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Enhanced (−)-α-Bisabolol Productivity by Efficient Conversion of Mevalonate in Escherichia coli

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Abstract: (−)-α-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 (−)-α-bisabolol by fed-batch fermentation using engineered Escherichia coli (E. coli) expressing the exogenous mevalonate (MVA) pathway genes. The productivity of (−)-α-bisabolol must be improved before industrial application. Here, we report enhancement of initial (−)-α-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 (−)-α-bisabolol in the engineered E. coli. The resulting strain produced 1.7-fold higher (−)-α-bisabolol relative to the strain expressing a feedback-inhibitory MvaK1 from Staphylococcus aureus (S. aureus). Next, to efficiently convert accumulated MVA to (−)-α-bisabolol, we additionally overexpressed genes involved in the lower MVA mevalonate pathway in E. coli containing the entire MVA pathway genes. (−)-α-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 (−)-α-bisabolol with an initial productivity of 0.12 g/L h in the optimal fed-batch fermentation. Thus, the microbial production of (−)-α-bisabolol would be an economically viable bioprocess for its industrial application.

Keywords: (−)-α-bisabolol; mevalonate (MVA); mevalonate kinase 1; Methanosarcina mazei; fed-batch fermentation

1. Introduction

A monocyclic sesquiterpene alcohol, (−)-α-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 (−)-α-bisabolol is expected to reach $73 million by 2020, with an annual growth rate of 5.9% from 2016 [6]. Commercially available (−)-α-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 (−)-α-bisabolol was obtained from the candeia tree with a yield of approximately 0.018 g/g candeia power through CO₂ supercritical extraction at 40 °C and 10 MPa [8]. Although a chemical
process has been developed to produce (−)-α-bisabolol, it forms diastereomers of
(−)-α-bisabolol ((+)-α-bisabolol and (±)-epi-α-bisabolol), and thus requires auxiliary purification steps [9]. In this
context, the biological production of naturally occurring (−)-α-bisabolol using engineered microbes
may be an attractive alternative to the current production processes of (−)-α-bisabolol.

(−)-α-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 (−)-α-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 (−)-α-bisabolol by the
(−)-α-bisabolol synthase (BBS, Figure 1).

![Biochemical Pathway Diagram](image_url)

**Figure 1.** Biosynthetic pathway of (−)-α-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 synthase).
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 pathway because it is inhibited by known feedback inhibitors: C5 (IPP and DMAPP), C15 (geranyl diphosphate 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 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\(^+\))-dependent GAPDH (glyceraldehyde-3-phosphate dehydrogenase) coded by *gapA* and nicotinamide adenine dinucleotide phosphate (NADP\(^+\))-dependent GAPDH coded by *gapC* from *Clostridium acetobutylicum*. The exogenous MVA pathway consists of MvaE (dual function of acetoyacet-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*).
0.9 g/L of (−)-α-bisabolol and 4.9 g/L of MVA along with consuming initially supplied glycerol. A total of 8.2 g/L of (−)-α-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 (−)-α-bisabolol production, a significant amount of MVA was still accumulated in the fed-batch fermentation.

![Graph showing (−)-α-bisabolol and MVA production](image)

**Figure 2.** Introduction of MvaK1 from *M. mazei* for the improvement of (−)-α-bisabolol production. (A) Plasmid constructs for expressing the entire MVA pathway, FPP synthase and (−)-α-bisabolol synthase. The plasmid pTSN-Bisa-Sa and pTSN-Bisa-Mm have mvak1 gene of *S. aureus* and *M. mazei*, respectively. (B) Improvement of (−)-α-bisabolol production in the engineered *E. coli* DH5α 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 β-D-1-thiogalactopyranoside (IPTG). The error bars represent the standard deviation of the concentrations of (−)-α-bisabolol and MVA from three biological replicates. (C) Fed-batch fermentation of *E. coli* DH5α 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 (−)-α-bisabolol biosynthesis in *E. coli* DH5α-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 (−)-α-bisabolol production and MVA accumulation in batch culture. When 0.025 mM IPTG was used for induction, (−)-α-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 (−)-α-bisabolol production, a pSEVA231-Bisa-Mm was generated using a medium copy number plasmid, pSEVA231 (pBBR1 ori) (Figure 3B). Interestingly, the *E. coli* DH5α-pSEVA231-Bisa-Mm strain produced 926 mg/L of (−)-α-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 (−)-α-bisabolol production compared to those in the presence of 0.025 mM IPTG (Figure 3A,B).
G concentrations to increase the pathway was reinforced. 

The concentrations of (−)-α-bisabolol and MVA increased (Figure 4B, C), indicating that enzymes for (−)-α-bisabolol and MVA from three biological replicates.

2.3. Reinforcement of the MVA Pathway

Considering the high accumulation of MVA in the production of (−)-α-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 (kcat) than that of MmMvaK1 [17]. In the absence of IPTG, E. coli DH5α harboring both pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA plasmids produced 1.2 g/L of (−)-α-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 DH5α 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 (−)-α-bisabolol production in the E. coli DH5α containing both pTSN-Bisa-Mm and pSSN12Didi-MrBBS-IspA plasmids. Unlike the results from the E. coli DH5α harboring the pTSN-Bisa-Mm alone (Figure 3A), production of both (−)-α-bisabolol and MVA decreased as the IPTG concentrations increased (Figure 4B, C), indicating that enzymes for (−)-α-bisabolol biosynthesis were sufficiently expressed in the absence of IPTG to increase the (−)-α-bisabolol production when the lower MVA pathway was reinforced.

Figure 3. Effect of IPTG on (−)-α-bisabolol production. The concentrations of (−)-α-bisabolol and MVA produced by the E. coli DH5α 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 (−)-α-bisabolol and MVA from three biological replicates.
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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 (−)-α-bisabolol biosynthetic pathway (Figure 1). Therefore, if the intracellular NADPH pool of engineered E. coli increases, it will improve (−)-α-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+ 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 (−)-α-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 (−)-α-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 (−)-α-bisabolol/OD₆₀₀ are similar between the two strains, it is likely that the increased production of (−)-α-bisabolol is due to increased cell mass.

Figure 4. (−)-α-Bisabolol production in E. coli DH5α 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 (−)-α-bisabolol biosynthetic pathway or lower MVA pathway. The concentrations of (−)-α-bisabolol (B) and MVA (C) produced by the engineered E. coli DH5α 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 (−)-α-bisabolol and MVA 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 5. Overexpression of gapC gene encoding NADP+-dependent GAPDH from C. acetobutylicum and repression of gapA gene coding for endogenous NAD+ 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 (pCas9-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_{600} from three biological replicates.
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 (−)-α-bisabolol production was not significantly improved (Figure 6B). It seems that acetyl-CoA was utilized for cell growth and other metabolism,

Figure 6. Fed-batch fermentation of the E. coli DH5α 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).
or metabolites downstream of MVA, accumulated. To overcome this problem, we sought to enhance the overall (−)-α-bisabolol flux through overexpressing all genes in the (−)-α-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 (−)-α-bisabolol were improved by 40% and 56%, respectively, as compared to the absence of IPTG, and the final titer of (−)-α-bisabolol reached 8.5 g/L (Figure 6C).

3. Discussion

In this study, E. coli was engineered for the efficient conversion of MVA to (−)-α-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 Methanoseta concilii (McMvaK) and Methanocella paludicola (MpMvaK) [20]. The McMvaK and MpMvaK enzymes not only showed feedback resistance to C. acetobutylicum but also exhibited 4.9- and 5.5-fold higher affinity to MVA, respectively, than MmvMvaK1 [20]. Therefore, these MvaK1 enzymes may enable the enhancement of (−)-α-bisabolol production in engineered E. coli.

To find the optimal conditions to efficiently convert MVA to (−)-α-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 (−)-α-bisabolol production without IPTG addition exhibited the highest production among all tested IPTG concentrations [12]. The IPTG-induced overexpression of genes for (−)-α-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 (−)-α-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 (−)-α-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 β-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+-dependent GADPH of E. coli with the NADP+-dependent GADPH 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 (−)-α-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 (−)-α-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 (−)-α-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 (−)-α-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, (−)-α-bisabolol was continuously produced after cell growth ceased. Therefore, recycling resting cells is a promising strategy to further improve (−)-α-bisabolol productivity. Although promising results in productivity were obtained in this study, it is necessary to improve the final (−)-α-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 (−)-α-bisabolol production.

**Table 1.** Summary of fed-batch fermentations of (−)-α-bisabolol by engineered E. coli.

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

* 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 DH5α strain (Enzynomics, Daejeon, Korea) was used for all experiments including gene cloning and (−)-α-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 K₂HPO₄, 2.2 g/L KH₂PO₄, and 1% (w/v) glycerol) was used for (−)-α-bisabolol production. All media were supplied with the appropriate antibiotics: ampicillin (100 µg/mL), chloramphenicol (34 µg/mL), and kanamycin (25 µg/mL). IPTG was used at concentrations of 0, 0.025, and 0.1 mM to induce gene expression involved in the (−)-α-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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>$\text{F}^-$, $\varnothing\text{B}\text{l}\text{K}15$ $\text{f}($$\text{lacZ}\alpha$ $\text{M}15$ $\text{f}($$\text{lacZY}A−\text{argF}1)\text{U}169$ $\text{deoR}$ $\text{recA}1$ $\text{endA}1$ $\text{hsdR}17$(rk−, mk+) $\text{phaA}$ $\text{supE}44$ $\text{thi}-1$ $\text{gyrA96}$ $\text{relA}1$</td>
<td>Enzynomics</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTrc99A</td>
<td>$\text{P}_{\text{trc}}$ promoter, Amp$^R$, lacF$,\text{pBR322 ori}$</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>pSTV28</td>
<td>$\text{P}_{\text{lac}}$ promoter, Cm$^R$, p15A ori</td>
<td>Takara</td>
</tr>
<tr>
<td>pSECRi</td>
<td>$\text{P}_{\text{phoAB}}\text{::cas9}$($\text{D}10\text{A}, \text{H}840\text{A}$) and constitutive sgRNA expression cassette in pSEVA221</td>
<td>[16]</td>
</tr>
<tr>
<td>pSNA-MrBBS-IspA</td>
<td>$\text{pTrc99A containing }\text{mvaE and mvaS of Enterococcus faecalis, mvaK1 and mvaK2 and mvaD of S. pneumoniae, idi, and ispA of E. coli, MrBBS of M. recutita}$</td>
<td>[12]</td>
</tr>
<tr>
<td>pTM-BBS</td>
<td>$\text{pTrc99A derivatives containing codon optimized Matricaria recutita}$</td>
<td>[16]</td>
</tr>
<tr>
<td>pSSN12Didi</td>
<td>$\text{pSTV28 containing mvaK1, mvaK2 and mvaD from Streptococcus pneumoniae, idi of E. coli}$</td>
<td>[12]</td>
</tr>
<tr>
<td>pTSN-Bisa-Sa</td>
<td>$\text{pTrc99A containing }\text{mvaE and mvaS of Enterococcus faecalis, mvaK1 of S. aureus, mvaK2 and mvaD of S. pneumoniae, idi, and ispA of E. coli, MrBBS of M. recutita}$</td>
<td>This study</td>
</tr>
<tr>
<td>pTSN-Bisa-Mm</td>
<td>$\text{pTrc99A containing }\text{mvaE and mvaS of Enterococcus faecalis, mvaK1 of M. masei, mvaK2, and mvaD of S. pneumoniae, idi, and ispA of E. coli, MrBBS of M. recutita}$</td>
<td>This study</td>
</tr>
<tr>
<td>pSEVA231-Bisa-Mm</td>
<td>$\text{pTSN-Bisa-Mm with pBBR1 ori instead of pBR322 ori}$</td>
<td>This study</td>
</tr>
<tr>
<td>pSSN12Didi-MrBBS-IspA</td>
<td>$\text{pSNA-MrBBS-IspA}$</td>
<td>This study</td>
</tr>
<tr>
<td>pTSN-Bisa-Mm-GapC</td>
<td>$\text{pTSN-Bisa-Mm containing gapC of C. acetobutylicum}$</td>
<td>This study</td>
</tr>
<tr>
<td>pdCas9-sgRNA-GapA</td>
<td>$\text{pSECRi containing gRNA targeting gapA gene}$</td>
<td>This study</td>
</tr>
</tbody>
</table>

The $\text{E. coli}$ codon-optimized $\text{mvaK1}$ gene of $\text{M. mazei}$ (GenBank accession number: KKI06753.1) was synthesized by Bioneer (Figure S1). The synthesized $\text{mvaK1}$ 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 $\text{mvaK1}$ from $\text{S. aureus}$ was amplified with SA-IF and SA-IR primers from pTSN-Bisa-Mm 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, $\text{ispA}$ and $\text{MrBBS}$ 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 $\text{MrBBS}$ and $\text{ispA}$ including $\text{trc}$ 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 $\text{E. coli}$ codon-optimized $\text{gapC}$ gene from $\text{C. acetobutylicum}$ (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 (−)-α-bisabolol biosynthetic pathway were verified by Sanger sequencing (Macrogen).

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 (−)-α-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 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), 0.98 g/L of MgSO4, 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 MgSO4, 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. (−)-α-Bisabolol Quantification

Extraction of (−)-α-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 (−)-α-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 μm, 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, (−)-α-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 (−)-α-bisabolol compound dissolved in n-dodecane. The (−)-α-bisabolol concentration produced was determined as follows:

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

4.5. Determination of Cell Growth and Metabolites

Cell growth was monitored by measuring the absorbance at 600 nm (OD600) 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 (−)-α-bisabolol productivity from engineered E. coli, which can serve as a promising platform strain for the microbial production of (−)-α-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, tableure S2: Fed-batch fermentation of the engineered E. coli DH5α, Table S1: List of primers used in this study.


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Conflicts of Interest: The authors declare no conflict of interest.

References
