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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, archaebacteria, 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		
E. coli BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm rne131*(*DE3*)	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 *mvaE* and HMG-CoA synthase gene *mvaS*, T7 promoter, Cm^R	[7]
pYJM14	pTrcHis2 B derivative carrying phosphomevalonate kinase gene *ERG8*, mevalonate kinase gene *ERG12*, mevalonate pyrophosphate decarboxylase gene *ERG19* and IPP isomerase gene *IDI1*, Trc promoter, Ap^R	[7]
pYJM8	pACYCDuet-1 derivative carrying isoprene synthase gene *ispS*, T7 promoter, Cm^R	[7]
pHB5	pACYCDuet-1 derivative carrying geranyl diphosphate synthase gene *GPPS2* and sabinene synthase gene *SabS1*, 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*, *Mangiferaindica*, and *Elaeocarpus photiniifolius* 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 βMδVL [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 ^{13}C-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 *IDI1,* 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 $Na_2HPO_4{\cdot}12H_2O$, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 20 g/L glucose, 0.24 g/L $MgSO_4$). The MVA fermentation medium consisted of