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Microbial synthesis of undec-9-enoic acid, heptyl ester from renewable fatty acids using recombinant *Corynebacterium glutamicum*-based whole-cell biocatalyst

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ABSTRACT

The conversion of ricinoleic acid from renewable sources to long-chain α,ω-dicarboxylic acids or w-hydroxyl carboxylic acids by microbial processes is constrained by toxicity issues. Here, we demonstrate the possible role of *Corynebacterium glutamicum* as a new microbial strategy for the biotransformation of fatty acids. The established strain *Escherichia coli* failed to grow at 5 mM *n*-heptanoic acid, while the specific growth rate of *C. glutamicum* declined by 28%. We partially constructed a previously designed multistep biocatalytic pathway in *C. glutamicum*, and confirmed that the *C. glutamicum* biocatalyst successfully converted ricinoleic acid to undec-9-enoic acid, heptyl ester via 12-keto-oleic acid. We investigated the effects of cultivation and reaction temperatures, and the type and concentration of non-ionic detergent on recombinant *C. glutamicum* whole-cell bioconversion. At a cultivation temperature of 30 °C and a reaction temperature of 35 °C, and in the presence of 0.09 g/L Triton X-100, the whole-cell *C. glutamicum* biocatalyst produced 0.8 mM undec-9-enoic acid, heptyl ester from 1.9 mM 12-ketooleic acid. It also generated 0.7 mM undec-9-enoic acid, heptyl ester from 5.5 mM ricinoleic acid. This is the first report of undec-9-enoic acid, heptyl ester production using a recombinant *C. glutamicum*-based biocatalyst.

1. Introduction

Since the first oil crisis of the 1970s, depletion of fossil fuels, fluctuating oil prices, and environmental challenges including global climate change, have become important issues [1–3]. Therefore, the development of ecofriendly biotechnological methods to produce renewable biofuels and important chemicals from renewable resources is imperative [1,2]. The most popular and important sources of renewable raw materials based on living organisms for the development of industrial chemicals include oil plants, starchy plants, sugar beets and sugar cane, wood, and waste as well as agricultural and industrial residues. In a previous study, a biocatalytic process was designed to produce long-chain α,ω-dicarboxylic acids or w-hydroxyl carboxylic acids from ricinoleic acid, renewable fatty acids of plant and animal origin, which are the most significant renewable feedstock in the chemical industry, and were evaluated in *Escherichia coli* BL21(DE3) [2]. The biocatalytic process entailed oxidation of ricinoleic acid to ketones by an alcohol dehydrogenase (ADH), catalytic oxidation to an ester by Baeyer–Villiger monooxygenase (BVMO), and further hydrolysis to yield *n*-heptanoic acid and ω-hydroxyundec-9-enoic acid, which are used in the production of a variety of chemical products and intermediates, such as nylons and other polyamides, polyesters, resins, hot-melt adhesives, powder coatings, corrosion inhibitors, lubricants, plasticizers, greases, and perfumes [2]. However, the severe toxicity of *n*-heptanoic acid significantly inhibits the growth of *E. coli* BL21(DE3). Furthermore, the recombinant *E. coli* BL21(DE3), produced the final product at very low concentration and yield [4].

Here, we suggest *Corynebacterium glutamicum* ATCC13032 as a potential multistep enzymatic producer of long-chain α,ω-dicarboxylic acids and ω-hydroxyl carboxylic acids. *C. glutamicum* is a Generally Recognized as Safe (GRAS) microorganism, and has been traditionally used for industrial fermentation [5]. Various studies demonstrated the efficiency of this strain for amino acid production, and also for the synthesis of valuable organic acids, such as lactate and succinate, diamines, polymers, diols and alcohols by industrial fermentation of *C. glutamicum* [6,7]. In this study, we confirmed that *C. glutamicum*

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showed superior tolerance in *n*-heptanoic acid to *E. coli* BL21(DE3). By constructing part of a previously-designed biocatalytic pathway, we developed *C. glutamicum* synthesizing undec-9-enoic acid, heptyl ester from 12-ketooleic acid [2]. In addition, we established the production of undec-9-enoic acid, heptyl ester from ricinoleic acid using whole-cell *C. glutamicum* expressing a hetrologous gene, Baeyer-Villiger monooxygenase (BVMO) of *Pseudomonas putida* KT2440 and secondary alcohol dehydrogenase (ADH) of *Micrococcus luteus* NCTC2665.

To the best of our knowledge, this is the first report showcasing the production of undec-9-enoic acid, heptyl ester by whole-cell *C. glutamicum*. Undec-9-enoic acid, heptyl ester is not only an intermediate in the previously-designed biocatalytic pathway, but also a high value-added industrial component, which is used in perfume, cosmetic, food, beverage, essential oil, organic solvents, surfactants, and plant protection products [8]. This study investigated the additional developmental potential of microbial sources utilizing renewable fatty acids and plant oils.

2. Material and methods

2.1. Microbial strains and culture media

The nutrient broth used as the growth medium for *C. glutamicum* ATCC 13032 comprised calf-derived brain-heart infusion (BHI) medium. The BHI medium contained 5 g/L beef heart, 12.5 g/L calf brain, 2.5 g/L disodium hydrogen phosphate, 2 g/L D (+)-glucose, 10 g/L peptone, and 5 g/L sodium chloride. A colony of recombinant *C. glutamicum* was inoculated into 3 mL of BHI medium, and cultivated at 30 °C for 12 h. Next, 1 mL of this starter culture was transferred into 100 mL of BHI medium containing 50 μg/mL kanamycin in a 500-mL baffled flask. The flask was then cultivated at 30 °C for 12 h with shaking at 200 rpm *Escherichia coli* strain DH5a (Real Biotech Corporation, Taiwan), which was used for the cloning and amplification of the expression vector, was cultivated in lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) at 37 °C.

2.2. n-Heptanoic acid tolerance assay

The tolerance of *C. glutamicum* ATCC 13032 and *E. coli* BL21(DE3) wild-type strains to *n*-heptanoic acid was assayed by adding *n*-heptanoic acid to the growth medium. The assay was performed in 500-mL baffled flask with 100 mL LB and BHI medium at 30 °C and 200 rpm. When the OD_{600} reached ~0.6, cells were exposed to 2 and 5 mM *n*-heptanoic acid. The control cultures were LB and BHI medium, respectively, without added *n*-heptanoic acid. Samples were taken periodically for the measurement of OD_{600}.

2.3. Gene cloning

The *E. coli- C. glutamicum* shuttle expression vectors were derivatives of pCES208 (KAIST, Daejeon, Korea). The pCES208:BVMO was constructed via polymerase chain reaction (PCR) of the KT2440 of *Pseudomonas putida* KT2440 [9], EthA of *Mycobacterium tuberculosis* [10], AFL210 of *Aspergillus flavus* NRRL3357 [11], AFL456 of *Aspergillus flavus* NRRL3357 [11], AFL838 of *Aspergillus flavus* NRRL3357 [11], and BVMO3 of *Dietzia* sp. D5 independently. Table 1 lists the forward and reverse primers (Macrogen, Inc., Seoul, Korea) used for BVMO gene amplification. *Bam*HI and *Nde*I represent the restriction sites used to clone the gene into pCES vectors. An in-fusion cloning kit (Clontech, USA) was used to ligate amplified BVMO into the pCES vectors. The expression plasmids pCESH5, pCESH17, pCESH28, pCESH36, pCESI16, pCESI64, and pCESL10 were constructed by inserting the *KT2440* gene (GenBank accession code: NP 744949.1) of *P. putida* KT2440 similarly. A two-step bioconversion strain was constructed by ligating *KT2240* gene into the vectors containing the *ADH* gene of *Micrococcus luteus* NCTC2665. All the PCR constructs were confirmed by sequencing (Macrogen, Inc.). The constructed vectors were transformed into *C. glutamicum* by electroporation and heat shock using a Gene Pulser Xcell Electroporator (Bio-Rad, USA) [12].

2.4. Biotransformation in a flask

Biotransformation with recombinant *C. glutamicum* was conducted based on our previous study [8]. In brief, the recombinant *C. glutamicum* strain was cultivated in BHI medium at 30 °C and 200 rpm. Cells were harvested at the stationary growth phase. The cells were washed with 50 mM Tris-HCl (pH 8.0) buffer. Biotransformation was induced by resuspending the cells (9 g/L cell dry weight) in a buffer containing 12-keto-oleic acid, and addition of a detergent. The reactions converting 12-keto-oleic acid to ester were performed in a shaking incubator at 35 °C and 200 rpm. The recombinant *C. glutamicum* strain for two-step bioconversion was constructed similar to the one-step bioconversion except that the reaction was initiated by adding 5.2 mM ricinoleic acid and 0.09 g/L Triton X-100.

2.5. Analytical methods

The possible end products including undec-9-enoic acid were analyzed using 5975 series mass spectrometry and Agilent 7890A gas chromatography as previously described [8]. Prior to the analyses, the whole-cell biotransformation reactions were terminated by acidification to pH 2 with HCl and the products were derivatized with a 3:1 mixture of pyridine:N methyl M (trimethylsilyl) trifluoroacetamide (TMS). The derivatives were separated on a nonpolar capillary column (30 m length, 0.25 μm film thickness; HP-5MS; Agilent Technologies, Palo Alto, CA, USA). Selected ion monitoring was used for the detection and fragmentation analysis of the reaction products.

2.6. Reagents

N-methyl-N-(trimethylsilyl) trifluoroacetamide (TMS) and ricinoleic acid were purchased from TCI (Japan). Ethyl acetate was obtained from Duksan (South Korea). Tween 80 was purchased from Riedel-de Haen (USA). Triton X-100 was supplied by Samchun (South Korea). Palmitic acid, Triton X-114, Tween 20, Tween 40, Span 20, Span 80, and other chemicals, unless otherwise indicated, were obtained from Sigma-Aldrich (USA). The 12-keto-oleic acid and undec-9-enoic acid heptyl ester were isolated in our laboratory.

3. Results

3.1. n-Heptanoic acid tolerance assay

Fig. 1 illustrates the previously designed biotransformation pathway [2]. Ricinoleic acid is converted to 12-keto-oleic acid by ADH, and BVMO catalyzed the oxidation to undec-9-enoic acid, heptyl ester, and hydrolysis of this ester yielded *n*-heptanoic acid and ω-hydroxyundec-9-enoic acid. *E. coli* is weak to *n*-heptanoic acid, which is the final product of the biosynthetic pathway. The toxicity of *n*-heptanoic acid leads to suppression of final product concentration and productivity of *E. coli* [4]. We demonstrated that only 2 mM *n*-heptanoic acid inhibited the *E. coli* growth rate by 66% (Fig. 2). *E. coli* growth was totally suppressed at the concentration of 5 mM *n*-heptanoic acid. However, *C. glutamicum* showed superior tolerance to *n*-heptanoic acid, compared *with E. coli.* Addition of 5 mM *n*-heptanoic acid induced a decrease in specific growth rate of 28%, compared with control. The toxicity of *n*-heptanoic acid significantly affected the growth of *E. coli,* but led to insignificant growth inhibition in *C. glutamicum*.

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