Advanced Synthesis & Catalysis

Accepted Article

Title: Simultaneous Enzyme/Whole-Cell Biotransformation of C18 Ricinoleic Acid into (R)-3-Hydroxynonanoic Acid, 9-Hydroxynonanoic Acid, and 1,9-Nonanedioic Acid

Authors: Hee-Jeong Cha, Eun-Ji Seo, Ji-Won Song, Hye-Jin Jo, Ravi Kumar A, and Jin-Byung Park

To be cited as: *Adv. Synth. Catal.* 10.1002/adsc.201701029

Link to VoR: http://dx.doi.org/10.1002/adsc.201701029

10.1002/adsc.201701029

FULL PAPER

DOI: 10.1002/adsc.201((will be filled in by the editorial staff))

Simultaneous Enzyme/Whole-Cell Biotransformation of C18 Ricinoleic Acid into (*R*)-3-Hydroxynonanoic Acid, 9-Hydroxynonanoic Acid, and 1,9-Nonanedioic Acid

Hee-Jeong Cha[+a], Eun-Ji Seo[+a], Ji-Won Song[a], Hye-Jin Jo[a], Akula Ravi Kumar[b], and Jin-Byung Park [a,c]*

[a] Department of Food Science and Engineering, Ewha Womans University, Seoul 03760, Republic of Korea
Fax: (+82)-2-3277-4213; phone: (+82)-2-3277-4509; e-mail: jbpark06@ewha.ac.kr
[b] Department of Chemistry and Nanoscience, Ewha Womans University, Seoul 03760, Republic of Korea
[c] Institute of Molecular Microbiology and Biosystems Engineering, Ewha Womans University, Seoul 03760, Republic of Korea
[+] H.-J. Cha and E.-J. Seo equally contributed to this work.

Received: ((will be filled in by the editorial staff))

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201######.((Please delete if not appropriate))

Abstract. Regiospecific oxyfunctionalization of renewable long chain fatty acids into industrially relevant C9 carboxylic acids has been investigated. One example was biocatalytic transformation of 10,12-dihydroxyocatadecanoic acid, which was produced from ricinoleic acid ((9*Z*,12*R*)-12-hydroxyoctadec-9-enoic acid) by a fatty acid double bond hydratase, into (*R*)-3-hydroxynonanoic acid, 9-hydroxynonanoic acid, and 1,9-nonanedioic acid with a high conversion yield of ca. 70%. The biotransformation was driven by enzyme/whole-cell biocatalysts, consisting of the esterase of *Pseudomonas fluorescens* and the recombinant *Escherichia coli* expressing the secondary alcohol dehydrogenase of *Micrococcus luteus*, the Baeyer-Villiger monooxygenase of *Pseudomonas putida* KT2440 and the primary alcohol/aldehyde dehydrogenases of *Acinetobacter* sp. NCIMB9871. The high conversion yields and the high product formation rates over 20 U/g dry cells with insoluble reactants indicated that various (poly-hydroxy) fatty acids could be converted into multi-functional products via the simultaneous enzyme/whole-cell biotransformations. This study will contribute to the enzyme-based functionalization of hydrophobic substances.

Keywords: Enzyme catalysis; Oxygenation; Carboxylic acids; Biotransformation; Fatty acids

Introduction

Recent developments in synthetic biology, metabolic engineering, and enzyme engineering enable us to expand the repertory of biocatalysis [1]. For instance, the engineered whole-cell based biocatalysis permitted the production of a variety of chemicals including medium chain ω-hydroxycarboxylic acids, α,ω-dicarboxylic acids, and ω-aminocarboxylic acids from renewable oils and fatty acids [2]. Here, we have included another type of biocatalysis to provide industrially relevant C9 carboxylic acids (i.e., (*R*)-3-hydroxynonanoic acid (**5**), 9-hydroxynonanoic acid (**6**), and 1,9-nonanedioic acid (**8**)) from renewable long chain fatty acids (i.e., ricinoleic acid (**1**)) (Scheme S1) to a high conversion yield. 9-Hydroxynonanoic acid and 1,9-nonanedioic acid, which are used as monomers for the production of polyesters and polyamides [3], are currently manufactured via ozonolysis of oleic acid [4] [3c]. 3-Hydroxynonanoic acid, which can be used as an antimicrobial agent [5] and a building block for the synthesis of polyhydroxyalkanoates [6] [7] and rhamnolipids [8], is difficult to produce by chemical means.

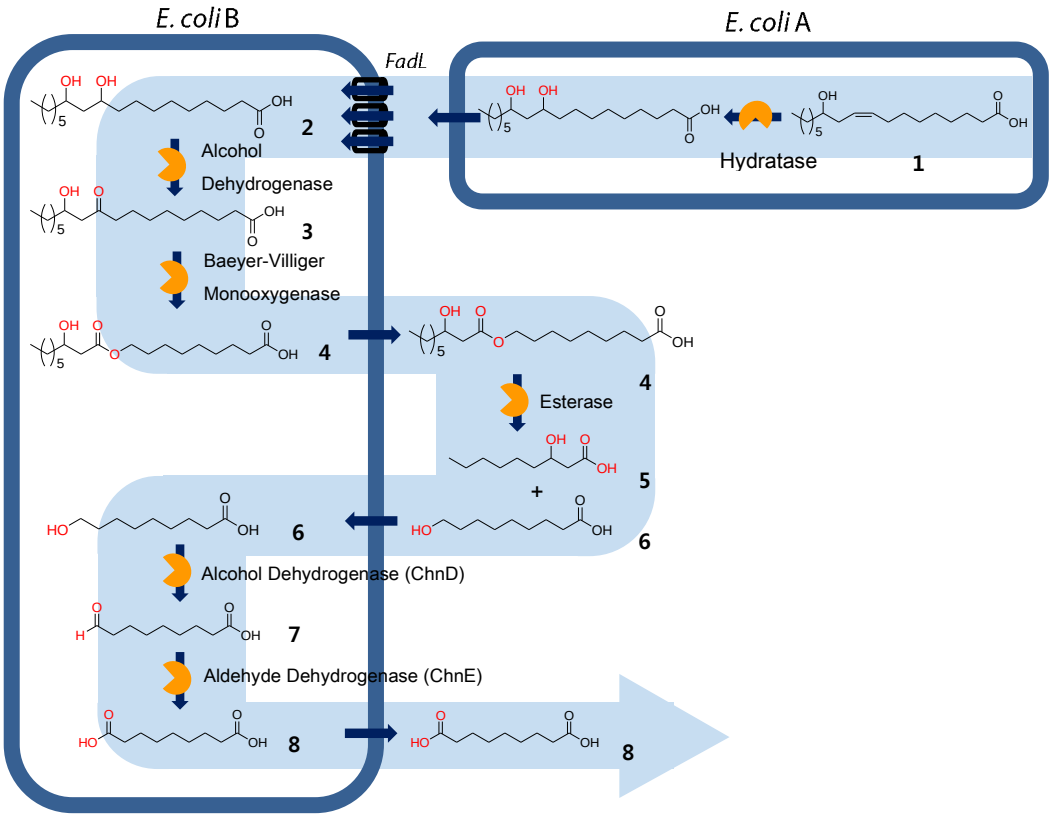
According to previous studies [2a,2e], oleic acid could be converted into n-nonanoic acid and 9-hydroxynonanoic acid by multi-step enzyme cascade reactions; oleic acid was transformed into an ester (i.e., 9-(nonanoyloxy)-nonanoic acid) via 10-hydroxyoctadecanoic acid and 10-ketooctadecanoic acid by a fatty acid double bond hydratase, a long chain secondary alcohol dehydrogenase, and a

Baeyer-Villiger monooxygenase (BVMO). The ester was then hydrolyzed into n-nonanoic acid and 9-hydroxynonanoic acid by a lipase. Alternatively, the ester was converted into n-nonanoic acid and 9-hydroxynonanoic acid or 1,9-nonanedioic acid by chemical reactions [2a,9].

In the present study, the biocatalytic system to produce the C9 carboxylic acids was established on a basis of a fatty acid double bond hydratase, a long chain secondary alcohol dehydrogenase (SADH), a BVMO, an esterase, a primary alcohol dehydrogenase (PADH), and an aldehyde dehydrogenase (AlDH) (Scheme 1). The nicotinamide cofactor-dependent enzymes (i.e., SADH, BVMO, PADH, and AlDH) have been expressed in *Escherichia coli* cells, while cofactor-independent enzymes (i.e., esterases) were directly added into the reaction medium to obtain high product formation rates per whole-cell biocatalyst. The long chain fatty acid transporter FadL [10] was also overexpressed in the outer membrane of the *E. coli* to facilitate mass transport of the insoluble reactants. In theory, the SADH and BVMO reactions will occur inside cells. The reaction products (**4**) will be excreted out of the cells and subjected to hydrolysis by the extracellular esterases (Scheme 1). The hydrolysis products (**6**) will enter into the *E. coli* cells and be further oxidized to 1,9-nonanedioic acid (**8**) by the intracellular PADH and AlDH. Ultimately, 3-hydroxynonanoic acid (**5**) and 1,9-nonanedioic acid (**8**) will accumulate in the reaction medium.

Results and Discussion

The first step for the biotransformation of ricinoleic acid into 3-hydroxynonanoic acid (**5**) and 9-hydroxynonanoic acid (**6**) (Scheme S1) was to discover suitable enzymes for the cascade reactions. Hydration of ricinoleic acid (**1**) into 10,12-dihydroxy-octadecanoic acid (**2**) has been reported in an earlier study [11]. However, the other steps were not described yet. The long chain secondary alcohol dehydrogenase of *Micrococcus luteus* NCTC2665 [2a,2e] was examined for oxidation of 10,12-dihydroxyoctadecanoic acid (**2**). The enzyme was able to oxidize the substrate into the target compound (**3**) with high regioselectivity (Fig. S1). The BVMOs from *Pseudomonas putida* KT2440 [12], *Pseudomonas fluorescens* DSM 50106 [13], and *Rhodococcus josti* [14], which were reported to catalyze oxygenation of long chain keto-fatty acids into esters [2a,2e], were investigated with 10-oxo-12-hydroxyoctadecanoic acid (**3**) as the substrate (Fig. S2). Remarkably, the BVMO from *P. putida* KT2440 catalyzed the Baeyer-Villiger oxygenation of 10-oxo-12-hydroxyoctadeca-noic acid (**3**) into the ester (**4**) (Fig. S2), whereas the BVMOs from *P. fluorescens* DSM 50106 and *R. josti* were not active with the compound (**3**). A lipase from *Thermomyces lanuginosus*, which was active with 9-(nonanoyloxy)-nonanoic acid [2e], was not so active in hydrolysis of the BVMO reaction product (i.e., 9-(12-hydroxynonanoyloxy)-nonanoic acid (**4**)). However, an esterase from *P. fluorescens* SIK WI, which was shown to hydrolyze long chain linear esters (e.g., *n*-



Scheme 1. Designed biotransformation pathway. Ricinoleic acid (**1**) is converted into (*R*)-3-hydroxynonanoic acid (**5**) and 1,9-nonanedioic acid (**8**) via 9-hydroxynonanoic acid (**6**).

10.1002/adsc.201701029

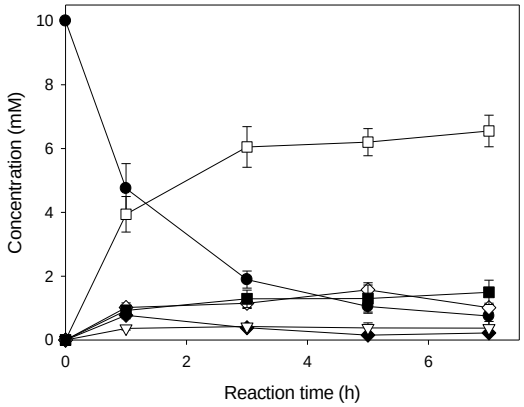


Figure 1. Time course of the biotransformation of ricinoleic acid by the recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL-OhyA, pJOE-E6BVMO, expressing a fatty acid double bond hydratase (OhyA) of *Stenotrophomonas maltophilia*, an alcohol dehydrogenase (ADH) of *Micrococcus luteus*, the engineered BVMO from *Pseudomonas putida* KT2440 (E6BVMO), and a long chain fatty acid transporter (FadL). The biotransformation was initiated by adding 15 mM ricinoleic acid and 0.5 g/L Tween80 at t = 0 into the recombinant *E. coli* culture broth (cell density: 3 g dry cells/L). Symbols indicate the concentrations of ricinoleic acid (**1**) (●), 10,12-dihydroxyoctadecanoic acid (**2**) (◆), 10-keto-12-hydroxy-octadecanoic acid (**3**) (▽), ester **4** (■), 12-ketooctadec-9-enoic acid (**9**) (◇), and ester **10** (□). The error bars indicate standard deviations.

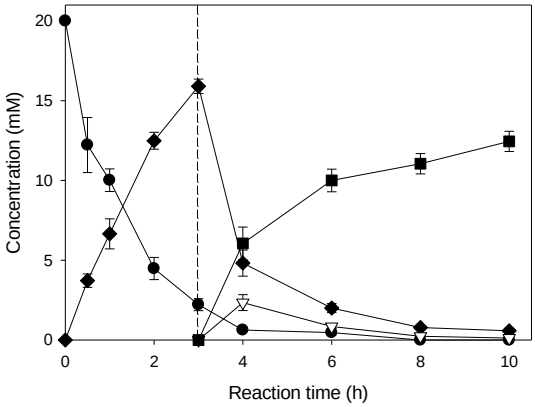


Figure 2. Time course of the biotransformation of ricinoleic acid into the ester (**4**) by two-whole cell systems, consisting of the recombinant *E. coli* BL21(DE3) pET-OhyA and the recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL, pJOE-E6BVMO. The recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL, pJOE-E6BVMO was added into the culture medium of the recombinant *E. coli* BL21(DE3) pET-OhyA after over 90% of the starting material (i.e., ricinoleic acid) was transformed into the dihydroxy fatty acid (**2**) (final cell density: 3 g dry cells/L). Symbols indicate the concentrations of ricinoleic acid (**1**) (●), 10,12-dihydroxyoctadecanoic acid (**2**) (◆), 10-keto-12-hydroxyoctadecanoic acid (**3**) (▽), and ester **4** (■). The error bars indicate standard deviations.

octylacetate) [15], turned out to be quite active with the ester (**4**) (Fig. S3).

The first trial to produce 3-hydroxynonanoic acid (**5**) and 9-hydroxynonanoic acid (**6**) from ricinoleic acid (**1**) (Scheme 1) was to use the recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL-OhyA, pJOE-E6BVMO as a biocatalyst based on our previous study [2e]. The recombinant strain expresses not only the fatty acid double bond hydratase of *Stenotrophomonas maltophilia* [16], the SADH of *M. luteus*, and the BVMO of *P. putida* KT2440 in the cytoplasm for the multi-step biotransformation of ricinoleic acid (**1**) into the ester (**4**), but also the long chain fatty acid transporter FadL in the outer membrane for the efficient substrate transport into the cascade enzymes. The whole-cell biotransformation of ricinoleic acid produced the ester (**10**) (Scheme S2) as the major product rather than the ester (**4**) targeted (Fig. 1). This was due to greater activity of the SADH of *M. luteus* with ricinoleic acid as compared to the fatty acid hydratase of *S. maltophilia* in the cytoplasm of *E. coli*.

Our strategy has been changed to use a serial combination of two-whole cell systems, consisting of the recombinant *E. coli* BL21(DE3) pET-OhyA for the biotransformation of ricinoleic acid into 10,12-dihydroxyoctadecanoic acid (**2**) and the recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL, pJOE-E6BVMO for the conversion of the dihydroxy fatty acid (**2**) into the ester (**4**) (Scheme 1). The recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL, pJOE-E6BVMO was added into the culture medium of the recombinant *E. coli* BL21(DE3) pET-OhyA just after approximately 90% of the starting material (i.e., ricinoleic acid) was transformed into the dihydroxy fatty acid (**2**) (final cell density: 3 g dry cells/L) (Fig 2). Ricinoleic acid was ultimately converted into the ester (**4**) via 10,12-dihydroxyoctadecanoic acid (**2**) and 10-oxo-12-hydroxyoctadecanoic acid (**3**) to a final concentration of 12.5 mM in the culture medium. The resulting ester product (**4**) was hydrolyzed into 3-hydroxynonanoic acid (**5**) and 9-hydroxynonanoic acid (**6**) (Scheme 1) by adding the esterase from *P. fluorescens* SIK WI into the reaction medium (Fig. S4), because the ester compound (**4**) was excreted out of the cells. Approximately 85% of the reaction intermediates including **3** and **4** were found in the extracellular space.

Overall, the results indicated that the industrially relevant C9 carboxylic acids could be produced from C18 ricinoleic acid by the serial reactions of whole-cell and enzyme catalysts.

The next experiment was focused on the production of the ester (**4**) to a high concentration in the reaction medium. This was exploited by using high cell density cultivation of the recombinant *E. coli* BL21(DE3) pET-OhyA and the recombinant *E. coli* BL21(DE3) pAPTm-E6BVMO-ADH, pCon-FadL on a basis of our previous studies [2e,17]. The first step was the fed-batch cultivation of the recombinant *E. coli* BL21(DE3) pET-OhyA to a cell density of 10 g dry cells/L. The whole-cell biotransformation of ricinoleic acid into 10,12-dihydroxyoctadecanoic acid

10.1002/adsc.201701029

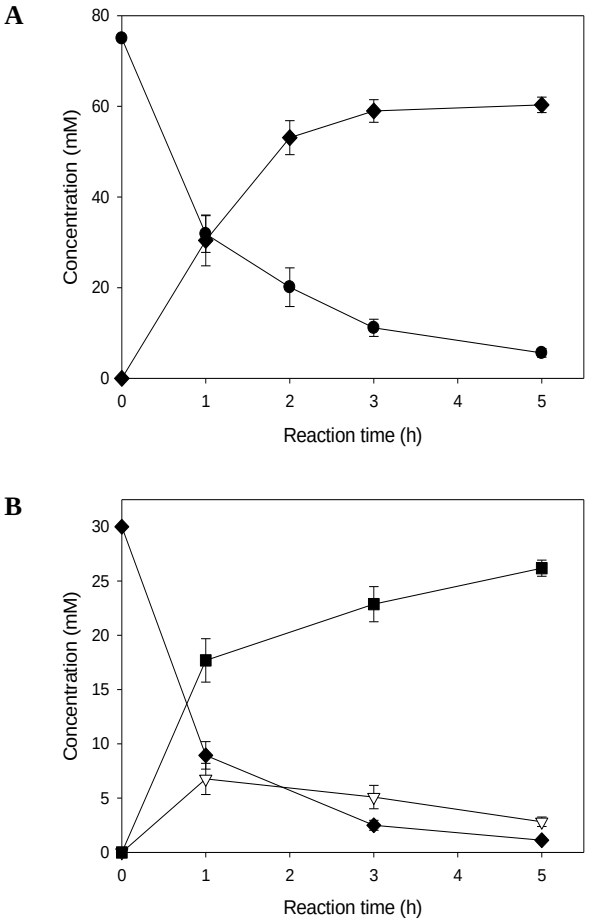


Figure 3. Time course of the biotransformation of ricinoleic acid at high cell density cultures. The biotransformation of ricinoleic acid into 10,12-dihydroxyoctadecanoic acid (**2**) was conducted by adding 70 mM ricinoleic acid into the recombinant *E. coli* BL21(DE3) pET-OhyA culture (A). Afterwards, the resulting reaction mixture was transferred into the culture of the recombinant *E. coli* BL21(DE3) pAPTm-E6BVMO-ADH, pCon-FadL (final cell density: 10 g dry cells/L) (B). Symbols indicate the concentrations of ricinoleic acid (**1**) (●), 10,12-dihydroxyoctadecanoic acid (**2**) (◆), 10-keto-12-hydroxyoctadecanoic acid (**3**) (▽), and ester **4** (■). The error bars indicate standard deviations.

(**2**) was conducted by adding 70 mM ricinoleic acid into the recombinant *E. coli* culture (Fig. 3A). 10,12-Dihydroxyoctadecanoic acid (**2**) was produced to 60 mM in the medium at t = 3 h. The resulting reaction mixture was then added into the culture broth of the recombinant *E. coli* BL21(DE3) pAPTm-E6BVMO-ADH, pCon-FadL (final cell concentration: 10 g dry cells/L). The dihydroxy fatty acid (**2**) was converted into the ester (**4**) to a concentration of 25 mM (8.3 g/L) in the culture medium with the volumetric productivity of 8.3 mM/h (2.8 g/L/h) (Fig. 3B). The molar conversion yield from ricinoleic acid, which was estimated by GC/MS analysis of the reactants, reached 68%. The isolation yield of the ester (**4**) from the reaction medium was ca. 70%; 320 mg ester (purity > 90%) was obtained as a colorless solid from 50 mL reaction medium containing 410 mg (1.24 mmol) ester (Fig. S5). These results indicated that the ester could be produced to a high concentration with a high productivity and a high product yield.

We have next investigated the biotransformation of 10,12-dihydroxyoctadecanoic acid (**2**) into 3-hydroxynonanoic acid (**5**) and 1,9-nonanedioic acid (**8**) (Scheme 1). This was carried out by using the alcohol/aldehyde dehydrogenases (ChnDE) of *Acinetobacter* sp. NCIMB9871 [18] in addition to the SADH of *M. luteus*, the BVMO of *P. putida* KT2440, and the esterase of *P. fluorescens*. The alcohol/aldehyde dehydrogenases of *Acinetobacter*sp. NCIMB9871 was chosen because the enzymes have shown the much greater activity with 9-hydroxynonanoic acid (**6**) as compared to the PADH (AlkJ) of *P. putida* GPo1[19], which had been used for oxidation of 9-hydroxynonanoic acid in our earlier study [2b] (Fig. S6). The biotransformation of the dihydroxy fatty acid (**2**) into 3-hydroxynonanoic acid (**5**) and 1,9-nonanedioic acid (**8**) was conducted by the recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL, pJOE-E6BVMO, pCOLA-ChnDE and the esterase of *P. fluorescens* (Scheme 1). The esterase was not expressed in cytoplasm of *E. coli* but just added into reaction medium, because the ester product (**4**) was secreted into extracellular space before hydrolysis into **5** and **6** by esterases in the cytoplasm.

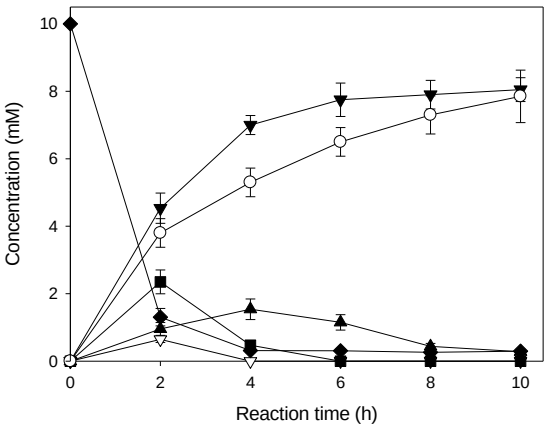


Figure 4. Time course of the biotransformation of 10,12-dihydroxy-octadecanoic acid (**2**) into 3-hydroxynonanoic acid (**5**) and 1,9-nonanedioic acid (**8**) by the recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL, pJOE-E6BVMO, pCOLA-ChnDE, expressing the ADH of *M. luteus*, the E6BVMO of *P. putida* KT2440, the alcohol/aldehyde dehydrogenases (ChnDE) of *Acinetobacter* sp. NCIMB9871, and a long chain fatty acid transporter (FadL). The biotransformation was initiated by adding 10 mM 10,12-dihydroxyoctadecanoic acid (**2**) and 0.5 g/L Tween80 at t = 0 into the recombinant *E. coli* culture broth (cell density: 3 g dry cells/L). Symbols indicate the concentrations of 10,12-dihydroxy-octadecanoic acid (**2**) (◆), 10-keto-12-hydroxyocta-decanoic acid (**3**) (▽), ester **4** (■), 3-hydroxynonanoic acid (**5**) (▼), 9-hydroxynonanoic acid (**6**) (▲), and 1,9-nonanedioic acid (**8**) (○). The error bars indicate standard deviations.

10.1002/adsc.201701029

Table 1. Comparison of the specific whole-cell biotransformation rates of fatty acids

	Biotransformation of **2** to ester (**4**) [a)]	Biotransformation of **6** to **8** [a)]	Biotransformation of ricinoleic acid into esters [b)]
Substrate concentrations (mM)	10 to 1.4 (16 to 4.8)	0 to 1.0	10 to 4.5
Specific whole-cell biotransformation rates (U/g dry cells) [c)]	37 (33)	21	27

a) The biotransformation shown in Fig 4. The numbers in the parenthesis indicate the values obtained from the experiment shown in Fig. 2.

b) The biotransformation of ricinoleic acid into esters shown in Fig. 1.

c) The specific whole-cell biotransformation rates were estimated with the cell concentrations and the initial product formation rates at t = < 1 h, which were measured by GC/MS analysis.

In more detail, the whole-cell biotransformation was initiated by adding the dihydroxy fatty acid (**2**) and the esterase of *P. fluorescens* into culture medium of the recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL, pJOE-E6BVMO, pCOLA-ChnDE (Fig. 4). The dihydroxy fatty acid (**2**) was transformed into 3-hydroxynonanoic acid (**5**) and 1,9-nonanedioic acid (**8**) to over 20 U/g dry cells (Table 1). The molar conversion yield, which was estimated by GC/MS analysis of the reactants, reached approximately 80%.

The isolation yield of 3-hydroxynonanoic acid (**5**) and 1,9- nonanedioic acid (**8**) was ca. 70%, respectively; 220 mg of 3-hydroxynonanoic acid and 240 mg of 1,9-nonedioic acid (purity > 90%) were obtained as a colorless solid from 200 mL reaction medium containing 282 mg (1.6 mmol) of 3-hydroxynonanoic acid and 308 mg (1.6 mmol) of 1,9-nonedioic acid (Fig. S7 and S8). These results indicated that the ester (**4**), which had been produced in the cytoplasm of the recombinant *E. coli* BL21(DE3) pACYC-ADH-FadL, pJOE-E6BVMO, pCOLA-ChnDE, was excreted out of cells and then converted into 3-hydroxynonanoic acid (**5**) and 9-hydroxynonanoic acid (**6**) by the esterase of *P. fluorescens* (Scheme 1). Afterwards, 9-hydroxynonanoic acid (**6**) entered into the cytoplasm of the recombinant *E. coli* BL21(DE3) and then oxidized into 1,9-nonanedioic acid (**8**) via 9-oxo-nonanoic acid (**7**) by ChnDE in the cytoplasm. The high product formation rates over 20 U/g dry cells (Table 1) pointed to the great performance of the simultaneous enzyme/whole-cell biocatalsysis.

The dihydroxy fatty acid (**2**) and other reaction intermediates are almost insoluble in the aqueous cultivation medium. They are present in a solid form in the culture medium, suggesting that mass transport can be problematic during the simultaneous enzyme/whole-cell biotransformation. However, the specific whole-cell biotransformation rates were comparable to those with liquid substrates (e.g., ricinoleic acid and its derivatives [17a] [20]) (Table 1). Therefore, it was assumed that solid-type fatty acid substrates could be also good substrates for the complex biotransformations.

Conclusion

The novel enzyme cascades (Scheme 1) including newly developed enzymes (e.g., ChnDE) enabled us to produce the industrially relevant C9 carboxylic acids (i.e., 3-hydroxynonanoic acid, 9-hydroxynonanoic acid, and 1,9-nonanedioic acid) from the renewable long chain fatty acid (i.e., ricinoleic acid) with a high product yield. Furthermore, the high specific whole-cell biotransformation rates with the insoluble reactants in a solid form indicated that solid-type fatty acids could be good substrates even for the complex biotransformations like the simultaneous enzyme/whole-cell biotransformation of dihydroxy fatty acid. This study can be used for the biotransformation of poly-hydroxy fatty acids such as 10,13-dihydroxyoctadecanoic acid [2d,21], 7,8-dihydroxy-9,12-octadecadienoic acid [22], 7, 10-dihydroxy-8-octadecenoic acid [23], and 5,8-dihydroxy-9,12,15-octadecatrienoic acid [24].

Experimental Section

Microbial strains and culture media

Recombinant *E. coli* BL21(DE3) cells were grown in Lysogeny broth (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with appropriate antibiotics (Table S1) for seed cultivation. Riesenberg medium [25] 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 into the cultivation medium. The recombinant genes in the pAPTm-E6BVMO-ADH was constitutively expressed by the synthetic promoter (J23100 (http://parts.igem.org/Part:BBa_J23100)).

Chemicals and materials

Ricinoleic acid, rhamnose, and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (TMS) were purchased from Tokyo Chemical Co (Tokyo, Japan). Glucose was obtained from Junsei Chemical Co (Tokyo, Japan). Antibiotics, trace elements for cell cultivation, IPTG, and Tween80 were purchased from Sigma (St. Louis, MO, USA). Ethyl acetate was obtained from Duksan Pure Chemical Co. (Ansan, Republic of Korea).

Gene cloning

For construction of pCOLA-ChnDE, genomic DNA was extracted from *Acinetobacter johnsonii* NCIMB 9871 grown in LB medium using the Qiagen Genomic DNA Purification kit (Valencia, CA, USA). The *chnD* gene (GenBank accession code: BAC80217.1) was amplified from genomic DNA by using primer 1 & 2 and sub-cloned into the *Nde*I and *Bgl*II site of pCOLAduet vector resulting in pCOLA-ChnD. Afterward, the *chnE* gene (GenBank accession code: BAA86294.1) was amplified from genomic DNA by using primer 3 & 4 and inserted into *EcoR*I and *Sal*I site of pCOLA-ChnD resulting in pCOLA-ChnDE. For constructio of pCon-FadL, the full length fadL gene (GenBank accession code: CAQ32746.1) was PCR-amplified by using pACYC-ADH-FadL as template and primer 5 & 6 and inserted into *BamH*I and *Eco88*I site of pCon plasmid with p15A origin and synthetic promoter (J23100). The plasmids and primers used in this study are presented in Table S1 and S2.

Whole-cell biotransformation

Biotransformation was carried out as reported previously [2a,2e]. Briefly, the biotransformation was initiated at the stationary growth phase (cell density: 3 g dry cells/L), usually 8 h after inducing gene expression with 0.1 mM IPTG and/or 2 g/L rhamnose. After changing the culture broth pH to 8.0 and shift of culture temperature to 35°C, ricinoleic acid (**1**) or 10,12-dihdyroxyoctadecanoic acid (**2**) and 0.5 g/L Tween80 were added to the culture medium. Cell cultivation and biotransformation were carried out in a 250 mL flask (working volume: 20 mL) in a shaking incubator (200 rpm). The high cell density culture of recombinant *E. coli* was conducted in a 1 L scale reactor (Biotron, Bucheon, Korea), as previously reported [17a]. Agitation speed and aeration rate were 400 to 1000 rpm and 1 vvm, respectively, to avoid oxygen limitation.

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

The concentrations of the remaining fatty acids and accumulating carboxylic acids in the medium (e.g., ricinoleic acid (**1**), 3-hydroxynonanoic acid (**5**), 9-hydroxynonanoic acid (**6**), 1,9-nonedioic acid (**8**)) were determined as described previously [2a,2e]. The reaction medium was mixed with an equal volume of ethyl acetate containing 0.5 g/L methyl palmitate as an internal standard. The organic phase was harvested after vigorous vortexing and subjected to derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide. The trimethylsilyl 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, 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. 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 detection and the fragmentation analysis of the reaction products.

Isolation of the ester (4) after biotransformation

The ester intermediate was isolated by simple extraction with ethyl acetate, based on our earlier studies [9,20]. The combined organic layer was washed with saline, dried over anhydrous Na_2SO_4, filtered, and evaporated *in vacuo*. The crude ester product was purified by silica gel column chromatography using 20% ethyl acetate-hexane as the eluent. The isolation/purification yield of the ester (**4**) was over 70%, based on the concentration of the ester intermediate after whole-cell biotransformation. The purified ester (320 mg) was a colorless solid; Mp 46-48°C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.86 (broad, COOH), 4.64 (broad, OH), 3.98-4.02 (2H, m), 3.79-3.81 (1H, m), 2.20-2.34 (2H, m), 2.17 (2H, m), 1.48-1.58 (4H, m), 1.18-1.26 (20H, m), 0.82-0.87 (3H, m).^{13}C NMR (125 MHz; DMSO-d_6) δ 174.40 (COOH), 171.32 (-COOR), 67.09 (C-OH), 63.57 (H_2C-OH), 42.62, 36.86, 33.65, 31.29, 28.71, 28.66, 28.54, 28.50, 28.14, 25.36, 24.98, 24.47, 22.07 (CH_3) (Fig. S5).

Isolation of 3-hydroxynonanoic acid (5) and 1,9-nonanedioic acid (8) after biotransformation

3-Hydroxynonanoic acid (**5**) and 1,9-nonedioic acid (**8**) were isolated from the biotransformation medium by simple extraction with ethyl acetate on a basis of our previous studies [9,20]. The combined organic layer was washed with saline, dried over sodium sulfate, filtered, and evaporated *in vacuo*. The crude compounds were separated and purified by column chromatography (silica gel) eluting with varying concentration of ethyl acetate-hexane. 3-Hydroxynonanoic acid (**5**) and 1,9-nonedioic acid (**8**) were recovered when 25% and 30% ethyl acetate-hexane, respectively, was used as the eluent. The isolation/purification yield of 3-hydroxynonanoic acid (**5**) and 1,9-nonedioic acid (**8**) were both over 70%, based on the concentrations of the compounds after enzyme/whole-cell biotransformation. The purified products (220 mg of 3-hydroxynonanoic acid and 240 mg of 1,9-nonedioic acid) were colorless solids. The structure of 1,9-nonedioic acid (**8**) was thoroughly characterized by GC/MS analysis using the authentic compound, which was purchased from Sigma (Fig. S8); Mp 109–110°C (lit.[9] Mp 109–110°C). The structure of 3-hydroxynonanoic acid (**5**) was characterized by GC/MS and NMR analysis. 3-Hydroxynonanoic acid (**5**): Mp 53-55°C; ^{1}H NMR (500 MHz; DMSO-d_6) δ 3.80-3.78 (m, 1H), 2.26 (dd, 1H, J= 15, 5 Hz), 2.21(dd, 1H, J= 15, 10 Hz), 1.34-1.32 (m, 4H), 1.27-1.20 (m, 6H), 0.85 (t, *J*=15 Hz, 3H); ^{13}C NMR (125 MHz; DMSO-d_6) δ 173.03 (COOH), 67.1 (C-OH), 42.72, 36.96, 31.34, 28.76, 25.06, 22.10, 13.95 (Fig. S7).

Acknowledgements

This study was supported by the Korea Research Foundation (KRF) grant funded by the Korea government and the Marine Biomaterials Research Center grant from the Marine Biotechnology Program funded by the Ministry of Oceans and Fisheries, Republic of Korea (No. D11013214H480000100).

References

[1] a) U. T. Bornscheuer, *Annu. Rev. Food Sci. Technol.* **2018**, *9(1), to be published*; b) M. Bucko, P. Gemeiner, A. Schenkmayerova, T. Krajcovic, F. Rudroff, M. D. Mihovilovic, *Appl. Microbiol. Biotechnol.* **2016**, *100(15)*, 6585-6599; c) J. H. Seo, S. M. Lee, J. Lee, J. B. Park, *J. Biotechnol.* **2015**, *216*, 158-166; d) J. H. Schrittwieser, S. Velikogne, M. Hall, W. Kroutil, *Chem. Rev.* **2017**, *DOI: 10.1021/acs.chemrev.7b00033* ; e) X. Chen, C. Gao, L. Guo, G. Hu, Q. Luo, J. Liu, J. Nielsen, J. Chen, L. Liu, *Chem. Rev.* **2017**, *DOI: 10.1021/acs.chemrev.6b00804*; f) M. J. Smanski, H. Zhou, J. Claesen, B. Shen, M. A. Fischbach, C. A. Voigt, *Nature Rev. Microbiol.* **2016**, *14*, 135-149.

[2] a) J. W. Song, E. Y. Jeon, D. H. Song, H. Y. Jang, U. T. Bornscheuer, D. K. Oh, J. B. Park, *Angew. Chem. Int. Ed.* **2013**, *52*, 2534-2537; b) J. W. Song, J. H. Lee, U. T. Bornscheuer, J. B. Park, *Adv. Synth. Catal.* **2014**, *356*, 1782-1788; c) H. Y. Jang, E. Y. Jeon, A. H. Baek, S. M. Lee, J. B. Park, *Process Biochem.* **2014**, *49*, 617-622; d) H. J. Oh, S. U. Kim, J. W. Song, J. H. Lee, W. R. Kang, Y. S. Jo, K. R. Kim, U. T. Bornscheuer, D. K. Oh, J. B. Park, *Adv. Synth. Catal.* **2015**, *357*, 408-416; e) E. Y. Jeon, J. H. Seo, W. R. Kang, M. J. Kim, J. H. Lee, D. K. Oh, J. B. Park, *ACS Catal.* **2016**, *6*, 7547-7553.

[3] a) B. Cornils, P. Lappe, in *Ullmann's Encyclopedia of Industrial Chemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, **2000**; b) K. Kroha, *INFORM -CHAMPAIGN-* **2004**, *15*, 568-571; c) A. Kockritz, A. Martin, *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 83-91.

[4] U. Schorken, P. Kempers, *Eur. J. Lipid Sci. Technol.* **2009**, *111*, 627-645.

[5] J. Sjögren, J. Magnusson, A. Broberg, J. Schnürer, L. Kenne, *Appl. Environ. Microbiol.* **2003**, *69*, 7554-7557.

[6] V. Mittendorf, E. J. Robertson, R. M. Leech, N. Krüger, A. Steinbüchel, Y. Poirier, *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13397-13402.

[7] X. Jiang, Z. Sun, R. H. Marchessault, J. A. Ramsay, B. A. Ramsay, *Biomacromolecules* **2012**, *13*, 2926-2932.

[8] a) K. K. Sekhon Randhawa, P. K. S. M. Rahman, *Front. Microbiol.* **2014**, *5*, 454; b) A. Wittgens, T. Tiso, T. T. Arndt, P. Wenk, J. Hemmerich, C. Müller, R. Wichmann, B. Küpper, M. Zwick, S. Wilhelm, R. Hausmann, C. Syldatk, F. Rosenau, L. M. Blank, *Microb. Cell Fact.* **2011**, *10*, 80-80.

[9] S. Koppireddi, J. H. Seo, E. Y. Jeon, P. S. Chowdhury, H. Y. Jang, J. B. Park, Y. U. Kwon, *Adv. Synth. Catal.* **2016**, *358*, 3084-3092.

[10] B. van den Berg, *Curr. Opin. Struct. Biol.* **2005**, *15*, 401-407.

[11] M. H. Seo, K. R. Kim, D. K. Oh, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 8987-8995.

[12] J. Rehdorf, A. Kirschner, U. T. Bornscheuer, *Biotechnol. Lett.* **2007**, *29*, 1393-1398.

[13] A. Kirschner, J. Altenbuchner, U. T. Bornscheuer, *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1065-1072.

[14] C. Szolkowy, L. D. Eltis, N. C. Bruce, G. Grogan, *Chembiochem* **2009**, *10*, 1208-1217.

[15]a) Y. A. Lee, E. Y. Jeon, S. M. Lee, U. T. Bornscheuer, J. B. Park, *Process Biochem.* **2014**, *49*, 2101-2106; b) N. Krebsfanger, F. Zocher, J. Altenbuchner, U. T. Bornscheuer, *Enzyme Microb. Technol.* **1998**, *22*, 641-646.

[16] a) Y. C. Joo, E. S. Seo, Y. S. Kim, K. R. Kim, J. B. Park, D. K. Oh, *J. Biotechnol.* **2012**, *158*, 17-23; b) E. Y. Jeon, J. H. Lee, K. M. Yang, Y. C. Joo, D. K. Oh, J. B. Park, *Process Biochem.* **2012**, *47*, 941-947.

[17] a) J. H. Seo, H. H. Kim, E. Y. Jeon, Y. H. Song, C. S. Shin, J. B. Park, *Sci. Rep.* **2016**, *6*, 28223.

[18] Q. Cheng, S. M. Thomas, K. Kostichka, J. R. Valentine, V. Nagarajan, *J. Bacteriol.* **2000**, *182*, 4744-4751.

[19] L. Kirmair, A. Skerra, *Appl. Environ. Microbiol.* **2014**, *80*, 2468-2477.

[20] H. Y. Jang, K. Singha, H. H. Kim, Y. U. Kwon, J. B. Park, *Green Chem.* **2016**, *18*, 1089-1095.

[21]K. R. Kim, H. J. Oh, C. S. Park, S. H. Hong, J. Y. Park, D. K. Oh, *Biotechnol. Bioeng.* **2015**, *112*, 2206-2213.

[22] M. J. Seo, W. R. Kang, K. C. Shin, D. K. Oh, *J. Agric. Food Chem.* **2016**, *64*, 8555-8562.

[23] J. H. Bae, D. S. Kim, M. J. Suh, S. R. Oh, I. J. Lee, S. C. Kang, C. T. Hou, H. R. Kim, *Appl. Microbiol. Biotechnol.* **2007**, *75*, 435-440.

[24] M. J. Seo, K. C. Shin, Y. J. Jeong, D. K. Oh, *J. Am. Oil Chem. Soc.* **2015**, *92*, 193-202.

[25] D. Riesenberg, *Curr. Opin. Biotechnol.* **1991**, *2*, 380-384.

[26] K. L. Rinehart, S. I. Goldberg, C. L. Tarimu, T. P. Culbertson, *J. Am. Chem. Soc.* **1961**, *83*, 225-231.

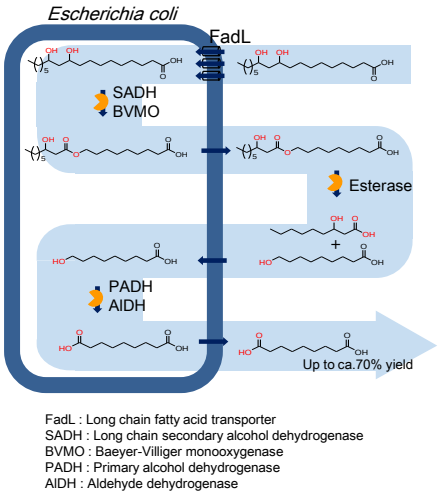
10.1002/adsc.201701029

FULL PAPER

Simultaneous Enzyme/Whole-Cell Biotransformation of C18 Ricinoleic Acid into (*R*)-3-Hydroxynonanoic Acid, 9-Hydroxynonanoic Acid, and 1,9-Nonanedioic Acid

Adv. Synth. Catal. **Year**, *Volume*, Page – Page

Hee-Jeong Cha, Eun-Ji Seo, Ji-Won Song, Hye-Jin Jo, Akula Ravi Kumar, and Jin-Byung Park*



Regiospecific oxyfunctionalization of renewable long chain fatty acids into industrially relevant C9 carboxylic acids was examined. (*R*)-3-Hydroxynonanoic acid, 9-hydroxynonanoic acid and 1,9-nonanedioic acid for polyesters and polyamides could be produced to a high yield from C18 ricinoleic acid by simultaneous enzyme/whole cell biocatalysis.