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Stereospecific production of 9*R*-hydroxy-10*E*,12*Z*-octadecadienoic acid from linoleic acid by recombinant *Escherichia coli* cells expressing 9*R*-lipoxygenase from *Nostoc* sp. SAG 25.82

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ABSTRACT

One of the most significant properties of lipoxygenase (LOX) as a biocatalyst is its stereo-selective oxygenation. In this study, the stereo-specific production of 9*R*-hydroxy-10*E*,12*Z*-octadecadienoic acid (9*R*-HODE) from linoleic acid was achieved using whole recombinant *Escherichia coli* cells expressing LOX from *Nostoc* sp. SAG 25.82. The optimal conditions for the production of 9*R*-HODE were pH 7.5, 25 °C, 40 g l^{-1} cells, 15 g l^{-1} linoleic acid, 2% (v/v) methanol, 1 working volume/oxygen volume/min (vvm) oxygenation rate, and 250 rpm agitation speed in 500 ml-baffled flask containing a working volume of 50 ml. Under these optimized conditions, whole recombinant cells expressing 9*R*-LOX protein produced 14.3 g l^{-1} 9*R*-HODE for 1 h, with a conversion yield of 95% (w/w) and a productivity of 14.3 g l^{-1} h^{-1}. The oxygen supply method significantly influenced stereo- and regio-selectivity of the oxygenation of linoleic acid. Among the oxygen supply methods tested, oxygenation (1 vvm) with agitation (250 rpm) resulted in the highest 9*R*/13*S*-HODE ratio of the products at 96:4. This is the first application using whole recombinant cells harboring *R*-specific LOX for the stereo-selective production of an *R*-specific hydroxy fatty acid.

1. Introduction

Lipoxygenase (LOX, EC 1.13.11.-) is a non-heme iron-containing dioxygenase, which catalyzes the oxidation of polyunsaturated fatty acids (PUFAs) with one or several (1*Z*,4*Z*)-pentadiene moieties to produce specific *E*,*Z*-conjugated-hydroperoxy PUFAs and their corresponding reduced hydroxy fatty acids (HFAs) [1,2]. These fatty acids are subsequently metabolized into important bioactive signaling compounds, such as leukotrienes and lipoxins in animals [3] and jasmonic acids in plants [4]. LOXs have been extensively studied in animals and plants, however, the existence of LOXs in coral, moss, algae, fungi, and bacteria has been recently reported [5].

HFAs are used as starting materials for the synthesis of polymers and additives in the manufacturing of lubricants, emulsifiers, and stabilizers in the chemical, food, cosmetic, and pharmaceutical industries because they possess higher reactivity, solvent miscibility, stability, and viscosity, compared to non-hydroxylated fatty acids [6–8]. HFAs have been found in bacterial, fungal, and mammalian lipids not only as signaling molecules, but also as bioactive compounds, such as antibacterial, antifungal, and anticancer agents [7]. Notably, the stereo-specific HFA 9*R*-hydroxy-10*E*,12*Z*-octadecadienoic acid (9*R*-HODE) is a known signaling molecule that directly binds to the human G-protein-coupled receptor [9]. 9*R*-HODE exhibits fungicidal and fat degradation activity and induces interleukin-1 beta expression [10,11]. The first reaction for the synthesis of fatty acid-derived bioactive compounds is the insertion of molecular oxygen into unsaturated fatty acid by cytochrome P450 and LOX. However, these enzymes have not been used as biocatalysts because of the difficulties associated in achieving high-level production of bioactive compounds and high-level expression in hosts. For the use of these enzymes as biocatalysts, the stereo-specific production of HFAs is essential.

LOXs have mainly been applied to the production of hydroperoxy fatty acids (HPFAs) instead of HFAs. For example, soybean LOXs, both free and immobilized enzymes in water and solvent systems (mono-, di-, and multi-phasic solvents), have been extensively utilized to produce 13-hydroperoxy octadecadienoic acid (13-HPODE) from linoleic acid and oils [12–20]. Bacterial [21] and fungal [22] LOXs produce 13-HPODE from linoleic acid, and

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potato LOX produces 9*S*-HPODE [15]. The production of HFAs has been reported in LOXs from only several sources, such as potato [23,24], fungi [25], and cyanobacteria [26]. Potato and fungal LOXs produce 9*S*-specific HFAs, whereas the cyanobacteria *Anabaena flos-aquae f. flos-aquae* and *Anabaena* sp. PCC 7120 produces 9*R*-specific HFAs [26,27], suggesting that cyanobacterial LOXs exhibit unusual stereo-selectivity. However, the biotechnological production of 9*R*-specific HFAs using *R*-specific LOXs has not been attempted.

In the present study, the stereo-selective biocatalyst 9*R*-LOX from *Nostoc* sp. was cloned and expressed in *Escherichia coli* to produce 9*R*-HODE from linoleic acid. The reaction conditions for the production of 9*R*-HODE via whole recombinant cells, including organic solvent, pH, temperature, oxygen supply method, and the concentrations of substrate, were optimized. Under the optimized conditions, the stereo-specific production of 9*R*-HODE was achieved.

2. Materials and methods

2.1. Chemicals

The 9*R*-, 9*S*-, 13*S*-HODE, and 9-HPODE standards were purchased from Cayman Chemical (98%; Ann Arbor, MI, USA). The linoleic acid standard and $SnCl_2$ were purchased from Sigma-Aldrich (>99%; St. Louis, MO, USA). The linoleic acid substrate was purchased from TCI (>95%; Tokyo, Japan). Tris(hydroxymethyl)aminomethane (Tris), NaH_2PO_4, Na_2HPO_4, and sodium $NaBH_4$ were purchased from Daejung (99%; Siheung, Korea). Ampicillin, boric acid, and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from USB (Cleveland, OH, USA). All high-performance liquid chromatography (HPLC) solvents were obtained from Duksan (HPLC grade; Ansan, Korea). PCR primers were ordered from Bioneer (Daejeon, Korea).

2.2. Microorganisms, media, and culture conditions

Nostoc sp. SAG 25.82, which was purchased from the culture collection of algae (SAG, Goettingen, Germany), was cultivated at 26 °C in BG-11 medium supplemented with 5 mM $NaHCO_3$ as the inorganic carbon source under 30 μmol photons m^{-2} s^{-1} as previously described [26,28,29]. After 3 weeks of cultivation, cells were harvested by centrifugation at 3000 × *g* for 15 min and used as a cloning source of 9*R*-LOX. *E. coli* ER 2566 (New England Biolabs, Hertfordshire, UK) was used as the expression host of the 9*R*-LOX gene and cultivated at 37 °C in Luria-Bertani (LB) medium containing 50 μg ml^{-1} ampicillin.

2.3. Gene cloning and expression of 9R-LOX

Genomic DNA was prepared using a genomic DNA micro kit (GeneAll, Seoul, Korea) according to the manufacturer manual. The gene encoding 9*R*-LOX was amplified by PCR using the genomic DNA of *Nostoc* sp. SAG 25.82 as a template. The primer sequences for gene cloning were based on the reported DNA sequence of *Nostoc* sp. PCC 7120 LOX (GenBank accession NC_003267; 17983-19293) [30]. Forward (5′-AC CAT ATG CAG TAT TTG TAT GGA AGT AAG GAT-3′) and reverse (5′-CA CTC GAGTAA ATG TTG ATA CTC ATC ATG AG-3′) primers for the insertion pET-15b vector (Novagen, Darmstadt, Germany) were designed to introduce the *Nde* I and *Xho* I restriction sites (underline), respectively. The amplified DNA fragment obtained by PCR with *ExTaq* polymerase (Takara, Shiga, Japan) was extracted using a gel extraction kit (Promega, Madison, WI, USA). The PCR product was ligated into the *Nde* I and *Xho* I restriction sites of pET-15b. The resulting plasmid was transformed into *E. coli* ER2566 cells by electroporation. The expression of the 9*R*-LOX gene was induced using 500 ml of LB medium in a 2000 ml flask containing 50 μg ml^{-1} ampicillin. To induce enzyme expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM at the optical density of the bacteria of 0.6 at 600 nm, and the culture incubated for an additional 16 h at 16 °C [31].

2.4. Effects of pH, temperature, and thermostability

Unless otherwise stated, reactions were performed at 25 °C for 20 min in 50 mM Tris–HCl buffer (pH 7.5) containing 10 g l^{-1} cells and 2 g l^{-1} linoleic acid. To examine the effect of pH on the production of 9*R*-HODE by whole recombinant cells expressing 9*R*-LOX from *Nostoc* sp., the pH was varied from 6.5 to 8.5 using 100 mM sodium phosphate buffer (pH 6.5–8.0) and 50 mM Tris–HCl buffer (pH 7.0–8.5). The effect of temperature on the production of 9*R*-HODE by whole recombinant cells expressing 9*R*-LOX was evaluated by varying the temperature from 10 to 45 °C. The stability of whole recombinant cells was measured after incubation for 3.5 h under the standard reaction conditions as described above. Samples were withdrawn at several time intervals and the activity for the production of 9*R*-HODE was determined.

2.5. Effects of solvent and detergent

The effect of solvent on the production of 9*R*-HODE was evaluated using ethanol, methanol, isopropanol, butanol, acetone, ethyl acetate, iso-octane, and hexane at the concentrations of 1% and 2% (v/v). After each solvent was added to the reaction buffer, linoleic acid was added. This mixture was sonicated for 2 min to disperse and then was rapidly added to the solution of whole cells resuspended in the same reaction buffer on ice. The effect of methanol concentration on the production of 9*R*-HODE was investigated by varying its concentration from 0.5 to 5% (v/v). The effect of detergent on the production of 9*R*-HODE was evaluated using Span 20, Span 80, Tween 20, Tween 40, and Tween 80 at the concentrations of 0.05 and 0.1% (w/v).

2.6. Effect of oxygen supplying method

Pure oxygen (O_2) was supplied to a 500 ml baffled flask containing a working volume of 50 ml through an air flow meter (capacity, 1–500 ml min^{-1}; Kojima, Kyoto, Japan) and double air regulators connected to oxygen bombe via tubing. An air stone was attached to the end of the tubing to achieve an even supply of bubbling oxygen. Atmospheric air was supplied to the flask using an air pump connected to the air flow meter. Oxygen or air supply was delivered at a 50 ml min^{-1} l^{-1} (1 working volume/oxygen or air volume/min, vvm) oxygenation or aeration rate without agitation or mixing. Agitation was carried out in a shaking incubator (Vision Scientific, Bucheon, Korea). Oxygenation or aeration with agitation took place at a 1 vvm oxygenation or aeration rate and a 250 rpm agitation speed. To investigate the effect of agitation speed on the production of 9*R*-HODE, the agitation speed in a 500 ml baffled flask was varied from 0 to 350 rpm in 50 rpm intervals.

2.7. Effects of cell and substrate concentrations

The cell concentration was varied from 10 to 60 g l^{-1} at a constant linoleic acid concentration of 2 g l^{-1} to determine the optimal cell concentration for the production of 9*R*-HODE using whole recombinant cells expressing 9*R*-LOX. The substrate concentration was varied from 5 to 45 g l^{-1} at a constant cell concentration of 40 g l^{-1} to determine the substrate concentration for the maximal production of 9*R*-HODE. The reactions were performed for 30 min

with a 1 vvm oxygenation rate and a 250 rpm agitation speed in a 500 ml baffled flask containing a working volume of 50 ml. Time course reactions of the production of 9*R*-HODE from linoleic acid using whole recombinant cells expressing 9*R*-LOX from *Nostoc* sp. were conducted at 25 °C for 90 min in 50 mM Tris–HCl buffer (pH 7.5) containing 40 g l^{-1} cells, 15 g l^{-1} linoleic acid, and 2% (v/v) methanol with a 1 vvm oxygenation rate and a 250 rpm agitation speed in a 500 ml baffled flask containing a working volume of 50 ml.

2.8. Analytical methods

The cell mass was determined using a linear calibration curve relating optical density at 600 nm to dry cell weight as previously described [31]. Protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with a bovine serum albumin as a standard. Whole cell conversion was determined as the amount of 9*R*-HODE from the initial linoleic acid using 9*R*-HODE as a standard. The specific activity (U mg^{-1}) of whole recombinant cells expressing 9*R*-LOX was defined as the amount of cells required to produce 1 μmol of 9*R*-HODE per min per unit amount of cells (mg dry cell weight) at 25 °C and pH 7.5 for 10 min. The protein expression of whole cells was examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples for SDS-PAGE analysis were prepared by mixing whole cells and SDS buffer as a ratio of 1:4, and then denatured for 10 min at 95 °C. After whole cell reactions, the reaction broth was acidified with 3 M acetic acid to stop the reactions, and the produced hydroperoxy fatty acid was reduced with $NaBH_4$ or $SnCl_2$ on ice to convert the product into hydroxy fatty acid. The reduced mixture was centrifuged at 12,000 × *g* at 4 °C for 5 min. The supernatant was extracted with a 2-fold volume of ethyl acetate twice, and dried using a nitrogen gas stream [26,32]. The dried extract was dissolved in ethanol for HPLC analysis.

2.9. Liquid chromatography-mass spectrometry

The formations of 9*R*- and 13*S*-HODE and 9*R*-HPODE were analyzed using an HPLC system (Agilent 1260, Palo Alto, CA, USA) coupled to a UV detector at the absorbance at 234 nm with a reverse phase Nucleosil C_{18} column (150 mm × 2.1 mm, 5 μm particle size; Phenomenex, Torrance, CA, USA) [28,32]. The column was eluted at 25 °C with 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). The chirality of the product was determined using HPLC with a chiral phase Chiralcel OD-H column (150 mm × 2.1 mm, 5 μm particle size; Daicel, Tokyo, Japan). The column was eluted at a flow rate of 0.15 ml min^{-1} with a solvent system of *n*-hexane/2-propanol/acetic acid (100:5:0.1, v/v/v).

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis of HFAs was performed using a ThermoFinnigan LCQ Deca XP plus ion trap mass spectrometer (Thermo Scientific, Pittsburg, PA, USA) with a Synergi fusion-RP 80 Å column (100 mm × 30 mm, 4 μm particle size; Phenomenex) at National Instrumentation Center for Environmental Management (NICEM, Seoul, Korea) using an electrospray ionization (ESI) interface. The flow rate was 250 μl min^{-1} and a gradient of 0.1% formic acid and acetonitrile was used. The instrument consisted of an LC pump, an auto sampler, and a photodiode array detector. The following operation parameters were used: capillary temperature, 275 °C; ion source voltage, 5 kV; nebulizer gas, 40 psi nitrogen; capillary voltage, 46 V in positive mode and 15 V in negative ionization mode; average scan time, 0.01 min; average time to change polarity, 0.02 min; and collision energy (CE), approximately 35% abundance of the precursor ion.

3. Results and discussion

3.1. Properties of recombinant cells expressing LOX from Nostoc sp.

The gene (1311 bp) encoding a LOX from *Nostoc* sp., with the same sequence as that previously reported gene [30], was cloned and expressed in the *E. coli* ER2566 host. The pET-15b plasmid harboring the LOX gene of *Nostoc* sp. was transformed into the host, and the LOX protein was expressed in the recombinant cells by IPTG induction. The expressed protein showed a single band on SDS-PAGE with a molecular mass of approximately 48 kDa, which is consistent with the calculated value of 48 kDa based on the 437 amino acids plus six histidine residues (data not shown). After 16 h of induction, the activity of recombinant cells harboring LOX was 0.017 ± 0.01 U mg^{-1} (dry cell weight).

3.2. Identification of the product obtained from linoleic acid by whole recombinant cells expressing LOX from Nostoc sp.

The reaction products of whole recombinant cells obtained from linoleic acid were analyzed by HPLC using reverse-phase and chiral-phase columns and LC-MS/MS (Fig. 1). Before reduction, two distinct reaction products were detected to have the same retention times as the standards 9*R*-HODE and 9*R*-HPODE by HPLC using a reverse-phase column (Fig. 1a). After reduction, a major reaction product was observed with the same retention time as the 9*R*-HODE standard. The product obtained after reduction gave the LC-MS/MS fragments as shown in the mass spectrum in Fig. 1b [32,33]. The total molecular mass of the product was represented by a peak at *m*/*z* 295 in full scan mode in LC-MS/MS. The molecular mass of HODE is 295. The peak at *m*/*z* 277 was formed by the loss of H_2O from the total molecular mass, and the peak at *m*/*z* 171 ($^-OOC(CH_2)_7C$—OH) was formed by cleavage of the adjacent hydroxylated carbon of the C_9—C_{10} bond. The small peak at *m*/*z* 251 was formed by the loss of CO_2 from the product. The peaks at *m*/*z* 295, *m*/*z* 277, and *m*/*z* 251 indicated that the product was HODE, and the peak at *m*/*z* 171 also indicated that the product was hydroxylated at the C_9 position. LC-MS/MS peaks of the 9*R*-HODE standard showed the same fragmentation pattern as the reaction product of whole recombinant cells obtained from linoleic acid (data not shown). The chirality of the 9-HODE product was confirmed by HPLC using a chiral-phase column with the 9*R*-, 9*S*-, and 13*S*-HODE standards. The major product 9*R*-HODE and the minor product 13*S*-HODE were detected using the same retention times of their standards (Fig. 1c). Therefore, the *Nostoc* sp. LOX was a 9*R*-LOX, and whole recombinant cells harboring *Nostoc* 9*R*-LOX converts linoleic acid to 9*R*-HODE.

3.3. Effects of pH, temperature, and stability on the production of 9R-HODE by whole recombinant cells expressing 9R-LOX from Nostoc sp.

The effects of pH and temperature on the production of 9*R*-HODE from linoleic acid as a substrate by whole recombinant cells expressing 9*R*-LOX were determined by using reverse phase-HPLC. The optimum pH of the whole cells was 7.5, and the production of 9*R*-HODE was rapidly declined at lower and higher pH. The activity in sodium phosphate buffer was approximately 80% of that in Tris–HCl buffer (Fig. 2a). The optimal pH of purified LOX from *Anabaena* sp. PCC 7120 expressed in *Bacillus subtilis* was 6.0 [34]. The activity of purified LOX from *Nostoc punctiforme* PCC 73102 for the production of 13*S*-HODE using linoleic acid as a substrate showed a broad optimum pH in the range of 4.5–8.0 [29]. Therefore, the optimum pH of recombinant cells expressing 9*R*-LOX from *Nostoc* sp. was different from those of other LOXs.

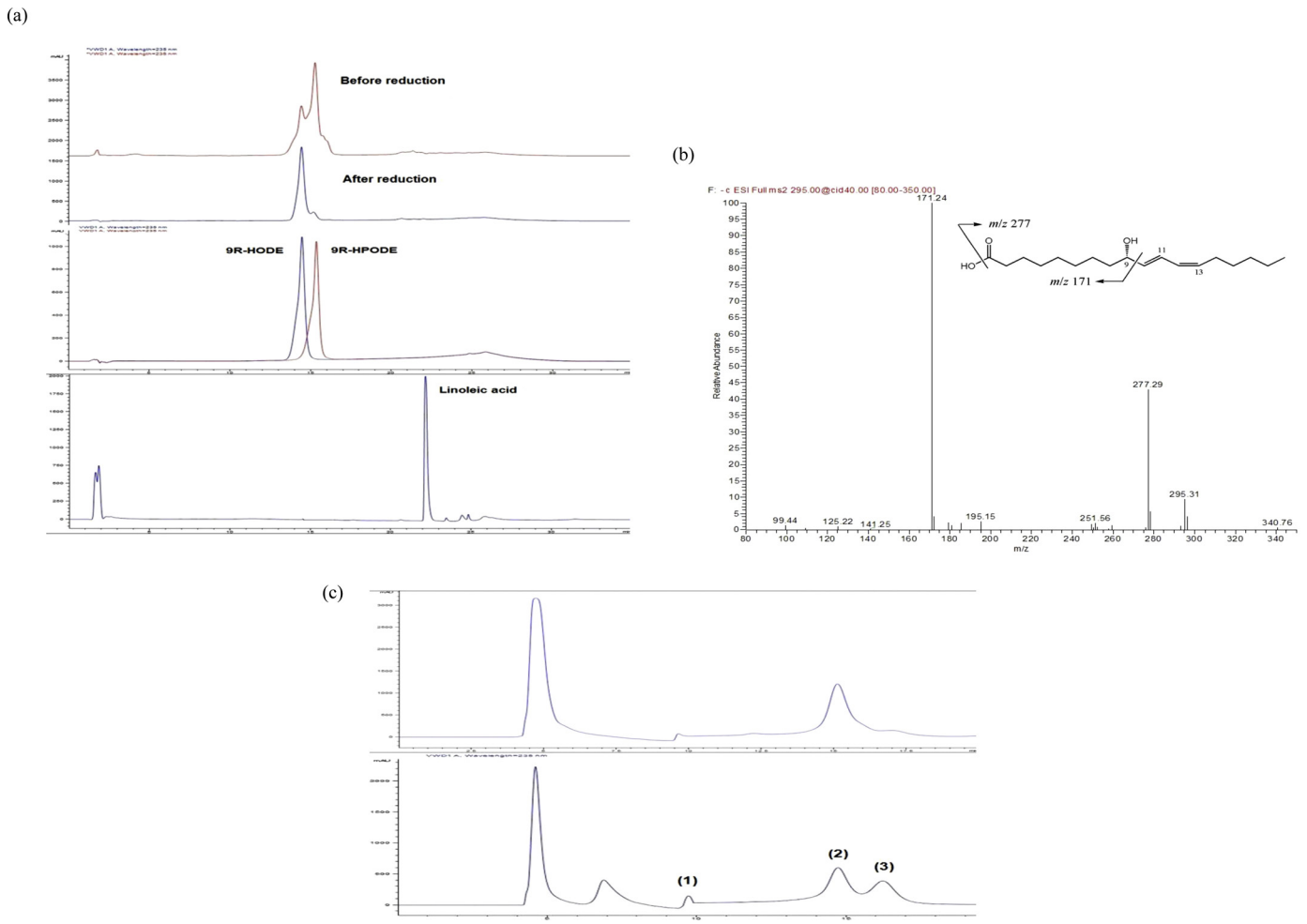

Fig. 1. Product analysis of whole recombinant cells harboring LOX from *Nostoc* sp. obtained in the presence of the linoleic acid substrate. (a) HPLC chromatogram using a reverse-phase column. Reaction products of *Nostoc* sp. LOX, upper; the 9*R*-HODE and 9*R*-HPODE standards, middle; and the linoleic acid substrate, lower. (b) LC-MS/MS chromatogram. The inset shows formation of the major fragments. (c) HPLC chromatogram using a chiral-phase column. Reaction products of *Nostoc* sp. LOX, upper; and the 9*R*- (2), 9*S*- (3), and 13*S*-HODE (1) standards, lower.

The optimum temperature of whole recombinant *E. coli* cells containing *Nostoc* sp. 9*R*-LOX was 25 °C, and its activity at 30 °C was approximately 95% of maximum activity at 25 °C (Fig. 2b). The optimum reported temperatures of LOXs were 45 °C for *Anabaena* sp. PCC 7120 LOX expressed in *B. subtilis*, [34] and 25 °C for *P. aeruginosa* 13*S*-LOX expressed in *E. coli* [35]. The thermal stability of whole recombinant cells expressing 9*R*-LOX was assayed after incubation for 3.5 h. The highest activity was obtained at 25 °C, and the enzyme activity at this temperature was not decreased after 3.5 h. Above 25 °C, the activity of recombinant cells expressing 9*R*-LOX decreased as the temperature increased and then the activity was approximately 50% of that at 40 °C (Fig. 2c).

3.4. Effects of solvent and detergent on the production of 9R-HODE by whole recombinant cells expressing 9R-LOX from Nostoc sp.

The effect of solvent on the production of 9*R*-HODE was investigated by 10 g l^{-1} whole recombinant cells expressing 9*R*-LOX with 2 g l^{-1} linoleic acid at 25 °C and pH 7.5 supplemented with 1 or 2% (v/v) solvent. The production of 9*R*-HODE from linoleic acid by whole recombinant cells expressing 9*R*-LOX was the highest using methanol among all of the solvents tested (Fig. 3a). Ethanol also showed similar enhancing effect for the production of 9*R*-HODE. The effect of methanol concentration on the production of 9*R*-HODE was examined by varying its concentration from 1 to 5% (v/v). Methanol at 2% was determined to be the optimum concentration, resulting in a 1.2-fold increase in the production of 9*R*-HODE, compared to that without methanol (Fig. 3b). Methanol supplementation may be helpful to solubilize the substrate linoleic acid and to enhance contact between substrate and enzyme, resulting in the increase of conversion rate. When methanol within 5% (v/v) was supplemented, no cell lysis was observed. However, the addition of detergent, including Span 20, Span 80, Tween 20, Tween 40, or Tween 80, at a concentration of 0.05 or 0.1% (w/v) did not increase the production of 9*R*-HODE (data not shown).

3.5. Effect of oxygen supply method on the production of 9R-HODE by whole recombinant cells expressing 9R-LOX

The effect of oxygen supply method on the production of 9*R*-HODE by whole recombinant cells expressing 9*R*-LOX was investigated without oxygenation, aeration, and agitation and with aeration (1 vvm), oxygenation (1 vvm), agitation (250 rpm), aeration (1 vvm) plus agitation (250 rpm), and oxygenation (1 vvm) plus agitation (250 rpm) in a 500 ml baffled flask (Fig. 4a). The production of 9*R*-HODE using all kinds of oxygen supply methods was over 5-fold higher than that when no oxygen supply was used. Among the oxygen supply methods tested, oxygen supplemented agitation method showed the highest production of 9*R*-HODE. The

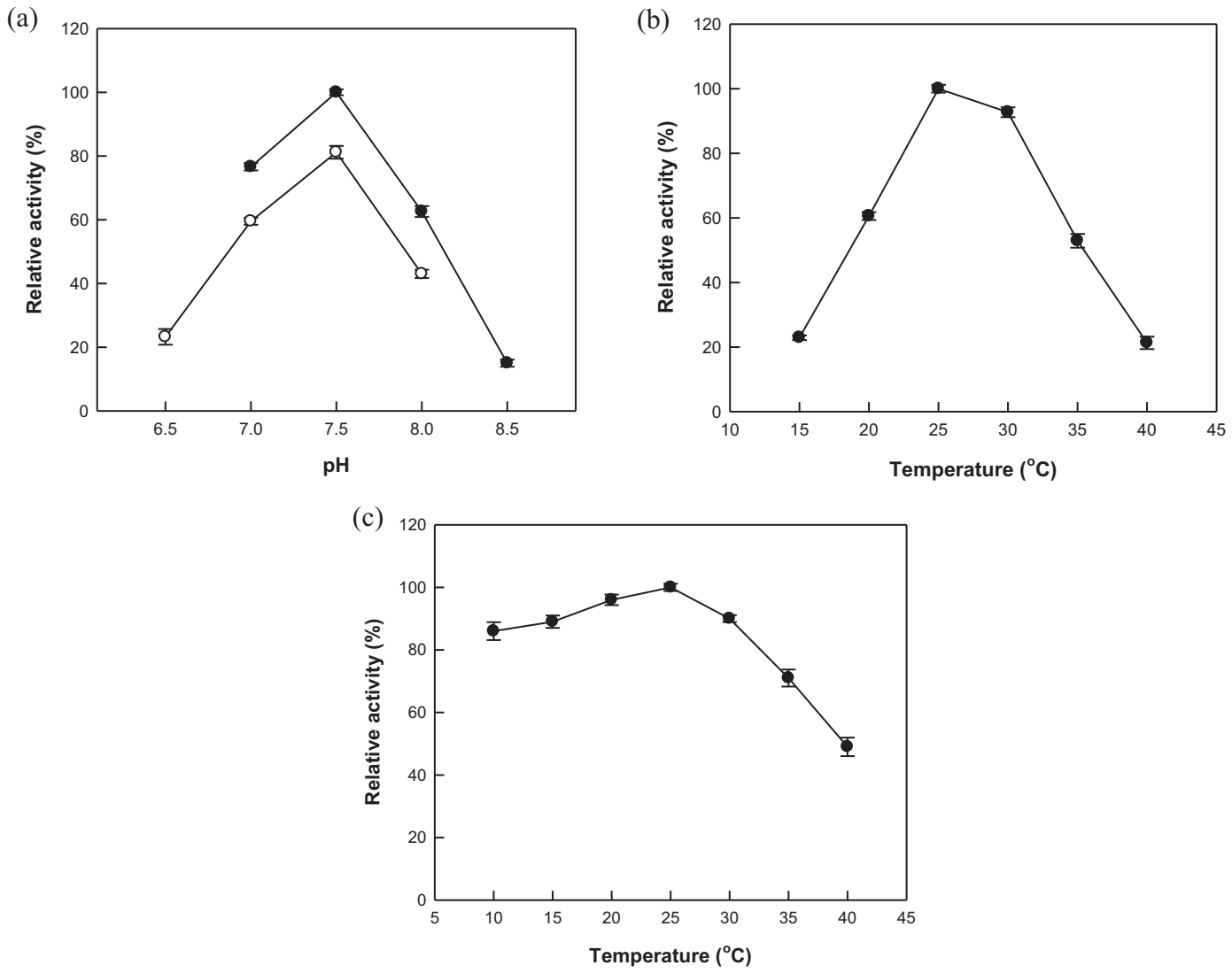

Fig. 2. Effects of pH (a), temperature (b), and stability (c) on the production of 9*R*-HODE by whole recombinant cells expressing 9*R*-LOX from *Nostoc* sp. The reactions for pH experiments were performed at 25 °C for 20 min using 100 mM sodium phosphate buffer (○, pH 6.5–8.0) and 50 mM Tris–HCl buffer (●, pH 7.0–8.5) containing 10 g l^{-1} cells and 2 g l^{-1} linoleic acid. The stability of whole recombinant cells was measured after incubation for 3.5 h. At 100% relative activity, 0.7 and 0.94 g l^{-1} of 9*R*-HODE was produced in the pH and temperature experiments, respectively.

effects of oxygenation with and without agitation on the production of 9*R*-HODE were slightly stronger than those of aeration with and without agitation, respectively. The conversion rate of soybean LOX by oxygenation (O_2 supply) was 1.4-fold higher than that by aeration (atmosphere air supply) [36]. Interestingly, agitation was more effective for the production of 9*R*-HODE than aeration or oxygenation because the used baffled flask may have enhanced the mixing and reduced the bubble sizes. At agitation speeds below 250 rpm, the production of 9*R*-HODE from linoleic acid increased with increasing agitation speed, however, the production plateaued above 250 rpm (Fig. 4b).

The effect of oxygen supply method on the 9*R*-/13*S*-HODE ratio was investigated (Table 1). Oxygen supplemented agitation showed the highest 9*R*/13*S*-HODE ratio (96:4) of the products, whereas no oxygen supply method showed the lowest ratio (87:13). Moreover, oxygen is a crucial co-substrate for the insertion of molecular oxygen into an unsaturated fatty acid. Therefore, oxygen supplemented agitation using whole recombinant cells harboring 9*R*-LOX was the most effective method to obtain a high 9*R*/13*S*-HODE ratio and to enhance the production of 9*R*-HODE. However, the increase of oxygen concentration in the reaction of soybean LOX, which produces 13-HPODE from linoleic acid, increases the 13/9-HPODE ratio [37].

3.6. Effects of cell and substrate concentrations on the production of 9R-HODE by whole recombinant cells expressing 9R-LOX from Nostoc sp.

The optimal concentration of whole recombinant cells expressing 9*R*-LOX to produce 9*R*-HODE was investigated by varying the

Table 1
Effect of oxygen supply method on the 9*R*-/13*S*-HODE ratio.

Air source	9*R*-HODE (%)	13*S*-HODE (%)	9*R*/13*S* Ratio
Oxygenation (1 vvm)	95.4	4.6	20.7
Aeration (1 vvm)	91.3	8.7	10.5
Oxygenation (1 vvm) with agitation (250 rpm)	96.2	3.8	25.3
Aeration (1 vvm) with agitation (250 rpm)	93.7	6.3	14.9
Agitation (250 rpm)	93.8	6.2	15.1
No aeration and agitation	87.3	12.7	6.3

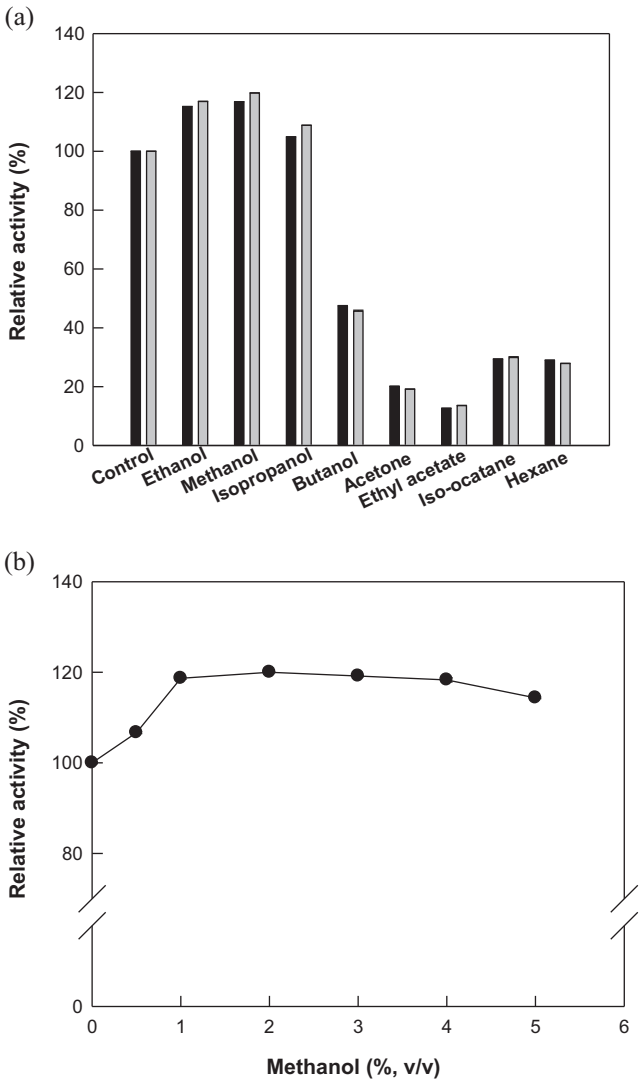

Fig. 3. Effects of solvent (a) and methanol concentration (b) on the production of 9*R*-HODE by whole recombinant cells expressing 9*R*-LOX from *Nostoc* sp. The reactions were performed at 25 °C for 20 min in 50 mM Tris–HCl buffer (pH 7.5) containing 10 g l^{-1} cells, 2 g l^{-1} linoleic acid, and 1 (■) or 2% (v/v) (■) solvent. A control reaction was performed without solvent. At 100% relative activity, 1.1 g l^{-1} of 9*R*-HODE was produced.

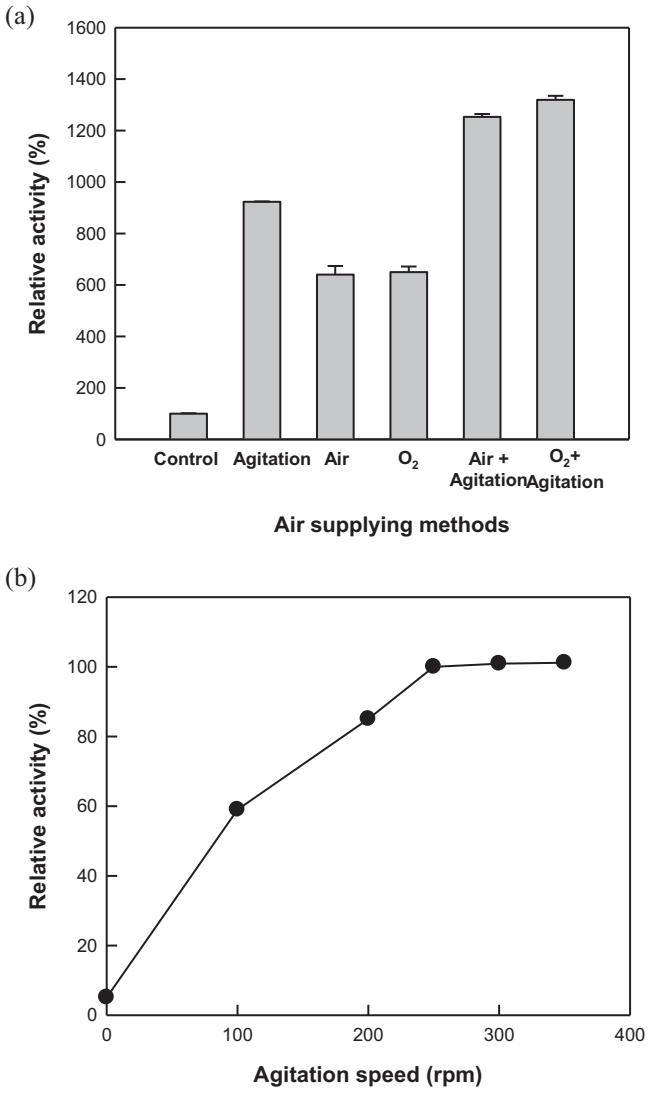

Fig. 4. Effects of oxygen supply method (a) and agitation speed (b) on the production of 9*R*-HODE by whole recombinant cells expressing 9*R*-LOX from *Nostoc* sp. The reactions were performed at 25 °C for 20 min under different oxygen supply conditions in 50 mM Tris–HCl buffer (pH 7.5) containing 10 g l^{-1} cells, 2 g l^{-1} linoleic acid, and 2% (v/v) methanol. The agitation speed for oxygen supply was 250 rpm and the aeration and oxygenation rates were 1 vvm in a 500 ml baffled flask containing a working volume of 50 ml. The control reaction was the production of 9*R*-HODE without oxygenation, aeration, and agitation. At 100% relative activity for blank, 0.1 g l^{-1} of 9*R*-HODE was produced.

cell concentration from 10 to 60 g l^{-1} with 2 g l^{-1} linoleic acid for 40 min. Below a cell concentration of 40 g l^{-1}, the production of 9*R*-HODE and conversion yield increased with increasing cell concentration, however, they decreased above 40 g l^{-1} (Fig. 5a). Therefore, the optimal cell concentration was determined to be 40 g l^{-1}. The production of 9*R*-HODE from linoleic acid was examined by varying the substrate concentrations from 5 to 45 g l^{-1} while holding the cell concentration constant at 40 g l^{-1} cells for 40 min. Below a substrate concentration of 30 g l^{-1}, increases in the substrate concentration led to increase in the production of 9*R*-HODE, however, these increases did not occur above 30 g l^{-1} linoleic acid (Fig. 5b). Above 15 g l^{-1} linoleic acid, the conversion yield decreased with increasing substrate concentration and then was 39% (w/w) at 45 g l^{-1} linoleic acid. When the substrate concentration was greater than 15 g l^{-1}, the conversion yield decreased significantly because the high viscosity of the reaction solution became high resistance to mass transfer. Thus, the substrate concentration was determined to be 15 g l^{-1}.

3.7. Production of 9R-HODE by whole recombinant cells expressing 9R-LOX from Nostoc sp. under the optimized conditions

The optimal conditions of whole recombinant *E. coli* cells expressing 9*R*-LOX from *Nostoc* sp. to produce 9*R*-HODE were pH 7.5, 25 °C, 40 g l^{-1} cells, 15 g l^{-1} linoleic acid, 2% (v/v) methanol, a 1 vvm oxygenation rate, and a 250 rpm agitation speed in a 500 ml baffled flask containing a working volume of 50 ml. Under these optimized conditions, whole recombinant cells expressing 9*R*-LOX produced 14.3 g l^{-1} 9*R*-HODE for 1 h with a conversion yield of 95% (w/w), volumetric productivity of 14.3 g l^{-1} h^{-1}, specific productivity 0.95 g l^{-1} h^{-1}, and 9*R*/13*S*-HODE ratio of 96:4 (Fig. 6).

The mechanism of stereo-selective oxygenation of LOX using linoleic acid as a substrate occurs by the stereo-specific hydrogen abstraction at the C_{11} position of linoleic acid, and then regio- and stereo-specific oxygen insertion takes place in antarafacial manner at either the C_9 or C_{13} position to produce 9- or

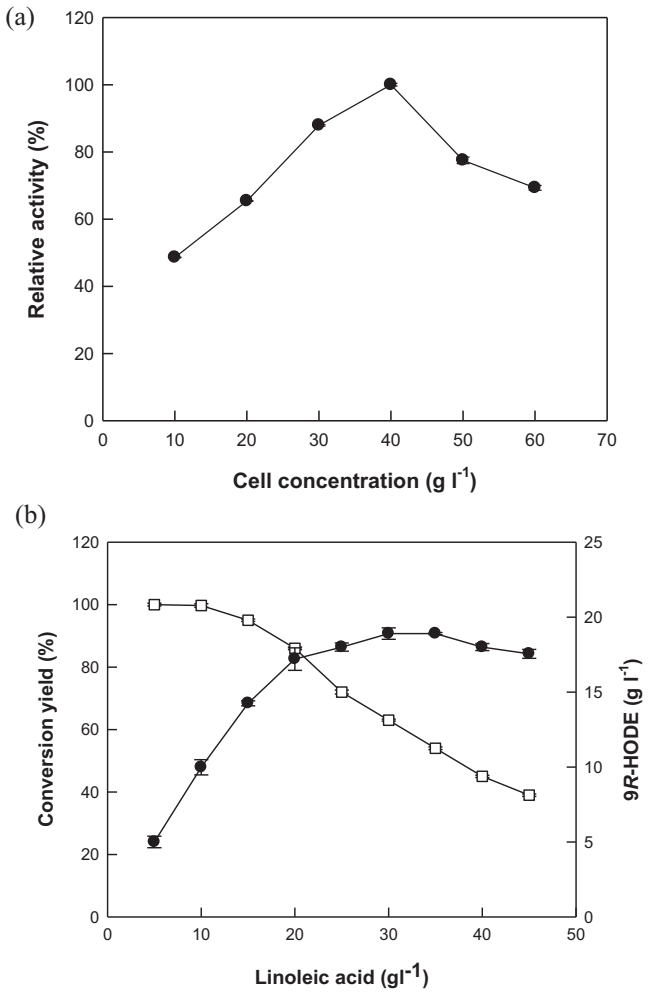

Fig. 5. Effects of cell (a) and substrate (b) concentrations on the production of 9*R*-HODE by whole recombinant cells expressing 9*R*-LOX from *Nostoc* sp. The agitation speed for oxygen supply was 250 rpm and the oxygenation rate was 1 vvm in a 500 ml baffled flask containing a working volume of 50 ml. Production of 9*R*-HODE (●) and conversion yield of 9*R*-HODE from linoleic acid (□). At 100% relative activity, 1.9 g l^{-1} of 9*R*-HODE was produced from 2 g l^{-1} linoleic acid.

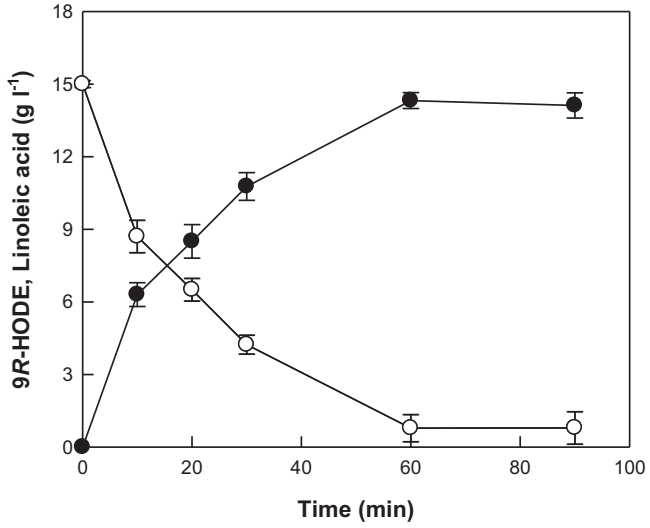

Fig. 6. Time course reactions of the production of 9*R*-HODE by whole recombinant cells expressing 9*R*-LOX from *Nostoc* sp. The agitation speed for oxygen supply was 250 rpm and the oxygenation rate was 1 vvm in a 500 ml baffled flask containing a working volume of 50 ml. Linoleic acid (○) and 9*R*-HODE (●).

13-hydroperoxide, respectively [2]. Plant LOXs catalyze the insertion of oxygen with *S* stereo-specificity to produce 9*S*-specific fatty acids, whereas cyanobacteria produce 9*R*-hydroxy fatty acids [26,27]. The determinant residues for *S*-LOX and *R*-LOX have conserved as valine and alanine residues [38,39], respectively. The 9*R*-LOX from *Nostoc* sp. SAG 25.82 used in the present study has a conserved alanine residue, indicating that the LOX is confirmed as an *R*-LOX.

HPFAs have been produced using either crude extracts or partial purified enzymes. However, these protein sources contain other enzymes and unknown proteins that result in the utilization of PUFA substrates or hydroperoxide products, leading to reduced activity [40]. Alternatively, purified recombinant LOX has been suggested to increase the catalysis of LOX with PUFA substrates [34,40]. However, the conversion using purified enzyme is laborious and requires the need for purification steps, such as cell lysis, precipitation, and dialysis. Thus, this is the first study in which whole recombinant cells have been used in the stereo-selective production of 9*R*-HODE. No LOX is present in *E. coli* and there are no by-products or unwanted reactions, thus, whole cell conversion is less laborious and eliminates the need for purification steps [41].

4. Conclusion

We successfully achieved the stereo-specific production of *R*-HFA using whole recombinant *E. coli* cells expressing *R*-specific LOX from *Nostoc* sp. Oxygen supplemented agitation was the most effective method not only to obtain a high 9*R*/13*S*-HODE ratio, but also to enhance the production of 9*R*-HODE. The pH, temperature, cell and substrate concentrations, and solvent to produce 9*R*-HODE from linoleic acid using oxygen supplemented agitation were optimized. Under these optimized conditions, whole recombinant cells expressing *R*-LOX produced 14.3 g l^{-1} 9*R*-HODE for 1 h, with a conversion yield of 95% (w/w), and an *R*/*S* ratio of 96:4. Therefore, our approach using whole cells expressing *R*-specific LOX is a cost-effective method for the stereo-specific production of HFA.

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