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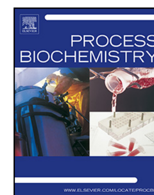

Production of ω-hydroxyundec-9-enoic acid and *n*-heptanoic acid from ricinoleic acid by recombinant *Escherichia coli*-based biocatalyst

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ABSTRACT

ω-Hydroxyundec-9-enoic acid and *n*-heptanoic acid are valuable building blocks for the production of flavors and antifungal agents as well as bioplastics such as polyamides and polyesters. However, a biosynthetic process to allow high productivity and product yield has not been reported. In the present study, we engineered an *Escherichia coli*-based biocatalytic process to efficiently produce ω-hydroxyundec-9-enoic acid and *n*-heptanoic acid from a renewable fatty acid (i.e., ricinoleic acid). Expression systems for catalytic enzymes (i.e., an alcohol dehydrogenase of *Micrococcus luteus*, a Baeyer–Villiger monooxygenase of *Pseudomonas putida* KT2440, an esterase of *Pseudomonas fluorescens* SIK WI) and biotransformation conditions were investigated. Biotransformation during stationary growth phase of recombinant *E. coli* in a bioreactor allowed to produce ω-hydroxyundec-9-enoic acid and *n*-heptanoic acid at a rate of 3.2 mM/h resulting in a final product concentration of ca. 20 mM. The total amount of ω-hydroxyundec-9-enoic acid and *n*-heptanoic acid produced reached 6.5 g/L (4.0 g/L of ω-hydroxyundec-9-enoic acid and 2.5 g/L of *n*-heptanoic acid). These results indicate that the high value carboxylic acids ω-hydroxyundec-9-enoic acid and *n*-heptanoic acid can be produced from a renewable fatty acid via whole-cell biotransformation.

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1. Introduction

ω-Hydroxyundec-9-enoic acid (**4**) is an antifungal substance found in the leaves of wild rice, *Oryza officinalis* [1]. Antifungal activity, evaluated based on spore-germinating inhibition of *Pyricularia oryza* P1, is ca. 70 μg/mL as an ED_{50} value. This level is promising as an antifungal agent. ω-Hydroxyundec-9-enoic acid can also be used as a precursor for synthesis of 11-aminoundecanoic acid, the monomer of polyamide 11 and 1,11-undecanedioic acid [2–4]. *n*-Heptanoic acid (**5**) is used to produce flavors and pharmaceuticals [5].

Medium chain (C9–C13) fatty acids (e.g., 1,9-nonanedioic acid, 1,10-decnaedioic acid, 1,13-tridecanedioic acid, 11-aminoundecanoic acid) are commercially produced from long chain (C18) fatty acids under harsh conditions (e.g., ozonolysis or caustic pyrolysis) [1,2,6,7]. Selective production of the specific medium chain fatty acids from renewable biomass at high yields by microbial biocatalysts was rarely reported. A representative example was production of ω-hydroxyundec-9-enoic acid (**4**) and *n*-heptanoic acid (**5**) from ricinoleic acid (**1**), which is a major constituent of castor oil, at high yields [8]. The C_{12}-hydroxyl group of the fatty acid is oxidized to an ester via the keto group in serial reactions of an alcohol dehydrogenase (ADH) from *Micrococcus luteus* and a Baeyer–Villiger monooxygenase (BVMO) from *Pseudomonas putida* KT2440 (Scheme 1). The ester group is then hydrolyzed by an esterase from *Pseudomonas fluorescens* SIK WI to generate ω-hydroxyundec-9-enoic acid and *n*-heptanoic acid. The first two reactions are catalyzed by a recombinant *Escherichia coli* expressing the genes coding for ADH and BVMO. The last step was performed by the isolated esterase. The biotransformation was conducted in a whole-cell assay condition after cell cultivation, harvest, and resuspension into a buffer solution.

The goal of this study was to engineer a biocatalytic process that allows efficient production of ω-hydroxyundec-9-enoic acid and *n*-heptanoic acid from ricinoleic acid in a simple process. We constructed a recombinant *E. coli* to catalyze all the reactions in a single cell, and developed a biotransformation coupling process and cell cultivation. Furthermore, the biotransformation and gene

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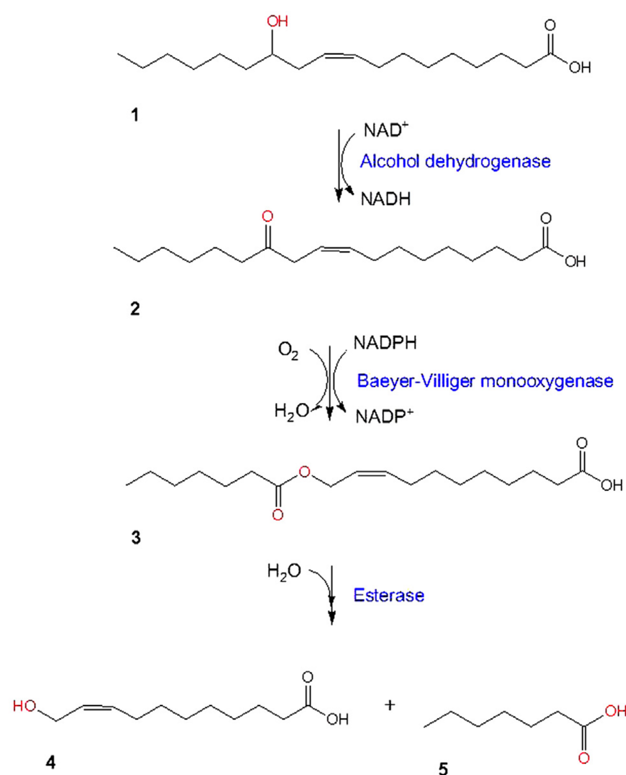

Scheme 1. The biotransformation pathway. Ricinoleic acid (**1**) is converted into ω-hydroxyundec-9-enoic acid (**4**) and *n*-heptanoic acid (**5**) in multistep enzyme reactions.

expression conditions were examined to maximize the whole-cell biotransformation rate.

2. Materials and methods

2.1. Microbial strains and culture media

Recombinant *E. coli* BL21(DE3) strains were cultivated in LB medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with appropriate antibiotics (Supplement Table S1) for plasmid DNA preparation and seed cultivation. The Riesenberg medium [9] supplemented with 10 g/L glucose and the appropriate antibiotics was used for the main cultivation and biotransformation. The Riesenberg medium consisted of 4 g/L $(NH_4)_2HPO_4$, 13.5 g/L KH_2PO_4, 1.7 g/L citric acid, 1.4 g/L $MgSO_4$, and 10 mL/L trace metal solution (10 g/L $FeSO_4$, 2.25 g/L $ZnSO_4$, 1.0 g/L $CuSO_4$, 0.5 g/L $MnSO_4$, 0.23 g/L $Na_2B_4O_7$, 2.0 g/L $CaCl_2$, and 0.1 g/L $(NH_4)_6Mo_7O_{24}$). Recombinant gene expression was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and/or 2 g/L rhamnose to the culture broth. Ricinoleic acid, palmitic acid, *n*-heptanoic acid, pyridine, hexane, ethyl acetate, and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide were obtained from Sigma (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA), respectively. ω-Hydroxyundec-9-enoic acid purified in the lab was used (see Section 2.7 for details).

2.2. Gene cloning

The pACYC-ADH-BVMO plasmid was constructed by transferring the BVMO gene (GenBank accession code: NP_744949.1) of *P. putida* KT2440 from the pJOE-BVMO [10] vector into the *Nde*I-*Pvu*I site of the plasmid pACYC-ADH [10]. The forward primer for BVMO gene amplification was 5′-GCTCATATGTCCTCTCACACTGCTCTTCCGG-3′ and the reverse primer was 5′-ACTCGATCGTCATCGGCGGCTACCTTGCTG-3′. The esterase gene (GenBank accession code: AAB60168) of *P. fluorescence* SIK WI was PCR-amplified from the vector pGASTON-PFE1 [10] and subcloned into the *Nde*I-*Xho*I site of the pCOLADuet-1 vector (Invitrogen, Carlsbad, CA, USA) resulting in the vector pCOLA-PFE1. The forward primer for esterase gene amplification was 5′-GCGCCATATGATGAGCACATTTGTTGCAAAA-3′ and the reverse primer was 5′-GCGCCTCGAGTCAGTGGTGATGGT-GATGATGGCGTTTCAAGAACGC-3′.

2.3. Biotransformation in a flask and bioreactor

The recombinant *E. coli* was cultivated in Riesenberg medium containing 10 g/L glucose at 30 °C. Biotransformation was initiated at the stationary growth phase, usually 8 h after induction of gene expression with 0.1 mM IPTG and/or 2 g/L rhamnose. Just after changing pH of culture broth to 8.0 and increasing temperature to 35 °C, 5–24 mM ricinoleic acid and 0.5 g/L Tween80 were added into the culture broth containing cell mass at 3 to 10 g CDW/L. Culture and biotransformation was performed in a 250 mL flask (working volume: 20 mL) in a shaking incubator (200 rpm). The bioreactor experiment was conducted in a 1 L scale reactor (Biotron, Bucheon, Korea). Cells for fed-batch cultivation were grown batch-wise until the glucose added initially was completely exhausted. Upon glucose depletion and concomitant elevation of pH >6.8, a mixture of glucose (600 g/L) and $MgSO_4·7H_2O$ (20 g/L) was fed using the pH-stat feeding strategy [11]. Culture pH was automatically maintained at pH 6.8 by feeding NaOH solution into the culture broth. Biotransformation was initiated after the pH was shifted to 8.0 and the culture temperature increased to 35 °C. Agitation speed and aeration rate were set at 400–1000 rpm and 1 vvm, respectively, to avoid any oxygen limitation during culture and biotransformation. The specific product formation rates were calculated based on the product concentrations in the reaction medium, which were determined by gas chromatography/mass spectrometry (GC/MS).

2.4. Determination of cell concentration

Cell concentration was determined by measuring the optical density (OD) at 600 nm and converting the value to cell dry weight using a conversion factor of 0.36 g/L/OD, which was determined in our previous study [12].

2.5. Product analysis by gas chromatography/mass spectrometry (GC/MS)

The concentrations of the fatty acids and carboxylic acids in the medium (e.g., ricinoleic acid, *n*-heptanoic acid, and ω-hydroxyundec-9-enoic acid) were determined as described previously [13]. The reaction medium was mixed with an equal volume of ethyl acetate containing 0.1 or 0.5 g/L methyl palmitate as an internal standard. The organic phase was harvested after vigorous vortexing and then subjected to derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (TMS). The TMS derivatives were analyzed using a Thermo Ultra Trace GC system connected to an ion trap mass detector (Thermo ITQ1100 GC-ion Trap MS, Thermo Scientific, and Indianapolis, IN, USA). The derivatives were separated on a non-polar capillary column (30 m length, 0.25 μm film thickness, HP-5MS, Agilent Technologies, Palo Alto, CA, USA). A linear temperature gradient was programmed as 90 °C, 5 °C/min to 280 °C. The injection port temperature was 230 °C. Mass spectra were obtained by electron impact ionization at 70 eV. Scan spectra were obtained within the range of 100–600 *m/z*. Selected ion monitoring was used for

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