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Enantioselective synthesis of (*S*)-phenylephrine by whole cells of recombinant *Escherichia coli* expressing the amino alcohol dehydrogenase gene from *Rhodococcus erythropolis* BCRC 10909

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ABSTRACT

(*R*)-phenylephrine [(*R*)-PE] is an α_1-adrenergic receptor agonist that is widely used in over-the-counter drugs to treat the common cold. We found that *Rhodococcus erythropolis* BCRC 10909 can convert detectable level of 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) to (*S*)-PE by high performance liquid chromatography tandem mass spectrometry analysis. An amino alcohol dehydrogenase gene (*RE_AADH*) which possesses the ability to convert HPMAE to (*S*)-PE was then isolated from *R. erythropolis* BCRC 10909 and expressed in *Escherichia coli* NovaBlue. The purified RE_AADH, tagged with 6×His, had a molecular mass of approximately 30 kDa and exhibited a specific activity of 0.19 μU/mg to HPMAE in the presence of NADPH, indicating this enzyme could be categorized as $NADP^+$-dependent short-chain dehydrogenase reductase. *E. coli* NovaBlue cell expressing the *RE_AADH* gene was able to convert HPMAE to (*S*)-PE with more than 99% enantiomeric excess (ee), 78% yield and a productivity of 3.9 mmol (*S*)-PE/L h in 12 h at 30 °C and pH 7. The (*S*)-PE, recovered from reaction mixture by precipitation at pH 11.3, could be converted to (*R*)-PE (ee > 99%) by Walden inversion reaction. This is the first reported biocatalytic process for the production of (*S*)-PE from HPMAE.

1. Introduction

Phenylephrine (PE), a sympathomimetic agonist, has a wide variety of surgical applications, such as counteracting hypotension. It also is an active ingredient used in eye drops to dilate the pupil and in topical nasal decongestants [1,2]. Although the systemic bioavailability of PE in nasal decongestants is only about 38% that of orally administered *d*-pseudoephedrine (*d*-PDE) [3,4], PE does not cause the release of endogenous noradrenaline [5] and is less likely to cause side effects like central nervous system stimulation, insomnia, anxiety, irritability and restlessness [2]. Moreover, the Combat Methamphetamine Epidemic Act of 2005 permits the banning of over-the-count sales of medications containing *d*-PDE, in order to control the clandestine manufacture of methamphetamine from *d*-PDE in the USA. This has resulted in the substitution of (*R*)-PE for *d*-PDE in many nasal decongestants [6,7].

PE contains a chiral center in the C_α of the side chain, and (*R*)-PE exhibits more potent than the *S* enantiomer in activating of α_1-adrenergic receptors [2]. (*R*)-PE can be synthesized by symmetric hydrogenation and chemical resolution [8–10]. However, a racemic mixture typically results from symmetric hydrogenation, and repeat kinetic resolution to obtain the desired optical product often is both expensive and tedious. In recent years, several chemical asymmetric hydrogenation methods have been developed to allow for more direct (*R*)-PE synthesis [11–14]; but these processes usually require high pressure, high temperature, and several organic solvents that are not environmentally friendly.

Chiral aryl alcohols are important chiral building blocks for the production of optically active drugs in the pharmaceutical and agrochemical industries [15–17]. They can be obtained by asymmetric reduction of prochiral aryl ketones using oxidoreductases. Chiral 2-chloro-1-phenylethanol is a key intermediate during the preparation of anti-depressants and potential therapeutic agents of cocaine-abuse [18]. It can be converted from 2-chloro-1-phenylethanone by asymmetric reduction, using *Candida magnoliae* $NADP^+$-dependent carbonyl reductase [19] or *Ralstonia* sp. $NADP^+$-dependent alcohol dehydrogenase [20]. *Corynebacterium* sp. ST-10 with NAD^+-dependent phenylacetaldehyde reductase activity can be used in the conversion of 2-chloro-1-(3-chlorophenyl)ethanone to 2-chloro-1-(3-chlorophenyl)ethanol, a precursor for the synthesis of β_3 adrenergic receptor agonists [21,22]. Very recently, *Escherichia coli* cells that express the L-1-

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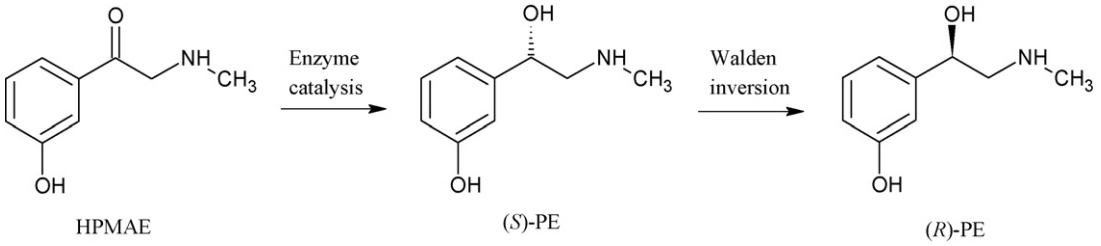

Fig. 1. Schematic diagram showing the conversion of HPMAE to (*S*)-PE by asymmetrical biocatalysis and the conversion of (*S*)-PE to (*R*)-PE by Walden inversion reaction.

amino-2-propanol dehydrogenase (*AADH*) gene from *Rhodococcus erythropolis* MAK154 have been demonstrated to possess the capacity to reduce (*S*)-1-phenyl-2-methylamino-propan-1-one (MAK) to *d*-PDE [23].

PE and *d*-PDE are almost identical in their structure, except that PE contains one hydroxyl group in the phenyl moiety, and *d*-PDE contains one methyl group at C_β in the side chain. To the best of our knowledge, no bioconversion method for the production of chiral PE from 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) has been reported. In this study, we describe that *E. coli* cells expressing amino alcohol dehydrogenase gene (*RE_AADH*) from *R. erythropolis* BCRC 10909 are able to produce (*S*)-PE from HPMAE, and the resulting (*S*)-PE could subsequently be converted to (*R*)-PE, a clinically useful sympathomimetic agonist, by Walden inversion reaction (Fig. 1).

2. Materials and methods

2.1. Chemicals, bacterial strains and media

(*R*)-PE and other general chemicals were purchased from Sigma–Aldrich (St. Louis, MO). All solvents used in HPLC analysis were of LC grade and purchased from Merck (Darmstadt, Germany). HPMAE and (*S*)-PE were obtained from Industrial Technology Research Institute (Hsinchu, Taiwan). The chemicals required for protein assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA). Culture media were obtained from Becton, Dickinson and Company (Sparks, MD). Bacterial strains were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). The expression vector pQE-30 was purchased from QIAGEN (Hilden, Germany).

2.2. Analysis of HPMAE and PE

A high performance liquid chromatography tandem mass spectrometry (HPLC–MS-MS) system consisting of two Perkin-Elmer Series 200 Micro LC pumps, a Series 200 Autosampler (Perkin-Elmer Co., Waltham, MA), and an AB-Sciex API-2000 triple quadrupole mass spectrometer with TurboIonSpray probe (Applied-Biosystems, Foster City, CA), was used to rapidly identify HPMAE and PE in the reaction mixture. Data processing was performed using Analyst version 1.3.1 software (Applied-Biosystems). HPLC analysis was performed on a reversed-phase Polaris C-18A column (2 mm i.d. × 50 mm; particle size, 3 μm) (Varian Inc. Palo Alto, CA). The mobile phase was 0.1% formic acid and methanol at a ratio of 15:85, and the flow rate was 100 μl/min. For MS-MS detection, the TurboIonSpray, orifice voltages, temperature, collision energy, and entrance potential were set at 5500 V, 80 V, 200 °C, 28 eV, and −9 V, respectively. The collision gas (nitrogen) was maintained at a pressure of 2.3×10^{-5} torr. The positive ion mode and multiple-reaction monitoring (MRM) mode were used to detect the HPMAE (*m*/*z* 166.1–148.1) and PE (*m*/*z* 168.1–150.1), respectively. Total analysis time was 5 min for each sample.

The chirality of PE was analyzed by HPLC with a chiral column (chiral-HPLC). Analytical HPLC was performed utilizing a Waters 2695 LC/Waters 996 Photodiode Array detector system with a CYCLOBOND I 2000 AC column (3.2 × 250 mm, Astec Inc., Whippany, NJ), the mobile phase consisted of methanol and 0.5% sodium acetate (pH 5.5) at a ratio of 5:95 under the following conditions at room temperature: flow rate of 0.7 ml/min, detection at 215 nm, scanning from 200 to 400 nm for compound identification, and retention times of 13.5 min for HPMAE, 14.8 min for (*R*)-PE, and 18.6 min for (*S*)-PE. Concentrations of HPMAE, (*S*)-PE and (*R*)-PE in the reaction mixture were also measured by chiral-HPLC, which was calibrated using several standard solutions of HPMAE, (*S*)-PE and (*R*)-PE, with their concentrations varied from 1 to 100 mM. The standard solutions were prepared freshly prior to each experiment. The values of enantiomeric excess of (*S*)-PE were calculated using the equation: $ee^{(S)\text{-PE}} = [(S\text{-enantiomer}) - (R\text{-enantiomer})]/[(S\text{-enantiomer}) + (R\text{-enantiomer})] \times 100\%$.

2.3. Screening of bacterial strains capable of converting HPMAE to PE

Bacterial colonies were inoculated into 50 ml Luria-Bertani (LB) medium and incubated at a given temperature for each microorganism with vigorous shaking until an OD_{600} of approximately 2.0 was achieved. Cells were harvested by centrifugation at 12,000 × *g* for 10 min. Cell pellets were resuspended in 10 ml of 100 mM sodium phosphate buffer (pH 7) or sodium citrate buffer (pH 6) containing 2% glucose and 10 mM HPMAE, and then incubated at 28 or 37 °C for 2 h. Once the reaction was completed, the cells were removed by centrifugation. The reaction solution was filtered through a 0.22-μm membrane and subjected to HPLC–MS-MS analysis.

2.4. Expression of the RE_AADH gene in E. coli

Genomic DNA of *R. erythropolis* BCRC 10909 was isolated using a MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). The open reading frame (ORF) of *RE_AADH* was obtained by PCR using *R. erythropolis* genomic DNA as the template, and primers designed on the basis of the *R. erythropolis* MAK154 *AADH* gene [23]: forward primer (5′-CGGGATCCATGTTCAACTCCATTGAAG), which introduces the sequence for a unique *Bam* HI site (underlined); and reverse primer (5′-CCCAAGCTTTTACAGTTCGCCGAGCGCCAT), which incorporates sequences from a unique *Hin*d III site (underlined). The PCR product was cloned as a *Bam* HI-*Hin*d III fragment into the corresponding site of pQE-30 to generate the plasmid pQE-*aadh*, thereby allowing high-level expression of the N-terminal 6×His-tagged RE_AADH in *E. coli*. The recombinant plasmid was introduced into *E. coli* NovaBlue. For high-level expression of the *RE_AADH* gene, *E. coli* NovaBlue (pQE-*aadh*) was grown in LB broth containing ampicillin (100 μg/ml) at 37 °C for 16 h with shaking. The culture was diluted (1:100) with 100 ml of LB and grown at 37 °C to an OD_{600} of approximately 0.6–0.8. To induce gene expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultivation continued at 30 °C for an additional 6 h. Cells were collected by centrifugation at 12,000 × *g* and 4 °C for 10 min. Bacterial pellets were washed twice with 25 ml of ice-cold 100 mM sodium phosphate buffer (pH 7.0) and resuspended in the same buffer. The IPTG-induced cells were disrupted by sonication and the cell debris pelleted by centrifugation at 4 °C for 15 min. The resulting supernatant was subjected to a Ni-NTA column (QIAGEN, Hilden, Germany), and the purification procedure carried out in accordance with the manufacturer's instructions. The expression level of the *RE_AADH* gene was analyzed by SDS-PAGE. The concentration of purified protein was measured with a protein assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard. Reduction activity of RE_AADH was measured by determining the production of PE from HPMAE by chiral-HPLC. The assay mixture (500 μl), containing the appropriate amount of enzyme, 5 mM HPMAE, and 1 mM NADPH in 100 mM sodium phosphate buffer (pH 6.0), was incubated at 30 °C. The enzyme activity of one unit (U) was defined as the amount of enzymes that result in the formation of 1 μmol PE from HPMAE per minute at 30 °C.

2.5. Production of (S)-PE using recombinant E. coli cells

A glass vessel (250 ml) equipped with a propeller-type impeller and an automatic pH controller (pH/ORP controller PC310, Suntex CO., Taipei, Taiwan) was used for the conversion of HPMAE to (*S*)-PE. A reaction mixture (100 ml) containing 1 g (wet weight) of IPTG-induced *E. coli* NovaBlue (pQE-*aadh*), 2% glucose, 20 mM KCl, 18 mM NH_4Cl, and various concentrations of HPMAE in 100 mM sodium phosphate buffer (pH 6.5 or 7.0) was incubated at 30 °C. The consumption of HPMAE and the production of (*S*)-PE were measured using chiral-HPLC.

2.6. Recovery of (S)-PE and Walden inversion reaction

Recovery of (*S*)-PE from the reaction mixture and conversion of (*S*)-PE to (*R*)-PE were performed mainly according to the method described by Dorokhova et al. [24]. Briefly, 200 ml solution containing 48 mM (*S*)-PE, which were prepared from the reaction mixture containing 60 mM HPMAE, was centrifuged at 12,000 × *g* and 4 °C for 20 min. The supernatants were filtered with a 0.22 μm membrane. The pH of the filtrate was adjusted to 10.5–11.5 using 10 N NaOH. As a result, the (*S*)-PE precipitated out, which was collected by centrifugation and then washed with cold water. Washed precipitates were dried by vacuum evaporator for Walden inversion reaction. To perform the Walden inversion reaction, (*S*)-PE, acetic anhydride, and

sulfuric acid were mixed in a molar ratio of 1:15:1. The reaction mixture was heated at 105 °C for 12 h, diluted to 25% of the original concentration using water, and then incubated at 95 °C for 3 h. Reaction products in the solution were recovered by alkaline precipitation as described above. Chirality and ee value of the product were determined by chiral-HPLC.

2.7. Sequence accession number

The nucleotide sequence of the *RE_AADH* gene from *R. erythropolis* BCRC 10909 has been deposited in the GenBank nucleotide database under accession number GQ280388.

3. Results and discussion

3.1. Analysis of HPMAE and PE using HPLC–MS-MS and chiral-HPLC

PE in pharmaceutical formulations usually is analyzed via the HPLC-UV method with a detection limit of 120 ng/ml [25]. However, HPLC-UV involves complicated purification procedures and requires a long separation time to avoid interference from other compounds. Recently, the HPLC–MS-MS method, which boasts higher sensitivity and specificity relative to HPLC-UV, was established for the determination of PE in human plasma [26]. In this study, a simple and fast HPLC–MS-MS method was developed to analyze HPMAE and PE in the reaction mixture.

In the preliminary experiment using the full scan mode for MS analysis, the most abundant $[M+H]^+$ ions for HPMAE and PE were m/z 166 and 168.1, respectively, which were selected as the precursor ions in MS-MS analysis. As shown in Fig. 2, the product ion spectra for HPMAE and PE were obtained by infusion in positive ion mode and the major product ions of HPMAE and PE were m/z 148 and 150.1, respectively. The MS-MS transitions of m/z 166–148 and m/z 168.1–150.1 then were selected for HPMAE and PE analysis using MS-MS with the MRM mode. To determine the concentrations of HPMAE and PE using HPLC–MS-MS analysis, the diluted reaction mixtures were separated by a Polaris C-18A column and introduced into MS-MS. The PE and HPMAE were eluted at retention

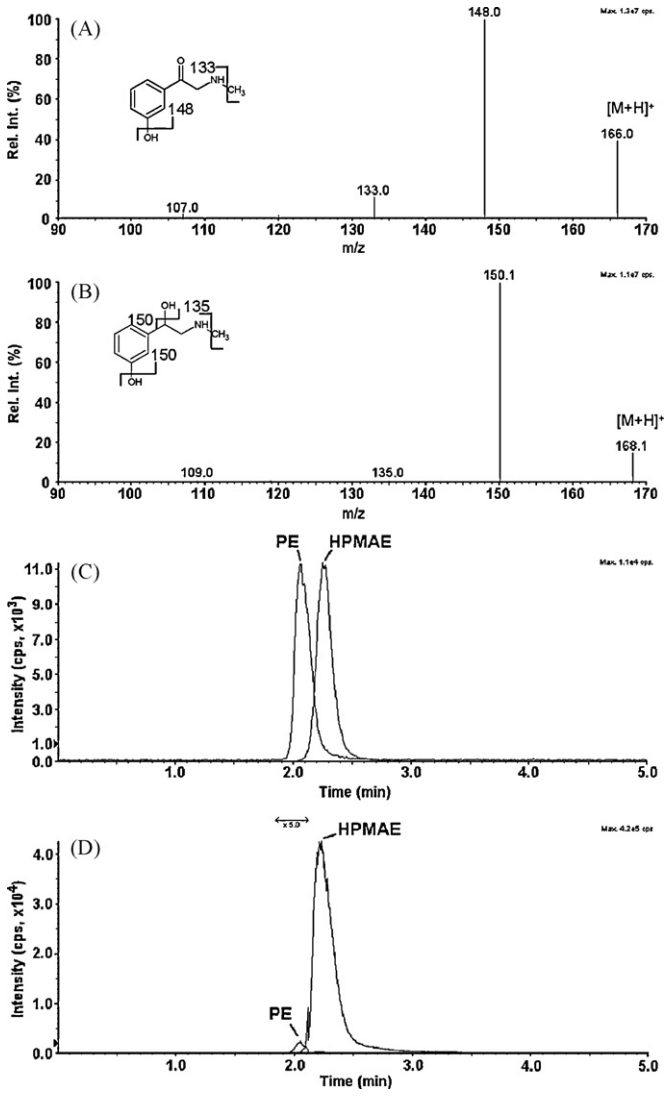

Fig. 2. Positive electron spray ionization product ion mass spectra of HPMAE (A) and PE (B) and mass chromatogram of HPMAE and PE (C). Standard solution (10 μM) of HPMAE and PE in 100 mM sodium phosphate buffer (pH 7.0) was separated by a Polaris C-18A column and detected under MRM mode (HPMAE: m/z 166–148; PE: m/z 168.1–150.1). Mobile phase: 0.1% formic acid/methanol 85:15 (v/v). (D) Mass chromatogram of PE converted from HPMAE by *R. erythropolis* BCRC 10909. The screening reaction using 100 mM phosphate buffer (pH 7.0) containing 2% glucose, 10 mM HPMAE, and 1% (wet weight) of cells was performed at 28 °C for 2 h.

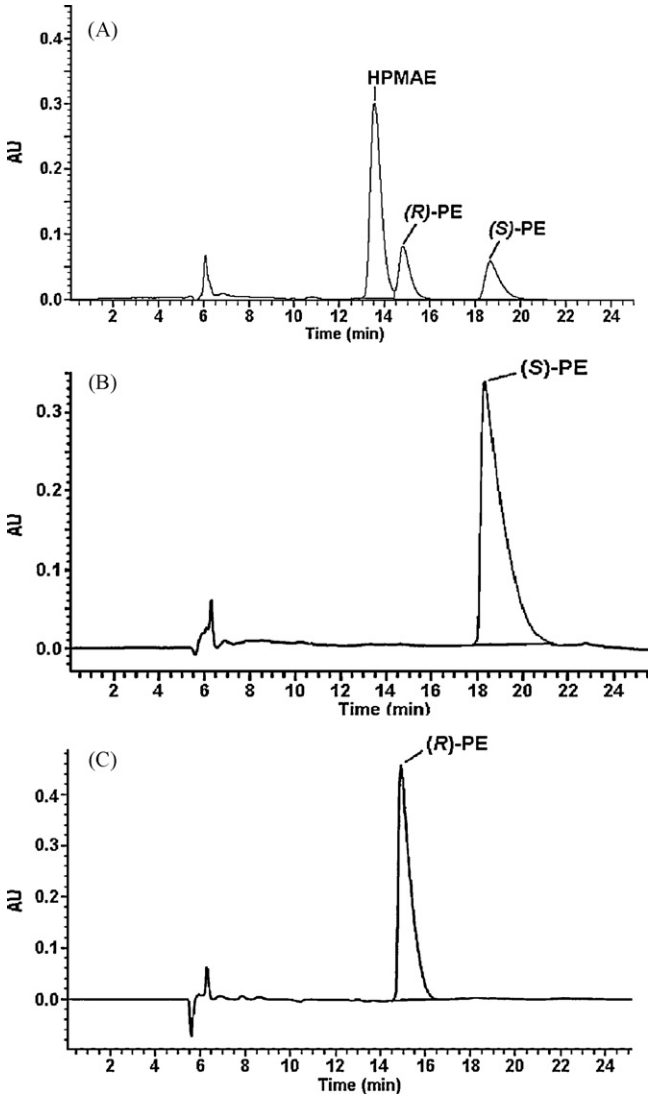

Fig. 3. Chiral-HPLC analysis of a PE enantiomer converted from HPMAE. (A) HPMAE, (*R*)-PE and (*S*)-PE standards. (B) Products recovered from reaction mixture by alkaline precipitation. (C) Inversion of (*S*)-PE to (*R*)-PE. To convert HPMAE to (*S*)-PE, the reaction mixture (200 ml) containing 100 mM phosphate buffer (pH 7.0), 2% glucose, 60 mM HPMAE and 1% (wet weight) of IPTG-induced *E. coli* NovaBlue (pQE-*aadh*) was incubated at 30 °C for 12 h. The resulting solution was used for the inversion of (*S*)-PE to (*R*)-PE.

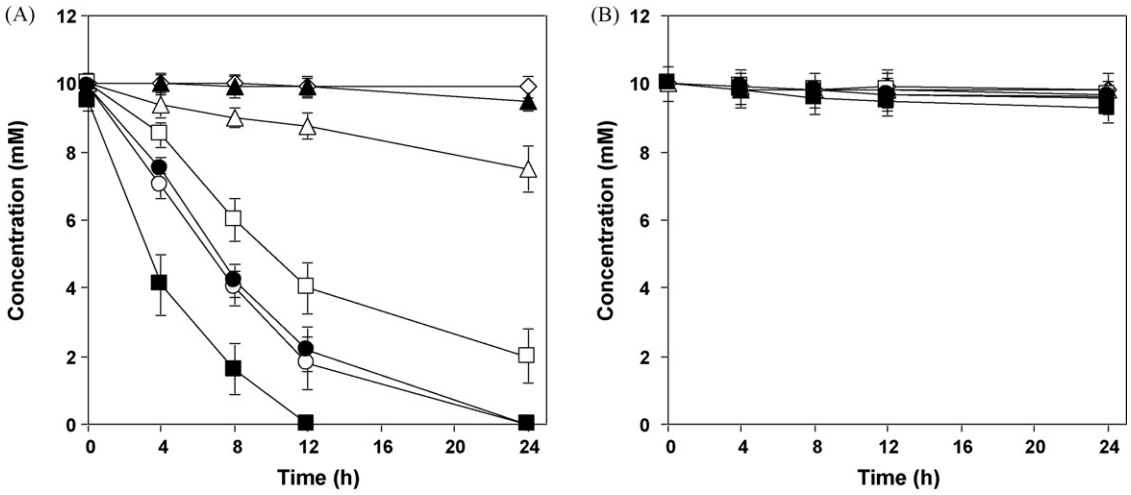

Fig. 4. Effect of pH on the stability of HPMAE (A) and (*S*)-PE (B). Buffer systems: (◊) sodium citrate (pH 4.0); (▲) sodium citrate (pH 5.0); (△) sodium citrate (pH 6.0); (□) sodium phosphate (pH 7.0); (○) sodium phosphate (pH 8.0); (●) Tris–HCl (pH 8.0); (■) Tris–HCl (pH 9.0). The reaction was performed in different buffers containing 10 mM HPMAE or (*S*)-PE at 30 °C for 24 h.

times of 2.05 and 2.3 min in the MRM chromatogram, respectively (Fig. 2C). The detection limit for PE was 0.1 μM (16.8 ng/ml, signal-to-noise ratio >3).

Although HPLC–MS-MS method is fast and high sensitive, it cannot determine the enantiomeric structure of PE. The absolute configurations of (*R*)-PE and (*S*)-PE were therefore determined by HPLC method using a CYCLOBOND I 2000 AC column. The CYCLOBOND I 2000 AC column, which is based upon the acetylated beta cyclodextrin form, has the potential to resolve many aromatic alcohols or amines that are chiral at the alpha or beta carbon [27–29]. According to the chromatogram of chiral-HPLC, the retention times for HPMAE, (*R*)-PE, and (*S*)-PE were 13.5, 14.8, and 18.6 min, respectively (Fig. 3A).

3.2. pH stability of substrate and product

The substrate, HPMAE, is synthesized under strong acid conditions and stable in solutions with a low pH. However, when the whole cells are used as a catalyst in the bioconversion reaction, the reaction is usually performed at pH 5–8 [30–32]. Thus, the effects of pH on the stabilities of HPMAE and PE were evaluated. As shown in Fig. 4A, HPMAE was stable at pH 4 and completely degraded in the buffer at pH higher than 8 after 24 h incubation. In contrast, PE was stable at all pH levels examined (Fig. 4B). PE has been reported to be stable in condition of stress caused by heat (80 °C), high acid (pH 2.0), high base (pH 10.0), UV radiation (254 nm) and oxidation (H_2O_2) treatments. Except under oxidative stress, less than 1.3% of PE is degraded over 24 h [33]. It is possible that the hydrogen of the hydroxyl group in the benzene ring of HPMAE can be removed in neutral or basic conditions following electron transfer between the benzene ring and the ketone in the C_α position, which may contribute to the structural rearrangement and instability of HPMAE. However, the ketone at C_α can be reduced to hydroxyl group to make PE stable.

3.3. Screening of bacterial strains capable of converting HPMAE to PE

Several microorganisms have been demonstrated to possess the capacity to reduce various kinds of ketone group to a hydroxyl group with enantioselectivity [34,35]. However, the bioprocess for the reduction of HPMAE to PE has never been reported. In this study, about 100 strains of bacteria from the genera of *Agrobacterium*, *Bacillus*, *Corynebacterium*, *Deinococcus*, *Enterobacter*, *Mesorhizobium*, *Pseudomonas*, *Rhodococcus* and *Streptomyces* were screened for their capacity to convert HPMAE to PE by HPLC–MS-MS analysis, and only *R. erythropolis* BCRC 10909 exhibited a detectable level of (*S*)-PE production (Fig. 2D).

3.4. Cloning and expression of the RE_AADH gene

NADP$^+$-dependent-L-1-amino-2 propanol dehydrogenase from *R. erythropolis* MAK154 possesses the ability to convert (*S*)-MAK to *d*-PDE, and the structure of HPMAE is similar to (*S*)-MAK. Thus, the ORF of the *R. erythropolis* BCRC 10909 *RE_AADH* gene was amplified by the PCR method using primers that were designed based upon the *R. erythropolis* MAK154 *AADH* gene. The amplified product was cloned into pQE-30 and introduced into *E. coli* NovaBlue to evaluate its capacity to convert HPMAE into PE.

The inserted DNA contained a 780-bp ORF encoding a 259-residue protein with a predicated molecular mass of 26.58 kDa. The DNA sequence of the *RE_AADH* gene demonstrated 99.5% homology with the *R. erythropolis* MAK154 *AADH* gene, with two amino acid residues Ala119 and Val188 in RE_AADH different from the Thr119 and Gly188 in *R. erythropolis* MAK154 AADH. RE_AADH exhibited 34–64% sequence identities with short-chain dehydrogenase reductase (SDR) from *Mycobacterium vanbaalenii* PYR-1; β-ketoacyl-(acyl carrier protein) reductase (Protein Data Bank code 2NMO) from *Streptomyces coelicolor* A3(2) [36]; 3-oxoacyl-(acyl carrier protein) reductase (Protein Data Bank code 2UVD) from *Bacillus anthracis* (Ba3989) [37]; and β-ketoacyl-(acyl carrier protein) reductase (Protein Data Bank code 1I01) from *E. coli* [38]. SDRs usually require a nicotinamide ribose, like NAD(H) or NADP(H), as a coenzyme in enzyme reactions. Alignment of the RE_AADH sequence with those of some NADP$^+$-dependent SDR enzymes of known three-dimensional structure revealed that the nucleotide-binding motif (TGxxxGxG) for coenzyme binding [39] is conserved in the N-terminal region (residues 13–20) of RE_AADH (Fig. 5). The conserved catalytic tetrad, Asn115-Ser143-Tyr157-Lys161, with the YxxxK active site motif for the SDRs [40,41], also was identified in RE_AADH. These results clearly imply that RE_AADH should be categorized among the NAD$^+$/NADP$^+$-dependent SDRs [42]. SDR genes are widely distributed in the genomes of humans, animals, plants and microorganisms, and are involved in the metabolism of steroid hormones, prostaglandins, carbohydrates, fatty acids and retinoids, as well as in the metabolism of xenobiotics, drugs and

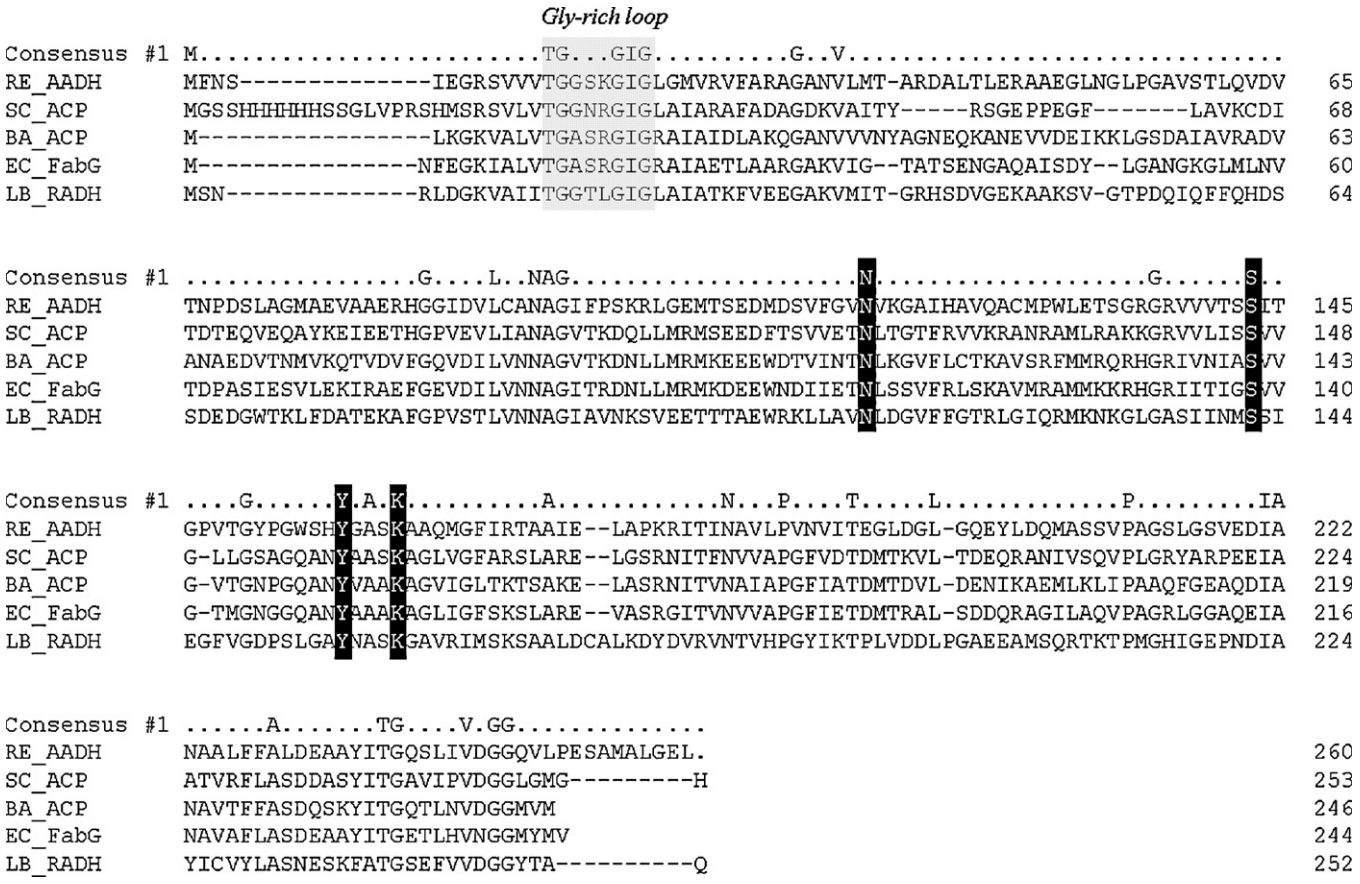

Fig. 5. Alignment of the RE_AADH with the short-chain dehydrogenase/reductases with known three-dimensional structures, from *Streptomyces coelicolor* (SC_ACP), *Bacillus anthracis* Ba3989 (BA_ACP), *E. coli* (EC_FabG) and *Lactobacillus brevis* (LB_RADH). The glycine-rich consensus sequence (TGxxxGxG) is shaded. The catalytic tetrad (Asn115, Ser143, Tyr157 and Lys161) is depicted on a black background.

carcinogens [43]. However, the physiological roles of this enzyme in *R. erythropolis* BCRC 10909 remain to be elucidated.

3.5. Expression of RE_AADH gene in E. coli

For high-level expression of the *RE_AADH* gene, the ORF of the *RE_AADH* gene was amplified by PCR and cloned into the *E. coli* expression vector pQE-30 to generate pQE-*aadh*. The recombinant *E. coli* NovaBlue (pQE-*aadh*) was incubated in LB medium containing ampicillin and induced with 0.5 mM IPTG at 30 °C for 6 h. The 6×His-tagged enzymes were purified to homogeneity by metal-chelate affinity chromatography on a Ni-NTA column. SDS-PAGE analysis of the crude extract and purified protein revealed a major protein band corresponding to a molecular mass of approximately 30 kDa (Fig. 6), comparing well with the calculated mass (28 kDa) of the 6×His-tagged translated product of the corresponding gene.

The purified recombinant RE_AADH catalyzed the conversion of HPMAE to (*S*)-PE with a specific activity of 0.19 μU/mg protein in the presence of NADPH, while no conversion activity was detected when NADH was used as the cofactor. The chiral-HPLC analysis revealed that the conversion product was (*S*)-PE with more than 99% ee. Structural study of $NADP^+$-dependent (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis* (LB_RADH) has shown that the residue Gly37 plays a critical role in determining the preference of SDRs for $NADP^+$ [41]. In several NAD^+-dependent SDRs, the residue corresponding to Gly37 of LB_RADH is highly conserved with aspartate, and the negative side-chain of aspartate forms hydrogen bonds with the 2′-hydroxyl group of the adenine ribose moiety of NAD^+ [44,45]. In $NADP^+$-dependent SDR enzymes, the protruding phosphor moiety of $NADP^+$ occupies more space and interacts with small amino acid residues, like Gly37 in LB_RADH [41]. The Ala39 residue of RE_AADH, corresponding to the Gly37 of LB_RADH, also is a small amino acid, suggesting that RE_AADH could be categorized as a $NADP^+$-dependent SDR.

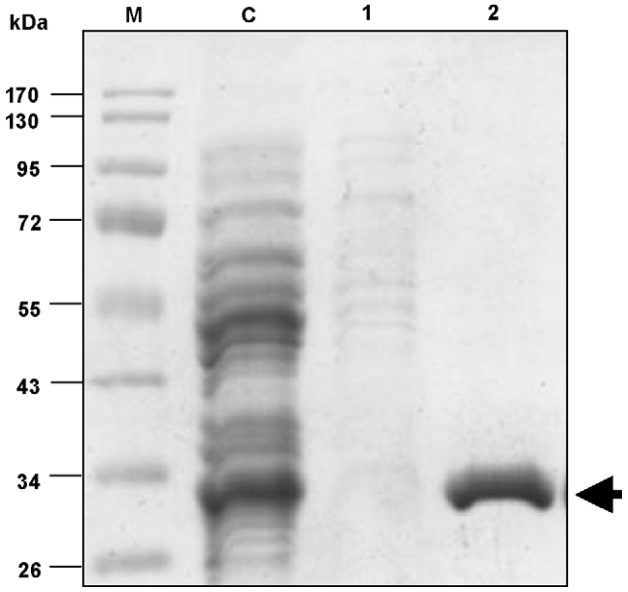

Fig. 6. SDS-PAGE analysis of RE_AADH purified from *E. coli* NovaBlue (pQE-*aadh*) by Ni-NTA column. Lane M: protein markers; lane C: crude extract; lane 1: wash flow; lane 2: purified RE_AADH.

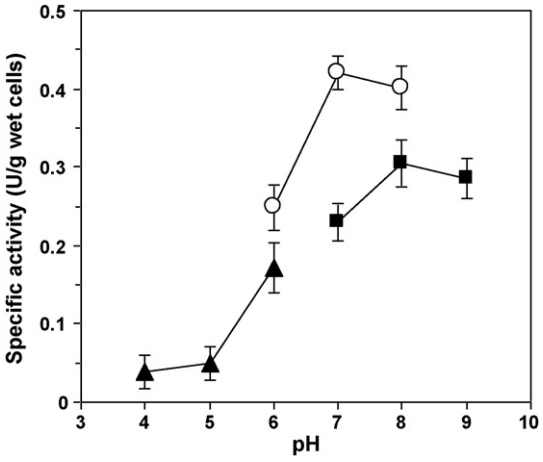

Fig. 7. Effect of pH on the cell enzyme activity of IPTG-induced *E. coli* NovaBlue (pQE-*aadh*) cells. Symbols: (▲) Sodium citrate buffer; (○) sodium phosphate buffer; (■) Tris–HCl buffer. The reaction was performed in 100 mM buffer containing 2% glucose, 10 mM HPMAE, and 1% (wet weight) of IPTG-induced *E. coli* NovaBlue (pQE-*aadh*) at 30 °C for 8 h.

3.6. Production of PE by recombinant E. coli cells

Since RE_AADH requires NADPH as a cofactor for its reduction activity, whole cells that express RE_AADH instead of purified RE_AADH would be the better choice for the bioconversion process, as NADPH may be continuously supplied by catabolic pathways of the cells during the course of the reaction [15]. To evaluate the feasibility of using whole cells expressing RE_AADH in the production of PE from HPMAE, the effect of pH on the RE_AADH activity of IPTG-induced *E. coli* NovaBlue (pQE-*aadh*) was determined. As shown in Fig. 7, the optimal pH for reduction was 7.0 in the sodium phosphate buffer, and the specific activity of whole cells expressing RE_AADH was 0.42 U/g wet cells.

To determine the enantioselectivity of the RE_AADH, the reaction mixture was centrifuged and filtered to remove cells and debris. The conversion products in the supernatant were analyzed using chiral-HPLC. The retention time of the examined PE was 18.8 min, indicating that the PE obtained from our biocatalysis process was *S*-form.

RE_AADH activity of recombinant *E. coli* cells was most active at pH 7 in the sodium phosphate buffer and decreased more than 50% at pH below 6 in the sodium citrate buffer (Fig. 7). However, HPMAE was unstable at the pH higher than 7.0 (Fig. 4A), but considerable stable at pH below 6.0. Therefore, the production of (*S*)-PE from HPMAE using IPTG-induced *E. coli* NovaBlue (pQE-*aadh*) was performed at pH 6.5 and 7.0 in 100 mM phosphate buffer. As shown in Fig. 8, about 7.5 mM PE was obtained in a 4-h reaction at pH 7.0, while only about 6.0 mM PE was obtained at pH 6.5. Obviously, pH 7.0 was better than pH 6.5 for the conversion of HPMAE to (*S*)-PE by cells expressing RE_AADH. We also found that the pH of the reaction mixture decreased over the course of the reaction (data not shown). To obtain a high yield of (*S*)-PE using a whole cell reaction system, the reaction mixture should be kept at a desired pH level. Therefore, a 250 ml glass vessel equipped with a propeller-type impeller and pH controller was used for (*S*)-PE production. The reaction was performed using 1% (wet weight) *E. coli* cells that express RE_AADH and 10 mM HPMAE at pH 7.0 and 30 °C. As shown in Fig. 9, the conversion of HPMAE to (*S*)-PE could be significantly improved by using a reaction mixture with pH control, and a productivity of 1.88 mmol PE/L h, with a conversion yield of about 75%, can be obtained in a 4-h reaction at pH 7.0. Obviously, cell enzyme activity would benefit from pH control, though maintaining a pH of 7.0 might cause HPMAE degradation.

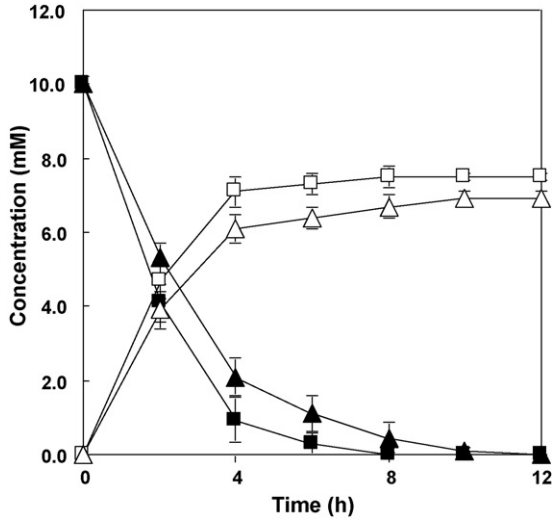

Fig. 8. Effect of pH on the synthesis of (*S*)-PE from HPMAE using *E. coli* NovaBlue (pQE-*aadh*) as catalysts. Symbols: (▲) HPMAE at pH 6.5; (△) (*S*)-PE at pH 6.5; (■) HPMAE at pH 7; (□) (*S*)-PE at pH 7. A reaction mixture (10 ml) containing 0.1 g *E. coli* NovaBlue (pQE-*aadh*) cells (wet wt.), 10 mM HPMAE and 2% glucose in 100 mM phosphate buffer was incubated at 30 °C.

To determine the effect of the substrate concentration on (*S*)-PE production, the HPMAE concentration was increased up to 70 mM and the pH of the reaction mixture maintained at 7.0 (Fig. 10). As shown in Fig. 10B, a maximal concentration of (*S*)-PE (46.8 mM) and productivity of 3.9 mmol/L h with a conversion yield of 78% could be obtained from 60 mM HPMAE in a 12 h reaction. When the concentration of HPAME was increased to 70 mM, the viable cell count of recombinant *E. coli* was decreased from 1.6×10^{10} to 9×10^{6} CFU/ml and the production of (*S*)-PE was also decreased significantly (Fig. 10C), indicating a toxicity of high HPMAE concentration to *E. coli* cells. Therefore, continuous feeding to maintain an appropriate substrate concentration of HPMAE in the reaction mixture would be a preferred strategy to optimize (*S*)-PE production.

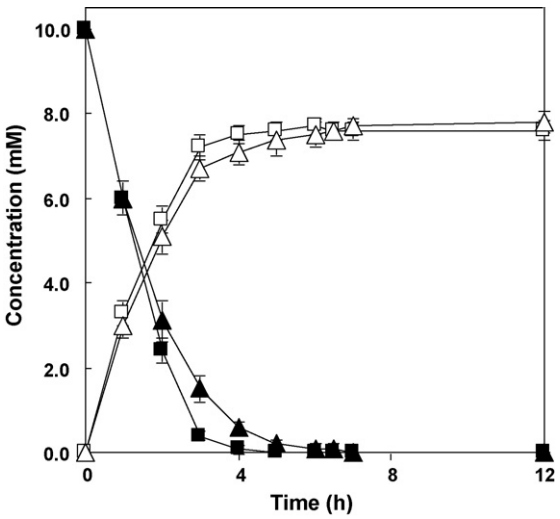

Fig. 9. Synthesis of (*S*)-PE from HPMAE using *E. coli* NovaBlue (pQE-*aadh*) cells as catalysts. Symbols: (▲) HPMAE consumption without pH control; (△) (*S*)-PE production without pH control; (■) HPMAE consumption with pH control at 7; (□) (*S*)-PE production with pH control at 7. A reaction mixture (100 ml) containing 1 g *E. coli* NovaBlue (pQE-*aadh*) cells (wet wt.), 10 mM HPMAE and 2% glucose in 100 mM phosphate buffer (pH 7) was incubated at 30 °C.

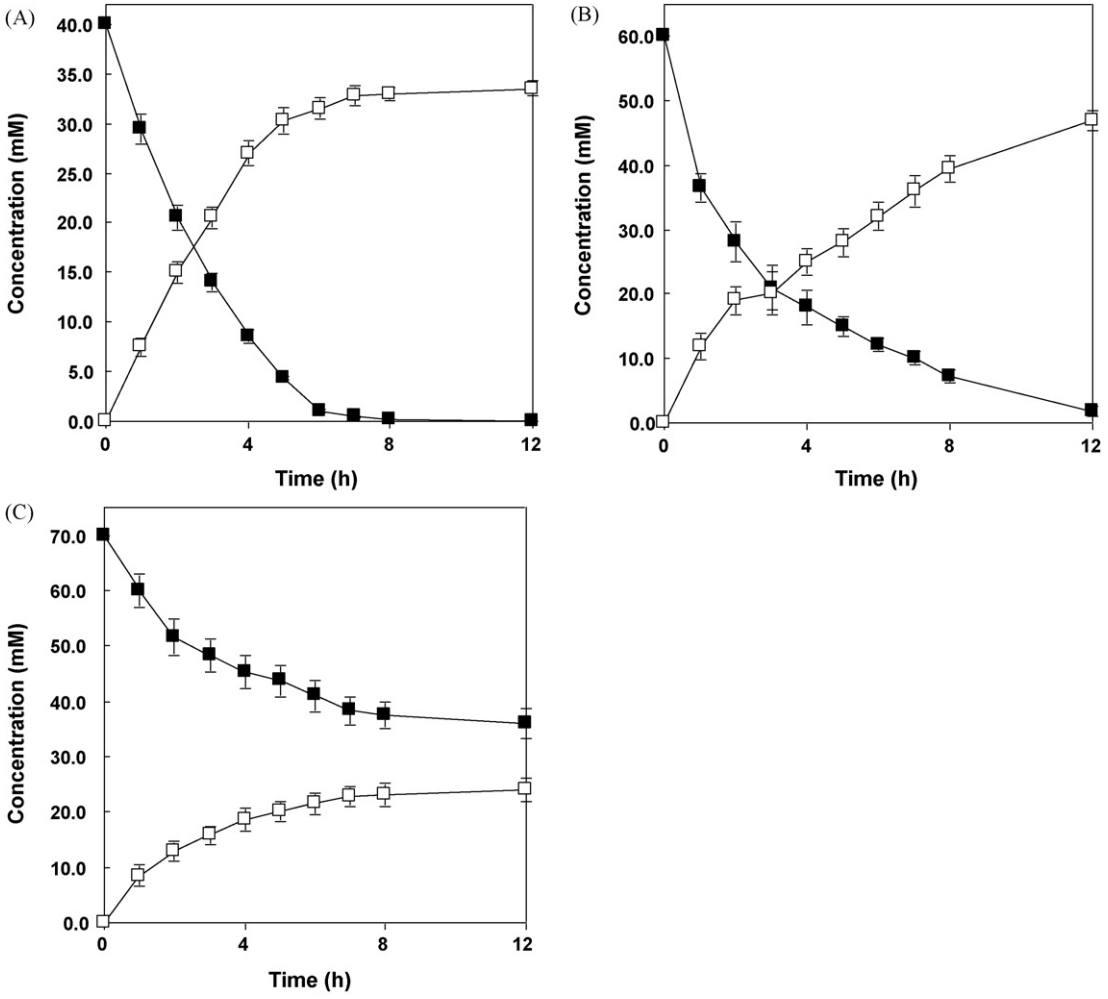

Fig. 10. Effect of HPMAE concentration on (*S*)-PE production by *E. coli* NovaBlue (pQE-*aadh*) cells. A reaction mixture (100 ml) containing 1 g IPTG-induced *E. coli* NovaBlue (pQE-*aadh*) cell (wet wt.), 2% glucose and different concentrations of HPMAE in 100 mM phosphate buffer (pH 7) was incubated at 30 °C. Reaction pH was maintained at 7.0 by a pH controller. Initial HPMAE concentrations: (A) 40 mM, (B) 60 mM, and (C) 70 mM. Symbols: (■) HPMAE consumption; (□) (*S*)-PE production.

3.7. Conversion of (S)-PE to (R)-PE by Walden inversion reaction

A reaction mixture (200 ml) containing 2 g (wet weight) of IPTG-induced *E. coli* NovaBlue (pQE-*aadh*), 2% glucose, 20 mM KCl, 18 mM NH_4Cl and 60 mM HPMAE in 100 mM sodium phosphate buffer (pH 7.0) was incubated at 30 °C for 12 h. To perform Walden inversion, recombinant *E. coli* cells in 200 ml resulting solution containing 48 mM of (*S*)-PE (1.60 g) were removed by centrifugation. About 81% of (*S*)-PE (1.42 g) was recovered from supernatant by alkaline precipitation (Fig. 3B). Resulting (*S*)-PE was subjected to inversion reaction. The (*R*)-PE was obtained in 99% reaction yield (1.41 g) and more than 99% ee (Fig. 3C). The overall yield of Walden inversion was about 80%, in which the decrease in the yield is almost resulted from the process of alkaline precipitation of (*S*)-PE from the reaction mixture.

4. Conclusion

Our study has shown that *E. coli* expressing RE_AADH can be used as biocatalyst for the enantioselective synthesis of (*S*)-PE (ee > 99%) from HPMAE, and resulting (*S*)-PE can be converted to give a reaction yield of 81% and an ee of >99% of (*R*)-PE using the Walden inversion reaction. Several improvements, such as the protein engineering of RE_AADH to increase biocatalytic activity of recombinant *E. coli* cells and the screening of recombinant *E. coli* cells with resistance to the high concentration of HPMAE, might lead to a bioconversion process with high yield of (*S*)-PE. Nevertheless, we found that about 20% of (*S*)-PE was lost in the alkaline precipitation step for the inversion of (*S*)-PE to (*R*)-PE. To avoid the need for Walden inversion reaction, experiments are currently in progress to develop a process for direct conversion of HPMAE to (*R*)-PE using other amino alcohol dehydrogenases.

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