

Article

# Enhanced (–)- $\alpha$ -Bisabolol Productivity by Efficient Conversion of Mevalonate in *Escherichia coli*

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**Abstract:** (–)- $\alpha$ -Bisabolol, a naturally occurring sesquiterpene alcohol, has been used in pharmaceuticals and cosmetics owing to its beneficial effects on inflammation and skin healing. Previously, we reported the high production of (–)- $\alpha$ -bisabolol by fed-batch fermentation using engineered *Escherichia coli* (*E. coli*) expressing the exogenous mevalonate (MVA) pathway genes. The productivity of (–)- $\alpha$ -bisabolol must be improved before industrial application. Here, we report enhancement of initial (–)- $\alpha$ -bisabolol productivity to 3-fold higher than that observed in our previous study. We first harnessed a farnesyl pyrophosphate (FPP)-resistant mevalonate kinase 1 (MvaK1) from an archaeon *Methanosarcina mazei* (*M. mazei*) to create a more efficient heterologous MVA pathway that produces (–)- $\alpha$ -bisabolol in the engineered *E. coli*. The resulting strain produced 1.7-fold higher (–)- $\alpha$ -bisabolol relative to the strain expressing a feedback-inhibitory MvaK1 from *Staphylococcus aureus* (*S. aureus*). Next, to efficiently convert accumulated MVA to (–)- $\alpha$ -bisabolol, we additionally overexpressed genes involved in the lower MVA mevalonate pathway in *E. coli* containing the entire MVA pathway genes. (–)- $\alpha$ -Bisabolol production increased by 1.8-fold with reduction of MVA accumulation, relative to the control strain. Finally, we optimized the fermentation conditions including inducer concentration, aeration and enzymatic cofactor. The strain was able to produce 8.5 g/L of (–)- $\alpha$ -bisabolol with an initial productivity of 0.12 g/L h in the optimal fed-batch fermentation. Thus, the microbial production of (–)- $\alpha$ -bisabolol would be an economically viable bioprocess for its industrial application.

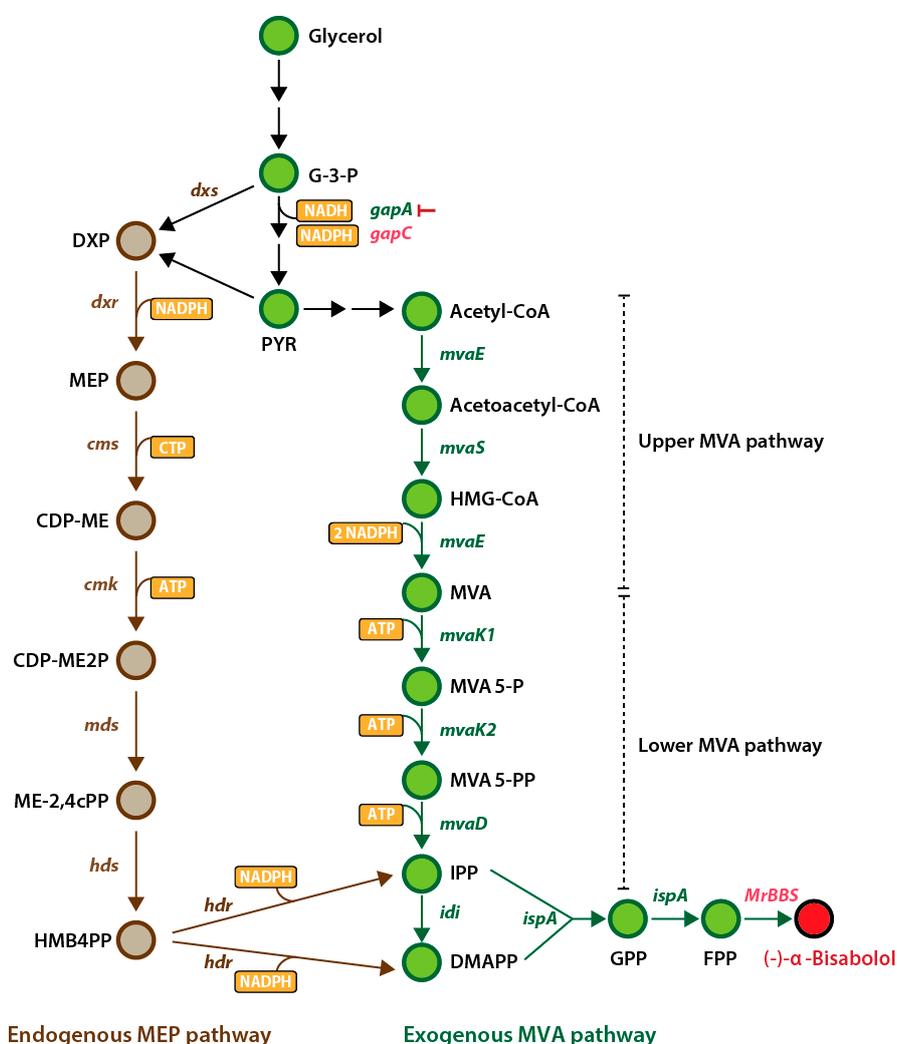
**Keywords:** (–)- $\alpha$ -bisabolol; mevalonate (MVA); mevalonate kinase 1; *Methanosarcina mazei*; fed-batch fermentation

## 1. Introduction

A monocyclic sesquiterpene alcohol, (–)- $\alpha$ -bisabolol, has been used in pharmaceuticals and cosmetics as it displays the beneficial effects of skin healing and anti-inflammation [1–5]. The global market of (–)- $\alpha$ -bisabolol is expected to reach \$73 million by 2020, with an annual growth rate of 5.9% from 2016 [6]. Commercially available (–)- $\alpha$ -bisabolol is currently produced by the steam-distillation method using oils extracted from German chamomile or Brazilian candeia tree [7,8]. This process, however, has caused environmental issues, as well as economic concerns owing to a low extraction yield [8]. Natural (–)- $\alpha$ -bisabolol was obtained from the candeia tree with a yield of approximately 0.018 g/g<sub>candeia power</sub> through CO<sub>2</sub> supercritical extraction at 40 °C and 10 MPa [8]. Although a chemical

process has been developed to produce (–)- $\alpha$ -bisabolol, it forms diastereomers of (–)- $\alpha$ -bisabolol ((+)- $\alpha$ -bisabolol and ( $\pm$ )-epi- $\alpha$ -bisabolol), and thus requires auxiliary purification steps [9]. In this context, the biological production of naturally occurring (–)- $\alpha$ -bisabolol using engineered microbes may be an attractive alternative to the current production processes of (–)- $\alpha$ -bisabolol.

(–)- $\alpha$ -Bisabolol can be synthesized from five-carbon building blocks of isopentenyl diphosphate (IPP) and its isomer dimethyl allylpyrophosphate (DMAPP) (Figure 1) [10]. Both universal precursors of terpenoids can be produced from the MVA or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Exogenous MVA or endogenous MEP pathway has been employed in engineered *E. coli* for production of various terpenoids. Although the MEP pathway exhibits higher theoretical yield than the MVA pathway, the exogenous MVA pathway showed generally higher production than the endogenous MEP pathway [11]. In particular, the MVA pathway has been harnessed to efficiently convert acetyl-CoA to several terpenoids including (–)- $\alpha$ -bisabolol [12]. Both universal isoprene units, IPP and DMAPP are converted into farnesyl pyrophosphate (FPP), which is catalyzed by FPP synthase encoded by the endogenous *ispA* gene, which is then used for production of (–)- $\alpha$ -bisabolol by the (–)- $\alpha$ -bisabolol synthase (BBS, Figure 1).



**Figure 1.** Biosynthetic pathway of (–)- $\alpha$ -bisabolol in engineered *E. coli*. The endogenous MEP) pathway consists of DXS (deoxyxylulose-5-phosphate synthase), DXR (deoxyxylulose 5-phosphate reductoisomerase), CMS (2-C-methylerythritol 4-phosphate cytidyl transferase), CMK (4-(cytidine 5'-diphospho)-2-C-methylerythritol kinase), MDS (2-C-methylerythritol 2,4-cyclodiphosphate synthase), HDS ((E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase), and HDR (hydroxymethylbutenyl

diphosphate reductase). The MEP pathway begins with the condensation of G-3-P (glyceraldehyde 3-phosphate) and pyruvate that is converted from G-3-P by endogenous nicotinamide adenine dinucleotide (NAD<sup>+</sup>)-dependent GAPDH (glyceraldehyde-3-phosphate dehydrogenase) coded by *gapA* and nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>)-dependent GAPDH coded by *gapC* from *Clostridium acetobutylicum* (*C. acetobutylicum*). The exogenous MVA pathway consists of MvaE (dual function of acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA reductase), MvaS (3-hydroxy-3-methylglutaryl-CoA synthase), MvaK1 (MVA kinase), MvaK2 (phosphomevalonate kinase), MvaD (mevalonate 5-pyrophosphate decarboxylase), Idi (isopentenyl diphosphate isomerase), IspA (geranyl diphosphate synthase or FPP synthase), and MrBBS ((-)- $\alpha$ -bisabolol synthase of *Matricaria recutita*). Endogenous and exogenous genes are depicted in brown and green, respectively.

The biological production of (-)- $\alpha$ -bisabolol has been explored using well-studied microbes such as *Saccharomyces cerevisiae* (*S. cerevisiae*) [9] and *E. coli* [12]. Because of the identification of BBS from German chamomile, *Matricaria recutita*, the microbes expressing the MrBBS enzyme can synthesize an (-)- $\alpha$ -bisabolol as a major terpenoid product [9]. Previously, we engineered an *E. coli* strain to express the MrBBS enzyme and exogenous MVA pathway. The resulting *E. coli* produced 9.1 g/L of (-)- $\alpha$ -bisabolol with a productivity of 0.04 g/L h at early stage of fermentation (0–42 h) [12], whereas *S. cerevisiae* expressing the MrBBS enzyme alone produced 8 mg/L of (-)- $\alpha$ -bisabolol during four days of cultivation [9]. These studies showed the potential of (-)- $\alpha$ -bisabolol production by microbial fermentation. However, productivity remains to be improved for the industrial production of (-)- $\alpha$ -bisabolol using engineered microbes. In our empirical fermentation studies, the initial productivity (0–48 h) of (-)- $\alpha$ -bisabolol was critical to improving its overall productivity, because after 2 days of fermentation, the production rate of (-)- $\alpha$ -bisabolol showed no significant differences among various production strains and fermentation conditions.

In this study, we improve (-)- $\alpha$ -bisabolol productivity in engineered *E. coli*, which can serve as a promising platform strain for development of an economically feasible bioprocess of (-)- $\alpha$ -bisabolol production. To this end, we first introduced a heterologous MvaK1 from *M. mazei* that is resistant to FPP feedback inhibition. We then added a copy of the lower MVA pathway genes to the whole MVA pathway for the efficient conversion of MVA to (-)- $\alpha$ -bisabolol. Finally, we optimized the fermentation conditions of the engineered *E. coli* by tuning the inducer concentrations and aeration for MVA pathway expression and sufficient ATP supply, respectively. Overall, a fed-batch fermentation produced 8.5 g/L of (-)- $\alpha$ -bisabolol with 0.12 g/L h of initial productivity (0–46 h) in the engineered *E. coli*.

## 2. Results

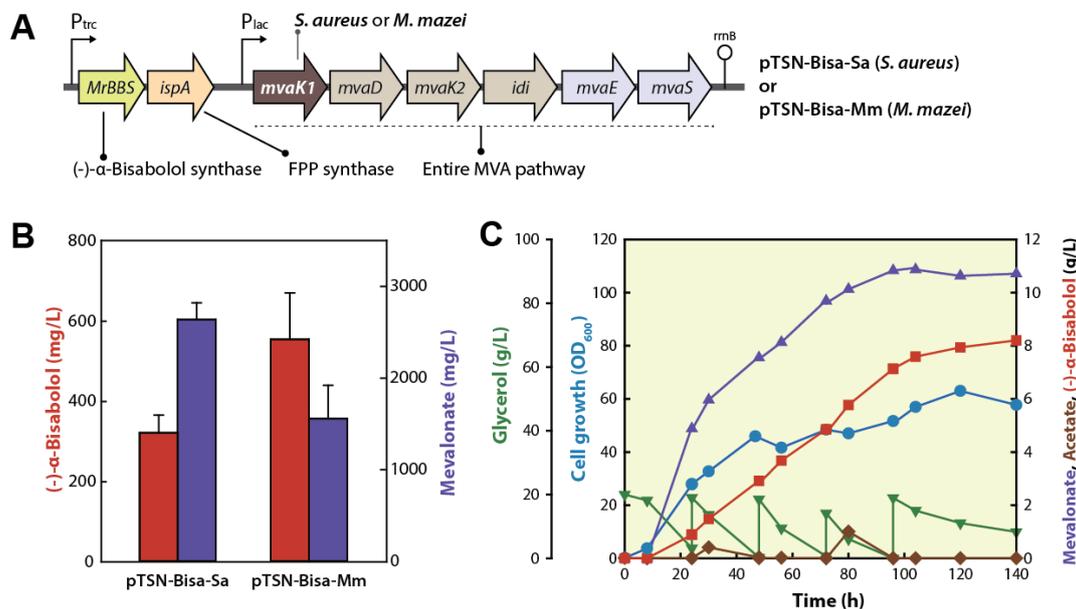
### 2.1. Feedback-Resistant MvaK1

MvaK1 is responsible for the first step of the lower MVA pathway by converting MVA to mevalonate phosphate (MVA 5-P in Figure 1) [10] and is important for the regulation of the entire MVA pathway because it is inhibited by known feedback inhibitors: C5 (IPP and DMAPP), C15 (geranyl pyrophosphate (GPP) and FPP), and longer chain terpenoids [13,14]. FPP is a feedback inhibitor of the widely used *Staphylococcus aureus* MvaK1 (*SaMvaK1*) for creating a heterologous MVA pathway [15]. Previously, we have also used *SaMvaK1* to produce (-)- $\alpha$ -bisabolol in engineered *E. coli* [16].

To avoid feedback inhibition of MvaK1 and subsequently improve (-)- $\alpha$ -bisabolol production, we replaced the *S. aureus mvaK1* gene of the pTSN-Bisa-Sa plasmid with a feedback-resistant *mvaK1* gene (Figure S1) from the versatile methanogen *M. mazei*, which resulted in a pTSN-Bisa-Mm plasmid. The *E. coli* DH5 $\alpha$ -pTSN-Bisa-Mm strain produced 555 mg/L of (-)- $\alpha$ -bisabolol, which is 1.7-fold higher than that of the *E. coli* DH5 $\alpha$ -pTSN-Bisa-Sa strain (Figure 2B). This is consistent with MVA accumulation of *MmMvaK1* showing 1.7-fold less than that of *SaMvaK1* (Figure 2B, right panel), suggesting that the feedback-resistant *MmMvaK1* leads to an increase the MVA utilization efficiency.

Using the *E. coli* DH5 $\alpha$ -pTSN-Bisa-Mm strain, we conducted a fed-batch fermentation by intermittently supplying glycerol (Figure 2C). Cells were grown exponentially for 24 h and produced

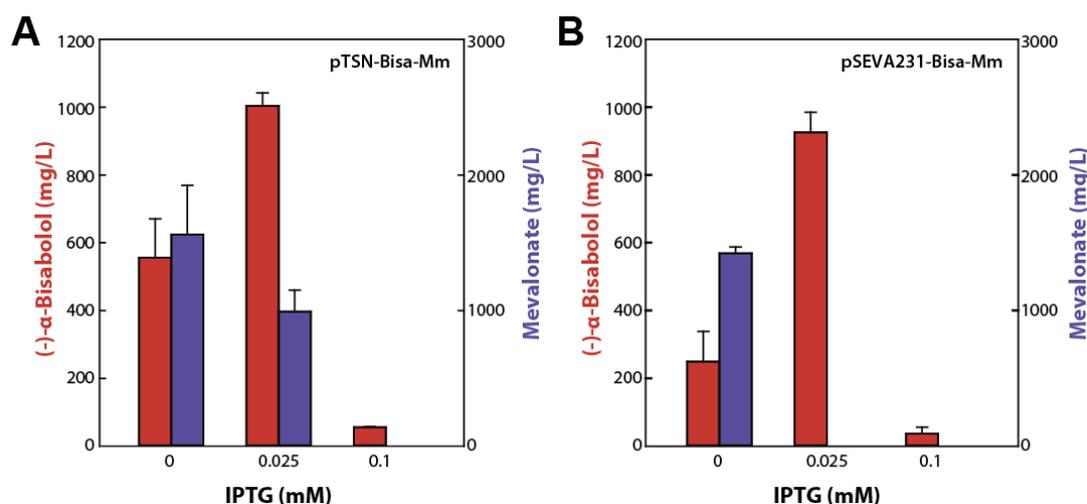
0.9 g/L of (–)- $\alpha$ -bisabolol and 4.9 g/L of MVA along with consuming initially supplied glycerol. A total of 8.2 g/L of (–)- $\alpha$ -bisabolol was yielded with a productivity of 0.06 g/L h, and 10.7 g/L of MVA was accumulated in 140 h. Overall, although feedback-resistant *MmMvaK1* was used for (–)- $\alpha$ -bisabolol production, a significant amount of MVA was still accumulated in the fed-batch fermentation.



**Figure 2.** Introduction of *MvaK1* from *M. mazei* for the improvement of (–)- $\alpha$ -bisabolol production. (A) Plasmid constructs for expressing the entire MVA pathway, FPP synthase and (–)- $\alpha$ -bisabolol synthase. The plasmid pTSN-Bisa-Sa and pTSN-Bisa-Mm have *mvaK1* gene of *S. aureus* and *M. mazei*, respectively. (B) Improvement of (–)- $\alpha$ -bisabolol production in the engineered *E. coli* DH5 $\alpha$  harboring pTSN-Bisa-Mm compared with the strain expressing pTSN-Bisa-Sa. Cells were grown in TB medium containing 10 g/L glycerol and 20% (v/v) of *n*-dodecane at 30 °C for 72 h without the addition of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). The error bars represent the standard deviation of the concentrations of (–)- $\alpha$ -bisabolol and MVA from three biological replicates. (C) Fed-batch fermentation of *E. coli* DH5 $\alpha$  harboring pTSN-Bisa-Mm. The fed-batch fermentation was performed in TB medium and 20% (v/v) of *n*-dodecane using two-phase culture in the absence of IPTG at 30 °C and pH 7.0. After depletion of glycerol initially added, glycerol was fed intermittently into the bioreactor during fermentation. An agitation speed of 280 rpm and an aeration rate of 1 vessel volume per minute (vvm) were maintained throughout the cultivation.

## 2.2. Overexpression of Entire MVA Pathway Genes

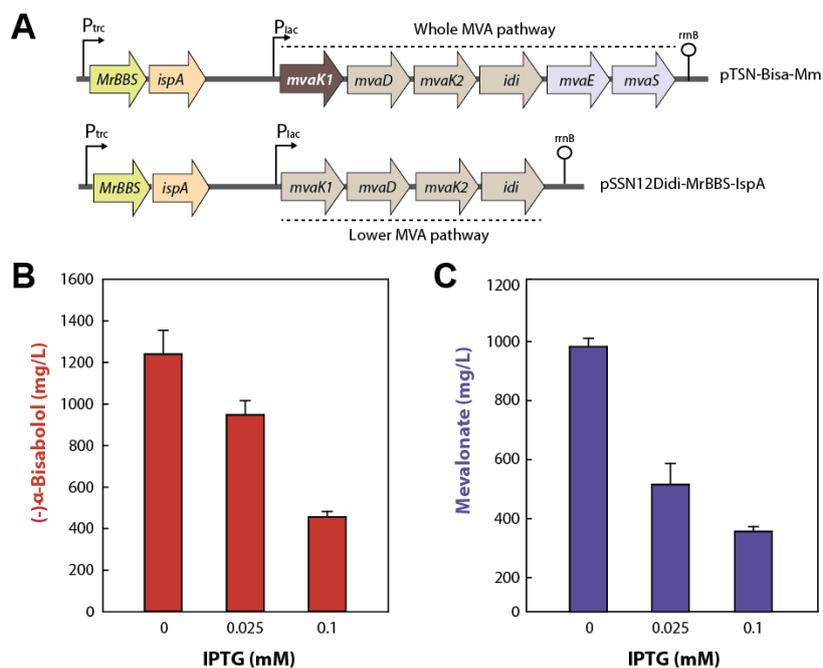
Enzymes responsible for (–)- $\alpha$ -bisabolol biosynthesis in *E. coli* DH5 $\alpha$ -pTSN-Bisa-Mm strain are controlled by IPTG-inducible promoters; *trc* promoter for *MrBBS*, and *ispA* genes, and *lac* promoter for all MVA pathway genes (Figure 2A). To this end, we explored the effect of IPTG amount on (–)- $\alpha$ -bisabolol production and MVA accumulation in batch culture. When 0.025 mM IPTG was used for induction, (–)- $\alpha$ -bisabolol production increased by 1.8-fold along with a 1.4-fold decrease of MVA accumulation compared to those of the control that were not induced by IPTG (Figure 3A). To scrutinize the effect of IPTG on (–)- $\alpha$ -bisabolol production, a pSEVA231-Bisa-Mm was generated using a medium copy number plasmid, pSEVA231 (pBBR1 ori) (Figure 3B). Interestingly, the *E. coli* DH5 $\alpha$ -pSEVA231-Bisa-Mm strain produced 926 mg/L of (–)- $\alpha$ -bisabolol without the accumulation of MVA under the induced condition (0.025 mM IPTG), which is 3.7-fold higher than the uninduced condition (Figure 3B). When the IPTG amount increased up to 0.1 mM, both pTSN-Bisa-Mm (high copy number), and pSEVA231-Bisa-Mm (medium copy number) showed a dramatic decrease (90%) in (–)- $\alpha$ -bisabolol production compared to those in the presence of 0.025 mM IPTG (Figure 3A,B).



**Figure 3.** Effect of IPTG on (–)- $\alpha$ -bisabolol production. The concentrations of (–)- $\alpha$ -bisabolol and MVA produced by the *E. coli* DH5 $\alpha$  harboring pTSN-Bisa-Mm, a high-copy plasmid (A) or pSEVA231-Bisa-Mm, a medium-copy plasmid (B). Cells were grown in TB medium containing 10 g/L glycerol and 20% (v/v) of *n*-dodecane in the presence of different IPTG concentrations (0, 0.025 and 0.1 mM) at 30 °C for 72 h. The error bars represent the standard deviation of the concentrations of (–)- $\alpha$ -bisabolol and MVA from three biological replicates.

### 2.3. Reinforcement of the MVA Pathway

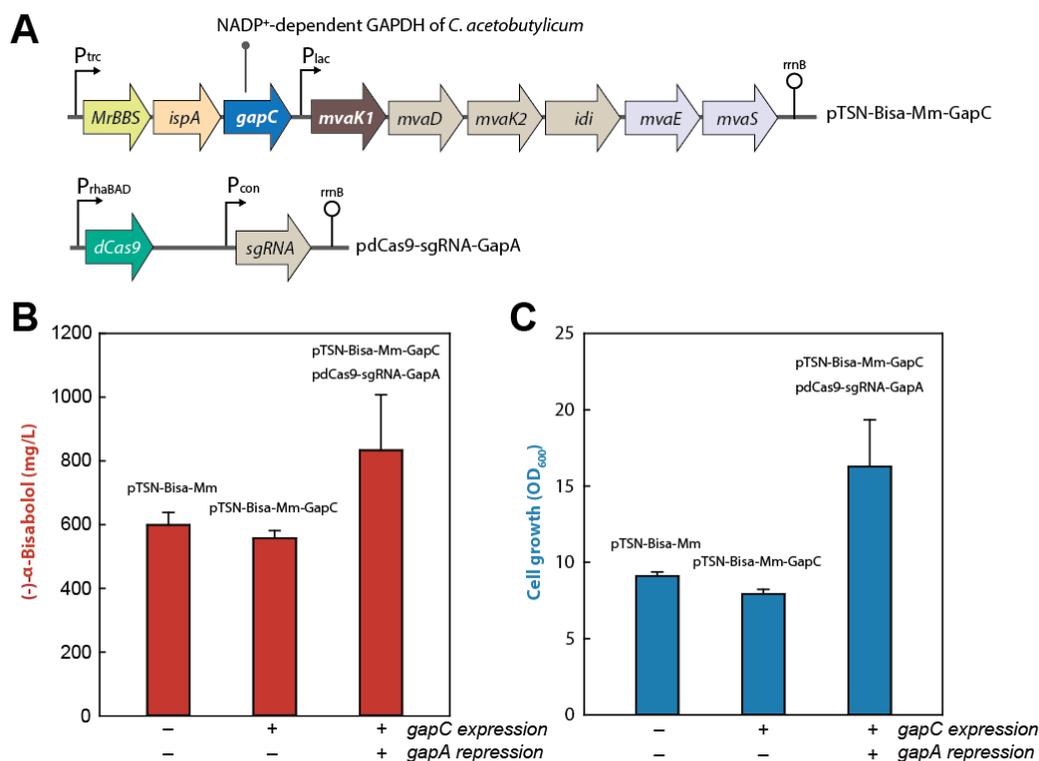
Considering the high accumulation of MVA in the production of (–)- $\alpha$ -bisabolol, we reinforced the whole MVA pathway through the expression of an additional copy of lower MVA pathway genes. A newly generated plasmid, pSSN12Didi-MrBBS-IspA, contains the lower MVA pathway genes (*mvaK1*, *mvaK2*, *mvaD*, *idi*), *MrBBS*, and *ispA* (Figure 4A). The *MvaK1* of pSSN12Didi-MrBBS-IspA plasmid was adopted from *Streptococcus pneumoniae* (*S. pneumoniae*), which has a 2.6-fold faster turnover number ( $k_{cat}$ ) than that of *MmMvaK1* [17]. In the absence of IPTG, *E. coli* DH5 $\alpha$  harboring both pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA plasmids produced 1.2 g/L of (–)- $\alpha$ -bisabolol and 988 mg/L of MVA (Figure 4B,C), which are 2.2-fold higher and 1.4-fold lower than those of the *E. coli* DH5 $\alpha$  containing the pTSN-Bisa-Mm plasmid alone, respectively. Because the metabolic flux was changed by the introduction of additional lower MVA pathway genes, we probed the effect of IPTG concentrations on the (–)- $\alpha$ -bisabolol production in the *E. coli* DH5 $\alpha$  containing both pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA plasmids. Unlike the results from the *E. coli* DH5 $\alpha$  harboring the pTSN-Bisa-Mm alone (Figure 3A), production of both (–)- $\alpha$ -bisabolol and MVA decreased as the IPTG concentrations increased (Figure 4B,C), indicating that enzymes for (–)- $\alpha$ -bisabolol biosynthesis were sufficiently expressed in the absence of IPTG to increase the (–)- $\alpha$ -bisabolol production when the lower MVA pathway was reinforced.



**Figure 4.** (–)- $\alpha$ -Bisabolol production in *E. coli* DH5 $\alpha$  containing pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA which has genes involved in the MVA lower pathway including *SpMvaK1*, *IspA* and *MrBBS*. (A) Plasmid constructs for expressing genes of entire (–)- $\alpha$ -bisabolol biosynthetic pathway or lower MVA pathway. The concentrations of (–)- $\alpha$ -bisabolol (B) and MVA (C) produced by the engineered *E. coli* DH5 $\alpha$  harboring pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA. Cells were cultivated in TB medium containing 10 g/L glycerol and 20% (v/v) of *n*-dodecane in the presence of different IPTG concentrations (0, 0.025 and 0.1 mM) at 30 °C for 72 h. The error bars represent the standard deviation of the concentrations of (–)- $\alpha$ -bisabolol and MVA from three biological replicates.

#### 2.4. Sufficient Supply of reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)

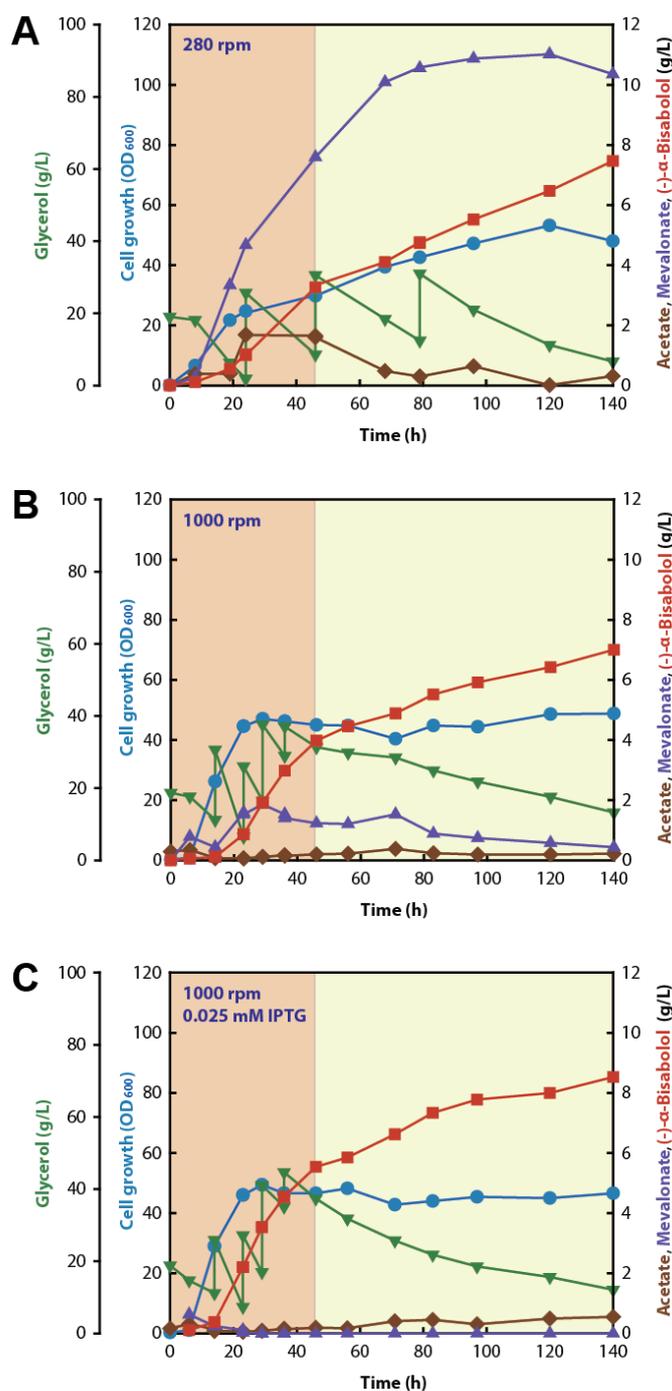
The engineered *E. coli* consumes 2 NADPH to convert 3-hydroxy-3-methyl-glutaryl-CoA (HMG)-CoA to MVA via the *MvaE* enzyme of the (–)- $\alpha$ -bisabolol biosynthetic pathway (Figure 1). Therefore, if the intracellular NADPH pool of engineered *E. coli* increases, it will improve (–)- $\alpha$ -bisabolol production. In *E. coli*, an endogenous GAPDH generates a reduced nicotinamide dinucleotide (NADH) to convert glyceraldehyde 3-phosphate into pyruvate. Therefore, we replaced the endogenous GAPDH gene (*gapA*) of *E. coli* with an NADP<sup>+</sup>-dependent GAPDH gene (*gapC*) of *C. acetobutylicum* to increase the intracellular NADPH pool in *E. coli*. To do this, we inserted the *gapC* gene of *C. acetobutylicum* into the downstream of the *ispA* gene of the pTSN-Bisa-Mm plasmid, which resulted in a pTSN-Bisa-Mm-GapC plasmid (Figure 5A). The *E. coli*-pTSN-Bisa-Mm-GapC strain showed similar (–)- $\alpha$ -bisabolol production and cell growth to the *E. coli*-pTSN-Bisa-Mm strain (Figure 5B,C). This might be caused by competition between endogenous GapA and heterologous GapC in the *E. coli*-pTSN-Bisa-Mm-GapC strain. To investigate this, the *gapA* gene was repressed by clustered regularly interspaced short palindromic repeats (CRISPR) interference (CRISPRi). The CRISPRi system comprises L-rhamnose-inducible deactivated Cas9 (dCas9) and a constitutively expressed single guide RNA targeting *gapA* gene (sgRNA-GapA) by J23119 promoter, respectively (Figure 5A). The *E. coli* strain harboring both pTSN-Bisa-Mm-GapC and pdCas9-sgRNA-GapA plasmids produced 1.4-fold higher (–)- $\alpha$ -bisabolol compared to the *E. coli* strain containing the pTSN-Bisa-Mm-GapC plasmid alone. Interestingly, the cell growth of the *E. coli* strain repressing *gapA* by CRISPRi showed better cell growth than the control *E. coli* (Figure 5C). Given that the (–)- $\alpha$ -bisabolol/OD<sub>600</sub> are similar between the two strains, it is likely that the increased production of (–)- $\alpha$ -bisabolol is due to increased cell mass.



**Figure 5.** Overexpression of *gapC* gene encoding NADP<sup>+</sup>-dependent GAPDH from *C. acetobutylicum* and repression of *gapA* gene coding for endogenous NAD<sup>+</sup>-dependent GAPDH from *E. coli* using the CRISPRi system. (A) Plasmid constructs for expressing genes of the entire (–)-α-bisabolol biosynthetic pathway introducing the *gapC* gene downstream of the *ispA* gene (pTSN-Bisa-Mm-GapC) and for expressing inactivated Cas9 (dCas9) and sgRNA targeting the *gapA* gene (pdCas9-sgRNA-GapA). (B) Comparison of (–)-α-bisabolol concentrations produced by the *E. coli* DH5α harboring pTSN-Bisa-Mm, pTSN-Bisa-Mm-GapC, or both pTSN-Bisa-Mm-GapC and pCas9-sgRNA-GapA. (C) Comparison of cell growth of the strains. Cells were grown in TB medium containing 10 g/L glycerol and 20% (v/v) of *n*-dodecane in the presence of different IPTG concentrations (0, 0.025 and 0.1 mM) at 30 °C for 72 h. The error bars represent the standard deviation of the concentrations of (–)-α-bisabolol and OD<sub>600</sub> from three biological replicates.

### 2.5. Effect of Aeration on (–)-α-Bisabolol Fermentation

We performed a fed-batch fermentation to produce the (–)-α-bisabolol in *E. coli* DH5α containing both pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA plasmids. The yield of (–)-α-bisabolol was improved by 16% in 46 h compared to when the lower MVA pathway was not additionally overexpressed. However, the MVA still accumulated from the beginning of fermentation and reached 10.1 g/L in 68 h at 280 rpm despite reinforcing the lower MVA pathway (Figure 6A). It seems that there are other bottlenecks when MVA is converted to (–)-α-bisabolol through the lower MVA pathway. The synthetic MVA pathway requires 3 moles of ATP to convert MVA to (–)-α-bisabolol (Figure 1) and competes for the ATP with other essential cellular reactions involved in cell growth [18]. Because ATPs are efficiently generated under aerobic conditions using NADHs in oxidative phosphorylation [19], aeration effects were examined by controlling the agitation speed in fed-batch fermentation.



**Figure 6.** Fed-batch fermentation of the *E. coli* DH5 $\alpha$  harboring both pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA plasmids. The fed-batch fermentation was performed in TB medium and 20% (v/v) of *n*-dodecane using two-phase culture at 30 °C and pH 7.0. After depletion of glycerol initially added, glycerol was fed intermittently into the bioreactor during fermentation. An agitation speed of 280 rpm (A) or 1000 rpm (B,C) and an aeration rate of 1 vvm were maintained throughout the cultivation. After 6 h cultivation, 0.025 mM of IPTG was added to the bioreactor (C).

In fed-batch fermentation at an agitation speed of 1000 rpm, the *E. coli* strain harboring pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA rapidly grew and reached the maximum cell growth within 23 h showing a 1.8-fold improved cell growth relative to those at 280 rpm. As expected, MVA accumulation was considerably reduced, but (-)- $\alpha$ -bisabolol production was not significantly improved (Figure 6B). It seems that acetyl-CoA was utilized for cell growth and other metabolism,

or metabolites downstream of MVA, accumulated. To overcome this problem, we sought to enhance the overall (–)- $\alpha$ -bisabolol flux through overexpressing all genes in the (–)- $\alpha$ -bisabolol biosynthetic pathway by the addition of IPTG. We carried out the fed-batch fermentation at 1000 rpm and supplied IPTG at a concentration of 0.025 mM after 6 h of incubation. Similar to the culture in the absence of IPTG, cells rapidly grew for 22 h, but MVA did not accumulate during fermentation, supporting the lower pathway was intensified by IPTG addition. The yield and productivity of (–)- $\alpha$ -bisabolol were improved by 40% and 56%, respectively, as compared to the absence of IPTG, and the final titer of (–)- $\alpha$ -bisabolol reached 8.5 g/L (Figure 6C).

### 3. Discussion

In this study, *E. coli* was engineered for the efficient conversion of MVA to (–)- $\alpha$ -bisabolol using the feedback-resistant MvaK1 and reinforcement of the lower MVA pathway. The feedback-resistant MvaK1 was firstly identified in the archaeon *M. mazei*. However, only a handful of studies have been carried out on terpenoid production in microbes. Recently, feedback-resistant MvaK1 enzymes were identified and characterized from *Methanosaeta concilii* (McMvaK) and *Methanocella paludicola* (MpMvaK) [20]. The McMvaK and MpMvaK enzymes not only showed feedback resistance to DMAPP, IPP, GPP, and FPP, but also exhibited 4.9- and 5.5-fold higher affinity to MVA, respectively, than *MmMvaK1* [20]. Therefore, these MvaK1 enzymes may enable the enhancement of (–)- $\alpha$ -bisabolol production in engineered *E. coli*.

To find the optimal conditions to efficiently convert MVA to (–)- $\alpha$ -bisabolol, we examined the inducer concentrations, cofactor, ATP, and reinforcement of the lower MVA pathway. Adding the inducers for overexpression of MVA or MEP pathway genes has been a controversial issue in the microbial production of terpenoids [21–23]. Lycopene production was reduced under all IPTG-induced conditions in *E. coli* expressing the lower MVA pathway [23]. The leaky expression of all enzymes involved in the (–)- $\alpha$ -bisabolol production without IPTG addition exhibited the highest production among all tested IPTG concentrations [12]. The IPTG-induced overexpression of genes for (–)- $\alpha$ -bisabolol production can inhibit the essential cellular metabolism due to a deficiency of FPP or accumulation of toxic intermediates (IPP and HMG-CoA) of the heterologous MVA pathway [12]. In contrast, isoprene production increased as the IPTG concentration increased from 0.2 to 1.2 mM [24]. In this study, a small amount of IPTG was effective to increase (–)- $\alpha$ -bisabolol production in the engineered *E. coli*. Concerning the complex regulation of the MVA pathway, balancing the expression of multiple heterologous enzymes is crucial for the optimal production of (–)- $\alpha$ -bisabolol [10].

The availability of reducing cofactors such as NADH and NADPH strongly affects the yield and productivity of terpenoids in bacteria. The strengthening of the reducing power for the increased production of terpenoids has been attempted; modulation of glutamate dehydrogenase increased the production of  $\beta$ -carotene and lycopene through the increased supply of NADPH [25–27]. The overexpression of GAPDH of *C. acetobutylicum* also resulted in the improvement of isoprene production [24]. Moreover, the replacement of NAD<sup>+</sup>-dependent GAPDH of *E. coli* with the NADP<sup>+</sup>-dependent GAPDH of *C. acetobutylicum* showed a 2.5-fold increase of lycopene productivity in the engineered *E. coli* [28]. We performed the fed-batch fermentation using the strain overexpressing *gapC* from *C. acetobutylicum* and repressing the *gapA* gene through the CRISPRi system under optimized conditions (1000 rpm and the addition of IPTG at a concentration of 0.025 mM) (Figure S2, Table 1). Contrary to the results in batch fermentations, both strains showed a negative effect on (–)- $\alpha$ -bisabolol production and had slightly reduced cell growth when compared with the strain that did not overexpress *gapC* and repress *gapA*. It appears that the overexpression of *gapC* and the repression of *gapA* were not effective when sufficient amounts of ATP and NADPH were supplied, owing to the activation of the citric acid cycle and respiration by increasing oxygen in the cells. This observation might be consistent with a previous study that lycopene production in *E. coli* was improved by decreasing the pentose phosphate pathway flux and increasing the tricarboxylic acid (TCA) cycle flux [26]. Additionally, NADPH has been shown to inhibit 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR),

which converts HMG-CoA to MVA [10]. Both the overexpression of *gapC* and aerobic condition might lead to an accumulation of excess NADPH, thereby repressing HMGR and causing a flux imbalance. As a result, overall (–)- $\alpha$ -bisabolol production decreased.

Results of fed-batch fermentations conducted in this study were summarized in Table 1. Compared with the previous report [12], the final titer of (–)- $\alpha$ -bisabolol (8.5 g/L) is similar in fed-batch fermentation using *E. coli* expressing pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA at an agitation speed of 1000 rpm with IPTG added at a concentration of 0.025 mM. In particular, 5.5 g/L of (–)- $\alpha$ -bisabolol was obtained within two days, indicating that the productivity was improved by 3-fold compared to previous research. In result of fed-batch fermentations, (–)- $\alpha$ -bisabolol was continuously produced after cell growth ceased. Therefore, recycling resting cells is a promising strategy to further improve (–)- $\alpha$ -bisabolol productivity. Although promising results in productivity were obtained in this study, it is necessary to improve the final (–)- $\alpha$ -bisabolol titer for industrial applications. To achieve this, high-cell density culture experiments using living cells to continuously supply cofactors and enzymes should be conducted. Moreover, metabolic modeling of the system used in this study might provide further insight into bottlenecks for (–)- $\alpha$ -bisabolol production.

**Table 1.** Summary of fed-batch fermentations of (–)- $\alpha$ -bisabolol by engineered *E. coli*.

Plasmids	Agitation (rpm)	IPTG (mM)	Final Titer (g/L)	Initial Yield * (g/g)	Initial Productivity * (g/L h)
pTSN-Bisa-Mm	280	0	8.2	0.08	0.06
pTSN-Bisa-Mm	280	0	7.5	0.10	0.07
pSSN12Didi-MrBBS-IspA	1000	0	7.0	0.07	0.09
	1000	0.025	8.5	0.11	0.12
pTSN-Bisa-Mm-GapC	1000	0.025	5.3	0.07	0.07
pSSN12Didi-MrBBS-IspA					
pTSN-Bisa-Mm-GapC	1000	0.025	3.6	0.05	0.05
pSSN12Didi-MrBBS-IspA					
pdCas9-sgRNA-GapA					

\* Initial yield and productivity are calculated based on values in the early cultivation period (0–46 h).

## 4. Materials and Methods

### 4.1. Strains and Culture Media

An *E. coli* DH5 $\alpha$  strain (Enzynomics, Daejeon, Korea) was used for all experiments including gene cloning and (–)- $\alpha$ -bisabolol production. A lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) (BD Bioscience, San Jose, CA, USA) was used for plasmid construction and pre-cultivation. Terrific broth (TB) medium containing glycerol (12 g/L enzymatic casein digest, 24 g/L yeast extract, 9.4 g/L K<sub>2</sub>HPO<sub>4</sub>, 2.2 g/L KH<sub>2</sub>PO<sub>4</sub>, and 1% (*w/v*) glycerol) was used for (–)- $\alpha$ -bisabolol production. All media were supplied with the appropriate antibiotics: ampicillin (100  $\mu$ g/mL), chloramphenicol (34  $\mu$ g/mL), and kanamycin (25  $\mu$ g/mL). IPTG was used at concentrations of 0, 0.025, and 0.1 mM to induce gene expression involved in the (–)- $\alpha$ -bisabolol biosynthetic pathway.

### 4.2. Plasmid Construction

The plasmids and primers used in this study are listed in Table 2 and Table S1, respectively. Standard molecular biological techniques including genomic DNA preparation, restriction digestions of DNA, plasmid transformation were performed as previously described [29]. T4 DNA ligase, and all restriction enzymes were obtained from New England Biolabs (NEB, Ipswich, MA, USA). Polymerase chain reaction (PCR) was carried out following the manufacturer's protocols with a high fidelity KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan). Kits for plasmid preparation and gel extraction

were purchased from Promega (Madison, WI, USA) and oligonucleotide synthesis were conducted by Bioneer (Daejeon, Korea).

**Table 2.** Strains and plasmids used in this study.

Name	Description	References
<b>Strains</b>		
DH5 $\alpha$	F <sup>-</sup> , $\Phi$ 80lacZ- $\Delta$ M15- <i>f(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1</i>	Enzygnomics
<b>Plasmids</b>		
pTrc99A	P <sub>trc</sub> promoter, Amp <sup>R</sup> , lacI <sup>q</sup> , pBR322 ori	GE Healthcare
pSTV28	P <sub>lac</sub> promoter, Cm <sup>R</sup> , p15A ori	Takara
pSECRi	P <sub>rhaBAD::cas9</sub> (D10A, H840A) and constitutive sgRNA expression cassette in pSEVA221	[16]
pSNA-MrBBS-IspA	pTrc99A containing <i>mvaE</i> and <i>mvaS</i> of <i>Enterococcus faecalis</i> , <i>mvaK1</i> and <i>mvaK2</i> and <i>mvaD</i> of <i>S. pneumoniae</i> , <i>idi</i> , and <i>ispA</i> of <i>E. coli</i> , <i>MrBBS</i> of <i>M. recutita</i>	[12]
pTM-BBS	pTrc99A derivatives containing codon optimized <i>Matricaria recutita</i> <i>MrBBS</i> , <i>mvaK1</i> of <i>S. aureus</i> , <i>mvaD</i> and <i>mvaK2</i> of <i>S. pneumoniae</i> , <i>idi</i> of <i>E. coli</i> , <i>mvaE</i> and <i>mvaS</i> of <i>E. faecali</i>	[16]
pSSN12Didi	pSTV28 containing <i>mvaK1</i> , <i>mvaK2</i> and <i>mvaD</i> from <i>Streptococcus pneumoniae</i> , <i>idi</i> of <i>E. coli</i>	[12]
pTSN-Bisa-Sa	pTrc99A containing <i>mvaE</i> and <i>mvaS</i> of <i>Enterococcus faecalis</i> , <i>mvaK1</i> of <i>S. aureus</i> , <i>mvaK2</i> , and <i>mvaD</i> of <i>S. pneumoniae</i> , <i>idi</i> , and <i>ispA</i> of <i>E. coli</i> , <i>MrBBS</i> of <i>M. recutita</i>	This study
pTSN-Bisa-Mm	pTrc99A containing <i>mvaE</i> and <i>mvaS</i> of <i>Enterococcus faecalis</i> , <i>mvaK1</i> of <i>M. masei</i> , <i>mvaK2</i> , and <i>mvaD</i> of <i>S. pneumoniae</i> , <i>idi</i> , and <i>ispA</i> of <i>E. coli</i> , <i>MrBBS</i> of <i>M. recutita</i>	This study
pSEVA231-Bisa-Mm	pTSN-Bisa-Mm with pBBR1 ori instead of pBR322 ori	This study
pSSN12Didi-MrBBS-IspA	pSSN12Didi containing <i>ispA</i> of <i>E. coli</i> and <i>MrBBS</i> of <i>M. recutita</i>	This study
pTSN-Bisa-Mm-GapC	pTSN-Bisa-Mm containing <i>gapC</i> of <i>C. acetobutylicum</i>	This study
pdCas9-sgRNA-GapA	pSECRi containing gRNA targeting <i>gapA</i> gene	This study

The *E. coli* codon-optimized *mvaK1* gene of *M. masei* (GenBank accession number: KKI06753.1) was synthesized by Bioneer (Figure S1). The synthesized *mvaK1* was PCR-amplified with MM-IF and MM-IR primers, and the plasmid backbone was amplified with the MM-VF and MM-VR primers from pSNA-MrBBS-IspA. The two PCR-amplicons were assembled via the Gibson Assembly Method [30] using Gibson Assembly Master Mix (NEB), resulting in the construction of the pTSN-Bisa-Mm plasmid.

The *mvaK1* from *S. aureus* was amplified with SA-IF and SA-IR primers from pTM-BBS, and the plasmid for the backbone was obtained from pSNA-MrBBS-IspA by PCR with a set of primers of SA-VF and SA-VR, followed by assembly with the Gibson assembly method. The resulting plasmid was named pTSN-Bisa-Sa.

For the construction of pSEVA231-Bisa-Mm, the first fragment containing the MVA pathway gene, *ispA* and *MrBBS* were amplified in pTSN-Bisa-Mm using primers of pBBR1-IF and pBBR1-IR. The second fragment harboring the kanamycin-resistant gene and pBBR1 origin was amplified from pSEVA231 as a template using pBBR1-VF and pBBR1-VR primers. The fragments were assembled via Gibson Assembly method.

To construct pSSN12Didi-MrBBS-IspA, the *MrBBS* and *ispA* including *trc* promoter gene were amplified using Didi-I-F and Didi-I-R primers from the pTSN-Bisa-Mm plasmid. The vector backbone containing genes encoding enzymes of the lower MVA pathway was amplified using Didi-V-F and Didi-V-R primers from the pSSN12Didi plasmid. Two amplified fragments were then assembled via the Gibson Assembly kit.

The *E. coli* codon-optimized *gapC* gene from *C. acetobutylicum* (GenBank accession number: NP\_347346) including the ribosome binding site and SpeI/XbaI restriction enzyme sites was synthesized

by Macrogen (Seoul, Korea). The synthesized DNA was then digested with SpeI/XbaI, and the fragment containing the *gapC* gene was gel-purified. The other fragment was prepared by digesting the pTSN-Bisa-Mm plasmid with XbaI. The two fragments were then ligated by T4 DNA ligase, which created the plasmid pTSN-Bisa-Mm-GapC.

We used the primers of *gapA*-gRNA-F and *gapA*-gRNA-R for amplification of the whole pSECRi plasmid by PCR. The amplified DNA fragment was gel-purified and treated with T4 polynucleotide kinase to phosphorylate it. T4 DNA ligase was used to ligate the PCR product. The sequences of all genes associated with the (–)- $\alpha$ -bisabolol biosynthetic pathway were verified by Sanger sequencing (Magcogen).

#### 4.3. Batch and Fed-Batch Fermentation

To prepare the pre-culture, recombinant *E. coli* was cultured in 5 mL of LB medium supplied with appropriate antibiotics at 30 °C and 200 rpm overnight. The batch fermentation was carried out by inoculating 1% (*v/v*) of the pre-culture into 3 mL of the TB medium with 1% (*w/v*) of glycerol in a 50 mL mini-bioreactor (SPL Life Sciences, Gyeonggi-do, Korea). 20% (*v/v*) of *n*-dodecane was overlaid to extract (–)- $\alpha$ -bisabolol from all fermentation broths. The cultures were incubated at 30 °C and 200 rpm for 72 h. For fed-batch fermentation, the pre-culture was prepared in 5 mL of TB medium with 10 g/L of glycerol at 30 °C and 200 rpm overnight. 1% (*v/v*) of the cells were inoculated into 300 mL of TB medium supplied with 0.1% (*v/v*) of trace metal solution (27 g/L FeCl<sub>3</sub>·6H<sub>2</sub>O, 2 g/L ZnCl<sub>2</sub>·4H<sub>2</sub>O, 2 g/L CoCl<sub>2</sub>·6H<sub>2</sub>O, 2 g/L Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 1 g/L CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.3 g/L CuCl<sub>2</sub>·6H<sub>2</sub>O, and 0.5 g/L H<sub>3</sub>BO<sub>3</sub>), 0.98 g/L of MgSO<sub>4</sub>, 1% (*v/v*) vitamin, appropriate antibiotics, 20% (*v/v*) of *n*-dodecane in a 1 L fermenter (CNS Inc. Daejeon, Korea). 60% (*w/v*) of glycerol containing 9.8 g/L MgSO<sub>4</sub>, 2% (*v/v*) of trace metal solution and 0.25% (*v/v*) of thiamine solution was fed intermittently during the fed-batch fermentation. The fed-batch fermentation was maintained at 30 °C, 1 vvm of air flow rate, and 280 or 1000 rpm of agitation. The pH was adjusted to pH 7.0 by adding 1 N HCl and 1 N NaOH solutions.

#### 4.4. (–)- $\alpha$ -Bisabolol Quantification

Extraction of (–)- $\alpha$ -bisabolol proceeded in the *n*-dodecane phase which is initially added to the culture broth throughout the cultivation. The overlaid *n*-dodecane phase was collected after the pellet, supernatant, and a layer of *n*-dodecane were fractionated from the culture broth using centrifugation at 13,000 rpm for 3 min. Subsequently, the collected *n*-dodecane was analyzed for the determination of (–)- $\alpha$ -bisabolol concentration using a gas chromatograph (GC, 7890B, Agilent, SC, USA) which is supplied with a flame ionization detector (FID) with HP-5 column (30 m × 0.320 mm × 0.25  $\mu$ m, Agilent, SC, USA). As the carrier gas, helium was used at a flow rate of 1 mL/min. Temperatures of an injector and an FID were maintained at 240 °C and 250 °C, respectively. The programmed temperature gradients controlled the column temperature: isotherm at 60 °C for 2 min; increase at a rate of 5 °C/min to 200 °C; isotherm at 200 °C for 2 min; increase at 50 °C/min to 300 °C; and isotherm at 300 °C for 5 min. For the generation of a standard curve, (–)- $\alpha$ -bisabolol was purchased from Sigma-Aldrich. In the GC analysis, there was a peak at 21.7 min in the *n*-dodecane phase sample of recombinant *E. coli* as a major peak (>95%) except for a peak of *n*-dodecane (11.5 min). The peak at 21.7 min corresponded to the standard (–)- $\alpha$ -bisabolol compound dissolved in *n*-dodecane. The (–)- $\alpha$ -bisabolol concentration produced was determined as follows:

$$(-)\text{-}\alpha\text{-Bisabolol (g/L)} = \frac{((-)\text{-}\alpha\text{-Bisabolol in } n\text{-dodecane}) \times (\text{Volume of } n\text{-dodecane})}{\text{Volume of medium}} \quad (1)$$

#### 4.5. Determination of Cell Growth and Metabolites

Cell growth was monitored by measuring the absorbance at 600 nm (OD<sub>600</sub>) using a spectrophotometer (Ultrospec 8000, GE Healthcare, Uppsala, Sweden). After the centrifugation of the culture broth at 13,000 rpm for 3 min, the overlaid *n*-dodecane phase was removed, and the

remaining supernatant was used for analyzing metabolite concentrations. The concentrations of glycerol, acetate, and MVA were measured by high-performance liquid chromatography (HPLC, Agilent Technologies 1200 series) equipped with a refractive index detector (RID) with an Aminex HPX-87H column (1300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA). The column was eluted with 4 mM of sulfuric acid at a flow rate of 0.5 mL/min at 50 °C. All reagents for the standard solution were purchased from Sigma-Aldrich.

## 5. Conclusions

We improved (–)- $\alpha$ -bisabolol productivity from engineered *E. coli*, which can serve as a promising platform strain for the microbial production of (–)- $\alpha$ -bisabolol at an industrial scale. Metabolic engineering strategies used in this study, including feedback-resistance of MvaK1 enzyme, reinforcement of lower MVA pathway flux, balance of the NADPH and ATP pools, and optimization of fermentation, could be applied to enhance the terpenoid production from engineered microbes. Moreover, metabolic modeling based on genome-wide omics data might provide clues to identify unknown bottlenecks and interpret the results. This experiment will be conducted as a further study.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4344/9/5/432/s1>, Figure S1: Nucleotide sequence of the *E. coli* codon-optimized *mvaK1* gene derived from *M. mazei*, Table S2: Fed-batch fermentation of the engineered *E. coli* DH5 $\alpha$ , Table S1: List of primers used in this study.

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## References

1. Brehm-Stecher, B.F.; Johnson, E.A. Sensitization of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by the sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone. *Antimicrob. Agents Chemother.* **2003**, *47*, 3357–3360. [[CrossRef](#)] [[PubMed](#)]
2. Forrer, M.; Kulik, E.M.; Filippi, 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*, 10–16. [[CrossRef](#)] [[PubMed](#)]
3. Kamatou, G.P.; Viljoen, A.M. A review of the application and pharmacological properties of  $\alpha$ -bisabolol and  $\alpha$ -bisabolol-rich oils. *J. Am. Oil Chem. Soc.* **2010**, *87*, 1–7. [[CrossRef](#)]
4. Leite, G.d.O.; Leite, L.H.; Sampaio, R.d.S.; Araruna, M.K.A.; de Menezes, I.R.A.; da Costa, J.G.M.; Campos, A.R. (–)- $\alpha$ -Bisabolol attenuates visceral nociception and inflammation in mice. *Fitoterapia* **2011**, *82*, 208–211. [[CrossRef](#)] [[PubMed](#)]
5. Russell, K.; Jacob, S.E. Bisabolol. *Dermatitis* **2010**, *21*, 57–58.
6. Global One-Stop Reports Center. Available online: <http://www.Gosreports.Com/global-%ce%b1-bisabolol-market-worth-73-million-by-2020/> (accessed on 30 march 2019).
7. Albertti, L.A.G.; Delatte, T.L.; de Farias, K.S.; Boaretto, A.G.; Verstappen, F.; van Houwelingen, A.; Cankar, K.; Carollo, C.A.; Bouwmeester, H.J.; Beekwilder, J. Identification of the bisabolol synthase in the endangered candeia tree (*Eremanthus erythropappus* (dc) mcleisch). *Front. Plant Sci.* **2018**, *9*, 1340. [[CrossRef](#)]

8. de Souza, A.T.; Benazzi, T.L.; Grings, M.B.; Cabral, V.; da Silva, E.A.; Cardozo-Filho, L.; Antunes, O.A.C. Supercritical extraction process and phase equilibrium of candeia (*Eremanthus erythropappus*) oil using supercritical carbon dioxide. *J. Supercrit. Fluids* **2008**, *47*, 182–187. [[CrossRef](#)]
9. Son, Y.J.; Kwon, M.; Ro, D.K.; Kim, S.U. Enantioselective microbial synthesis of the indigenous natural product (–)- $\alpha$ -bisabolol by a sesquiterpene synthase from chamomile (*Matricaria recutita*). *Biochem. J.* **2014**, *463*, 239–248. [[CrossRef](#)]
10. Chatzivasileiou, A.O.; Stephanopoulos, G.; Ward, V.C.A. Metabolic engineering of *Escherichia coli* for the production of isoprenoids. *FEMS Microbiol. Lett.* **2018**, *365*, fny079.
11. Ajikumar, P.K.; Xiao, W.H.; Tyo, K.E.; Wang, Y.; Simeon, F.; Leonard, E.; Mucha, O.; Phon, T.H.; Pfeifer, B.; Stephanopoulos, G. Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science* **2010**, *330*, 70–74. [[CrossRef](#)]
12. Han, G.H.; Kim, S.K.; Yoon, P.K.-S.; Kang, Y.; Kim, B.S.; Fu, Y.; Sung, B.H.; Jung, H.C.; Lee, D.H.; Kim, S.W. Fermentative production and direct extraction of (–)- $\alpha$ -bisabolol in metabolically engineered *Escherichia coli*. *Microb. Cell Fact.* **2016**, *15*, 185. [[CrossRef](#)]
13. Voynova, N.E.; Rios, S.E.; Miziorko, H.M. *Staphylococcus aureus* mevalonate kinase: Isolation and characterization of an enzyme of the isoprenoid biosynthetic pathway. *J. Bacteriol.* **2004**, *186*, 61–67. [[CrossRef](#)]
14. Andreassi, J.L.; Dabovic, K.; Leyh, T.S. *Streptococcus pneumoniae* isoprenoid biosynthesis is downregulated by diphosphomevalonate: An antimicrobial target. *Biochemistry* **2004**, *43*, 16461–16466. [[CrossRef](#)] [[PubMed](#)]
15. Kim, S.K.; Kim, S.H.; Subhadra, B.; Woo, S.G.; Rha, E.; Kim, S.W.; Kim, H.; Lee, D.H.; Lee, S.G. A genetically encoded biosensor for monitoring isoprene production in engineered *Escherichia coli*. *ACS Synth. Biol.* **2018**, *7*, 2379–2390. [[CrossRef](#)]
16. Kim, S.K.; Han, G.H.; Seong, W.; Kim, H.; Kim, S.W.; Lee, D.H.; Lee, S.G. CRISPR interference-guided balancing of a biosynthetic mevalonate pathway increases terpenoid production. *Metab. Eng.* **2016**, *38*, 228–240. [[CrossRef](#)] [[PubMed](#)]
17. Primak, Y.A.; Du, M.; Miller, M.C.; Wells, D.H.; Nielsen, A.T.; Weyler, W.; Beck, Z.Q. Characterization of a feedback-resistant mevalonate kinase from the archaeon *Methanosarcina mazei*. *Appl. Environ. Microbiol.* **2011**, *77*, 7772–7778. [[CrossRef](#)]
18. Kang, A.; George, K.W.; Wang, G.; Baidoo, E.; Keasling, J.D.; Lee, T.S. Isopentenyl diphosphate (IPP)-bypass mevalonate pathways for isopentenol production. *Metab. Eng.* **2016**, *34*, 25–35. [[CrossRef](#)] [[PubMed](#)]
19. Pontrelli, S.; Chiu, T.Y.; Lan, E.I.; Chen, F.Y.; Chang, P.C.; Liao, J.C. *Escherichia coli* as a host for metabolic engineering. *Metab. Eng.* **2018**, *50*, 16–46. [[CrossRef](#)]
20. Kazieva, E.; Yamamoto, Y.; Tajima, Y.; Yokoyama, K.; Katashkina, J.; Nishio, Y. Characterization of feedback-resistant mevalonate kinases from the methanogenic archaeons *Methanosaeta concilii* and *Methanocella paludicola*. *Microbiology* **2017**, *163*, 1283–1291. [[CrossRef](#)]
21. Martin, V.J.; Pitera, D.J.; Withers, S.T.; Newman, J.D.; Keasling, J.D. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* **2003**, *21*, 796–802. [[CrossRef](#)] [[PubMed](#)]
22. Kizer, L.; Pitera, D.J.; Pfleger, B.F.; Keasling, J.D. Application of functional genomics to pathway optimization for increased isoprenoid production. *Appl. Environ. Microbiol.* **2008**, *74*, 3229–3241. [[CrossRef](#)] [[PubMed](#)]
23. Dahl, R.H.; Zhang, F.; Alonso-Gutierrez, J.; Baidoo, E.; Batth, T.S.; Redding-Johanson, A.M.; Petzold, C.J.; Mukhopadhyay, A.; Lee, T.S.; Adams, P.D. Engineering dynamic pathway regulation using stress-response promoters. *Nat. Biotechnol.* **2013**, *31*, 1039–1046. [[CrossRef](#)]
24. Liu, C.L.; Dong, H.G.; Zhan, J.; Liu, X.; Yang, Y. Multi-modular engineering for renewable production of isoprene via mevalonate pathway in *Escherichia coli*. *J. Appl. Microbiol.* **2019**, *126*, 1128–1139. [[CrossRef](#)]
25. Zhao, J.; Li, Q.; Sun, T.; Zhu, X.; Xu, H.; Tang, J.; Zhang, X.; Ma, Y. Engineering central metabolic modules of *Escherichia coli* for improving  $\beta$ -carotene production. *Metab. Eng.* **2013**, *17*, 42–50. [[CrossRef](#)]
26. Choi, H.S.; Lee, S.Y.; Kim, T.Y.; Woo, H.M. *In silico* identification of gene amplification targets for improvement of lycopene production. *Appl. Environ. Microbiol.* **2010**, *76*, 3097–3105. [[CrossRef](#)] [[PubMed](#)]
27. Alper, H.; Jin, Y.S.; Moxley, J.F.; Stephanopoulos, G. Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. *Metab. Eng.* **2005**, *7*, 155–164. [[CrossRef](#)] [[PubMed](#)]
28. Martinez, I.; Zhu, J.; Lin, H.; Bennett, G.N.; San, K.Y. Replacing *Escherichia coli* NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with a NADP-dependent enzyme from *Clostridium acetobutylicum* facilitates NADPH dependent pathways. *Metab. Eng.* **2008**, *10*, 352–359. [[CrossRef](#)] [[PubMed](#)]

29. Sambrook, J.; Russell, D.W. *Molecular Cloning: A Laboratory Manual*; Sambrook, J., Russell, D.W., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2001; Volume 3.
30. Gibson, D.G.; Young, L.; Chuang, R.Y.; Venter, J.C.; Hutchison III, C.A.; Smith, H.O. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **2009**, *6*, 343. [[CrossRef](#)]



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