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Production of a Novel 9,12-Dihydroxy-10(*E*)-eicosenoic Acid from Eicosenoic Acid by *Pseudomonas aeruginosa* PR3

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ABSTRACT: Microbial conversions of unsaturated fatty acids often generate polyhydroxy fatty acids, giving them new properties such as higher viscosity and reactivity. A bacterial strain *Pseudomonas aeruginosa* (PR3) has been intensively studied to produce mono-, di-, and trihydroxy fatty acids from different 9-cis-monoenoic fatty acids such as oleic acid, ricinoleic acid, and palmitoleic acid. However, from the results and the postulated similar metabolic pathways involved in these transformations, it was assumed that the enzyme system involved in transformation of the monoenoic fatty acid by strain PR3 could utilize fatty acids with different chain lengths and locations of the double bond. In this study was used as a substrate for bioconversion by strain PR3 eicosenoic acid (C20:1, ω-9) containing a singular cis double bond at different positions from the carboxyl end as oleic acid, and it was confirmed that PR3 could produce a novel 9,12-dihydroxy-10(*E*)-eicosenoic acid (DED) with 6.2% yield from eicosenoic acid. The structure of DED was confirmed using GC-MS, FTIR, and NMR analyses. DED production was maximized at 72 h after the substrate was added to the 24 h culture. Some other nutritional factors were also studied for optimal production of DED.

KEYWORDS: eicosenoic acid, 9,12-dihydroxy-10(*E*)-eicosenoic acid, bioconversion, *Pseudomonas aeruginosa*

INTRODUCTION

Hydroxy fatty acids (HFAs) are known as functional oxylipins containing hydroxyl groups on fatty acid backbones. Because of the hydroxyl groups, HFAs gain special properties such as higher viscosity and reactivity compared to normal fatty acids.[1] These special properties enable HFAs to have a wide range of industrial applications in the manufacture of resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings. In addition, HFAs were reported to have antimicrobial activities against plant pathogenic fungi and some bacteria.[2−5]

Natural HFAs are mostly found in plants as trace elements[6] except ricinoleic acid, which occurs as a major monohydroxy fatty acid in mature castor seeds. Recently, much effort has been focused on microbial production of various hydroxy fatty acids from several fatty acid substrates. Among the microbial strains tested for HFA production, *Pseudomonas aeruginosa* PR3 has been well studied to produce mono-, di-, and trihydroxy fatty acids from mono- and dienoic unsaturated fatty acid substrates. The PR3 strain converted linoleic acid into an equimolar mixture of 9,10,13-trihydroxy-11(*E*)-octadecenoic acid (9,10,13-THOD) and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (9,12,13-THOD).[7,8] The PR3 strain also produced 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) from oleic acid[9,10] and 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) from ricinoleic acid.[11,12] 10-Hydroxy-8(*E*)-octadecenoic acid was identified as an intermediate compound leading to the formation of DOD by *P. aeruginosa* PR3.[13,14] Recently, rather than free oleic acid, the triglycerides containing oleic acid were successfully used as substrates for DOD production.[15−18]

The bioconversion pathway of DOD production by *P. aeruginosa* PR3 from oleic acid containing a cis-double bond at carbon 9 was postulated as the conversion of oleic acid to 10-hydroxy-8(*E*)-octadecenoic acid (HOD) during which one hydroxyl group was introduced at carbon 10 and a double bond was shifted from C9-cis to C8-trans. The resulting plausible intermediate HOD was then subjected to another hydroxylation at carbon 7, resulting in complete formation of DOD.[14] Bioconversion of palmitoleic acid (C16:1, ω-7-cis) by *P. aeruginosa* PR3 introduced two hydroxyl groups at carbons 7 and 10 with a C9-cis double bond being shifted to C8-trans,[19] which was observed in the case of DOD production from oleic acid.[14] However, the conversion yield of palmitoleic acid was much lower than that of oleic acid. These results suggested that the enzyme system involved in DOD production from oleic acid had different substrate specificities for oleic acid and palmitoleic acid. This difference could be caused by different chain lengths or different locations of the double bond from the methyl end of the fatty acid.

These results prompted us to examine whether eicosenoic acid (C20:1, ω-9-cis) could be utilized by *P. aeruginosa* PR3 as a substrate for production of a novel dihydroxy fatty acid. Eicosenoic acid is a ω-9-cis fatty acid containing 20 carbons and 1 cis-double bond at carbon 11, which is the same location from the methyl end as oleic acid. In this study we first report that a novel dihydroxy fatty acid is produced from eicosenoic acid by *P. aeruginosa* PR3.

MATERIALS AND METHODS

Chemicals. Eicosenoic acid (C20:1, 11-cis) with >99% purity by GC and heptadecanoic acid (C17:0) were purchased from Nu-Chek Prep (Elysian, MN). A mixture of trimethylsilylimidazole (TMSI) and

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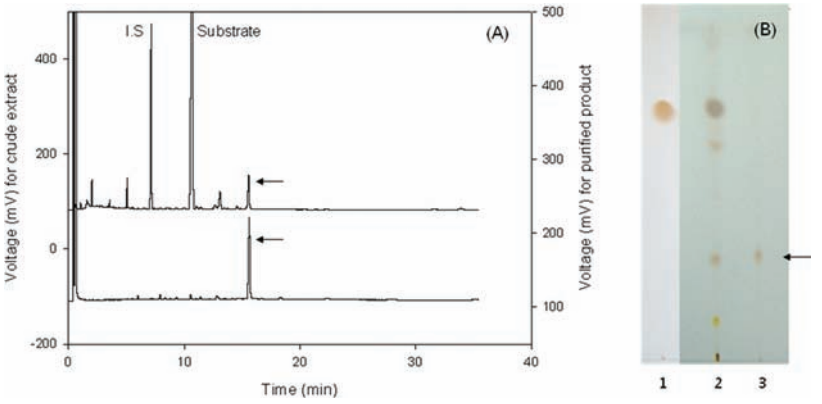

Figure 1. GC (A) and TLC (B) analyses of the crude extract and the purified target compound produced from eicosenoic acid by *P. aeruginosa* PR3. The upper and lower GC chromatograms of panel A represent crude extract and purified compound, respectively. In panel B, lane 1 represents eicosenoic acid; lane 2, crude extract; and lane 3, purified compound. Target compound is indicated by the arrow. Reaction and analysis conditions are explained under Materials and Methods.

pyridine (1:4, v/v) was purchased from Supelco (Bellefonte, PA). All other chemicals were of reagent grade and were used without further purification. Thin-layer precoated Kieselgel 60F_{254} plates were obtained from EM Science (Cherry Hill, NJ). Other chemicals were purchased from Sigma Chemical (St. Louis, MO), unless mentioned otherwise.

Microorganism and Bioconversion. *P. aeruginosa* NRRL strain B-18,602 (PR3) was kindly provided by Dr. Hou of NCAUR (National Center for Agricultural Utilization Research, Peoria, IL). The strain was aerobically grown at 28 °C with shaking at 200 rpm in a 125 mL Erlenmeyer flask containing 50 mL of standard medium. The standard medium used hereafter contained (per liter) 4 g of dextrose, 2 g of K_2HPO_4, 2 g of $(NH_4)_2HPO_4$, 1 g of NH_4NO_3, 1 g of yeast extract, 0.056 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $MgSO_4$, and 0.01 g of $MnSO_4 \cdot 7H_2O$. The medium was adjusted to pH 7.0 with diluted phosphoric acid. For bioconversion, as a standard condition, eicosenoic acid (0.3 g) was added to the 24-h-old preculture, followed by additional incubation for 72 h. At the end of cultivation, the culture was acidified to around pH 2.0 with 6 N HCl, followed by immediate extraction twice with an equal volume of ethyl acetate and diethyl ether. The solvent was evaporated from the combined extract with a rotary evaporator. For optimization study, individual nutritional components were replaced as needed from the standard medium and conversion was carried out under standard conditions, unless mentioned otherwise. Cell growth was determined spectrophotometrically by measuring the absorbance of cell culture at 610 nm.

Analysis of Products. The reaction products were analyzed by thin-layer chromatography (TLC) and gas chromatography (GC). The TLC was developed with a solvent system consisting of toluene/dioxane/acetic acid (79:14:7, v/v/v). Spots were visualized by spraying the plate with 50% sulfuric acid and heating in a 100 °C oven for 10 min. For GC analysis, the samples were first methylated with diazomethane for 5 min at room temperature, followed by derivatization with a mixture of TMSI and pyridine (1:4, v/v) for at least 20 min at room temperature. The derivatized sample was analyzed with a Younglin AMCE 6100 GC system (Younglin, Seoul, Korea) equipped with a flame ionization detector and a capillary column (SPB-1, 15 m × 0.32 mm i.d., 0.25 μm thickness, Supelco Inc.). GC was run with a temperature gradient of 20 °C/min from 70 to 200 °C, 1 min hold at 200 °C, and then 0.7 °C/min from 200 to 240 °C followed by 15 min hold at 240 °C (nitrogen gas flow rate = 0.67 mL/min). Injector and detector temperatures were held at 250 and 270 °C, respectively. Heptadecanoic acid (C17:0) was used as an internal standard for quantification. For purification of the target product from crude extract, the extract was fractionated by column chromatography (1.2 cm i.d. × 25 cm length) packed with silica gel 60. Elutions were carried out sequentially using 50 mL of hexane, 50 mL of hexane/ethyl acetate (80:20 v/v), 50 mL of hexane/ethyl acetate (50:50 v/v), and 100 mL of chloroform/methanol (50:50 v/v). Most of the target product (>95%) was eluted from the chloroform/methanol (50:50 v/v) fraction.

The chemical structure of the purified target compound was determined by GC−mass spectrometry (GC-MS), NMR, and Fourier transform infrared (FTIR) spectroscopy. Electron-impact (EI) mass spectra were obtained with a Hewlett-Packard (Avondale, PA) 5890 GC coupled to a Hewlett-Packard 5972 series mass selective detector. The column outlet was connected directly to the ion source. Separation was carried out in a methylsilicone column (30 m × 0.25 mm i.d., 0.25 μm film thickness) with a temperature gradient of 20 °C/min from 70 to 170 °C, a 1 min hold at 170 °C, and a gradient of 5 °C/min to 250 °C followed by 15 min hold at 250 °C (helium gas flow rate = 0.67 mL/min). Proton and ^{13}C NMR spectra were determined in deuterated chloroform with a Varian 400 spectrometer (Billerica, MA), operated at frequencies of 400 and 100 MHz, respectively. FTIR analysis of the free acid product was run as films on KBr on a Perkin-Elmer infrared Fourier transform model 1750 spectrometer (Perkin-Elmer, Oakbrook, IL). All of the experiments were duplicated, unless mentioned otherwise.

RESULTS

Production and Isolation of a Target Compound. From incubation with fatty acid substrate for 72 h at 28 °C in standard medium, *P. aeruginosa* PR3 converted the eicosenoic acid into a mixture of several products including a compound with a GC retention time around 16 min, indicated by the arrow in Figure 1A (upper chromatogram). This crude extract revealed several spots including a spot indicated by the arrow with the R_f value being 0.33 in TLC analysis (Figure 1B, lane 2). The R_f value of this spot was similar to that of DOD,[15,17] suggesting that this spot might be a dihydroxy fatty acid. This product was isolated from the chloroform/methanol (50:50 v/v) fraction of the column chromatography (data not shown). The purified product was identified as a single major peak (>95%) by GC (Figure 1A, lower chromatogram) and revealed a single spot on TLC analysis (Figure 1B, lane 3).

Structure Determination. The purified target compound was subjected to GC-MS, NMR, and FTIR analyses for structure determination. The EI GC-MS spectrum of the TMS derivative of the methylated sample is given in Figure 2. The GC-MS spectrum was consistent with the TMS derivative of the methylated

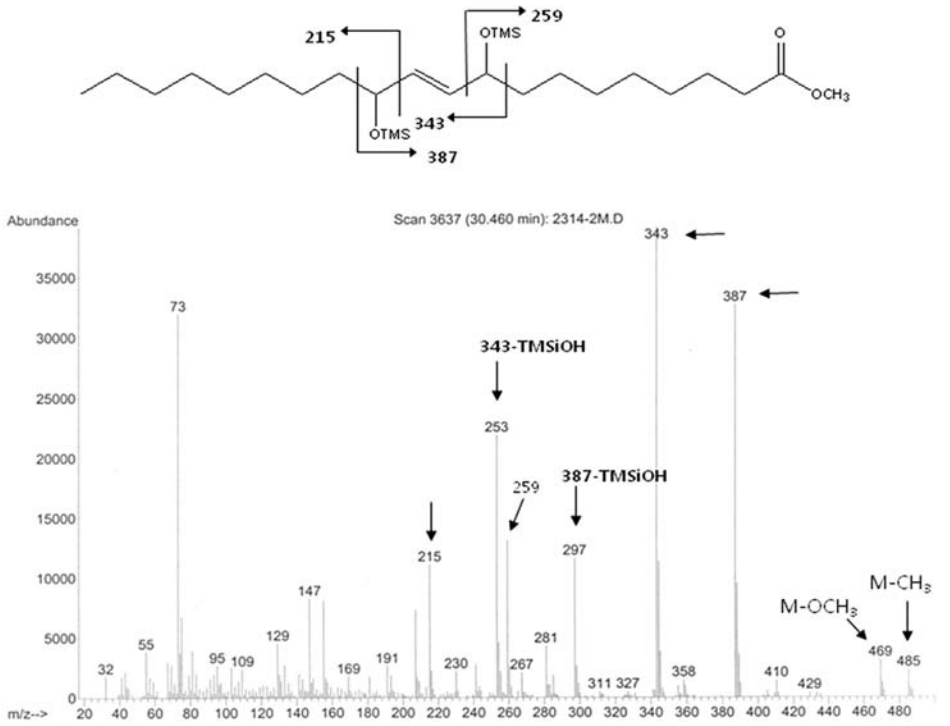

Figure 2. EI mass spectrum of TMS derivative of the methylated target compound. Conditions for compound separation are explained under Materials and Methods.

C20 dihydroxy monoenoic fatty acid with the molecular mass of 500. Locations of the hydroxyl groups were apparent from the fragments observed in the spectrum of the sample. The intense fragment arising from α cleavage to the derivatized hydroxyl group toward the methyl end gave fragments containing TMS at *m*/*z* 215 and both two TMS and a double bond at *m*/*z* 343. Two other intense fragments arising from α cleavage to the derivatized hydroxyl group toward the methylated carboxyl end were observed at *m*/*z* 259 containing TMS and at *m*/*z* 387 containing both two TMS and a double bond. These fragments allocated the hydroxyl groups at carbons 9 and 12 and a double bond between carbons 10 and 11.

The purified compound was subjected to IR analysis (Figure 3). The presence of a hydroxyl group was indicated by the broad strong IR absorption at the 3400 cm^{-1} region. The absorption observed at 1710 cm^{-1} represented a carbonyl group, and the absorption at 970 cm^{-1} indicated that the double bond was trans.[14,19] The purified free acid was subjected to proton and ^{13}C NMR analyses to confirm the elucidated structure. Resonance signals (ppm) and corresponding molecular assignments are given in Table 1. A resonance signal of the olefinic protons (−CH═CH−) obtained from proton NMR was observed at 5.55 ppm with a coupling constant of 15.4 Hz, strongly indicating trans-configuration across the double bond. Two tertiary protons (−CH—O−) were observed at 4.75 ppm. Because of the downfield shift, compared to alcohols adjacent to saturated carbons, and the doublet of triplet multiplicity, the hydroxyl-bearing carbons were suggested to be vicinal to the double bond. ^{13}C NMR confirmed the presence of the following carbons: carbonyl carbon at 177.0 ppm (C1), a double bond between 134.47 (C10) and 134.49 (C11), and two −CHOH− carbons at 73.01 ppm (C9) and at 73.05 (C12). Other protons and carbons were as shown in the table. The data obtained from GC-MS,

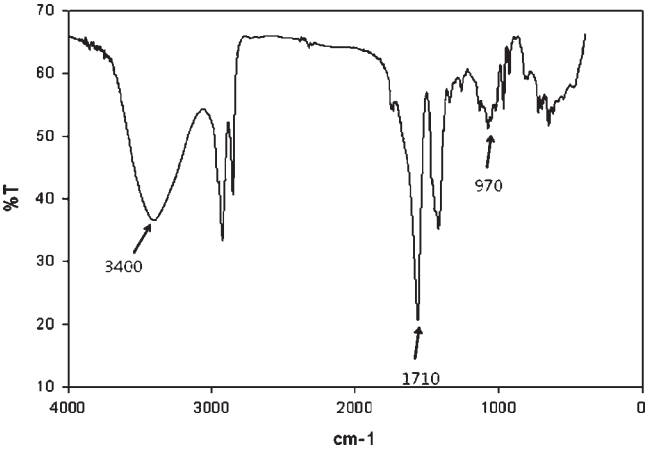

Figure 3. FTIR analysis of the purified target compound. Analytical conditions are explained under Materials and Methods.

FTIR, and proton and ^{13}C NMR analyses confirmed that the purified compound was 9,12-dihydroxy-10(*E*)-eicosenoic acid (DED). On the basis of these results, the bioconversion pathway of DED production from eicosenoic acid by *P. aeruginosa* PR3 was postulated (Figure 4). As shown in the figure, two hydroxyl groups were introduced at carbons 9 and 12 and the 11−12 cis double bond was shifted to 10−11 trans configuration. The final product became DED. This product showed a structure similar to those of other dihydroxy fatty acids such as DOD from oleic acid and DHD from palmitoleic acid produced by *P. aeruginosa* PR3 except for the chain length and position of the double bond.[11,19]

Optimization of DED Production. Time-course production of DED was studied and showed that production of DED was

Table 1. Proton and ^{13}C Nuclear Magnetic Resonance Signals and Molecular Assignments for the Purified Compound

	resonance signal (ppm)	
carbon no.	^{1}H	^{13}C
1		177.0
2	3.35	37.95
3	1.56	27.37
4	1.26	30.06
5	1.30	30.31
6	1.39	30.73
7	1.40	26.31
8	2.16	38.01
9	4.75	73.01
10	5.55 ($J_{10,11}$ = 15.4 Hz)	134.47
11	5.56	134.49
12	4.75	73.05
13	2.32	38.83
14	1.91	24.27
15	1.42	30.37
16	1.42	30.41
17	1.43	30.50
18	1.44	32.71
19	1.50	23.43
20	0.87	14.49

time-dependently increased and maximized between 60 and 72 h after the addition of eicosenoic acid as substrate, after which production was remarkably decreased (Figure 5). The maximum amount of DED at 72 h was 10.7 mg per 50 mL of culture, representing 3.6% production yield over substrate. On the basis of this result, the optimal incubation time was fixed as 72 h after substrate addition for further experiments. The peak profile of DED production indicated that DED was further degraded or consumed by bacterial cells after 72 h, which was also observed in the cases of DOD and DHD.[15,19]

Several well-known carbon sources were tested individually for DED production using standard medium under standard conditions (Table 2). Among nine carbon sources tested, glucose, galactose, fructose, and maltose were effective for DED production and cell growth. Among those carbon sources, fructose was most effective for DED production with a maximum amount of 18.7 mg per 50 mL of culture, representing 6.2% production yield. Mannitol and glycerol were effective for cell growth but not for DED production. These results were quite different from those of DOD production from triolein and DHD production from palmitoleic acid by the same strain.[15,20] Glucose and fructose were similarly relatively effective for production of all dihydroxy fatty acids mentioned, although their production yields were different. However, galactose, which was effective for DOD and DED production, did not show any DHD production.

Several organic and inorganic nitrogen sources were tested for DED production. The combined nitrogen source of the control standard medium containing yeast extract and ammonium phosphate was replaced individually with various nitrogen sources satisfying the same nitrogen concentration as control medium. As shown in Table 3, yeast extract, malt extract, and glutamine showed DED production representing 2.1, 2.7, and 1.6 mg per 50 mL of culture, respectively, although their production yields

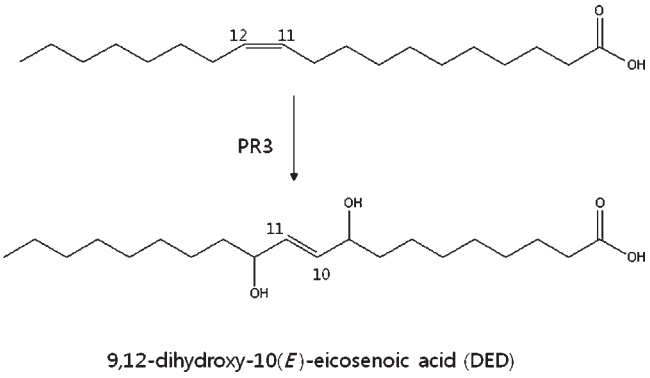

Figure 4. Postulated bioconversion pathway of eicosenoic acid leading to formation of DED by *P. aeruginosa* PR3.

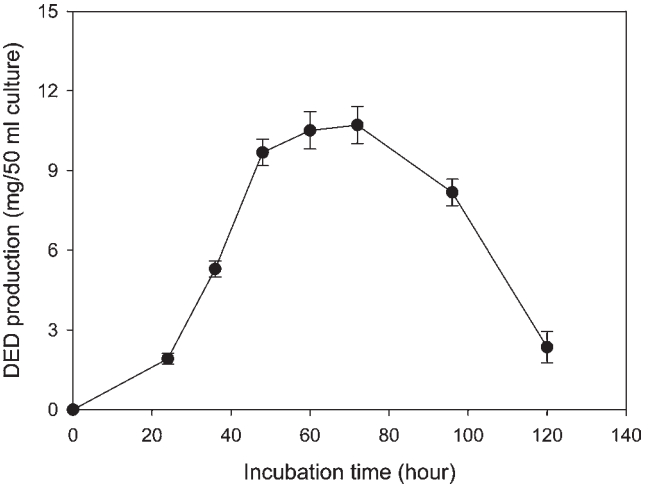

Figure 5. Time-course production of DED from eicosenoic acid by *P. aeruginosa* PR3. Eicosenoic acid (300 mg) was added to the culture at 24 h after incubation started. See Materials and Methods for other reaction conditions.

were relatively much lower than that of control medium (13.2 mg). Peptone, tryptone, urea, and ammonium phosphate were effective for cell growth but not for DED production. These results suggested that a single nitrogen source was not effective for DED production, although its concentration retained was the same as the combined nitrogen source in control medium.

DISCUSSION

Microbial modification of naturally occurring materials such as carbohydrates and lipids often generates new properties for the compounds, rendering new industrial applicability. There have been many trials to modify fatty acids or lipids to produce novel oxylipins including hydroxy fatty acids by microbial oxygenation. However, no effort was contributed to usage of eicosenoic acid as a substrate for the production of HFA. In this study, we first used eicosenic acid as a substrate for the production of a novel dihydroxy fatty acid by *P. aeruginosa* PR3. Structural analysis of the new product converted from eicosenoic acid by *P. aeruginosa* PR3 confirmed that strain PR3 could introduce two hydroxyl groups on carbons 9 and 12 with shifted migration of the 11-cis double bond to 10-trans configuration, resulting in the formation

Table 2. Effect of Carbon Source on DED Production from Eicosenoic Acid

carbon source (0.4%)	cell growth[a] (abs at 610 nm)	total DED production (mg/50 mL culture)
glucose	3.34 ± 0.32	13.2 ± 1.1
galactose	2.62 ± 0.24	15.7 ± 1.7
fructose	3.02 ± 0.21	18.7 ± 0.9
xylose	1.97 ± 0.17	nd[b]
sucrose	0.93 ± 0.15	nd
maltose	2.94 ± 0.25	18.2 ± 1.2
lactose	1.15 ± 0.21	1.1 ± 0.1
mannitol	3.46 ± 0.29	1.8 ± 0.2
glycerol	2.91 ± 0.25	nd

[a] Cell growth was determined after 24 h of incubation prior to substrate addition. [b] Not detected.

Table 3. Effect of Nitrogen Source on DED Production from Eicosenoic Acid

nitrogen source	cell growth[a] (abs at 610 nm)	total DED production (mg/50 mL culture)
control[b]	3.34 ± 0.32	13.2 ± 1.1
yeast extract	2.64 ± 0.26	2.1 ± 0.2
malt extract	0.43 ± 0.17	2.7 ± 0.2
peptone	2.04 ± 0.12	nd[c]
tryptone	2.15 ± 0.15	nd
glutamine	2.31 ± 0.18	1.6 ± 0.1
NH_4NO_3	0.15 ± 0.11	nd
$(NH_4)_2HPO_4$	3.02 ± 0.22	nd
$(NH_4)_2SO_4$	0.98 ± 0.12	nd
urea	2.63 ± 0.15	0.3 ± 0.1

[a] Cell growth was determined after 24 h incubation prior to substrate addition. [b] Control represented the standard medium. [c] not detected.

of 9,12-dihydroxy-10(*E*)-eicosenoic acid (DED) (Figure 4). However, the production yield of DED from eicosenoic acid (6.2%) was relatively low compared to those of DHD production from palmitoleic acid (23%)[19] and DOD production from olein (70%),[15] although the overall bioconversion pathways were similar for all of those fatty acids in that the cis-double bond was shifted by one carbon toward the carboxyl end with trans configuration and two hydroxyl groups were introduced at both sides of the shifted double bond. These results strongly suggested that the enzyme system involved in double hydroxylation on a monoenoic fatty acid retained a high preference for the 18 carbon ω-9-cis fatty acid, that is, oleic acid. Also, the position of the double bond from the carboxyl end was more important than that from the methyl end of the fatty acid substrate for this event because the position of the double bond of eicosenoic acid is the same as that of oleic acid from the methyl end but not from carboxyl end.

In conclusion, a fatty acid, eicosenoic acid, was successfully used as a substrate to produce a novel dihydroxy fatty acid, 9,12-dihydroxy-10(*E*)-eicosenoic acid, by *P. aeruginosa* PR3. Although the production yield of DED was relatively low compared to those of other dihydroxy fatty acids, it is worthy of further study, on the basis of this paper, to elucidate the chemical and biological properties of DED for possible industrial applications because DED is a novel dihydroxy fatty acid. Hence, further study should be focused on elucidation of novel functionalities of DED and scaled-up production in a fermentor.

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