

# 5,8-Dihydroxy-9,12,15(Z,Z,Z)-Octadecatrienoic Acid Production by Recombinant Cells Expressing *Aspergillus nidulans* Diol Synthase

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**Abstract** Whole cells of recombinant *Escherichia coli* expressing diol synthase from *Aspergillus nidulans* produced 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from  $\alpha$ -linolenic acid via 8-hydroperoxy-9,12,15(Z,Z,Z)-octadecatrienoic acid as an intermediate. The optimal conditions for 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid production using whole recombinant cells were exhibited at pH 7.0, 40 °C, and 250 rpm with 40 g/L cells, 12 g/L  $\alpha$ -linolenic acid, and 5 % (v/v) dimethyl sulfoxide in a 250-mL baffled flask containing 50 mL reaction solution. Under these conditions, whole recombinant cells produced 9.1 g/L 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid for 100 min, with a conversion yield of 75 % (w/w), a volumetric productivity of 5.5 g/L/h, and specific productivity of 137 mg/g-cells/h. As an intermediate, 8-hydroperoxy-9,12,15(Z,Z,Z)-octadecatrienoic acid was observed at approximately 1.4 g/L after 100 min. With regard to dihydroxy fatty acid production, this is the highest reported volumetric and specific productivities thus far. This is the first report on the biotechnological production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid.

**Keywords** *Aspergillus nidulans* · Diol synthase · 5,8-Dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid production ·  $\alpha$ -Linolenic acid · Recombinant *Escherichia coli*

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## Introduction

Hydroxy fatty acids comprise of one or more hydroxyl groups and a carbon chain with a carboxyl group at the  $\alpha$ -carbon position. Hydroxy fatty acids show a higher reactivity, solvent miscibility, hydrophilicity, and viscosity than non-hydroxylated fatty acids [1, 2]. Therefore, they are widely used as starting materials for the synthesis of resins, nylons, polyurethanes, plastics, and polymers, as additives for the manufacture of lubricants, surfactants, and stabilizers [3, 4], and as precursors for the synthesis of lactones [5]. Hydroxy fatty acids have been shown to function as signaling compounds in host-pathogen interactions in fungi [6], and have pharmaceutical activities, including anti-bacterial, anti-fungal, and anti-diabetic properties [2, 4, 7, 8]. Among hydroxy fatty acids, dihydroxy fatty acids are used in the manufacturing polyurethane rigid foams and skin care products [9], and they are known to be better surfactants than monohydroxy fatty acids [10]. Some dihydroxy fatty acids, called precocious sexual inducer (psi) factors, regulate the sexual and asexual life cycles in filamentous fungi [11].

$\alpha$ -Linolenic acid (18:3 $\Delta^{9,12,15}$ ), polyunsaturated fatty acid, and omega-3 fatty acid, are an essential dietary nutrient for vertebrates [12]. Dihydroxy fatty acid derived from  $\alpha$ -linolenic acid such as 9,16-dihydroxyoctadecatrienoic acid exhibits anti-aggregatory and anti-inflammatory properties [13]. Thus, the production of dihydroxy fatty acid from  $\alpha$ -linolenic acid via biotransformation is important. Several microorganisms, including *Agaricus bisporus* [14], *Clavibacter* sp. ALA2 [15], *Flavobacterium* sp. DS5 [16], *Fusarium oxysporum* [17], *Gaeumannomyces graminis* [18], *Lysinibacillus fusiformis* [19], *Magnaporthe grisea* [20], *Nocardia cholesterolicum* [21], and

*Stenotrophomonas maltophilia* [22], are capable of converting  $\alpha$ -linolenic acid into several kinds of hydroxy fatty acids. Specially, *Agaricus bisporus* [14], *Clavibacter* sp. ALA2 [15], *Fusarium oxysporum* [17], and several strains of *Pseudomonas aeruginosa* such as PR3, 42A2, and 32T3 [23–29], have been shown to qualitatively convert fatty acids into dihydroxy fatty acids. The quantitative production of diverse dihydroxy fatty acids from different fatty acids, including palmitoleic acid, oleic acid, eicosenoic acid, ricinoleic acid, and linoleic acid, has been reported in *P. aeruginosa* PR3 and 42A2 containing diol synthase [30–36] and *E. coli* expressing *A. nidulans* diol synthase [37]. However, the quantitative production of dihydroxy fatty acid from  $\alpha$ -linolenic acid by diol synthase or cells containing diol synthase has not yet been attempted.

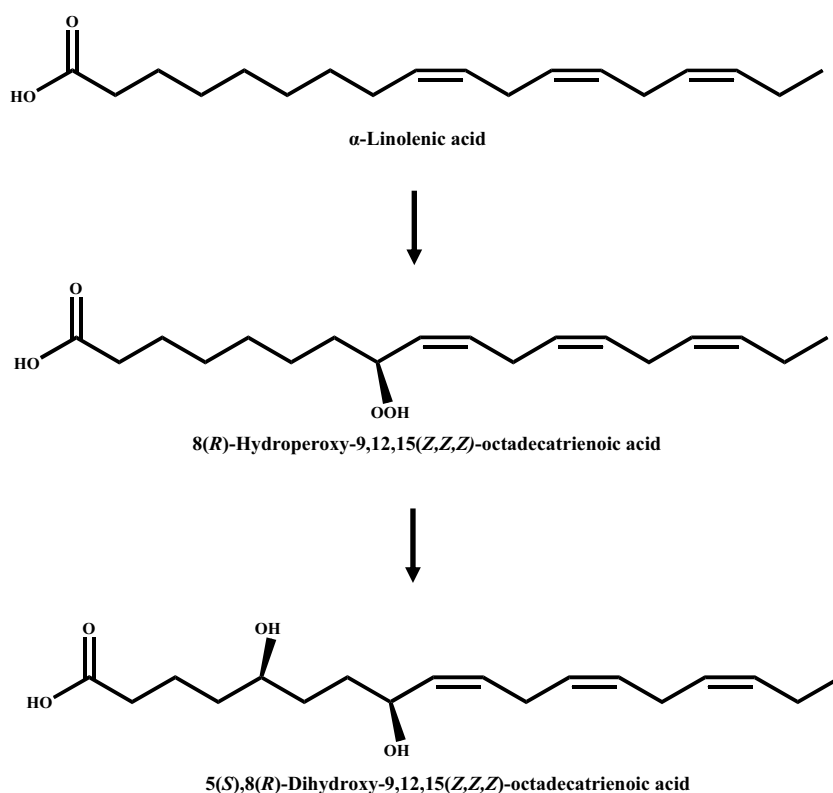
In the present study, the biotechnological production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid was achieved using whole recombinant cells expressing diol synthase from *A. nidulans* (Fig. 1). To increase the production, the reaction conditions such as pH, temperature, solvent, agitation speed, and concentrations of cell and substrate were optimized. Under the optimized conditions, an increase in the production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid was achieved.

## Materials and Methods

### Preparation of Dihydroxy Fatty Acid Standards

A double-site (H1004A-C1006S) variant of *A. nidulans* 5,8-diol synthase produced only 8-hydroperoxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid (not yet published). Recombinant cells expressing the wild-type and double-site variant of *A. nidulans* diol synthase were used for the preparation of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid and 8-hydroperoxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid, respectively. The reactions were performed as described previously [37]. After the reactions, the fractions containing the reaction products were collected by solvent fractional crystallization at low temperature [38]. The partially purified products were extracted with two volumes of ethyl acetate. The solvent was removed from the extracts using a rotary evaporator. After evaporation, methanol was added to the solvent-free solutions, which were then applied to an HPLC system (Agilent 1260, Santa Clara, CA, USA) coupled with a UV detector and analyzed at a detection wavelength of 202 nm using a Nucleosil C18 semi-prep column (10.0  $\times$  250 mm; Phenomenex, Torrance, CA, USA) and a separate fraction collector. The column was eluted at 40  $^{\circ}$ C, with a flow rate of 0.25 mL/min. The product fractions were collected and the solvent using

**Fig. 1** Production of 5*S*,8*R*-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid via 8*R*-hydroperoxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid intermediate by whole recombinant cells expressing diol synthase from *A. nidulans*. The stereospecificity of these compounds produced by diol synthase from *A. nidulans* has been reported previously [11]



a rotary evaporator was removed, leaving solid extracts. 5,8-Dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid and 8-hydroperoxy-9,12,15(Z,Z,Z)-octadecatrienoic acid with high purity (>99 %) were obtained and used as standard compounds in subsequent analyses.

#### Microorganisms, Plasmids, Gene Cloning, and Culture Conditions

*A. nidulans* ATCC 10074, *E. coli* ER2566 (New England Biolabs, Hertfordshire, UK), and pET-21a(+) plasmid (Novagen, Madison, WI, USA) were used as the sources of DNA template for the diol synthase gene, host cells, and expression vector, respectively. *P. aeruginosa* PR3 was kindly provided by Professor Hak-Ryul Kim at the Kyungpook National University (Daegu, South Korea). Although the production of dihydroxy fatty acids by *P. aeruginosa* PR3 has been published [23, 30–32, 39], the cell mass of *P. aeruginosa* PR3 in the production was not reported. *P. aeruginosa* PR3 was cultivated as described previously [39], and the strain was used for determining the cell mass of *P. aeruginosa* PR3 using a linear calibration curve relating optical density at 610 nm versus dry cell weight. The cell mass of *P. aeruginosa* PR3 was used for the calculation of volumetric and specific productivities for dihydroxy fatty acids. The diol synthase gene (GenBank accession number AY502073) from *A. nidulans* was obtained from a previous construction in *E. coli* [37]. The recombinant *E. coli* cells expressing diol synthase from *A. nidulans* were cultivated in a 2-L flask containing 500 mL Luria–Bertani (LB) medium and 50 µg/mL ampicillin at 37 °C with shaking at 200 rpm. When the optical density of the culture reached 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. The culture was incubated at 16 °C with shaking at 150 rpm for 16 h to express the enzyme. The whole cells obtained were used for 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid production.

#### Effects of pH, Temperature and Thermostability

Unless otherwise stated, the reactions were performed in 50 mM HEPES (pH 7.0) containing 2 g/L cells and 0.14 g/L α-linolenic acid, which was purchased from Sigma-Aldrich (St. Louis, MO, USA), at 40 °C for 5 min with agitation at 250 rpm in a 250-mL baffled flask containing 50 mL reaction solution. Agitation was carried out in a shaking incubator (Vision Scientific, Bucheon, Korea). The effects of pH and temperature on the production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from α-linolenic acid were investigated by varying the pH from 6.0 to 8.0 using 50 mM HEPES buffer at a constant temperature of 40 °C, and by varying the temperature from 30

to 60 °C at a constant pH of 7.0. The thermal stability of recombinant cells expressing diol synthase from *A. nidulans* for the production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from α-linolenic acid was determined after incubating the cells at four different temperatures (35, 40, 45, and 50 °C). Samples were withdrawn at regular time intervals, and the activity was assessed by measuring 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid production under the standard reaction conditions described above. The half-life of recombinant cells expressing diol synthase from *A. nidulans* was calculated using the Sigma Plot 9.0 software (Systat Software, San Jose, CA, USA).

#### Effect of Solvent

The effect of solvent on 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid production was examined using ethanol, methanol, ethyl acetate, cyclohexane, diethyl ether, 1-propanol, dimethyl sulfoxide, 1-butanol, hexane, and isopropanol at the concentrations of 5 and 10 % (v/v) in 50 mM HEPES buffer (pH 7.0) with 2 g/L cells and 0.14 g/L α-linolenic acid at 40 °C for 5 min. To determine the optimum concentration of 1-propanol or dimethyl sulfoxide for the production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from α-linolenic acid at the high concentrations of cells and substrate, the reactions were performed in 50 mM HEPES buffer (pH 7.0) with 20 g/L cells and 3 g/L α-linolenic acid at 40 °C for 10 min with agitation at 250 rpm by varying the concentration from 0 to 13 % (v/v) in a 250-mL baffled flask containing 50 mL reaction solution.

#### Optimization of Reaction Conditions

The effect of agitation speed on the production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from α-linolenic acid by whole recombinant cells expressing diol synthase from *A. nidulans* was examined in 50 mM HEPES buffer (pH 7.0) containing 20 g/L cells, 3 g/L α-linolenic acid, and 5 % (v/v) dimethyl sulfoxide by varying the agitation speed from 0 to 270 rpm in a 250-mL baffled flask containing 50 mL reaction solution at 40 °C for 10 min.

To determine the optimal cell concentration required for the maximum production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from α-linolenic acid, the reactions were performed in 50 mM HEPES buffer (pH 7.0) containing cells with a constant substrate concentration of 9 g/L α-linolenic acid and 5 % (v/v) dimethyl sulfoxide at 40 °C for 60 min with agitation at 250 rpm by varying the cell concentration from 10 to 50 g/L in a 250-mL baffled flask containing 50 mL reaction solution. To determine the optimal substrate concentration, the reactions were performed at 40 °C in 50 mM HEPES buffer (pH 7.0) containing with

a constant cell concentration of 40 g/L cells and 5 % (v/v) dimethyl sulfoxide with shaking at 250 rpm for 60 min by varying the substrate concentration from 3 to 18 g/L in a 250-mL baffled flask containing 50 mL reaction solution.

#### Time-course of Production of 5,8-Dihydroxy-9,12,15(Z,Z,Z)-Octadecatrienoic Acid from $\alpha$ -Linolenic Acid

The time-course reactions of the production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from  $\alpha$ -linolenic acid by whole recombinant cells expressing diol synthase from *A. nidulans* via 8-hydroperoxy-9,12,15(Z,Z,Z)-octadecatrienoic acid as an intermediate were conducted under optimized conditions at 40 °C in 50 mM HEPES buffer (pH 7.0) containing 40 g/L cells, 12 g/L  $\alpha$ -linolenic acid, and 5 % (v/v) dimethyl sulfoxide with shaking at 250 rpm in a 250-mL baffled flask containing 50 mL reaction solution for 100 min. A sample of 0.5 mL reaction solution was withdrawn at time interval and was analyzed with the HPLC system described below.

#### Analytical Methods

The cell mass was determined using a linear calibration curve relating optical density at 600 nm of recombinant *E. coli* expressing diol synthase from *A. nidulans* versus dry cell weight. The reaction solution was extracted with two volumes of ethyl acetate, and then the solvent was removed from the extract. The ethyl acetate fraction was then evaporated to dryness using a rotary evaporator, and methanol was added. 5,8-Dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid and  $\alpha$ -linolenic acid were quantitatively analyzed using an HPLC system (Agilent 1260, Santa Clara, CA, USA) equipped with a UV detector at a detection wavelength of 202 nm and a reverse phase Nucleosil C18 column (3.2  $\times$  150 mm; Phenomenex). The column was eluted at 40 °C using a gradient of solvent A (acetonitrile/water/acetic acid, 50/50/0.1, v/v/v) and solvent B (acetonitrile/acetic acid, 100/0.1, v/v) as follows: 100 % solvent A at a flow rate of 0.25 mL/min for 0–5 min; solvent B for 5–21 min at 0.25 mL/min; for 21–22 min at 0.4 mL/min; 100 % solvent B at 0.4 mL/min for 22–27 min; solvent A at 0.4 mL/min for 27–32 min; and 100 % solvent A at 0.25 mL/min for 32–35 min.

Liquid chromatography-mass spectrometry/mass spectrometry (LC–MS/MS) analysis of dihydroxy fatty acids was performed using an electrospray ionization (ESI) interface with a Thermo-Finnigan LCQ Deca XP plus ion trap mass spectrometer (Thermo Scientific, Pittsburgh, PA, USA) at the National Instrumentation Center for Environmental Management (NICEM) facility (Seoul National University, Seoul, South Korea). The instrument consisted

of an LC pump, an auto sampler, and a photodiode array detector. The operation parameters were as follows: 275 °C capillary temperature, 5 kV ion source voltage, 30 psi nebulizer gas, 46 V capillary voltage in positive mode and 15 V in negative ionization mode, 0.01 min average scan time, 0.02 min average time to change polarity, and collision energy of approximately 35 % abundance of the precursor ion.

## Results and Discussion

### Identification of the Reaction Product and Intermediate Obtained from the Conversion of $\alpha$ -Linolenic Acid by Whole Recombinant Cells

The reaction product and intermediate obtained from the conversion of  $\alpha$ -linolenic acid using whole recombinant cells expressing the wild-type and double-site variant (H1004A-C1006S) diol synthase of *A. nidulans*, respectively, were analyzed by LC–MS/MS, and their mass spectra were obtained. The total molecular mass of the product was represented by a peak at  $m/z$  309 in LC/MS (Supplementary data, Fig. S1A). The peaks have been known to be formed from the cleavage of the hydroxylated carbon at  $\alpha$ -carbon of hydroxy fatty acid [40]. The major peak at  $m/z$  291 was formed by the loss of H<sub>2</sub>O from the total molecular mass. The peaks at  $m/z$  115 and 173 resulted from the cleavage of the hydroxyl groups at the C5 and C8 positions, respectively. The product was identified as 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid on the basis of these fragment peaks. The reaction intermediate obtained from the conversion of  $\alpha$ -linolenic acid using whole recombinant cells expressing the double-site variant diol synthase gave the mass spectrum of LC–MS/MS as shown in Supplementary data, Fig. S1B. The total molecular mass of the product was represented by a peak at  $m/z$  309, and the peak at  $m/z$  173 resulted from the cleavage of hydroperoxy group at the C8 position. These peaks indicate that the product was 8-hydroperoxy-9,12,15(Z,Z,Z)-octadecatrienoic acid.

### Effects of pH and Temperature on the Production of 5,8-Dihydroxy-9,12,15(Z,Z,Z)-Octadecatrienoic Acid from $\alpha$ -Linolenic Acid by Whole Recombinant Cells

The maximum conversion rate of  $\alpha$ -linolenic acid to 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid by whole cells of recombinant *E. coli* expressing diol synthase from *A. nidulans* was observed at pH 7.0 and 40 °C (Fig. 2a, b). The maximum conversion rate of linoleic acid to 5,8-dihydroxy-9,12(Z,Z)-octadecadienoic acid by recombinant cells expressing diol synthase from *A. nidulans* was observed previously at pH 7.5 and 35 °C [37]. These different optimum pH and temperature

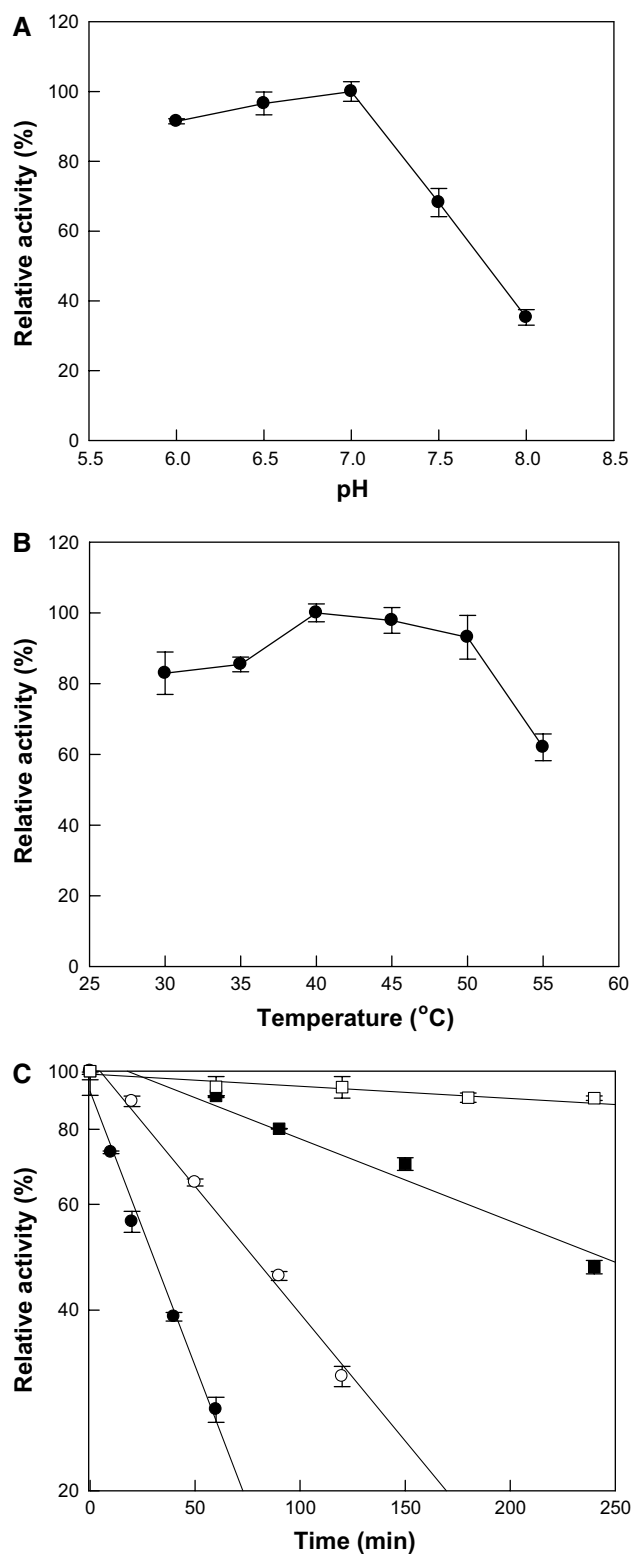
**Fig. 2** Effects of pH and temperature on the production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid by whole recombinant cells expressing diol synthase from *A. nidulans*. **a** Effect of pH. The reactions were performed in 50 mM HEPES buffer with 2 g/L cells and 0.14 g/L  $\alpha$ -linolenic acid at 40 °C for 5 min by varying the pH from 6.0 to 8.0. **b** Effect of temperature. The reactions were performed in 50 mM HEPES (pH 7.0) containing 2 g/L cells and 0.14 g/L  $\alpha$ -linolenic acid for 5 min by varying the temperature from 30 to 60 °C. **c** Effect of temperature on enzyme stability. Recombinant cells were incubated at 35 (unfilled squares), 40 (filled square), 45 (unfilled circles), and 50 °C (filled circles) in 50 mM HEPES buffer (pH 7.0) for various periods of time. A sample was withdrawn at each time point and assayed in 50 mM HEPES (pH 7.0) containing 2 g/L cells and 0.14 g/L  $\alpha$ -linolenic acid at 40 °C for 5 min. Data present the means of three experiments and error bars represent standard deviations

values may be due to different substrate solubility. The stability of recombinant cells expressing diol synthase from *A. nidulans* was measured, and the half-lives of the cells at 35, 40, 45, and 50 °C were 1,133, 232, 77, and 28 min, respectively (Fig. 2c).

#### Effect of Solvent on the Production of 5,8-Dihydroxy-9,12,15(*Z,Z,Z*)-Octadecatrienoic Acid from $\alpha$ -Linolenic Acid by Whole Recombinant Cells

The effect of organic solvent on the hydroxylation activity of recombinant cells expressing diol synthase from *A. nidulans* was examined at the concentrations of 5 and 10 % (v/v) with 2 g/L cells and 0.14 g/L  $\alpha$ -linolenic acid. Among the solvents tested, the activity was the highest in the presence of 5 % (v/v) 1-propanol, followed by 5 % (v/v) dimethyl sulfoxide, and their activities were 1.4 and 1.3-fold higher than the hydroxylation activity in the absence of an organic solvent, respectively (Fig. 3a). The effects of solvent concentrations of the two solvents for the production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid were investigated with the high concentrations of cells (20 g/L) and substrate (3 g/L). The optimal concentrations of 1-propanol and dimethyl sulfoxide for 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid production were 1 and 5 % (v/v), respectively (Fig. 3b, c). At these concentrations, the activities were 1.2- and 1.4-fold higher than that in the absence of solvent, respectively. The higher production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid using dimethyl sulfoxide was resulted from the higher substrate solubility at high concentrations in dimethyl sulfoxide than those in 1-propanol. Thus, 5 % (v/v) dimethyl sulfoxide was used for 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid production by whole recombinant cells expressing diol synthase from *A. nidulans*.

Organic solvents are widely used to dissolve free fatty acids in aqueous solution. As dimethyl sulfoxide was used to increase the concentration of fatty acid as a substrate [41] in the production of 13-hydroxy-9,11-octadecadienoic acid with immobilized soybean lipoxigenase,



the conversion yield was 1.4-fold higher than that in the absence of any treatment [42]. These results indicate that the increased production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid by the addition of dimethyl sulfoxide was due to an increase in substrate solubility.

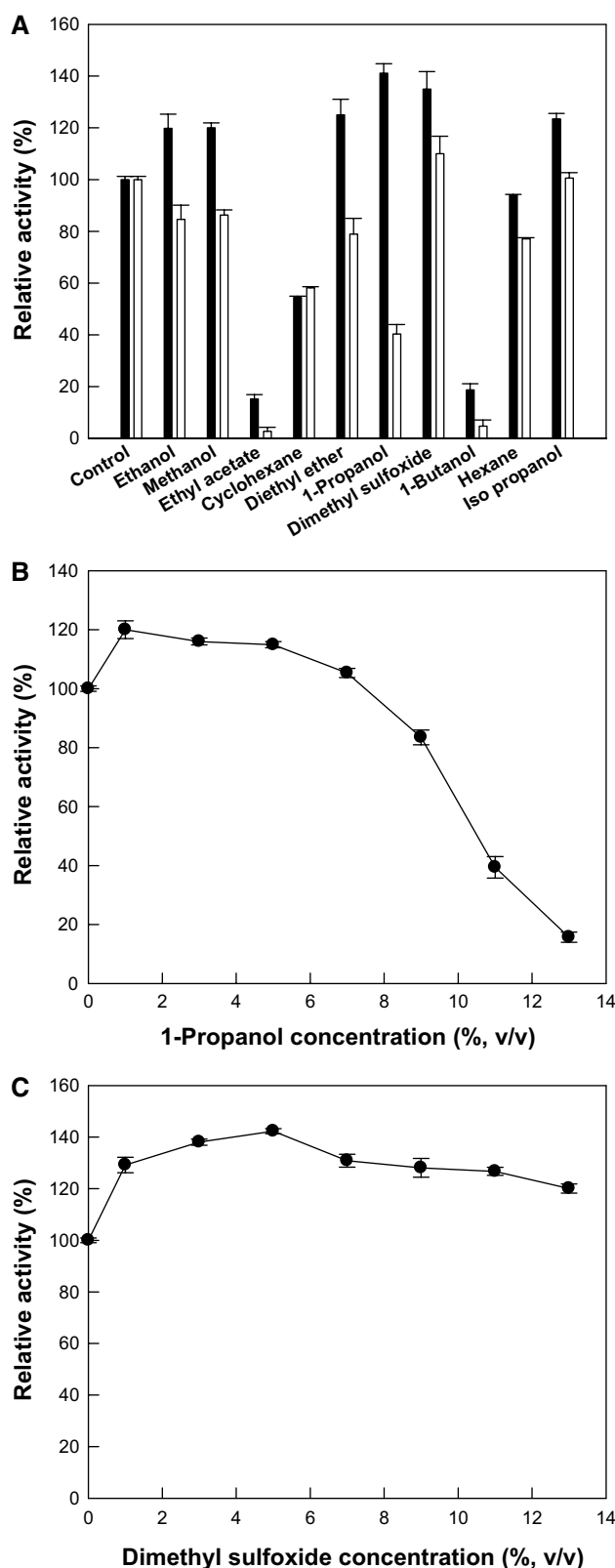
**Fig. 3** Effect of solvent on the production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid by whole recombinant cells expressing diol synthase gene from *A. nidulans*. **a** Effect of solvent type. The reactions were performed in 50 mM HEPES buffer (pH 7.0) with 2 g/L cells, 0.14 g/L  $\alpha$ -linolenic acid, and 5 % (v/v) (filled square) or 10 % (v/v) (unfilled square) solvent at 40 °C for 5 min. **b** Effect of 1-propanol concentration. The reactions were performed in 50 mM HEPES buffer (pH 7.0) containing 20 g/L cells, 3 g/L  $\alpha$ -linolenic acid, and 1-propanol at 40 °C for 10 min. **c** Effect of dimethyl sulfoxide concentration. The reactions were performed in 50 mM HEPES buffer (pH 7.0) containing 20 g/L cells, 3 g/L  $\alpha$ -linolenic acid, and dimethyl sulfoxide at 40 °C for 10 min. Data present the means of three experiments and error bars represent standard deviations

#### Optimization of Reaction Conditions for the Production of 5,8-Dihydroxy-9,12,15(*Z,Z,Z*)-Octadecatrienoic Acid from $\alpha$ -Linolenic Acid by Whole Recombinant Cells

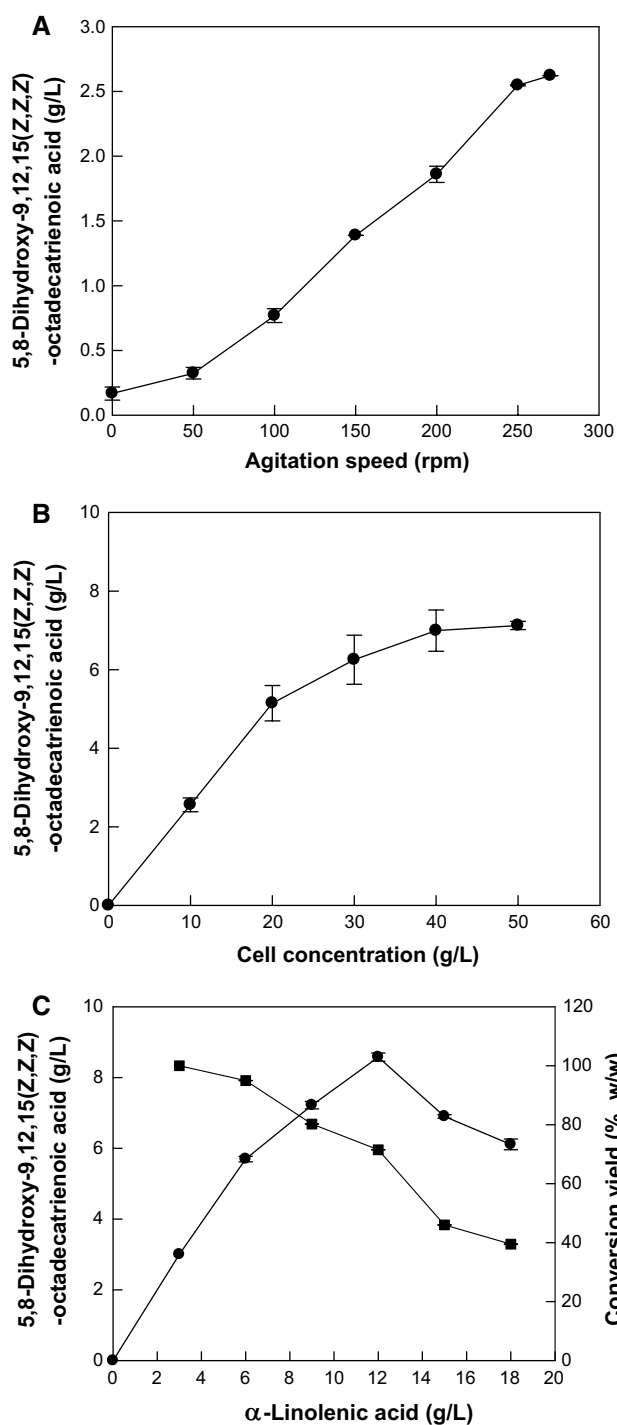
The production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid by whole recombinant cells increased with an increase in the agitation speed (Fig. 4a). However, above 250 rpm, the increase in 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid production with increasing the agitation speed was not critical. Thus, the agitation speed was determined as 250 rpm. The optimal cell concentration was evaluated at agitation speed of 250 rpm with 9 g/L  $\alpha$ -linolenic acid by varying the cell concentration from 10 to 50 g/L after 60 min. When the concentration was less than 40 g/L cells, the production increased with an increase in the cell concentration. However, above 40 g/L concentration, it reached a plateau (Fig. 4b), indicating that the optimum concentration of recombinant cells was 40 g/L. The production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid was assessed over a 60-min period by varying the substrate concentration from 3 to 18 g/L while keeping the cell concentration constant at 40 g/L cells. Below 12 g/L  $\alpha$ -linolenic acid, the production increased with an increase in the concentration of  $\alpha$ -linolenic acid (Fig. 4c). However, above 12 g/L  $\alpha$ -linolenic acid, the production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid decreased as the concentration of  $\alpha$ -linolenic acid increased. Thus, the optimum substrate concentration for 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid production was 12 g/L  $\alpha$ -linolenic acid, and the conversion yield at this concentration was approximately 72 % (w/w).

#### Production of 5,8-Dihydroxy-9,12,15(*Z,Z,Z*)-Octadecatrienoic Acid from $\alpha$ -Linolenic Acid by Whole Recombinant Cells Under the Optimized Conditions

The production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid by whole recombinant cells expressing diol



synthase from *A. nidulans* was optimal at pH 7.0, 40 °C, 12 g/L  $\alpha$ -linolenic acid, and 40 g/L cells in the presence of 5 % (v/v) dimethyl sulfoxide in a 250-mL baffled

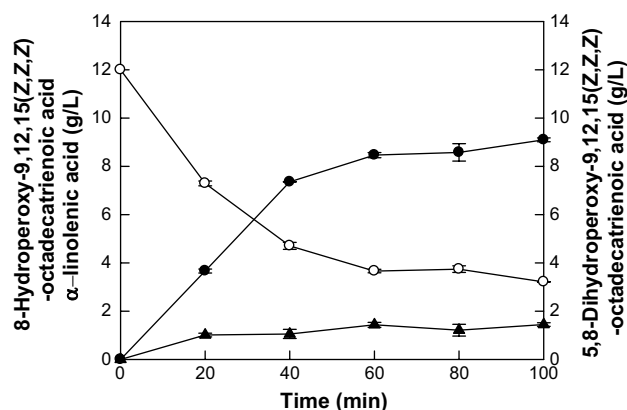


**Fig. 4** Effects of agitation speed and concentrations of cells and substrate for the production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from  $\alpha$ -linolenic acid by whole recombinant cells expressing diol synthase from *A. nidulans*. **a** Effect of agitation speed. The reactions were performed in 50 mM HEPES buffer (pH 7.0) with 20 g/L cells, 3 g/L  $\alpha$ -linolenic acid, and 5 % (v/v) dimethyl sulfoxide at 40 °C for 10 min. **b** Effect of cell concentration. The reactions were performed in 50 mM HEPES buffer (pH 7.0) containing cells, 9 g/L  $\alpha$ -linolenic acid, and 5 % (v/v) dimethyl sulfoxide at 40 °C for 60 min with agitation at 250 rpm. **c** Effect of substrate concentration on the production (filled circles) and conversion yield (filled squares) of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid. The reactions were performed in 50 mM HEPES buffer (pH 7.0) containing 40 g/L cells,  $\alpha$ -linolenic acid, and 5 % (v/v) dimethyl sulfoxide at 40 °C for 60 min with agitation at 250 rpm. Data present the means of three experiments and error bars represent standard deviations

9.1 g/L 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from 12 g/L  $\alpha$ -linolenic acid after 100 min, with a conversion yield of 75 % (w/w), a volumetric productivity of 5.5 g/L/h, and specific productivity of 137 mg/g-cell/h. Whole recombinant cells also produced 1.0 g/L 8-hydroperoxy-9,12,15(Z,Z,Z)-octadecatrienoic acid as an intermediate at 20 min. After the time, the concentration of the monohydroxy fatty acid was maintained at 1.0–1.4 g/L during the time-course reactions.

Whole recombinant cells expressing hydratase from *S. maltophilia* produced 14.3 g/L 10-hydroxy-12,15(Z,Z)-octadecadienoic acid from 17.5 g/L  $\alpha$ -linolenic acid with volumetric and specific productivities of 0.79 g/L/h and 16 mg/g-cell/h, respectively [22]. This is the highest

flask containing 50 mL reaction mixture with shaking at 250 rpm. Under these optimized conditions, time-course reactions for the production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid by whole recombinant cells were performed for 100 min (Fig. 5). The cells were stable at 40 °C for 100 min because the half-life of the cells at this temperature for 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid production was 232 min (Fig. 4). Whole recombinant cells produced



**Fig. 5** Time-course reactions for the production of 5,8-dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid from  $\alpha$ -linolenic acid by whole recombinant cells expressing diol synthase from *A. nidulans* under the optimized conditions. 5,8-Dihydroxy-9,12,15(Z,Z,Z)-octadecatrienoic acid (filled circles),  $\alpha$ -linolenic acid (unfilled circles), and 8-hydroperoxy-9,12,15(Z,Z,Z)-octadecatrienoic acid (filled triangles). The reactions were performed in 50 mM HEPES buffer (pH 7.0) containing 40 g/L cells, 12 g/L  $\alpha$ -linolenic acid, and 5 % (v/v) dimethyl sulfoxide at 40 °C for 100 min with agitation at 250 rpm. Data present the means of three experiments and error bars represent standard deviations

**Table 1** Quantitative production of dihydroxy fatty acid from fatty acid by microorganisms

Microorganism	Substrate (g/L)	Product (g/L)	Volumetric productivity (g/L/h)	Specific productivity (mg/g-cells/h)	Yield (% w/w)	References
<i>Pseudomonas aeruginosa</i> PR3	Oleic acid (10.2)	7,10-Dihydroxy-8( <i>E</i> )-octadecenoic acid (9.1)	0.19	NR	89	[23]
	Triolein (6.0)	7,10-Dihydroxy-8( <i>E</i> )-octadecenoic acid (1.88)	0.05	22	32	[31]
	Palmitoleic acid (6.0)	7,10-Dihydroxy-8( <i>E</i> )-hexadecenoic acid (1.6)	0.04	20	27	[32]
	Eicosenoic acid (6.0)	9,12-Dihydroxy-10( <i>E</i> )-eicosenoic acid (0.4)	0.01	4	6	[30]
	Olive oil (7.4)	7,10-Dihydroxy-8( <i>E</i> )-octadecenoic acid (4.0)	0.08	47	54	[39]
<i>E. coli</i> expressing <i>Aspergillus nidulans</i> diol synthase	Linoleic acid (5.0)	5,8-Dihydroxy-9,12( <i>Z,Z</i> )-octadecadienoic acid (4.9)	2.5	85	99	[37]
	$\alpha$ -Linolenic acid (12.0)	5,8-Dihydroxy-9,12,15( <i>Z,Z,Z</i> )-octadecadienoic acid (9.1)	5.5	137	75	This study

Specific productivity of *P. aeruginosa* PR3 was determined by the cell mass calibrated from the reported optical density at 610 nm  
NR not reported

reported concentration, yield, and volumetric and specific productivities for the production of hydroxy fatty acid from  $\alpha$ -linolenic acid. The volumetric and specific productivities of recombinant cells in the present study were 7.0- and 8.6-fold higher than those obtained by whole recombinant cells expressing oleate hydratase from *S. maltophilia*, respectively. However, the concentration and yield in the present study were 1.6-fold and 7 % lower, respectively.

The quantitative production of dihydroxy fatty acids from fatty acids has been reported in only two microorganisms, namely *P. aeruginosa* PR3 and recombinant *E. coli* expressing diol synthase from *A. nidulans* (Table 1). *P. aeruginosa* PR3 produced 7,10-dihydroxy-8(*E*)-octadecenoic acid from oleic acid [23], 7,10-dihydroxy-8(*E*)-octadecenoic acid from triolein [31], 7,10-dihydroxy-8(*E*)-hexadecenoic acid from palmitoleic acid [32], 9,12-dihydroxy-10(*E*)-eicosenoic acid from eicosenoic acid [30], and 7,10-dihydroxy-8(*E*)-octadecenoic acid from olive oil [39]. The specific productivity of *P. aeruginosa* PR3 was determined by calibrating 1.0 optical density at 610 nm to 0.54 g/L cell mass. Among the production of these dihydroxy fatty acids, the production of 7,10-dihydroxy-8(*E*)-octadecenoic acid from oleic acid exhibited the highest concentration, conversion yield, and volumetric productivity, which were 9.1 g/L, 89 % (w/w), and 0.19 g/L/h, respectively. Moreover, the production of 7,10-dihydroxy-8(*E*)-octadecenoic acid from olive oil showed the highest specific productivity of 47 mg/g-cell/h. The volumetric and specific productivities of recombinant cells in the present study were 29- and 3-fold higher than those obtained using whole *P. aeruginosa* PR3, respectively. However, the concentration and conversion yield in the present study was same and 1.2-fold lower, respectively. Whole recombinant cells expressing diol synthase from *A. nidulans* produced 4.98 g/L 5,8-dihydroxy-9,12(*Z,Z*)-octadecadienoic acid from 5.0 g/L linoleic acid for 150 min with a volumetric productivity of 2.5 g/L/h and a specific productivity of 85 mg/g-cell/h

[37]. The volumetric and specific productivities for the production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid by whole recombinant cells expressing diol synthase from *A. nidulans* were 2.2- and 1.6-fold higher than those for the production of 5,8-dihydroxy-9,12(*Z,Z*)-octadecadienoic acid from linoleic acid.

## Conclusions

To increase the production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid from  $\alpha$ -linolenic acid by whole recombinant cells expressing diol synthase from *A. nidulans*, the reaction conditions such as pH, temperature, thermostability, solvent, agitation speed, and cell and substrate concentrations were optimized. Under the optimized conditions, whole recombinant cells produced 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid with the highest specific and volumetric productivities as compared to dihydroxy fatty acid production described previously. To the best of our knowledge, this is the first report on the biotechnological production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid. These results will contribute to the improved industrial production of 5,8-dihydroxy-9,12,15(*Z,Z,Z*)-octadecatrienoic acid via a biological process.

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