Continuous supply of glucose and glycerol enhances biotransformation of ricinoleic acid to (E)-11-(heptanoyloxy) undec-9-enoic acid in recombinant Escherichia coli

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ABSTRACT

This study aimed at the development of biotransformation strategies with feeding of energy sources for bioconversion of ricinoleic acid to (E)-11-(heptanoyloxy) undec-9-enoic acid (11-HOUA), a key intermediate of brassylic acid, by recombinant Escherichia coli overexpressing an alcohol dehydrogenase from Micrococcus luteus and a Baeyer-Villiger monooxygenase from Pseudomonas putida KT2440. Feeding of glucose or glycerol facilitated both the preparation of high-density cell biocatalyst and supply of the NAD^+ and NADPH cofactors. By the glucose feeding strategy, 30.8 g/L of the engineered E. coli cells produced 29.7 mM of 11-HOUA with 1.9 mM/h of productivity, which were 1.8 and 1.6 times higher than the same biotransformation without the glucose feeding, respectively. Intermittent addition of glycerol increased 11-HOUA productivity by 16% compared to that by the glucose feeding. Finally, 34.5 mM of 11-HOUA concentration, 77% conversion and 2.2 mM/h productivity were obtained using 31.6 g/L of cell biocatalyst along with the glycerol addition. It was concluded that supplementation of additional carbon sources in biotransformation process would be a potent strategy to increase the performance of fatty acid conversion.

1. Introduction

Long-chain fatty acids are abundant natural resources composed of aliphatic tail 13 to 21 carbons, and has gained attractive interest as a starting material for value-added products. For example, ricinoleic acid with eighteen carbons is a major fatty acid in castor oil (Ogunniyi, 2006) and was used for production of unnatural α,ω-dicarboxylic acids such as 1,11-undecanedioic acid (brassylic acid) (Seo et al., 2015) which is able to be utilized to produce commodity products of polyamides and polyester (Kockritz and Martin, 2011; Zhang et al., 2009). Currently, production of α,ω-dicarboxylic acids from petrochemical feedstocks or fatty acids are based on chemical routes (Biermann et al., 2011; Huf et al., 2011). These chemical processes are operated under harsh conditions such as high temperature and pressure, and mediated by toxic reagents (e.g., ozone and Pd(II) catalyst), which are affordable to cause serious problems in terms of safety and environment (Giri and Yu, 2008).

As an alternative, biological routes utilizing microorganisms have been developed (Huf et al., 2011). Recombinant Candida tropicalis without the β-oxidation pathway was able to convert fatty acids to long-chain α,ω-dicarboxylic acids (Eschenfeldt et al., 2003; Lu et al., 2010). Production of 1,11-undecanedioic acid from ricinoleic acid by utilizing recombinant Escherichia coli was also reported (Song et al., 2014). Since conversion of ricinoleic acid to (E)-11-(heptanoyloxy) undec-9-enoic acid (11-HOUA), a key intermediate of 1,11-undecanedioic acid, was considered as a rate-limiting step in the biological process, genetic engineering and process design were adopted for efficient biotransformation of 11-HOUA (Jang et al., 2016; Song et al., 2013). As shown in Fig. 1, expression of two heterologous enzymes in recombinant E. coli should be required for conversion of ricinoleic acid into 11-HOUA: an alcohol dehydrogenase (Adhp) from Micrococcus luteus and a Baeyer-

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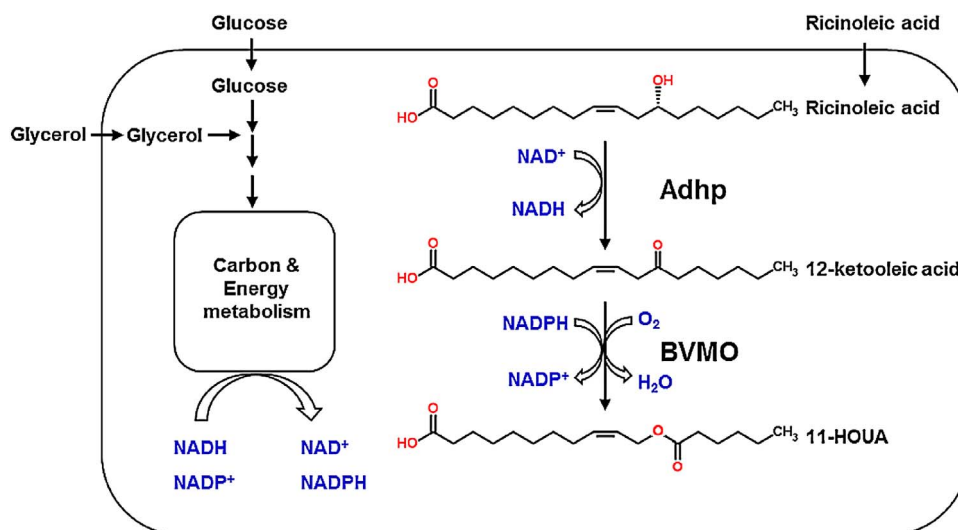 *E-mail address:* ycpark@kookmin.ac.kr (Y.-C. Park).

Fig. 1. Reaction scheme for biotransformation of ricinoleic acid to (*E*)-11-(heptanoyloxy) undec-9-enoic acid (11-HOUA) in recombinant *Escherichia coli* BL21(DE3) expressing an alcohol dehydrogenase (Adhp) from *Micrococcus luteus* and a Baeyer-Villiger monooxygenase (BVMO) from *Pseudomonas putida* KT2440.

Villiger monooxygenase (BVMO) from *Pseudomonas putida* KT2440. Adhp catalyzes hydrogenation reaction from ricinoleic acid to 12-ketooleic acid with an aid of NAD^+ cofactor. 12-Ketooleic acid is further oxidized into 11-HOUA by BVMO using O_2 and NADPH cofactor. In a previous report, whole-cell biotransformation using recombinant *E. coli* expressing *M. luteus* Adhp and *P. putida* KT2440 BVMO resulted in 0.76 mM of 11-HOUA from ricinoleic acid with 76% conversion (Song et al., 2013). While the Adhp was expressed highly and functionally in recombinant *E. coli*, the BVMO was known to be rarely expressed as an active form. To overcome this problem, protein fusion between the Adhp and the BVMO (ADH-Gly-BVMO) was attempted using a glycine-rich peptide as linker, resulted in a more than 40% improvement in 11-HOUA productivity as compared to the separate expression of the two enzymes (Jeon et al., 2015). To increase the final concentration of 11-HOUA, high concentrations of substrate (63 mM of ricinoleic acid) and recombinant cells (20 g/L of dry cell weight) were utilized in biotransformation. As a result, final concentration and productivity of 11-HOUA increased by 5.7- and 3.9-folds, respectively, indicating that whole-cell biotransformation using high density of resting cells contributed to efficient conversion of ricinoleic acid to 11-HOUA (Jang et al., 2016).

In most cases, cell preparation and biotransformation were separated into two processes. After batch or fed-batch cultivation, the resting cells were prepared by concentration or dilution to adjust their concentration, followed by changing fresh reaction medium or conditioning biotransformation environments such as pH and temperature (Jang et al., 2016; Jeon et al., 2015; Song et al., 2013). And biotransformation was carried out without addition of energy sources, causing the deactivation of biotransformation activity of the resting cells because of limited amounts of intracellular cofactors. To overcome these obstacles in biotransformation of ricinoleic acid to 11-HOUA, in this study, high-density cell cultivation stage for biocatalyst preparation was connected to biotransformation stage without change of environmental process parameters, and continuous or intermittent supplementation of energy sources was adopted in the biotransformation stage. Fed-batch cultivation of recombinant *E. coli* overexpressing *M. luteus* Adhp and *P. putida* KT2440 BVMO was carried out to achieve high-density of cell biocatalyst. Without any change of acidity and temperature, biotransformation was initiated in the same bioreactor by addition of IPTG inducer and a high concentration of ricinoleic acid. To investigate the effects of additional energy source supply on 11-HOUA bioconversion, concentrated glucose or glycerol solution was fed continuously or intermittently into the bioreactor in the stage of biotransformation.

2. Materials and methods

2.1. Strains and plasmids

E. coli DH5α and BL21(DE3) (Invitrogen Co., Carlsbad, CA, USA) strains were used as hosts for gene manipulation and 11-HOUA production, respectively. Plasmid pACYC-ADH-BVMO harboring two genes coding for *M. luteus* alcohol dehydrogenase (Adhp) and *P. putida* KT2440 Baeyer-Villiger monooxygenase (BVMO) was provided by Prof. Jin-Byung Park at Ewha Womans University (Seoul, Korea) (Jang et al., 2014) and utilized to produce 11-HOUA. Plasmid pACYC-ADH-BVMO was transformed into *E. coli* BL21(DE3) by the $CaCl_2$ method (Kim et al., 2011). Expression of all recombinant genes was under the control of the *T7* promoter.

2.2. Culture and biotransformation conditions

Recombinant *E. coli* was grown in LB medium (5 g/L yeast extract, 10 g/L bacto-tryptone and 10 g/L NaCl) with appropriate antibiotics for genetic manipulation and seed culture. For pre-culture, *E. coli* was cultured in a 500 mL baffled flask (Nalgene, Rochester, NY, USA) containing 100 mL of defined R medium with 5 g/L yeast extract and 10 g/L glucose at 200 rpm and 37 °C. The R 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 $(NH_4)_6Mo_7O_{24}$) (Kim et al., 2014).

For biotransformation using resting cells in a flask, the recombinant *E. coli* was cultivated in R medium containing 5 g/L yeast extract and 10 g/L glucose at 30 °C and 250 rpm. At the mid-exponential growth phase, isopropyl β-D-1-thiogalactopyranoside (IPTG) at the final concentration of 0.1 mM was added into the culture broth, and culture temperature was changed to 20 °C. After depletion of glucose initially added, the cells were harvested, and the cell pellets were added into a 125 mL-scale baffled flask with 20 mL of fresh R medium. Biotransformation was initiated by addition of ricinoleic acid and tween 80 at the final concentrations of 6.7 mM and 0.1% (v/v), respectively. Initial acidity was set at pH 6.8 or pH 8.0, and agitation speed and temperature were maintained at 250 rpm and 35 °C, respectively, during biotransformation.

To prepare high-density biocatalyst, fed-batch cultivations were performed in a 2.5 L jar bioreactor (Kobiotech, Incheon, Korea) with a 1 L working volume of R medium with 5 g/L yeast extract and 20 g/L glucose. After the depletion of glucose initially added, a feeding