

Production of Bio-Based Isoprene by the Mevalonate Pathway Cassette in *Ralstonia eutropha*

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Isoprene has the potential to replace some petroleum-based chemicals and can be produced through biological systems using renewable carbon sources. *Ralstonia eutropha* can produce value-added compounds, including intracellular polyhydroxyalkanoate (PHA) through fatty acid and lipid metabolism. In the present study, we engineered strains of *R. eutropha* H16 and examined the strains for isoprene production. We optimized codons of all the genes involved in isoprene synthesis by the mevalonate pathway and manipulated the promoter regions using pLac and pJ5 elements. Our results showed that isoprene productivity was higher using the J5 promoter ($1.9 \pm 0.24 \mu\text{g/l}$) than when using the lac promoter ($1.5 \pm 0.2 \mu\text{g/l}$). Additionally, the use of three J5 promoters was more efficient ($3.8 \pm 0.18 \mu\text{g/l}$) for isoprene production than a one-promoter system, and could be scaled up to a 5-L batch-cultivation from a T-flask culture. Although the isoprene yield obtained in our study was insufficient to meet industrial demands, our study, for the first time, shows that *R. eutropha* can be modified for efficient isoprene production and lays the foundation for further optimization of the fermentation process.

Keywords: *Ralstonia eutropha*, isoprene production, promoter, fermentation, MVA pathway

Introduction

Ralstonia eutropha is an industrial strain used in the production of various industrial materials, biodegradable plastics, biofuels as alternatives to gasoline, and other bio-based chemicals because of the ability of *R. eutropha* to use a sole carbon source such as sugar, amino acid, carbon dioxide, fatty-acids, and various oils for cell growth [1–4]. Specifically, *R. eutropha* is known as a model bacterium for biodegradable polyhydroxyalkanoate (PHA) biopolymer production [5–7]. However, PHB, a type of PHA, has been shown to accumulate up to 70% of dry cell weight (DCW) by adequate carbon metabolism under unfavorable culture conditions of nutrient limitation [8]. Recently, *R. eutropha* has gained much attention as a candidate for biofuel production, which is an alternative to petroleum-based products. The bacterium can be engineered to produce, C4

and C5 alcohols such as isobutanol, and 3-methyl-1-butanol by electromicrobial conversion of CO₂ [9]. In addition, *R. eutropha* has been used to produce various industrial materials including isopropanol, methyl ketone, and isoprene using acetyl-CoA as a precursor of PHA biosynthesis. Batch cultivation of *R. eutropha* (Re2133/pEG7c) has been carried out using fructose as a sole carbon source to produce 3.44 g/l isopropanol [10]. In the case of medium-chain hydrocarbons, 180 mg/l methyl ketones were obtained by heterologous expression of the acyl coenzyme A oxidase under chemolithoautotrophic growth conditions with CO₂ and H₂ as the sole carbon source and electron donor, respectively [11].

Isoprene hydrocarbons are a useful chemical product widely used as feedstock in fuel additives for gasoline or jet fuel as well as in the synthetic rubber industry [12–14]. The production of isoprenoids including isoprene is

divided into two biosynthetic pathways, the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway from isopentenyl diphosphate pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP), with each having been studied using engineered *E. coli* systems [15–17]. Several reports have also described bio-based isoprene production using novel engineered strains of *Bacillus*, cyanobacteria, and *Saccharomyces cerevisiae* species [18, 19]. The MVA pathway is used by eukaryotes and archaeobacteria, and occurs in the cytosols of higher plants, while the MEP pathway is mainly used by eubacteria and green algae [20, 21]. The MVA pathway requires acetyl-CoA as the primary precursor, whereas the MEP pathway requires pyruvate and glyceraldehyde-3-phosphate as initial molecules for IPP and DMAPP biosynthesis. The MEP pathway has been the target of genetic engineering in efforts to increase the supply of IPP and DMAPP substrates for increased synthesis of isoprenoids [22, 23]. However, this approach still has limitations because the MEP pathway may be associated with unknown physiological regulation elements in the native host [24]. Heterologous expression of the MVA pathway can contribute to the increase in metabolic flux and synthesis of IPP and DMAPP and can be used as an alternative to the MEP pathway [25].

In our previous study, we achieved 12.7 g/l isoprene accumulation through heterologous co-expression of the *Populus trichocarpa ispS* gene and MVA pathway genes using *E. coli* DH5 α [17]. Despite the use of various hosts, genetic elements, and culture conditions in previous studies, the yield produced is still insufficient to meet the industrial demand for cost-effective productivity.

Although *R. eutropha* appears to be a suitable host for isoprene production due to its autotrophic growth, it has never been engineered as a host strain for this purpose. Therefore, we demonstrate here for the first time the production of isoprene using the MVP pathway in *R. eutropha* H16. A three-promoter system was introduced for isoprene production by the mevalonate pathway. In previous reports, the expression level of eGFP under various promoters has been evaluated in *R. eutropha* H16 transconjugants, and the results showed that eGFP expression using the J5 promoter was 5 times higher than that using the lac promoter [26]. Further, highly efficient overproduction of the *E. coli* protein complex has been achieved using a two-promoter system [27]. To improve isoprene production, all the genes involved in isoprene synthesis by the mevalonate pathway were optimized according to the codon usage of *R. eutropha*, and a novel cassette with Plac and PJ5 promoter elements was used.

Materials and Methods

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* S17 and *R. eutropha* H16 harboring pTS-sPt-MVA were used in this study. The plasmid was obtained from pS-NA plasmid derived from pSTV28, as described previously [17] and contained the following: *mvaS* and hydroxymethylglutaryl-CoA reductase (*mvaE*) from *Enterococcus faecalis*; mevalonate kinase (*mvaK1*), phosphomevalonate kinase (*mvaK2*), and mevalonate diphosphate decarboxylase (*mvaD*) from *Streptococcus pneumoniae*; *idi* from *E. coli*; and *ispS* from *Populus trichocarpa*. *ispS* is an isoprene synthase gene to catalyze the formation of isoprene from DMAPP. All plasmids constructed in this study contain the backbone from the broad-host-range vector pBBR1-MCS2 with the kanamycin resistance gene [28]. pBBR1MCS-2-P_{J5} was constructed by replacing the P_{LAC} promoter region of the pBBR1MCS-2 vector, and the sequences of the promoters and terminators are listed in Table S1. DNA sequence amplification was achieved using Phusion Flash High-Fidelity PCR Master Mix (New England Biolabs, USA). Restriction enzymes were obtained from New England Biolabs. The primers used in this study are listed in Table S2, and all constructed plasmids are described in Table 1. Codon usage tables of mevalonate pathway and isoprene synthase genes in *R. eutropha* are listed in Table S3.

Culture Conditions

Conjugated *R. eutropha* strains for isoprene production were pre-cultured in 2 ml of tryptic soy broth (TSB) supplemented with 10 μ g/ml gentamicin for 24 h at 30°C. The pre-cultured cells were transferred to a 250-ml, sealed T-flask containing 20 ml of TSB broth with 2 g/l fructose to compare isoprene production of conjugated *R. eutropha* cells. For 5-L batch fermentation, the seed media were transferred to a 5-L fermenter containing 100 ml of TSB broth as a seed culture. After 12 h of cultivation at 37°C on a rotary shaker (200 rpm), the culture was inoculated into a 5-L fermenter (KFC LA-150; Kobiotech Co., Ltd., Korea) containing 2 L of the initial medium [20 g/l fructose, 20 g/l yeast extract, 10 g/l tryptone peptone, 5 g/l (NH₄)₂SO₄, 3 g/l KH₂PO₄, 3 g/l Na₂HPO₄, 1 g/l MgSO₄·7H₂O, 0.4 ml/l antifoam, 10 mg/ml gentamicin] and 1 ml of trace element solution. The trace element solution was prepared as previously described [29]. The phosphate-containing compounds and magnesium sulfate were sterilized separately from the main medium. Aeration was maintained at 0.2 vvm and the stirring speed was gradually increased to 200–1,000 rpm to maintain the dissolved oxygen (DO) concentration at \geq 20% by adjusting the rate of agitation and maintaining the pH at 7.0 with 10 N NaOH. For 5-L batch cultivation using the three-promoter system, cell growth was monitored by measuring the optical density at 600 nm, starting from an initial value of 0.42.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Reference
<i>E. coli</i>		
S-17	recA pro hsdR RP42Tc::Mu-Km::Tn7 integrated into the chromosome	Simon et al., 1983
TS-sPt-MVA	S17 with pTS-sPt-MVA	This study
RE-MVA1	S17 with pRE-MVA1	This study
RE-MVA2	S17 with pRE-MVA2	This study
RE-MVA3	S17 with pRE-MVA3	This study
RE-MVA4	S17 with pRE-MVA4	This study
<i>R. eutropha</i>		
H16 DSM28	wild type	DSM
H16-MCS2	H16 with pBBR1MCS-2	This study
TS-sPt-MVA1	H16 with pTS-sPt-MVA	This study
RE-MVA5	H16 with pRE-MVA1	This study
RE-MVA6	H16 with pRE-MVA2	This study
RE-MVA7	H16 with pRE-MVA3	This study
RE-MVA8	H16 with pRE-MVA4	This study
Plasmid		
pBBR1MCS-2	Km ^r , pBBR1 replicon, mob ⁺	Kovach et al.
pTS-sPt-MVA		Kim et al.
pRE-MVA1		This study
pRE-MVA2		This study
pRE-MVA3		This study
pRE-MVA4		This study

Analytical Methods

An auto-sampler (CNS, Korea) was used to measure both the optical density and the fructose concentration in the culture broth at specified time points. Cell growth was monitored by measuring the OD at 600 nm (OD₆₀₀) using a spectrophotometer (Uvicon 941 Plus; Kontron Instruments Co., Switzerland). The residual fructose concentration was analyzed on a high-performance liquid chromatography (HPLC) system with an RID detector (RID-7515A, ERC Instrument Co., Japan) equipped with an

Aminex 87H ion-exclusion column (Bio-Rad, Hercules, CA, USA). The column temperature was maintained at 85°C, and the mobile phase was deionized water applied at a flow rate of 0.5 ml/min. The isoprene concentration was measured with a gas chromatograph (Varian X-3300, USA) equipped with a flame ionization detector. The analysis was performed at previously reported conditions [17]. The temperature program used was 3 min at 50°C followed by an increase to 150°C for 10 min; the column was maintained at this temperature for 12 min before lowering to 50°C again. Isoprene

production was identified using a gas chromatography-mass spectrometry (GC-MS) system (Agilent GC/MSD 5975 coupled with an Agilent 7890A) equipped with a quadrupole mass selective detector on electro-ionization (EI) operated at 70 eV. The sim-mode was used to detect the desired peaks. The Agilent HP-5MS column (30 m length, 0.25 μm inner diameter, 0.25 μm film thickness) was used at a 10:1 split ratio, helium was used as a carrier gas, and the oven temperature ranged from 150 to 172°C.

Nucleotide Accession Numbers

The *ispS*, *mvaK1*, *mvaD*, *mvaK2*, *idi*, *mvaE*, *mvaS*, and *LipA* DNA sequences codon-optimized in *R. eutropha* have been deposited at GenBank under the accession numbers of MK775336, MK775337, MK775338, MK775339, MK775340, MK775341, MK775342, and MK775343, respectively.

Results

Identification of Isoprene Production Using GC and GC-MS from T-Flask Cultivation of Engineered *R. eutropha* Strains

Vapor samples from the sealed T-flask cultivation of engineered *R. eutropha* strains carrying the plasmid (pRE-MVA5 to -MVA8) with the P_L (lac promoter), P_J (J5 promoter), P_{LLL} (three lac promoters), and P_{JJJ} (three J5 promoters) were examined by GC and GC-MS techniques (Table 1). For GC-MS analysis, 1 ml of gas from the headspace of sealed cultures was manually injected into the GC inlet port, based on a calibration curve constructed from serial dilutions of vaporized isoprene standard. For the calibration

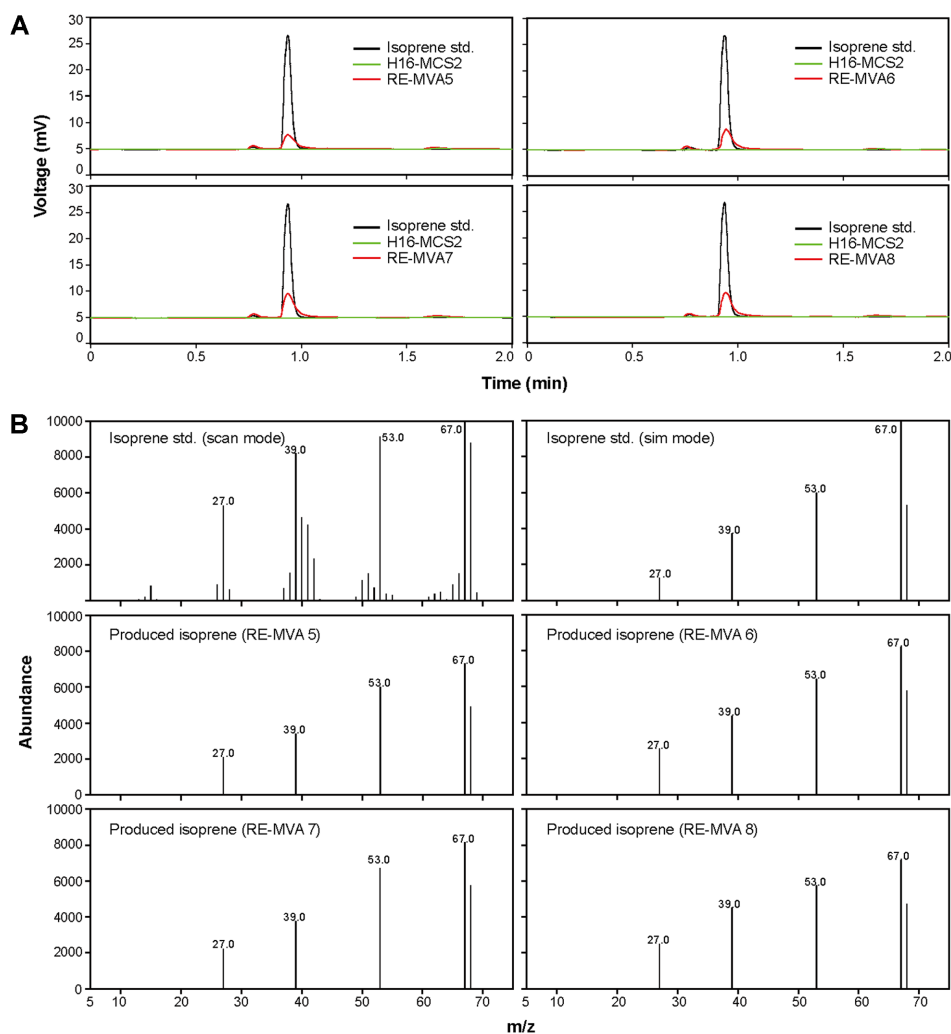


Fig. 1. Production of isoprene in *R. eutropha*.

Detection of produced isoprene by (A) GC peak, (B) GC-Mass peak during T-flask cultivation of recombinant *R. eutropha* strains harboring plasmids pTS-sPT-MVA, pRE-MVA1, pRE-MVA2, pRE-MVA3, and pRE-MVA4.

curve, a 1 ml sample from diluted standard was injected in the GC and the peak amplitude of the sample was recorded (Fig. 1A). The GC peak areas were plotted against the known amount of isoprene, and the slope of the standard curve was used to determine the amount of isoprene produced by the engineered *R. eutropha* strains in T-flask and 5-L batch cultivation. The headspace samples including isoprene standard showed two peaks with retention times of 0.75 and 0.94 min (Fig. 1A). For more accurate identification of the isoprene produced by the recombinant *R. eutropha* strains, the samples were subjected to GS-MS. The Agilent GC/MSD 5975 was coupled with an Agilent 7890A device, which allowed selection of scanning or selected ion monitoring (SIM) mode. The scan mode photograph showed all ionized isoprene peaks having a molecular weight of 68.1 in reference (Fig. 1B). The scan mode can represent a mixed spectrum of the target and complex compounds. On the other hand, the chromatographic run of SIM-scan mode has the ability to obtain powerful information at very low detection limits of the target compound. Therefore, the SIM mode of GC-MS was used to identify the isoprene produced by the recombinant *R. eutropha* strains. GC-MS analysis of vapor samples from the headspace of the transformants is shown in Fig. 1B. The peaks displayed dominant 27.0, 39.0, 53.0, and 67.0 lines, which could be attributed to isoprene. The isoprene from all four *R. eutropha* transformants examined appeared as separate and distinct GC-MS peaks (Fig. 1B). Together, these results clearly showed that the recombinant *R. eutropha* could actively produce isoprene as well as various bio-based products.

Cultivation of *R. eutropha* H16 Transconjugants Harboring Each of Five Plasmids

In order to achieve optimal carbon flow into isoprene biosynthetic pathway, *R. eutropha* H16 strains harboring mevalonate pathway genes and isoprene synthase were constructed. The strains H16-MCS2 and TS-sPt-MVA1 were conjugated with the plasmids codon-optimized in *E. coli*, namely, pBBR1MCS2 and pTS-sPt-MVA, respectively, as control strains for isoprene production in this study. All strains were cultivated on fructose (2 g/l, for protection against decrease in pH) as the sole carbon source with TSB media without dextrose during the sealed T-flask cultures. As shown in Fig. 2, isoprene was not detectable in the H16-MCS2 strain transformed with the empty plasmid pBBR1MCS2. In the case of TS-sPt-MVA1 strain harboring pTS-sPt-MVA, the isoprene production ($0.45 \pm 0.16 \mu\text{g/l}$) was slightly lower than that in the strains harboring codon-

optimized genes in *R. eutropha* in the T-flask cultivation. Next, we compared the efficiency of the Lac and J5 promoters by comparing the expression level of alkaline lipase (LipA). LipA derived from *Proteus vulgaris* consists of 287 amino acids. *R. eutropha* H16 transformants containing pLac-LipA and pJ5-LipA were screened using 1% tributyrin-emulsion YPD plates using fructose instead of glucose [30]. The clear zone on the opaque tributyrin emulsion identified the lipase-secreting transformants. A clear zone was formed around pLac-LipA and pJ5-LipA fermentation broth and supernatant after cell disruption. The diameter of the transparent ring formed by the pJ5-LipA was larger than that formed by the pLac-LipA, indicating that the J5 promoter was the stronger of the two promoters in *R. eutropha* (Fig. S1). The isoprene produced by the *R. eutropha* conjugates with the one-promoter system of pLac and pJ5 was $1.5 \pm 0.2 \mu\text{g/l}$ and $1.9 \pm 0.24 \mu\text{g/l}$, respectively (Fig. 2). We also examined the efficacy of a three-promoter system of P_{LLL} and P_{JJJ} for isoprene production (Table 1). The P_{JJJ} system showed higher isoprene production than P_{LLL} in sealed T-flask cultivation, and the amounts of isoprene produced by P_{JJJ} and P_{LLL} were $3.4 \pm 0.05 \mu\text{g/l}$ and $3.8 \pm 0.18 \mu\text{g/l}$, respectively. In addition, the specific yield and carbon yield of the three-promoter system was higher than that of the one-promoter system. The maximum specific yield ($2.53 \mu\text{g isoprene/OD}$) and carbon yield ($1.9 \mu\text{g isoprene/g c-source}$) was obtained by the strain containing the P_{JJJ} promoter (Fig. 2). The results of the sealed T-flask cultivation are summarized in Table 2.

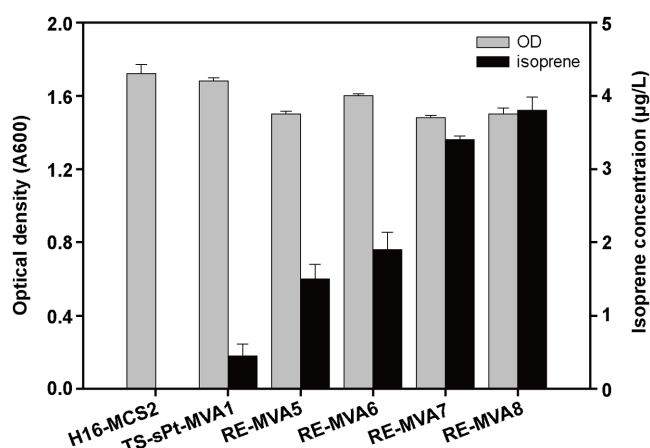


Fig. 2. T-flask cultivation of *R. eutropha* H16. *R. eutropha* H16 transconjugants harboring plasmids pTS-sPt-MVA, pRE-MVA1, pRE-MVA2, pRE-MVA3, and pRE-MVA4. Error bars indicate SD; $n = 3$ experiments.

Cultivation of *R. eutropha* H16 Transconjugants Containing Three-Promoter Systems in 5-L Batch Cultivation

As described above, *R. eutropha* H16 harboring the three-promoter system demonstrated the highest isoprene productivity in sealed T-flask cultures. Therefore, these strains were further evaluated as candidate strains for isoprene production by 5-L batch culture. Fructose and NaOH (10 N) were used as the single carbon source and the pH control reagent, respectively. The time profiles for isoprene production by *R. eutropha* H16 was determined in the complex culture medium (Fig. 3). To improve the isoprene detection using on-line GC, 0.2 vvm of aeration and the DO level (20%) were maintained with agitation. The lag phase was terminated after approximately 6 h of culture, and cell growth entered the exponential growth

phase. The optical density, produced isoprene, specific yield, and carbon yield of *R. eutropha* strains harboring the three-promoter system are summarized in Table 2. As shown in Figs. 3A and 3B, maximal cell growth of *R. eutropha* RE-MVA7 and RE-MVA8 reached an OD_{600} of 32.9 and 26.32 at 54 h of culture, and the isoprene produced was 22.9 mg/l and 28.45 mg/l, respectively. Isoprene productivity exhibited a rapid decrease upon depletion of the carbon source. Overall, strain RE-MVA8 showed 1.24 times higher isoprene production than RE-MVA7 in 5-L batch culture. In addition, the specific yield and the carbon yield produced relative to the consumed fructose was higher with RE-MVA8 than that obtained with the RE-MVA8 strain (Table 2, Fig. 3). Based on the results of sealed T-flask cultivation, we could successfully scale-up to a 5-L batch fermenter for isoprene production using *R. eutropha* containing the three-plasmid system.

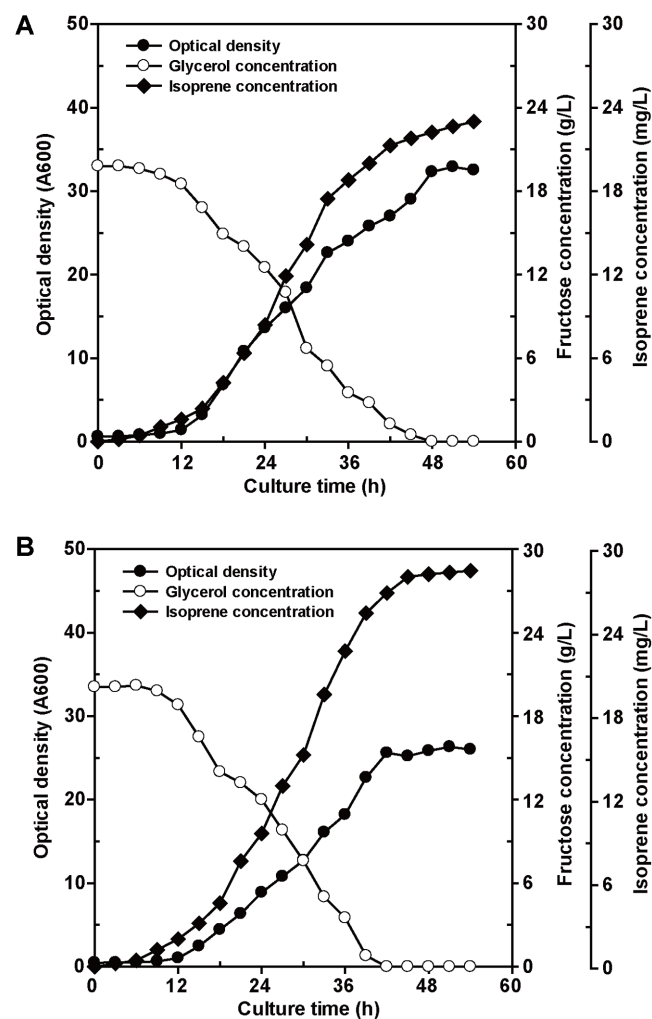


Fig. 3. Time course of 5-L batch cultivation of *R. eutropha* H16 harboring (A) pRE-MVA3 and (B) pRE-MVA4.

Discussion

Isoprene, a volatile hydrophobic molecule, is one of the major chemical feedstocks in the synthetic chemical industry required for the production of synthetic rubber or plastics and is a future biofuel candidate. The bio-based production of isoprene has a number of advantages; one such advantage is that it can be generated from various kinds of biomass [13, 14]. Isoprene was mainly produced from renewable carbohydrates such as glucose or glycerol by the engineered *E. coli*, *Bacillus*, and *S. cerevisiae*. However, in recent years, isoprene synthesis studies have been conducted using various strains in order to obtain a production efficiency for commercial use. During the production of isoprene in the photosynthetic apparatus by cyanobacteria, the highest isoprene production yield of 12.3 mg per gram biomass has been reported (4.2 mg/l for 96 h cultivation) [31]. However, biomass or accumulated isoprene could be obtained at a slightly slower rate when using carbon dioxide as a carbon source. Isoprene has been produced by *Clostridium ljungdahlii* using syngas as an inexpensive carbon source [32]. However, the quantities of produced isoprene and biomass were far lower than that commercially required. *R. eutropha* is commercially employed to produce industrially valuable products in small- or large-scale fermentation processes.

R. eutropha is able to convert a significant amount of carbon source into PHB under unfavorable nutrient limitation conditions such as deficiency of nitrogen, phosphate, and oxygen [33]. This ability to produce PHB using excess carbon source may be applied in isoprene production

because isoprene and PHB share the same production pathway precursor acetyl-CoA. If the conversion pathway of acetyl-CoA toward PHB can be altered to isoprene by genetic modification, *R. eutropha* may be considered an important strain for isoprene bio-production.

Here, we first demonstrated that all the heterologous genes of the mevalonate pathway and isoprene synthase could be utilized for isoprene synthesis by redirecting carbon flow in *R. eutropha*. The transformants harboring the isoprene production plasmids were evaluated for isoprene production in sealed T-flask and 5-L batch cultures utilizing fructose as the carbon source. In this study, the codon usage of all genes involved in the synthesis was optimized for the efficient production of isoprene through the mevalonate pathway. Using the Lac and J5 promoters, a novel cassette was screened to increase the isoprene production in *R. eutropha*. Codon usage difference for isoprene production between *E. coli* and *R. eutropha* was observed in sealed T-flask cultures. Strain RE-MVA5 with codon optimization in *R. eutropha* showed 3.33-fold higher production of isoprene than strain TS-sPt-MVA1 with codon optimization in *E. coli* using the single Lac promoter system.

Previously, the use of the P_{J5} promoter instead of the P_{LAC} promoter increased the protein expression level in *R. eutropha* [26]. Indeed, the constructed strains RE-MVA5 and RE-MVA6 with P_{LAC} and P_{J5} promoters, respectively, produced 1.5 ± 0.2 µg/l and 1.9 ± 0.24 µg/l of isoprene, respectively, indicating that increasing the promoter strength enhanced isoprene production. In order to further evaluate the high conversion of carbon flow from acetyl-CoA to isoprene, the three-promoter system was investigated, and the RE-MVA7 encoding P_{LLL} or P_{JJJ} were successfully constructed on the basis of the results of recent studies on two-promoter systems to increase protein expression levels or produce PHAs [27, 34]. Notably, the study on the gene expression efficiency of promoter and ribosome binding sites (RBSs) in *R. eutropha* showed that constitutive promoters with more than 700-fold activity may be located in *R. eutropha*, compared to the native *PphaC* and RBSs associated with isoprene production by the MEP pathway [35]. In our study, we showed that the maximum titers of 3.8 ± 0.18 µg/l and 28.45 mg/l of isoprene were obtained with the P_{JJJ} system under conditions of sealed T-flask and 5-L batch cultures, respectively, and this promoter system may be applied to produce bio-based products as well as isoprene in engineered *R. eutropha*.

Although the productivity of isoprene produced in this study is far below that required for commercial production,

we have shown that *R. eutropha* may produce potential fuel-based products using various carbon sources such as fatty acids or oils. In particular, the use of non-edible oil such as jatropha is expected to contribute to food or energy reduction issues [36].

To our knowledge, this is the first report on the production of isoprene in *R. eutropha*. To increase isoprene production, we propose enhancing levels of heterologous enzymes, carbon flux optimization, and supply of ATP. In addition, an optimum method of cultivation such as fed-batch, continuous cultivation, and scale-up can be adopted after a thorough cost-benefit analysis in order to make the process technically and economically feasible for industrial application.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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