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Short communication

Purification of biomevalonate from fermentation broth and conversion of biomevalonate into biomevalonolactone

Jin Won Kim, Hee Chan Yoon, Soon Jae Kwon, Bun Yeol Lee, Pyung Cheon Lee*

Department of Molecular Science and Technology and Department of Applied Chemistry and Biological Engineering, Ajou University, Woncheon-dong, Yeongtong-gu, Suwon 16499, South Korea

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ABSTRACT

Mevalonate (MVA) is a key compound of living organisms including bacteria, plants, and humans. MVA and mevalonolactone (MVL), a lactonized form of MVA, are important for pharmaceutical, cosmeceutical, and biotechnological applications. Although (*R*, *S*)-MVA with 50% enantiomeric purity is mainly produced by chemical synthesis, recently, microbial fermentation processes for MVA production have been considered as an alternative to the chemical synthesis because of high enantiomeric purity [(*R*)-MVA] and high titer. In the present study, bio-MVA produced by a fermentative process was decolorized by a charcoal-based method and then chemically transformed into bio-MVL without byproducts by means of phosphoric acid as an acid catalyst. The final bio-MVL was (*R*)-MVL with over 99% enantiomeric purity according to ^{1}H NMR analysis.

Mevalonic acid (β-hydroxy-β-methyl-δ-valerate; MVA) and its lactonized form (mevalonolactone; MVL) were discovered as growth factors for *Lactobacillus* strains by independent research groups (Tamura, 1956). Later studies revealed that MVA is a metabolic intermediate of cholesterol biosynthesis in eukaryotes and a key compound of living organisms including bacteria, plants, and humans (Lynen, 1967; Tabata and Hashimoto, 2004). In most eukaryotes and some prokaryotes, MVA is synthesized via the MVA pathway (Lee and Schmidt-Dannert, 2002), where three acetyl-CoA molecules are condensed to form hydroxylmethyl-glutaryl-CoA (HMG-CoA) through enzymatic steps of acetoacetyl-CoA synthase and HMG-CoA synthase. HMG-CoA is then reduced to MVA by HMG-CoA reductase (Fig. 1A). MVA and MVL have major pharmaceutical, cosmeceutical, and biotechnological significance. For instance, MVL is known as a monomer of biodegradable polymers (Xiong et al., 2014) and a bioactive antiwrinkle agent for cosmetic creams (http://www.adeka.co.jp/en/). At present (*R*, *S*)-MVL with 50% enantiomeric purity is mainly produced by chemical synthesis. On the other hand, the chiral purity and the purification step increase production costs. Recently, microbial fermentation processes for MVA production have been considered as an alternative to the chemical synthesis because of high enantiomeric purity [(*R*)-MVA] and high titer (Li et al., 2016; Pitera et al., 2007; Tabata and Hashimoto, 2004; Wang et al., 2016; Xiong et al., 2014; Yang et al., 2012). Although upstream process development—including strain improvement and fermentation process optimization—is important, downstream processes, especially conversion of MVA to MVL, are also crucial for the whole process. However, so far, little information is available on purification of bio-MVA from culture broth and conversion of bio-MVA into bio-MVL (Pitera et al., 2007; Xiong et al., 2014). In the present study, we developed an efficient method for conversion of bio-MVA, which was produced by a fermentative process, into bio-MVL with high chiral purity.

Two MVA pathway genes, bifunctional *mva*E (GenBank accession No. KF286540.1) encoding acetoacetyl-CoA synthase and HMG-CoA reductase together, and *mva*S (GenBank accession No. KF286539.1) encoding HMG-CoA synthase, were amplified by PCR from the genome of *Enterococcus* sp. (unpublished data) and subcloned into two expression vectors, pUCM and pSTVM (Kim et al., 2010), resulting in vectors pUCM_mvaE and pSTVM_mvaS. *Escherichia coli* strain MG1655 was used for the production of bio-MVA. The recombinant *E. coli* strain harboring pUCM_mvaE and pSTVM_mvaS was aerobically grown in 50 mL of the SB medium (35 g/L tryptone, 20 g/L yeast extract, and 5 g/L NaCl) supplemented with 10 g/L glycerol as a carbon source at 250 rpm for 12 h at 37 °C in 500-mL flasks. Ampicillin (100 mg/L) and chloramphenicol (50 mg/L) were added into the SB medium to implement selective pressure. Fed-batch fermentation was performed in a 2.5-L BIOSTAT B plus bioreactor (Sartorius, Germany) with a working volume of 1 L of the SB medium supplemented with 20 g/L glycerol. When cell growth reached optical density (OD_{600}) of 8, pH-stat feeding was started with a 600 g/L glycerol solution. The dissolved oxygen (DO) level was maintained at above 30% by controlling an impeller speed and by supplying pure oxygen (99.9%). Culture pH was

* Corresponding author.
E-mail address: pclee@ajou.ac.kr (P.C. Lee).

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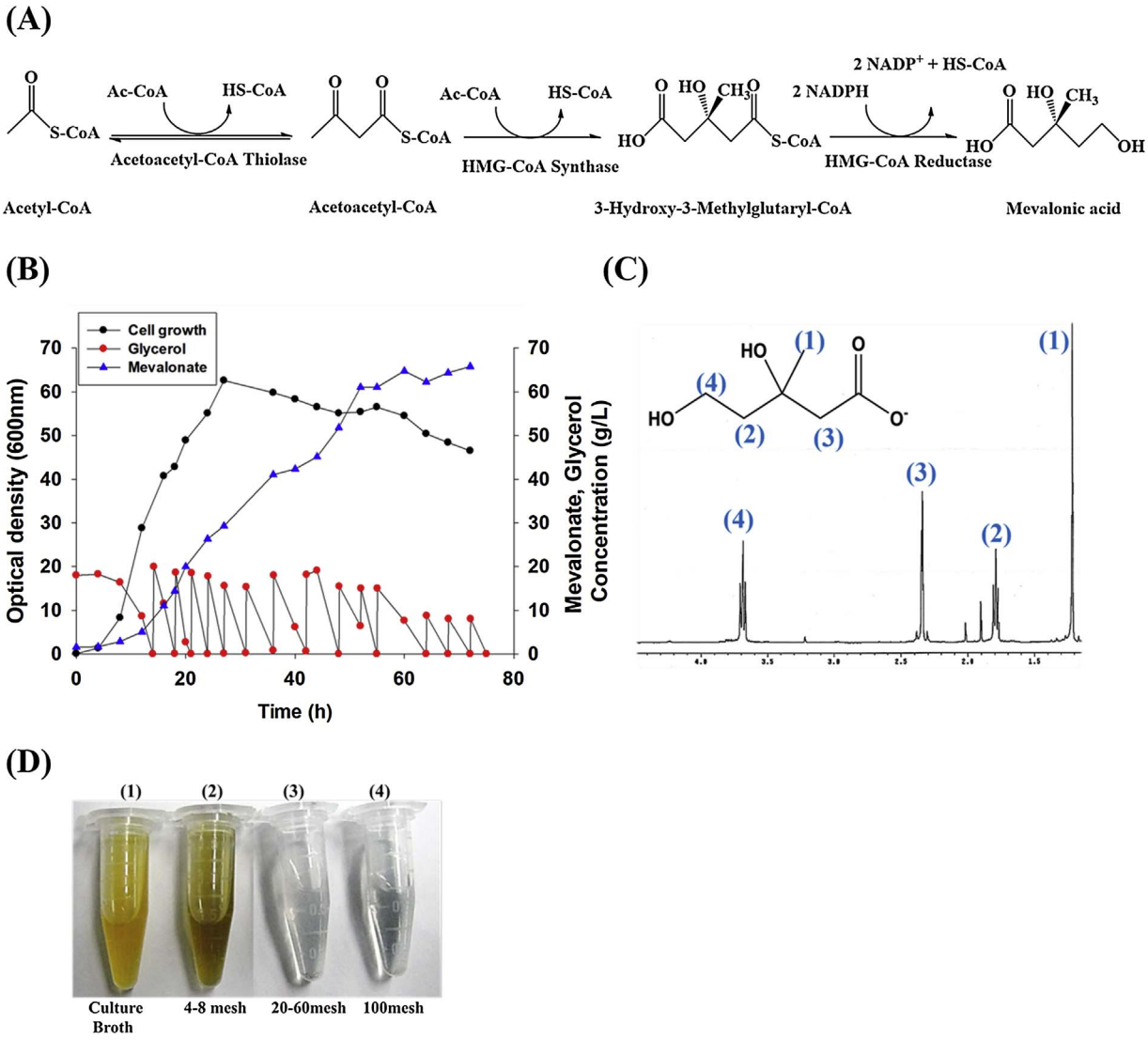

Fig. 1. The MVA biosynthetic pathway (A), fed-batch fermentation of recombinant *Escherichia coli* expressing enzymes of the MVA synthetic pathway (B), ^{1}H NMR analysis of bio-MVA present in fermentation broth (C), and decolorization of cell-free fermentation broth (D). (A) The MVA biosynthetic pathway consists of 3 pathway enzymes in nature. (B) Black circles represent cell growth, red squares denote bio-MVA concentration, and open circles indicate residual glycerol concentration. (C) Numbers 1, 2, 3, and 4 in the NMR spectrum indicate positions of the corresponding protons in the structure of bio-MVA. (D) (1) Untreated fermentation broth; (2) filtrate from 5% (v/w) granular activated charcoal of 4–8 mesh size; (3) a filtrate from 5% (v/w) powder activated charcoal of 20–60 mesh size; (4) a filtrate from 5% (v/w) powder activated charcoal of 100-mesh size. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

maintained at 6.8 ± 0.1 by addition of a 30% ammonia solution, and the temperature was maintained at 30 °C. Cell growth was monitored by measuring OD_{600} on a spectrophotometer (SPECTRAmax Plus384, Molecular Device). To remove colored contaminants present in the fermentation broth, the latter was separated from cells by centrifugation, and then the cell-free fermentation broth was filtered by passing it through a glass column (4 × 50 cm) packed with activated charcoal (in granular and powder form) of varying mesh sizes: 4–8, 20–60, and 100 (Sigma-Aldrich). Bio-MVA and glycerol were quantified relative to standards with known concentrations of commercial MVA (Sigma-Aldrich) and glycerol (Sigma-Aldrich) by means of a high-performance liquid chromatography (HPLC) system (Agilent 1200, USA) that was equipped with an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad) and a RI detector (Agilent, USA). The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.7 mL/min at 50 °C (Xiong et al., 2014). To investigate the presence of bio-MVA and bio-MVL in fermentation broth, 1 mL of cell-free culture broth was completely dried at 50 °C in a vacuum distillation apparatus and with $MgSO_4$, and the resulting dried residues were dissolved in CD_3OD and then subjected to ^{1}H NMR analysis (400 MHz, Bruker Avance 400 system). To determine the optimal proportion of an acid catalyst in the medium (phosphoric acid or sulfuric acid) to be added into cell-free fermentation broth during conversion of bio-MVA into bio-MVL, three molar ratios (1:1, 1:5, and 1:20) of concentrated phosphoric acid (85%, Sigma-Aldrich) or sulfuric acid (97%, Sigma-Aldrich) were added into the fermentation broth containing bio-MVA on the basis of concentration of bio-MVA, mixed vigorously, and were kept at 25 °C for 12 h. Next, 10 mL of the reaction mixture was completely dried at 50 °C in the vacuum distillation apparatus. The dried samples were dissolved in CD_3OD and subjected to ^{1}H NMR spectroscopy. For a kinetic analysis of the conversion of bio-MVA into bio-MVL, a 5-fold higher molar amount of phosphoric acid was added into the decolored cell-free fermentation broth, mixed vigorously, and kept at 25 °C for 24 h. After that, a 10-mL aliquot of the reaction mixture was taken at 0, 3, 6, 9, and 24 h and completely dried at 50 °C in the vacuum distillation apparatus. Completely dried bio-MVA and bio-MVL were dissolved in CD_3OD and subjected to ^{1}H NMR spectroscopy. For chirality analysis, commercial racemic (*R*, *S*)-MVL (Sigma-Aldrich) and purified bio-MVL were dissolved in CD_3OD, mixed