

High titer mevalonate fermentation and its feeding as a building block for isoprenoids (isoprene and sabinene) production in engineered *Escherichia coli*



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ABSTRACT

Isoprenoids are important fine chemicals as material monomers, advanced fuels and pharmaceuticals. A variety of natural isoprenoids can be synthesized by engineered microbial strains. This work established a process by dividing the current isoprenoid pathway into the upstream fermentation process, from sugar to mevalonate (MVA), and the downstream process, from MVA to the target isoprenoids. The results showed that significant differences existed in the process conditions between the upstream and downstream fermentations. After individually optimizing the process conditions, the upstream MVA production (84.0 g/L, 34.0% and 1.8 g/L/h) and downstream isoprene production (11.0 g/L and 0.23 g/L/h) were greatly improved in this two-step process. Flask fermentation experiments also confirmed that two-step route can significantly improve the sabinene titer to 150 mg/L (6.5-fold of the sabinene titer in an earlier flask study of our lab). Therefore, the two-step route proposed in this study may have potential benefits towards the current isoprenoids production directly from glucose. The high titer and yield of MVA indicate that MVA has great potential to be more broadly utilized as starting precursor in synthetic biology.

1. Background

With the fast development of synthetic biology and applied microbiology, many natural secondary metabolites can now be synthesized by engineered microbes [1]. A variety of value added isoprenoids already have their biosynthesis methods, including material monomers, advanced fuels and pharmaceuticals [2–5]. Dimethylallyl pyrophosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP) are the C5 building units of terpenes and higher isoprenoids. These C5 precursors (DMAPP and IPP) are synthesized via two naturally occurring pathways, MVA pathway and methylerythritol 4-phosphate (MEP) pathway. The MVA pathway mainly exists in eukaryotes, archaeobacteria, and cytosols of higher plants [6,7]. The MEP pathway is present in many eubacteria, green algae, and chloroplasts of higher plants [6,8]. The MVA pathway is more studied than the recently discovered MEP pathway, and usually achieved higher titers of isoprenoids [5]. Moreover, MVA and the MVA pathway are correlated to a variety

of physiological functions [9].

The multistep fermentation route is a promising alternative to the conventional one-step microbial production in dealing with the bottlenecks existed in de novo synthesis of structurally complex compounds such as isoprenoids, vitamins, coenzymes and antibiotics. The most successful method for vitamin C microbial production is the two-step fermentation process in industry instead of the one-step biosynthesis route [10,11]. Sustainable renewable fuels and chemicals such as alkanes, fatty alcohols and isoprenoids could also be manufactured through the two-step route with free fatty acid serving as the intermediate platform chemical [12]. It is different from the chemical synthesis strategy which usually demands multiple steps towards the structurally complex chemicals formation, the one-step microbial production (engineering of all endogenous/heterologous enzymes in one chassis cell) has advantage for its “all in one feature” to avoid the intermediates purification steps [13]. However, the “all in one” strategy may also lead to low productivity and high cellular stress in the chassis

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Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant genotype/property	Source/reference
Strains		
<i>E. coli</i> BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm rne131(DE3)</i>	Invitrogen
MVA producer MP	BL21(DE3)/pYJM16	[7]
Isoprene producer IP	BL21(DE3)/pYJM14 and pYJM8	[7]
Sabinene producer SP	BL21(DE3)/pYJM14 and pHB5	[35]
Plasmids		
pYJM16	pACYCDuet-1 derivative carrying acetyl-CoA acetyltransferase/hydroxymethylglutaryl-CoA (HMG-CoA) reductase gene <i>mvaE</i> and HMG-CoA synthase gene <i>mvaS</i> , T7 promoter, Cm ^R	[7]
pYJM14	pTrcHis2B derivative carrying phosphomevalonate kinase gene <i>ERG8</i> , mevalonate kinase gene <i>ERG12</i> , mevalonate pyrophosphate decarboxylase gene <i>ERG19</i> and IPP isomerase gene <i>ID11</i> , Trc promoter, Ap ^R	[7]
pYJM8	pACYCDuet-1 derivative carrying isoprene synthase gene <i>ispS</i> , T7 promoter, Cm ^R	[7]
pHB5	pACYCDuet-1 derivative carrying geranyl diphosphate synthase gene <i>GPPS2</i> and sabinene synthase gene <i>SabS1</i> , T7 promoter, Cm ^R	[35]

strains especially for those having unbalanced engineered pathways due to: (i) the more nutrition and energy consumption; and (ii) the accumulation of heterogeneous intermediate metabolites or products influencing the native physiology of the cell. Although many tools have been developed to cope with these issues [1,14–17], two-step or multistep microbial production should also be investigated from the process engineering point of view.

In the case of the biosynthesis of isoprenoids, the involved enzymes of the whole biosynthesis pathway are usually engineered in one chassis strain as a microbial factory [18,19]. However, some pioneering studies suggested that over-expression of isoprenoid pathway in one engineered strain could lead to feedback inhibition of key enzymes and severe growth inhibition due to the nutrient or energy limitation and the accumulation of toxic intermediate metabolites [3,17,20–25]. Yang et al. reported that the complementary reducing equivalent demand and ATP requirement of MVA and MEP pathway play important roles in the isoprene production by *E. coli* [24]. For isoprenoids production, the control of prenyldiphosphates (IPP and/or DMAPP) level is critical [20]. The expression of the MVA pathway in *E. coli* led to severe growth inhibition due to the toxicity resulting from the accumulation of prenyldiphosphates (IPP and/or DMAPP) [20]. Isoprene synthase is the key enzyme in the biosynthesis pathway of isoprene, which catalyzes the elimination of pyrophosphate from DMAPP [26,27]. The variant plant isoprene synthase with improved catalytic activity could increase the rate of conversion of DMAPP to isoprene and alleviate the toxicity. Jung et al. found novel isoprene synthases from *Ipomoea batatas*, *Mangifera indica*, and *Elaeocarpus photinifolius* among terpene synthases by the sequence homology searches [28]. The isoprene synthase from *Ipomoea batatas* produced the highest titer of isoprene, which exceeded the isoprene levels obtained by the well-known isoprene synthases from *P. alba* and *P. montana* [28]. Wang et al. improved the isoprene production in *Saccharomyces cerevisiae* by combining Gal4p-mediated expression enhancement and directed evolution of isoprene synthase [29]. So far, the titers of many microbial-produced isoprenoids have not reached the industry levels [16].

The multistep fermentation route should be further employed to improve the microbial production of isoprenoids in our opinion. The isoprenoid pathway can be divided into two or more microbial strains to separately optimize each sub-level building block. The key point for the proposed strategy is to screen a feasible intermediate, which can satisfy the following requirements: (i) economic process (high production titer and yield, easy to be purified) to increase the feasibility, (ii) stable *in vitro* and can be transported in the downstream strain as a precursor, (iii) weak inhibition effects towards its host strains. Among the key intermediate metabolites of the MVA and MEP pathways, MVA is one of the promising intermediate building blocks. This compound is biocompatible towards common chassis strains and can be excreted and kept stable *in vitro* once produced by engineered strains with accessible titer of 47 g/l [30]. Moreover, MVA can also be transported inside the

cell as a precursor for the biosynthesis of downstream isoprenoids [25]. Xiong et al. developed a bio- and chemo-integrated approach to a rubbery polymer through mevalonate fermentation and subsequent transformation of mevalonate to β m δ VL [31]. To increase the production of MVA, the genes of *mvaE* and *mvaS* from several organisms were tested and the *E. coli* strains carrying genes from *Lactobacillus casei* achieved the MVA titer of 88 g/L with the yield of 0.26 g/g glucose [31]. Wang et al. constructed a highly efficient *E. coli* strains through chromosomal integration and the engineered strain exhibited high maximal productivity of 1.01 g/L/h and high yield of 0.49 g MVA/g glucose in a shake flask [32]. The effects of MVA production on the central metabolism in the gene engineered *E. coli* strain were also investigated by ¹³C-metabolic flux analysis [33].

Although the pioneering studies tested the chemo-synthesized MVA as intermediate building block in isoprenoids biosynthesis [7,25], the whole process (MVA fermentation, purification and feeding as a precursor for the downstream isoprenoids fermentation) has not been systematically established. To evaluate the feasibility of MVA as a building precursor in isoprenoids biosynthesis, we improved in this study the fermentation process for high titer production of MVA through betaine supplementation and late induction. Betaine is a trimethylated derivative of glycine, which can act as stress protectant or methyl donor involved in the energy metabolism and the biosynthesis of vitamins, ethanol, coenzymes and pyruvate [34]. Furthermore, we set up a methodology to purify and feed MVA as a precursor for the fermentation of selected isoprenoids, isoprene and sabinene.

2. Materials and methods

2.1. Strains, plasmids and media

Bacterial strains and plasmids used in this study were listed in Table 1. *E. coli* BL21 (DE3, Invitrogen) was used as a host for the production of MVA, isoprene or sabinene. The MVA producer strain (MP) harboring plasmid pYJM16 with acetyl-CoA acetyltransferase/hydroxymethylglutaryl-CoA (HMG-CoA) reductase gene *mvaE* and HMG-CoA synthase gene *mvaS* [7]. The isoprene producer strain (IP) harboring plasmids pYJM14, with phosphomevalonate kinase gene *ERG8*, mevalonate kinase gene *ERG12*, mevalonate pyrophosphate decarboxylase gene *ERG19* and IPP isomerase gene *ID11*, and pYJM8 with isoprene synthase gene *ispS* [7]. In the sabinene producer strain (SP), it carried the plasmid pYJM14 like IP-strain but the pYJM8 plasmid is replaced by pHB5 with geranyl diphosphate synthase gene *GPPS2* and sabinene synthase gene *SabS1* [35].

The seed culture of the MVA producer (MP), isoprene producer (IP) or sabinene producer (SP) was grown in LB medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) and M9 minimal medium (15.12 g/L Na₂HPO₄·12H₂O, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl, 20 g/L glucose, 0.24 g/L MgSO₄). The MVA fermentation medium consisted of

1 g/L $(\text{NH}_4)_2\text{SO}_4$, 3 g/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.24 g/L MgSO_4 , 1.9 g/L KCl, 1 g/L sodium citrate, 1 g/L citric acid, 20 g/L glucose, 5 g/L yeast extract, 1 g/L betaine (according to different purposes) and 1 ml/L trace elements (each 100 ml solution containing 0.37 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.29 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.47 g H_3BO_3 , 0.25 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.58 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$). The isoprene fermentation medium consisted of 1 g/L $(\text{NH}_4)_2\text{SO}_4$, 3 g/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.24 g/L MgSO_4 , 1.9 g/L KCl, 1 g/L sodium citrate, 1 g/L citric acid, 20 g/L glucose, 5 g/L yeast extract, 34 $\mu\text{g}/\text{ml}$ chloramphenicol, 100 $\mu\text{g}/\text{ml}$ ampicillin and 1 ml/L trace elements (each 100 ml solution containing 0.37 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.29 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.47 g H_3BO_3 , 0.25 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.58 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$). The sabinene fermentation medium consisted of 9.8 g/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 2.1 g/L citric acid monohydrate, 0.3 g/L ferric ammonium citrate, 5 g/L beef extract, 20 g/L glucose, 0.06 g/L MgSO_4 , 34 $\mu\text{g}/\text{ml}$ chloramphenicol, 100 $\mu\text{g}/\text{ml}$ ampicillin and 1 ml/L trace elements (each 100 ml solution containing 0.37 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.29 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.47 g H_3BO_3 , 0.25 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.58 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$).

2.2. Fed-batch fermentation for MVA production

A single colony of MP-strain was picked and used to inoculate 5 ml LB media supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol and was cultivated at 37 °C, 180 rpm over-day. Afterwards, 1 ml of the over-day culture was used to inoculate a 100 ml M9 minimal medium supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol in a 500-mL shake flask and was cultivated at 37 °C, 180 rpm for 12 h. This seed culture was used in a ratio of 3% (V/V) to inoculate a 5-L jar fermentor (BIOSTAT Bplus 5 l, Sartorius stedim) containing 3 l MVA fermentation medium. The fermentation process was operated under the following conditions: temperature 32 °C, pH was controlled at 7.0 ± 0.1 by automatic addition of 25% (w/w) ammonia water, the aeration rate was 1 vvm, and dissolved oxygen (DO) level was maintained at $30 \pm 1\%$ by adjusting stirring rate. When the initial glucose was depleted, 50% (w/w) concentrated glucose was intermittently fed into the fermentor to maintain the residual glucose below 0.5 g/L. The culture was induced when the OD_{600} (measured by a Cary 50 UV–vis spectrophotometer, VARIAN) was 15 by the addition of 0.5 mM IPTG unless specified according to different purposes. Samples were collected at certain intervals for MVA analysis.

2.3. Extraction and pretreatment of MVA

Fermentation broth containing MVA was centrifuged at 8000 rpm for 10 min at room temperature. Next, the supernatant was adjusted to pH 2.0 with 3 M HCl and incubated at 45 °C for 1 h to convert MVA to mevalonic acid lactone. Then this solution was saturated with Na_2SO_4 , and extracted three times by equal volume of ethyl acetate. The mevalonic acid lactone was regained from the ethyl acetate via vacuum rotary evaporation at 0.1 MPa/40 °C and stored at -20 °C for later use. The mevalonic acid lactone was neutralized to pH 7.0 by the addition of 1 M NaOH before using it as a feeding precursor for the fermentation of isoprene and sabinene.

2.4. Fed-batch fermentation for isoprene production from MVA

A single colony of IP-strain was picked and used to inoculate 5 ml LB media supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol and 100 $\mu\text{g}/\text{ml}$ ampicillin for over-day cultivation at 37 °C, 180 rpm. Then it was used to inoculate 100 ml M9 medium supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol and 100 $\mu\text{g}/\text{ml}$ ampicillin. This seed culture was used in a ratio of 3% (V/V) to inoculate a 5-L jar fermentor (BIOSTAT Bplus 5 l, Sartorius stedim) containing 2 l isoprene fermentation medium. The cultivation temperature was maintained at 34 °C, the pH was controlled at 7.0 ± 0.1 by automatic addition of 25% (w/w) ammonia water, the aeration rate was 1 vvm, and DO level was maintained at $20 \pm 1\%$ by

adjusting stirring rate. When the initial glucose was depleted, 50% (w/w) concentrated glucose was intermittently fed into the fermentor to maintain the residual glucose below 0.5 g/L. The cells were induced at OD_{600} 15 by the addition of 0.5 mM IPTG unless specified according to different purposes. The total amount of MVA (30 g MVA for 1 l of fermentation broth) was added to the fermentor using three feeding strategies: A, addition of 10 g/L MVA once every four hours and a total of 3 times, feeding glucose intermittently into the fermentor to maintain the residual glucose below 0.5 g/L; B, addition of MVA at a constant rate of 1 g/L/h from 0 h to 30 h, feeding glucose intermittently into the fermentor to maintain the residual glucose below 0.5 g/L; C, co-feeding glucose and MVA together to maintain DO level at 20%. Samples were collected at certain intervals for isoprene analysis.

2.5. Fed-batch fermentation for the two-step isoprene production without the purification of MVA

A single colony of IP-strain was picked and used to inoculate 5 ml LB media supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol and 100 $\mu\text{g}/\text{ml}$ ampicillin for over-day cultivation at 37 °C, 180 rpm. Then it was used to inoculate 100 ml M9 medium supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol and 100 $\mu\text{g}/\text{ml}$ ampicillin. This seed culture was used in a ratio of 3% (V/V) to inoculate a 5-L jar fermentor (BIOSTAT Bplus 5 l, Sartorius stedim) containing 2 l isoprene fermentation medium. The cultivation temperature was maintained at 34 °C, the pH was controlled at 7.0 ± 0.1 by automatic addition of 25% (w/w) ammonia water, the aeration rate was 1 vvm, and DO level was maintained at $20 \pm 1\%$ by adjusting stirring rate. When the initial glucose was depleted, 50% (w/w) concentrated glucose was intermittently fed into the fermentor to maintain the residual glucose below 0.5 g/L. The cells were induced at OD_{600} 15 by the addition of 0.5 mM IPTG.

A single colony of MP-strain was picked and used to inoculate 5 ml LB media supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol and was cultivated at 37 °C, 180 rpm over-day. Afterwards, 1 ml of the over-day culture was used to inoculate a 100 ml M9 minimal medium supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol in a 500-mL shake flask and was cultivated at 37 °C, 180 rpm for 12 h. This seed culture was used in a ratio of 3% (V/V) to inoculate a 5-L jar fermentor (BIOSTAT Bplus 5 l, Sartorius stedim) containing 3 l MVA fermentation medium. The fermentation process was operated under the following conditions: temperature 32 °C, pH was controlled at 7.0 ± 0.1 by automatic addition of 25% (w/w) ammonia water, the aeration rate was 1 vvm, and dissolved oxygen (DO) level was maintained at $30 \pm 1\%$ by adjusting stirring rate. When the initial glucose was depleted, 50% (w/w) concentrated glucose was intermittently fed into the fermentor to maintain the residual glucose below 0.5 g/L. The culture was induced when the OD_{600} (measured by a Cary 50 UV–vis spectrophotometer, VARIAN) was 80 by the addition of 0.5 mM IPTG.

The MVA fermentation broth was directly added to the downstream fermentation broth at the ratio of 1:3 (v/v) after the MVA and downstream fermentations were induced for 6 h respectively. Samples were collected at certain intervals for isoprene analysis.

2.6. Flask fermentation of sabinene from MVA

A single colony of SP-strain was inoculated to 5 ml LB supplemented with 34 $\mu\text{g}/\text{ml}$ chloramphenicol and 100 $\mu\text{g}/\text{ml}$ ampicillin, and cultured overnight at 37 °C, 180 rpm. One percent (v/v) inoculant was added aseptically to a 600-ml sealed shake flask containing 100 ml sabinene fermentation medium and was cultivated at 37 °C, 180 rpm. The cultures were induced at OD_{600} of 0.6 by the addition of 0.5 mM IPTG at 30 °C, followed by the addition of 1 g/L MVA to the fermentation medium. 1 ml gas sample was collected after 24 h post-induction and quantitatively analyzed by Gas Chromatography.

2.7. Gas chromatography (GC) analysis of MVA

The MVA produced by the engineered strains was quantitatively analyzed as described previously [36]. 5 ml sample was taken and centrifuged at 8000 rpm for 10 min at room temperature, the supernatant was adjusted to pH 2.0 with 3 M HCl and incubated at 45 °C for 1 h to convert MVA to mevalonic acid lactone. Then this solution was saturated with Na₂SO₄, and extracted with ethyl acetate containing 0.05% (v/v) caryophyllene as an internal standard. The ethyl acetate phase containing mevalonic acid lactone was analyzed by GC. GC analysis was performed in an Agilent 7890A system equipped with a flame ionization detector (FID) and a HP-AL/S column (25 m × 320 μm × 8 μm). N₂ was used as carrier gas with a linear velocity of 1 ml/min. The column temperature profile was 75 °C for 0.5 min, 25 °C/min to 150 °C, 15 °C/min to 200 °C, 30 °C/min to 250 °C, and 250 °C for 5 min. The ratio of MVA to the internal standard (caryophyllene) was converted to MVA concentration by comparing with a standard curve plotted with a set of known concentration of MVA.

2.8. GC analysis of isoprene

1 ml of off-gas samples from the headspace of the fermentor were analyzed as described earlier [7] using a GC (Agilent 7890A, America) equipped with FID and a HP-AL/S column (25 m × 320 μm × 8 μm). N₂ was used as carrier gas with a linear velocity of 1 ml/min. The product was characterized by direct comparison with standard isoprene (TCI-EP, Tokyo, Japan). The peak area was converted to isoprene concentration by comparing with a standard curve plotted with a set of known concentration of isoprene. Then isoprene accumulation was measured every 30 min by GC.

2.9. GC analysis of sabinene

1 ml of gas samples were taken from the headspace of the sealed cultures and analyzed by GC as described previously [35]. The GC analysis was performed on an Agilent 7890A equipped with FID. The separation of sabinene was performed using an HP-INNOWAX column (25 m × 250 μm × 0.2 μm). N₂ was used as carrier gas with a linear velocity of 1 ml/min. The column temperature profile was 50 °C for 1 min, 5 °C/min to 100 °C, 25 °C/min to 250 °C, and 250 °C for 5 min. The temperatures of injector and detector were held at 250 °C and 260 °C, respectively. The peak area was converted into sabinene concentration by comparison with a standard curve plotted with a set of known concentrations of sabinene which was bought from Sigma-Aldrich.

3. Results

3.1. Betaine supplementation improved the MVA yield and productivity

To investigate the effects of betaine supplementation on MVA biosynthesis, the current work was designed to have a control (without betaine), and a betaine supplemented (1 g/L) fermentation by engineered MP-strain (Table 1). On one hand betaine supplementation only slightly increased the titer of MVA (from 37.0 g/L to 41.3 g/L) compared to the control, on the other hand it doubled the productivity of MVA (from 0.5 g/L/h to 1.1 g/L/h) and the maximum titer of MVA was finally achieved 36 h earlier than the control (Fig. 1). Moreover, the OD₆₀₀ value of betaine supplementation group (OD₆₀₀ value around 30) was lower than the control (OD₆₀₀ value around 70). The increased productivity of MVA and the decreased carbon consumption for cell growth significantly improved the MVA yield from 0.230 g MVA/g glucose to 0.368 g MVA/g glucose.

3.2. Induction at late exponential phase improved the production of MVA

To test the effects of induction on the MVA production, three processes (A-C) were induced with IPTG at different OD₆₀₀ values of 15, 80 and 60, respectively. As shown in Fig. 2, when the induction was performed at higher OD₆₀₀ of 80 and 60, both the maximum biomass and MVA titer were greatly improved compared to the control (induction at OD₆₀₀ of 15 in the process A). Among them, process B has the best titer of 84.0 g/L with the yield of 34.0% and OD₆₀₀ of 100 at 48 h, which were all significantly higher than the previous published results [30]. Similar with process B, the titer of process C was also increased comparing to process A, while the MVA yield was greatly decreased to 30.0%. Therefore, the low cell density imposed restrictions on MVA production, and late induction can improve the MVA production through increasing cell density. However, the high cell density also has negative effects on MVA yield for the higher maintenance metabolism.

3.3. Induction at early exponential phase improved the production of isoprene

We further purified the fermented MVA and utilized it as a precursor to produce isoprene by engineered IP-strain (Table 1, which harbored the biosynthesis pathway to convert MVA to isoprene). Three testing processes were induced with IPTG and fed with MVA at different OD₆₀₀ values of 15, 30 and 60, respectively. Contrary to the induction mode of MVA production, the isoprene production showed a declining trend with the increase of induction OD. The highest isoprene titer of 2.8 g/L was achieved when the induction was performed at OD₆₀₀ 15 at early exponential phase (Fig. 3). The isoprene production totally disappeared when the induction and MVA feeding was conducted at OD₆₀₀ 60. Therefore, the serious contradiction existed in the induction modes between the upstream fermentation process, from sugar to MVA, and the downstream process, from MVA to isoprene, which might result in low productivity and titer of isoprene via one-step biosynthesis route.

3.4. DO-stat based feeding strategy improved the isoprene productivity and titer from MVA

The accumulation of intermediate metabolites could inhibit the isoprenoids production, so the expression levels of key enzymes and metabolic flux need to be thoroughly balanced to diminish the inhibiting effects [14,17]. We proposed here another strategy to balance the metabolic flux by optimizing the precursor supply in the isoprene production.

As shown in Fig. 4, the feeding strategies apparently affected the cell growth and isoprene production. Due to the unlimited precursor supply, the cell growth stopped early at around OD₆₀₀ of 30 in A, and the OD₆₀₀ in B gradually decreased from the highest value 50–30. Under the glucose and MVA limitation conditions, the cell growth in C increased slowly and finally reached OD₆₀₀ about 40. As isoprene concentration of 0.3 mg/L in the off-gas sample was regarded as the threshold value, the downstream fermentation processes A, B and C were respectively stopped due to the low productivity of isoprene when the isoprene concentration in the off-gas decreased gradually below this threshold value. The final isoprene titer in C reached 11.0 g/L at the end of the fermentation, which was 3.7- and 1.2-fold of isoprene titer in A and B, respectively.

The inhibition phenomenon happened in the downstream fermentation during the conversion of MVA to isoprene, which could be reflected by the process control parameters DO and the stirring rate (Fig. 5). The stirring rate began to decrease sharply after the second addition of MVA in the feeding strategy A. Subsequently, DO began to rise quickly to 70% and OD₆₀₀ stopped increasing at the same time, which suggested that the respiratory metabolisms of cells were greatly inhibited. In contrast with A, DO could be controlled at 20% within the whole process using the feeding strategy B. However, the stirring rate

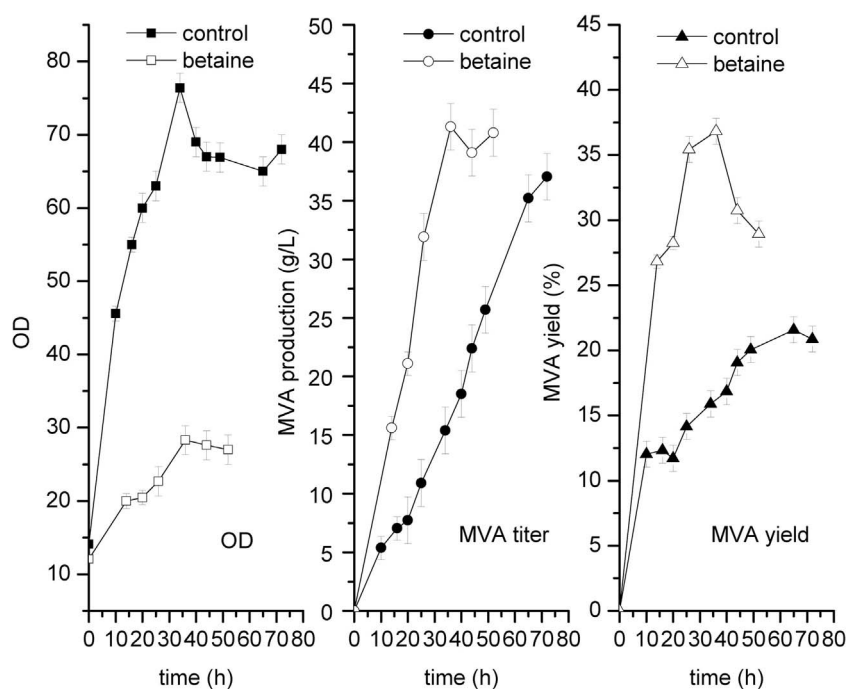


Fig. 1. Effects of betaine on MVA production in engineered *E. coli*. Square, circle and upright triangle indicate OD₆₀₀, MVA production and MVA yield, respectively. The value and error bar represent the mean and S.D. of three biological replicates.

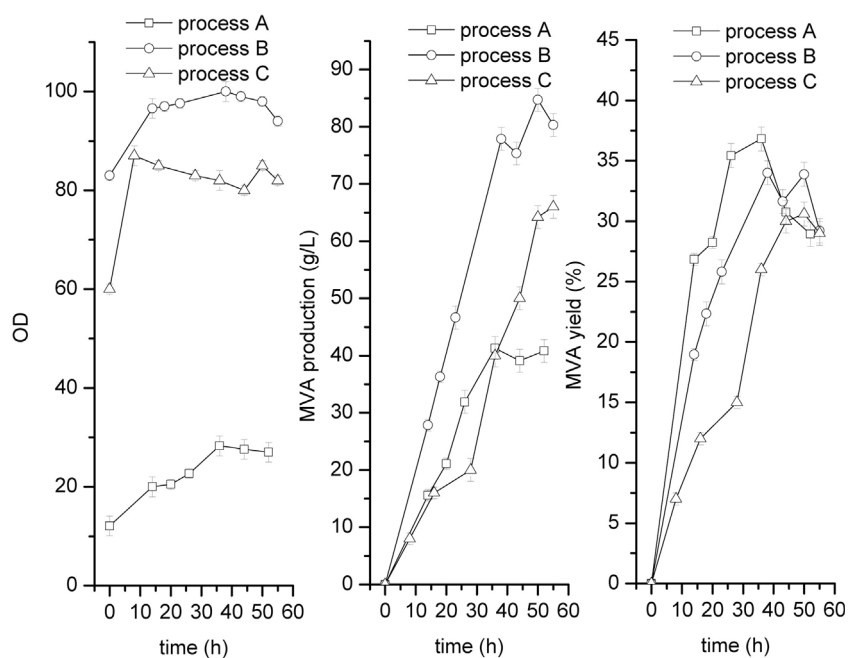


Fig. 2. Effects of induction on MVA production in engineered *E. coli*. Empty square, empty circle and empty upright triangle indicate fermentation processes (A–C) induced with IPTG at different levels of OD 15, 80 and 60, respectively. The value and error bar represent the mean and S.D. of three biological replicates.

decayed slowly because DO was also associated with stirring rate simultaneously, and the OD₆₀₀ decreased too. As far as the feeding strategy C was concerned, the lower DO level and higher stirring rate were maintained due to the precursor limitation during the fermentation process.

3.5. Comparison of the two-step isoprene production with the one-step process

In order to evaluate the two-step isoprene production using three MVA feeding strategies A–C described above, the titer, overall yield and productivity of isoprene were determined for these two-step fermentation processes (Table 2). Furthermore, the feasibility of skipping the purification of MVA in the two-step isoprene production was

investigated in the present paper by directly adding the MVA fermentation broth to the downstream fermentation broth after induction at OD₆₀₀ of 80 and 15 respectively. The titer, overall yield and productivity of isoprene were also determined for this preliminary attempt (Table 2).

Compared to the one-step isoprene titer of 6.3 g/L [7], a higher isoprene titer of 11.0 g/L was achieved in the two-step process using MVA feeding strategy C. The isoprene titer of 5.9 g/L in the two-step process without the purification of MVA was close to that of the one-step process. The overall yields of isoprene from glucose in the two-step processes B and C were close to that of one-step process. However, the yield of the two-step process without the purification of MVA was much lower than that of one-step process. This was probably because the MVA and isoprene fermentations were performed simultaneously with

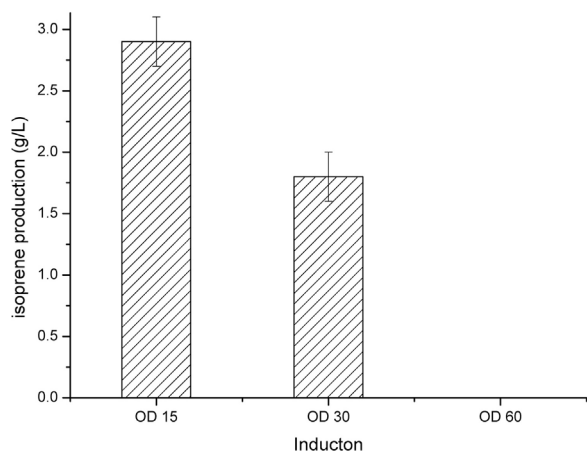


Fig. 3. Effects of IPTG induction on isoprene production in engineered *E. coli*. The value and error bar represent the mean and S.D of three biological replicates.

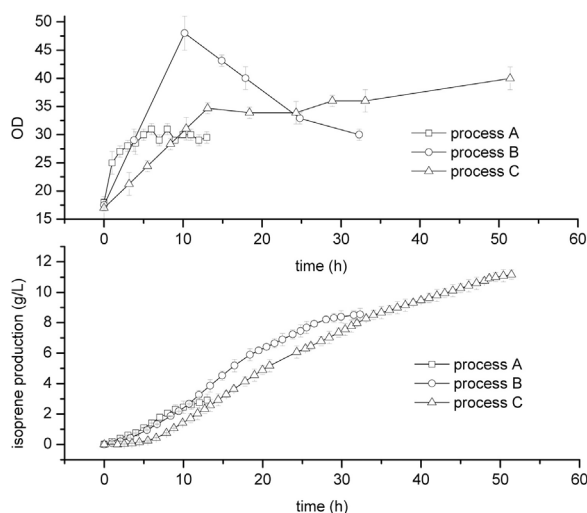


Fig. 4. Effects of the feeding strategies of MVA on isoprene production in engineered *E. coli*. Empty square, empty circle and empty upright triangle indicate fermentation processes (A–C) using three feeding strategies respectively as follows: A, adding MVA of 10 g/L once every four hours, a total of 3 times; B, adding MVA at constant rate of 1 g/L/h from 0 h to 30 h; C, co-feeding glucose and MVA together to maintain dissolved oxygen (DO) level at 20% or so. The value and error bar represent the mean and S.D of three biological replicates.

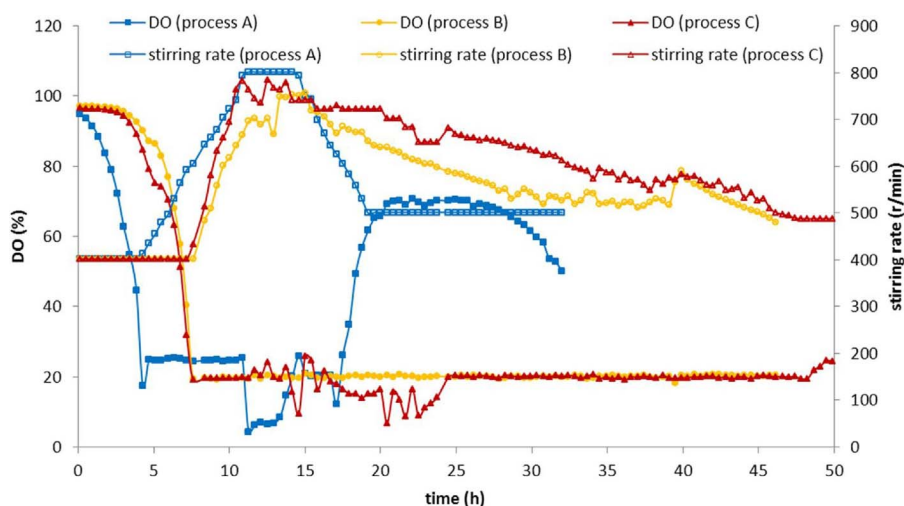


Fig. 5. Time courses of process control parameters DO and stirring rate in the fermentation process (A–C). Filled and empty squares, filled and empty circles, filled and empty upright triangles indicate DO and stirring rate in the processes (A–C), respectively.

Table 2 Comparison of the two-step isoprene production with the one-step process.

Item	Two-step process ^a			One-step process [7]	Two-step process without the purification of MVA ^b
	A	B	C		
Titer of isoprene (g/L)	3.0	8.7	11.0	6.3	5.9
Yield of isoprene from glucose (%)	5.2	6.2	6.5	7.0	4.1
Productivity of one batch (g/L/h)	0.05	0.11	0.11	0.16	0.20
Maximal productivity of multi-batch (g/L/h)	0.23	0.27	0.23	0.16	0.20

^a The processes (A–C) using three feeding strategies respectively as follows: A, adding MVA of 10 g/L once every four hours, a total of 3 times; B, adding MVA at constant rate of 1 g/L/h from 0 h to 30 h; C, co-feeding glucose and MVA together to maintain dissolved oxygen (DO) level at 20% or so.

^b The two-step process without the purification of MVA was performed by directly adding the MVA fermentation broth to the downstream fermentation broth at the ratio of 1:3 (v/v) after the MVA and downstream fermentations were induced for 6 h respectively.

an unoptimized ratio in the two-step process without the purification of MVA. In order to improve the isoprene production and make the two-step process without the purification of MVA more economically feasible, the optimal ratio of the upstream MVA and downstream isoprene fermentation should be further optimized.

The overall productivity of one batch fermentation (A–C) for the two-step isoprene production was below 0.11 g/L/h, which was relatively lower than that of the one-step process due to the longer period of the sequential two-step process. However, the maximal productivity of multi-batch fermentation (A–C) for the two-step isoprene production was above 0.23 g/L/h, which was higher than that of the one-step process. This was because the productivity of the upstream MVA fermentation (1.8 g/L/h) was much higher than that of the downstream isoprene fermentation (0.23 g/L/h). And the overall period was finally determined by the downstream fermentation in the multi-batch process and the maximal productivity of isoprene could be equal to that of the downstream fermentation. Furthermore, the productivity of one batch fermentation for the two-step isoprene production could be increased to 0.20 g/L/h through the co-production of MVA and isoprene in the same fermentor without the purification of MVA.

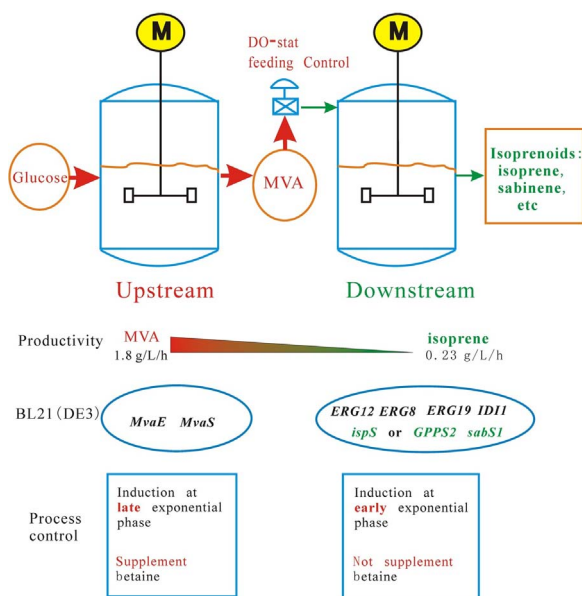


Fig. 6. The two-step fermentation process for isoprenoids production and the significant differences in the process control between the upstream and downstream fermentations.

3.6. Feasibility of the proposed strategy for other isoprenoids: a case study of sabinene fermentation

We further tested the effects of MVA feeding on sabinene production under flask level. The SP-strain was cultured in a 600 ml sealed shake flask and induced at OD_{600} of 0.6 as described previously [35], followed by the addition of 1 g/L MVA to the fermentation medium. The results showed that the sabinene titer from MVA by engineered SP-strain was significantly improved to 150 mg/L, which was 6.5-fold of the sabinene titer in an earlier flask production of sabinene in our lab (20 mg/L, [35]). This study indicated that the MVA feeding-strategy could be more broadly utilized in the biosynthesis of isoprenoids.

4. Discussion

It is hard to set optimal fermentation conditions for both upstream and downstream pathways in one strain because the significant differences existed in the process control between the upstream and downstream fermentations (Fig. 6). Firstly, the induction mode in the upstream MVA fermentation was contrary to the downstream isoprenoids fermentation. Secondly, the betaine supplementation was essential to the upstream fermentation, while it was not required in the downstream fermentation at all. Finally, the productivity of the upstream MVA fermentation (1.8 g/L/h) was much higher than that of the downstream isoprene fermentation (0.23 g/L/h), which suggested that the upstream pathway might be hard to be balanced with the downstream pathway in one strain. Therefore, the two-separated-step (glucose to MVA, then MVA to the objective isoprenoids) route proposed in this study may have potential benefits towards the current one-step (glucose to isoprenoids) fermentation.

Betaine supplementation and the induction mode were found to be the key factors for high titer MVA and isoprene production in the two-step process through the systematical optimization of fermentation conditions. In order to evaluate the two-step fermentation route, the effects of betaine supplementation and induction mode on the one-step production of isoprene were also investigated. The betaine supplementation was essential to the upstream MVA fermentation in the two-step process, while it was not required in the one-step isoprene production. Furthermore, the induction mode of the one-step isoprene fermentation was contrary to that of the upstream fermentation in the two-step process. However, it was consistent with that of the

downstream isoprene fermentation. When the induction was conducted above OD_{600} 60, the isoprene production totally disappeared in the one-step process. The isoprene production could be improved when the induction was conducted at early exponential phase (OD_{600} 15 or so), and the final isoprene titer of 6.3 g/L was achieved in the one-step fermentation in our lab [7]. Under the same induction condition, the same strain only containing the downstream MVA pathway (from MVA to isoprene) produced a higher isoprene titer of 11.0 g/L in the present paper. Therefore, the two-step fermentation route could improve the intermediate building block MVA production and the downstream isoprene production simultaneously.

The decoupled MVA production from cell growth was achieved by betaine supplementation and the late induction mode, which greatly improved the titer and yield of MVA. As shown in Figs. 1 and 2, the MVA production was coupled with cell growth when the engineered MP-strain was induced at early exponential phase without betaine supplementation in the medium. However, the engineered MP-strains entered the stationary phase soon after the initial MVA synthesis in process A with betaine supplementation and late induction at OD_{600} 80. In contrast with our strategies, many attempts had been made previously to improve the fermentation performance by the separation of cell growth from the product synthesis through nutrient limitations such as carbon, nitrogen, dissolved oxygen, and so on [37]. The effects of essential nutrient starvations on the MVA production were investigated and the sulfur starvation play important roles in the improvement of MVA yield [38,39]. High-level production of amorpho-4, 11-diene, a precursor of the antimalarial agent artemisinin, was achieved through glucose limitation and nitrogen feed [40]. L-lysine production was improved by restrained growth fed-batch strategies [41]. The yield of L-lysine on glucose approximately was linearly increased with decreasing the specific growth rate [42].

Compared with no betaine supplementation in the medium, the betaine titer of 1 g/L could greatly improve MVA yield and productivity. When the betaine titer was further increased to 3 g/L, no differences were observed between 1 g/L and 3 g/L (the data of the primitive experiment is not shown in this manuscript). The beneficial effect of betaine in MVA fermentation should be similar to the previous studies of lactate, ethanol, lysine, and pyruvate fermentations [34]. The unique feature in all of these studies was the high titer of the final products with betaine supplementation which can help to neutralize the inhibiting stress of the intermediate metabolites or the products towards its corresponding strains or key enzymes [34]. We proposed here in this study that betaine supplementation may help to eliminate the inhibition effects of the intermediate metabolites such as 3-hydroxy 3-methyl glutaryl coenzyme A (HMG-CoA), which is known as a disturbed metabolite in MVA biosynthesis pathway [17]. However, it was not clear why the betaine supplementation could help the cells entering the stationary phase in this study. The metabolism of strains at stationary phase is also the focus of several researches, and it is not well characterized so far [43–46].

The preferred induction mode was actually determined by the relationship between cell growth and product formation. The decoupled product formation from cell growth can be achieved by induction at late exponential phase if the product formation like MVA was not strictly related with the cell growth. Considering that the OD_{600} at the stationary phase was 100 or so, the MVA production was induced at OD_{600} value of 80 in the present paper. In contrast, if the product formation like isoprene was greatly related with the cell growth, the induction was usually conducted at early or middle exponential phase (OD_{600} value of 15 or 30), otherwise the product formation would be weakened or even totally disappeared. Therefore, three different induction experiments were conducted at different OD values for MVA and isoprene production respectively in the present paper.

The high titer and yield (84.0 g/L, 34.0%) production of MVA showed its feasibility and potential as a feeding precursor in isoprenoids biosynthesis. The improvement of MVA fermentation could decrease

the production and purification cost of MVA and makes it feasible to build up a downstream isoprenoids process depended on feeding of MVA as a precursor. The high titer of MVA (above 80 g/L) in the broth also indicated that the produced MVA was not catabolized again by the engineered *E. coli* and was excreted to the broth during the fermentation process. This behavior has been observed before with butyrate production by the engineered butyrogenic *E. coli* [47] which both reflects the un-reverse functionality of 3-hydroxy 3-methyl glutaryl-CoA reductase and the butyryl-CoA dehydrogenase in MVA and butyrate biosynthesis pathways, respectively.

The strategy of DO-stat based glucose and MVA co-feeding was designed according to the tight relationship between DO and the conversion of MVA and glucose. When the glucose was depleted and the level of DO exceeded 20% in the fed-batch fermentation, the concentrated solution of glucose and MVA was added to maintain the level of DO at 20% or so. Contrary to the relationship of glucose feeding with DO level, high MVA feeding rate can result in the slow increment of DO and the decrease of glucose consumption rate possibly due to the inhibition of the intermediate metabolite. Therefore, the feeding rate of MVA should be designed to be positively related with the glucose consumption rate of isoprene producing strains to alleviate the inhibition phenomenon. When the inhibition phenomenon happened and the glucose consumption rate decreased, the feeding rate of MVA should also be decreased. Although the inhibition was not cancelled completely, considering that the relevant parameters in C could be further improved, C was still a potential solution to solve the inhibition of the intermediate metabolite produced in isoprenoids pathways.

Compared to the one-step fermentation methods for isoprenoids [7,35], the advantages of two-step route are listed as followings: (1) The platform chemical MVA can be produced efficiently and cost effectively in two-step fermentation. It can serve as the precursor for all of the isoprenoids besides isoprene and sabinene. (2) Higher productivities are achieved for both upstream MVA fermentation and downstream isoprene or sabinene fermentation after the division of the MVA pathway. (3) More stable engineered strains can be constructed due to low cellular stress after the division of the MVA pathway. Furthermore, the disadvantage of two-step route is that more purification steps of the platform chemical MVA are required. The feasibility of skipping the purification of MVA in the two-step isoprene production was investigated in the present paper by directly adding the MVA fermentation broth to the downstream fermentation broth. Optimization of the isoprenoids fermentation without the purification of MVA would further reduce the production costs of isoprenoids through the two-step process.

5. Conclusions

The high titer and yield (84.0 g/L, 34.0%) production of mevalonate showed its feasibility and potential as a feeding precursor in isoprenoids biosynthesis. Comparing to the one-step isoprene or sabinene biosynthesis from glucose [7,35], the modified process (from mevalonate to isoprene or sabinene) improved the production of isoprene and sabinene. The proposed strategy offers a potential methodology to set up a robust biosynthesis process for structurally complex isoprenoids with increased production. Future studies on mevalonate derivatives can further expand the use of mevalonate.

Competing interests

All authors have no conflict of interest to declare.

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