell growth decreased by 25% (OD₆₀₀ 8.7, Fig. 6d).

Extraction and fed-batch fermentation of (–)- α -bisabolol

n-Dodecane has been mostly used for the extraction of terpenoids produced in microorganisms due to its relatively low volatility, enabling continuous extraction over multiple days [40]. The (–)- α -bisabolol produced in flask culture and batch fermentation could be efficiently extracted from the broth to the overlaid *n*-dodecane phase. The in situ recovery of (–)- α -bisabolol from the *n*-dodecane phase during culture can reduce production costs by simplifying both the harvest and purification processes. However, *n*-dodecane is not an economically viable extracting solvent. We assessed various natural and inexpensive vegetable oils for selective (–)- α -bisabolol extraction from fermentation broth. As shown in Table 2, all tested vegetable oils showed extraction yields ranging from 96.6 to 98.8%, which are comparable to that of *n*-dodecane (99.5%). Among them, canola oil (98.8%) was the best extracting oil and was used for the extraction of (–)- α -bisabolol during fed-batch fermentation. A greener extraction of (–)- α -bisabolol plays a key role in the application of (–)- α -bisabolol produced from fermentation broth to pharmaceutical and cosmetic products. We successfully used vegetable oils as natural, cost-effective, and biodegradable extractors for in situ extraction of



(-)- α -bisabolol during fermentation. Moreover, due to the difference in polarity between the fermentation broth (water based) and vegetable oils, there is a phase separation, resulting in isolation of oil from the broth containing cell debris. This in situ (-)- α -bisabolol separation from the fermentation broth can also alleviate production inhibition and improve (-)- α -bisabolol production, which will be of interest in industrial processes. To examine the production performance of (-)- α -bisabolol under conditions more relevant to industrial processes, we conducted (-)- α -bisabolol fermentation in a 50 L fermenter

with a fed-batch mode. *E. coli* DH5 α , containing pSNA-MrBBS-IspA, was grown at 25 °C with 10% (v/v) canola oil as an extracting solution. The final (-)- α -bisabolol titer reached 3.3 g/L after 90 h of fermentation, which was almost 8.4-fold higher than that obtained from the batch fermentation at 25 °C (Fig. 7). At the beginning of fermentation, MVA was accumulated until depletion of the initial 10 g/L glycerol. After complete depletion of initial glycerol, MVA was consumed and (-)- α -bisabolol accumulated continuously up to 9.1 g/L at the end of fermentation (Fig. 7). The specific (-)- α -bisabolol production

Table 2 Recovery yield of (–)- α -bisabolol using common vegetable oils

Extracting solution	Extraction yield (%)
Soybean oil	98.5
Canola oil	98.8
Corn oil	98.5
Sunflower oil	96.7
<i>n</i> -Dodecane	99.5

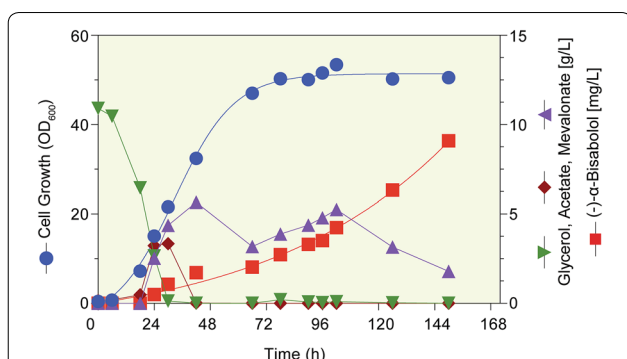


Fig. 7 Fed-batch fermentation of *E. coli* DH5 α in TB medium supplemented with 10 g/L of glycerol as initial carbon source. After complete depletion of glycerol, glycerol was fed at 3 g/L/h. Concentrations of acetate, mevalonate, and glycerol were determined by HPLC and (–)- α -bisabolol content was measured using GC. Canola oil 10% (v/v) instead of *n*-dodecane 20% (v/v) was used to overlay 30 L TB medium in a 50 L fermenter

and productivity reached 0.18 g/g dry cell weight (DCW) and 1.24 g/L/day, respectively. A previous study reported the production of 8 mg/L of (–)- α -bisabolol from *S. cerevisiae* by overexpressing the *MrBBS* gene for 4 days [15]. In this study, two-phase fed-batch fermentation of the DH5 α strain expressing the *MrBBS*, entire MVA pathway, and *ispA* genes yielded 9.1 g/L of (–)- α -bisabolol after 150 h, a 1137-fold increase from that reported in the previous study. Since the (–)- α -bisabolol intermediate, mevalonate, accumulated after glycerol depletion, (–)- α -bisabolol production would increase if we increase expression of the genes of the lower MVA pathway. Such adjustments will be the content of further work to improve (–)- α -bisabolol production.

Conclusions

In this study, we have engineered *E. coli* for *de novo* production of (–)- α -bisabolol for the first time. Introduction of the MVA pathway, (–)- α -bisabolol synthase from German chamomile, and FPP synthase turned *E. coli* into a microbial cell factory for the *de novo* production of (–)- α -bisabolol from the renewable carbon source, glycerol. Using Canola oil as an extraction

solvent, the engineered *E. coli* strain produced 9.1 g/L of (–)- α -bisabolol from glycerol in a fed-batch fermentation system, and the specific (–)- α -bisabolol production and productivity reached 0.18 g/g DCW and 1.24 g/L/day, respectively. To the best of our knowledge, this is the first report demonstrating that a large amount of (–)- α -bisabolol could be produced by metabolically engineered *E. coli*. Although more work is needed to optimize the fermentation process, the strains developed in this study will serve as promising platform hosts for development of microbial production of (–)- α -bisabolol on a large scale. Moreover, a more environmentally friendly means to extract (–)- α -bisabolol, using vegetable oils, will reduce the production cost in an industrial process.

Additional file

Additional file 1. Figure S1. Nucleotide sequence of the *E. coli* codon-optimized *MrBBS* gene. **Figure S2.** Extraction efficiency of (–)- α -bisabolol using *n*-dodecane. **Figure S3.** Cell growth during optimization of (–)- α -bisabolol production in *E. coli* DH5 α expressing *MrBBS* and entire MVA pathway genes.

Abbreviations

TPS: terpenoid synthase; IPP: isopentenyl diphosphate; DMAPP: dimethyl allyldiphosphate; MVA: mevalonate; MEP: 2-C-methyl-D-erythritol 4-phosphate; GPP: geranyl diphosphate; FPP: farnesyl diphosphate; GGPP: geranyl geranyl diphosphate; GRAS: generally regarded as safe; MrBBS: German chamomile (–)- α -bisabolol synthase gene; LB: Luria-Bertani; TB: terrific broth; IPTG: isopropyl β -D-1-thiogalactopyranoside; OD₆₀₀: optical density at a wavelength of 600 nm; CFU: colony-forming unit; PCR: polymerase chain reaction; vvm: volume of air per volume of medium per min; GC: gas chromatography; GC-MS: GC-mass spectrometry; FID: flame ionization detector; HPLC: high performance liquid chromatography; HMG-CoA: 3-hydroxy-3-methyl-glutaryl-CoA; DCW: dry cell weight.

Authors' contributions

GHH and SKK performed most of the bacterial fermentation and plasmid construction experiments. PKY, YK, and BSK conducted the optimization experiments, their linked analyses, and fermentation. YF carried out the solubility test of the MrBBS protein. BHS, H CJ, and DL planned experiments, and analyzed and interpreted the data. DL, SK, and SL supervised the study, designed experiments and analyzed and interpreted the results. GHH, SKK, PKY, and DL wrote the manuscript. All authors read and approved the final manuscript.

Author details

¹ Synthetic Biology and Bioengineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Republic of Korea. ² Biosystems and Bioengineering Program, University of Science and Technology (UST), Daejeon 34113, Republic of Korea. ³ Department of Chemical and Biological Engineering, Korea University, Seoul 02841, Republic of Korea. ⁴ Department of Biotechnology, Chonnam National University, Yeosu 550749, Republic of Korea. ⁵ Bioenergy and Biochemical Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Republic of Korea. ⁶ Division of Applied Life Science (BK21 Plus), PMBBRC, Gyeongsang National University, Jinju 52828, Republic of Korea.

Acknowledgements

The authors would like to thank the members of the Synthetic Biology Laboratory in the Synthetic Biology and Bioengineering Center at KRIBB for their valuable comments and helpful discussions.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Funding

This work was supported by the Intelligent Synthetic Biology Center of Korea (2011-0031944) through the Global Frontier Research Program, a Grant (NRF-2010-0029087) from the National Research Foundation of the Ministry of Science, ICT and Future Planning (MSIP), and the KRIBB Research Initiative Program.

Received: 9 June 2016 Accepted: 2 November 2016

Published online: 08 November 2016

References

- Buckingham J. Dictionary of natural products. London: Chapman and Hall/CRC; 2011.
- Berndt N, Hamilton AD, Sebt SM. Targeting protein prenylation for cancer therapy. *Nat Rev Cancer*. 2011;11:775–91.
- Bohlmann J, Keeling CI. Terpenoid biomaterials. *Plant J*. 2008;54:656–69.
- Chappell J. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. *Ann Rev Plant Physiol Plant Mol Biol*. 1995;46:521–47.
- Tholl D. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Curr Opin Plant Biol*. 2006;9:297–304.
- Kuzuyama T. Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. *Biosci Biotechnol Biochem*. 2002;66:1619–27.
- Bohlmann J, Meyer-Gauen G, Croteau R. Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proc Natl Acad Sci*. 1998;95:4126–33.
- Cane DE. Enzymic formation of sesquiterpenes. *Chem Rev*. 1990;90:1089–103.
- Vuorela H, Holm Y, Hiltunen R, Harvala T, Laitinen A. Extraction of the volatile oil in chamomile flowerheads using supercritical carbon dioxide. *Flavour Fragr J*. 1990;5:81–4.
- de Souza AT, Benazzi TL, Grings MB, Cabral V, Antônio da Silva E, Cardozo-Filho L, Ceva Antunes OA. Supercritical extraction process and phase equilibrium of Candeia (*Fremantulus erythropappus*) oil using supercritical carbon dioxide. *J Supercrit Fluids*. 2008;47:182–7.
- Brehm-Stecher BF, Johnson EA. Sensitization of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by the sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone. *Antimicrob Agents Chemother*. 2003;47:3357–60.
- Russell K, Jacob SE. Bisabolol. *Dermatitis*. 2010;21:57–8.
- Forrer M, Kulik EM, Filipi A, Waltimo T. The antimicrobial activity of alpha-bisabolol and tea tree oil against *Solobacterium moorei*, a Gram-positive bacterium associated with halitosis. *Arch Oral Biol*. 2013;58:10–6.
- Kamatou GP, Viljoen A. A review of the application and pharmacological properties of α -bisabolol and α -bisabolol-rich oils. *J Am Oil Chem Soc*. 2010;87:1–7.
- Son YJ, Kwon M, Ro DK, Kim SU. Enantioselective microbial synthesis of the indigenous natural product (–)- α -bisabolol by a sesquiterpene synthase from chamomile (*Matricaria recutita*). *Biochem J*. 2014;463:239–48.
- Kim CM, Goldstein JL, Brown MS. cDNA cloning of MEV, a mutant protein that facilitates cellular uptake of mevalonate, and identification of the point mutation responsible for its gain of function. *J Biol Chem*. 1992;267:23113–21.
- Sambrook J, Russell DW. Molecular cloning: a laboratory manual, vol 1, 3rd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2001.
- Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA 3rd, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 2009;6:343–5.
- Yoon S-H, Park H-M, Kim J-E, Lee S-H, Choi M-S, Kim J-Y, Oh D-K, Keasling JD, Kim S-W. Increased β -carotene production in recombinant *Escherichia coli* harboring an engineered isoprenoid precursor pathway with mevalonate addition. *Biotechnol Prog*. 2007;23:599–605.
- Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A. Engineering microbial biofuel tolerance and export using efflux pumps. *Mol Syst Biol*. 2011;7:487.
- Yoon S-H, Lee S-H, Das A, Ryu H-K, Jang H-J, Kim J-Y, Oh D-K, Keasling JD, Kim S-W. Combinatorial expression of bacterial whole mevalonate pathway for the production of β -carotene in *E. coli*. *J Biotechnol*. 2009;140:218–26.
- Jang H-J, Ha B-K, Zhou J, Ahn J, Yoon S-H, Kim S-W. Selective retinol production by modulating the composition of retinoids from metabolically engineered *E. coli*. *Biotechnol Bioeng*. 2015;112:1604–12.
- Yang L, Wang C, Zhou J, Kim SW. Combinatorial engineering of hybrid mevalonate pathways in *Escherichia coli* for protoilludene production. *Microb Cell Fact*. 2016;15:14.
- Leon R, Martin M, Vigarà J, Vilchez C, Vega JM, Vega JM. Microalgae mediated photoproduction of beta-carotene in aqueous-organic two phase systems. *Biomol Eng*. 2003;20:177–82.
- Newman JD, Marshall J, Chang M, Nowroozi F, Paradise E, Pitera D, Newman KL, Keasling JD. High-level production of amorpho-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. *Biotechnol Bioeng*. 2006;95:684–91.
- Verhoef S, Wierckx N, Westerhof RGM, de Winde JH, Ruijsenaars HJ. Bioproduction of *p*-hydroxystyrene from glucose by the solvent-tolerant bacterium *Pseudomonas putida* S12 in a two-phase water-decanol fermentation. *Appl Environ Microbiol*. 2009;75:931–6.
- Attia M, Kim S-U, Ro D-K. Molecular cloning and characterization of (+)- α -bisabolol synthase, catalyzing the first step in the biosynthesis of the natural sweetener, hernandulcin, in *Lippia dulcis*. *Arch Biochem Biophys*. 2012;527:37–44.
- Nakano C, Kudo F, Eguchi T, Ohnishi Y. Genome mining reveals two novel bacterial sesquiterpene cyclases: (–)-germacradien-4-ol and (–)- α -bisabolol synthases from *Streptomyces citricolor*. *ChemBioChem*. 2011;12:2271–5.
- Li JX, Fang X, Zhao Q, Ruan JX, Yang CQ, Wang LJ, Miller DJ, Faraldos JA, Allemann RK, Chen XY, Zhang P. Rational engineering of plasticity residues of sesquiterpene synthases from *Artemisia annua*: product specificity and catalytic efficiency. *Biochem J*. 2013;451:417–26.
- Wang C, Yoon S-H, Shah AA, Chung Y-R, Kim J-Y, Choi E-S, Keasling JD, Kim S-W. Farnesol production from *Escherichia coli* by harnessing the exogenous mevalonate pathway. *Biotechnol Bioeng*. 2010;107:421–9.
- Yoon SH, Lee YM, Kim JE, Lee SH, Lee JH, Kim JY, Jung KH, Shin YC, Keasling JD, Kim SW. Enhanced lycopene production in *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate. *Biotechnol Bioeng*. 2006;94:1025–32.
- Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat Biotechnol*. 2003;21:796–802.
- Zahiri HS, Yoon SH, Keasling JD, Lee SH, Won Kim S, Yoon SC, Shin YC. Coenzyme Q10 production in recombinant *Escherichia coli* strains engineered with a heterologous decaprenyl diphosphate synthase gene and foreign mevalonate pathway. *Metab Eng*. 2006;8:406–16.
- Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Battath TS, Redding-Johnson AM, Petzold CJ, Mukhopadhyay A, Lee TS, Adams PD, Keasling JD. Engineering dynamic pathway regulation using stress-response promoters. *Nat Biotechnol*. 2013;31:1039–46.
- Kizer L, Pitera DJ, Pflieger BF, Keasling JD. Application of functional genomics to pathway optimization for increased isoprenoid production. *Appl Environ Microbiol*. 2008;74:3229–41.
- Withers ST, Gottlieb SS, Lieu B, Newman JD, Keasling JD. Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity. *Appl Environ Microbiol*. 2007;73:6277–83.
- Schein CH, Noteborn MHM. Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. *Nat Biotechnol*. 1988;6:291–4.
- Schein CH. Production of soluble recombinant proteins in bacteria. *Nat Biotechnol*. 1989;7:1141–9.
- Zhou K, Zou R, Stephanopoulos G, Too H-P. Enhancing solubility of deoxyxylulose phosphate pathway enzymes for microbial isoprenoid production. *Microb Cell Fact*. 2012;11:1–8.
- Davies FK, Work VH, Beliaev AS, Posewitz MC. Engineering limonene and bisabolene production in wild type and a glycogen-deficient mutant of *Synechococcus* sp. PCC 7002. *Front Bioeng Biotechnol*. 2014;2:21.