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Enantioselective synthesis of (*R*)-phenylephrine by *Serratia marcescens* BCRC10948 cells that homologously express *SM_SDR*

Yi-Chia Kuan[a], Yue-Bin Xu[a], Wen-Ching Wang[b], Ming-Te Yang[a,*]

[a] Institute of Molecular Biology, National Chung Hsing University, Taichung 40227, Taiwan
[b] Institute of Molecular and Cellular Biology and Department of Life Sciences, National Tsing-Hua University, Hsinchu, 30013, Taiwan

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ABSTRACT

A short-chain dehydrogenase/reductase from *Serratia marcescens* BCRC10948, SM_SDR, has been cloned and expressed in *Escherichia coli* for the bioconversion of 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) to (*R*)-phenylephrine[(*R*)-PE]. However, only 5.11 mM (*R*)-PE was obtained from 10 mM HPMAE after a 9 h conversion in the previous report. To improve the biocatalytic efficiency, the homologous expression of the *SM_SDR* in *S. marcescens* BCRC10948 was achieved using the T5 promoter for expression. By using 2% glycerol as carbon source, we found that 8.00 ± 0.15 mM of (*R*)-PE with more than 99% enantiomeric excess was produced from 10 mM HPMAE after 12 h conversion at 30 °C and pH 7.0. More importantly, by using 50 mM HPMAE as the substrate, 23.78 ± 0.84 mM of (*R*)-PE was produced after a 12 h conversion with the productivity and the conversion yield of 1.98 mmol (*R*)-PE/l h and 47.50%, respectively. The recombinant *S. marcescens* cells could be recycled 6 times for the production of (*R*)-PE, and the bioconversion efficiency remained at 85% when compared to that at the first cycle. Our data indicated that a high conversion efficiency of HPMAE to (*R*)-PE could be achieved using *S. marcescens* BCRC10948 cells that homologously express the *SM_SDR*.

1. Introduction

Optically active phenylephrine (PE) plays a rapidly growing role as a building block for the synthesis of pharmaceuticals and fine chemicals. PE is a sympathomimetic, and it contains an α_1-adrenergic receptor of the phenethylamine class, which is widely used as a nasal decongestant in common cold and flu medicines [1], as an anti-hemorrhoid agent, for priapism treatment, as a vasopressor and for pupil dilatation. Other sympathomimetics, such as phenylpropanolamine, pseudoephedrine (PDE) and ephedrine, are also used in cough and cold medicines [2]. The over-the-counter (OTC) medicines containing phenylpropanolamine and pseudoephedrine have no longer been recommended for use by the Food and Drug Administration since the year 2000 and 2005, respectively, because these drugs are associated with an increasing risk of hemorrhagic stroke and the production of methamphetamine [3].

The structure of (*R*)-PE and (*S*)-PDE are almost identical, except that (*R*)-PE contains a hydroxyl group in the phenyl moiety, and (*S*)-PDE contains one methyl group at C_β in the side chain. Bioconversion methods for the production of chiral PE from 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) by *E. coli* expressing short chain dehydrogenase/reductase (SDR) or amino alcohol dehydrogenase have been reported [3–5] (Fig. S1). Compared to chemical synthesis, the enzymatic synthesis of chiral compounds is highly enantioselective and eco-friendly. Moreover, many SDRs require the expensive cofactor NAD(H) or NADP(H) for the stereoselectively reduction enzyme activity [4,6]. Due to the internal cofactor regeneration and low cost for downstream processing [4,7], whole-cell bioconversion is a favorable method for industrial chiral compound production. Many microorganisms have been applied as whole-cell bioconversion hosts for bioreduction processes, e.g., *E. coli* cells, which were used to synthesize (*R*)-phenylethanol [8] and (*S*)-phenylephrine [3,5], and *Candida parapsilosis* cells, which were used to reduce aromatic prochiral ketones [9].

In our previous report, SM_SDR, a NADPH-preferred short-chain alcohol dehydrogenase/reductase from *S. marcescens* BCRC10948, was isolated, characterized and successfully expressed in *E. coli* cells for the production of active (*R*)-PE from HPMAE. However, a low conversion yield and productivity limited the application of this method in

Abbreviations: E. coli, *Escherichia coli*; HPLC, high performance liquid chromatography; HPMAE, 1-(3-hydroxyphenyl)-2-(methylamino) ethanone; PDE, pseudoephedrine; PE, phenylephrine; *S. marcescens*, *Serratia marcescens*; SDR, short-chain dehydrogenase/reductase; SM_SDR, *S. marcescens* short-chain dehydrogenase/reductase; *SM_SDR*, *S. marcescens* short-chain dehydrogenase/reductase gene; OTC, over-the-counter

* Corresponding author.
E-mail address: mtyang@dragon.nchu.edu.tw (M.-T. Yang).

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Table 1
Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Sources or references
E. coli NovaBlue	*endA1 hsdR17*($r_{K12}^{-}m_{K12}^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac F'*[*proA* + *B* + *lacI*^{q} *ZΔM15:Tn10*] (*Tet*^{R})	Novagen Co.
S. marcescens BCRC10948	Production of L-asparaginase–U. S. Pat. 3,627,639; follicle stimulating hormone (FSH) binding inhibitor–U. S. Pat. 4,652,450	Bioresource Collection Research Center
pET30a-*sdr10*	pET30a containing *SM_SDR* gene	Peng et al. [4]
pQE-30	Amp^{r}; 6x His tag; T5 promoter	Qiagen Co.
pUC57-kan	Kan^{r}; *lac* promoter	GenScript
pUC-T5-*sdr*10	pUC57-kan containing *SM_SDR* gene with N-terminal His-tag under the control of T5 promoter	This study

industrial processes [4]. It is reasonable to assume that the heterologous expression of the SM_SDR in *E. coli* cells affected their metabolic homeostasis. In this study, the homologous expression of SM_SDR in its native host *Serratia marcescens* BCRC10948 was carried out to produce optically pure (*R*)-PE from HPMAE with improved biotransformation efficiency, yield and productivity.

2. Materials and methods

2.1. Chemicals, media and bacterial strains

All solvents used in high performance liquid chromatography (HPLC) were LC grade and purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA). HPMAE and (*S*)-PE were obtained from Cheng Fong Chemical (Taoyuan, Taiwan). (*R*)-PE and other chemicals were purchased from Sigma-Aldrich. *Taq* DNA polymerase and T4 DNA ligase were purchased from TaKaRa (Tokyo, Japan). Culture media were obtained from Becton, Dickinson and Company (Sparks, MD, USA). The bacterial strains and plasmids used in this study were listed in Table 1. *S. marcescens* BCRC10948 was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The plasmids pET-30a (Novagen, Inc., Madison, WI, USA), pUC57-kan (GenScript, NJ, USA) and pQE-30 (QIAGEN, Hilden, Germany) were used as expression vectors in *E. coli* and *S. marcescens*. *S. marcescens* BCRC10948 was cultivated in a YP medium (yeast extract 10 g/l, peptone 10 g/l and NaCl 5 g/l) at pH 7.0, 30 °C with shaking at 150 rpm for 24 h. *E. coli* NovaBlue was cultivated in Luria-Bertani (LB) medium at 37 °C.

2.2. Analysis of HPMAE and PE

A reverse-phase INERTSIL 10 ODS column (3.2 × 250 mm; VERCOPAK, Taipei, Taiwan) was used for the HPLC analysis of HPMAE and PE. The flow rate of the mobile phase [methanol and 0.5% sodium acetate buffer (pH 5.5) at a ratio of 2:98] was 0.8 ml/min, and the detection wavelength was 215 nm [4,5].

The PE enantiomers were analyzed by HPLC with the chiral column CYCLOBOND I 2000 AC (4.6 × 250 mm; Aztec, NJ, USA). The flow rate of the mobile phase [methanol and 0.5% sodium acetate buffer (pH 5.5) at a ratio of 5:95] was 0.4 ml/min, and the detection wavelength was 215 nm. The retention times were 11.8 min for (*R*)-PE and 13.2 min for (*S*)-PE. The values of the enantiomeric excess of (*R*)-PE were calculated using the following equation: $ee^{(R)-PE}$ = [(*R*-enantiomer)-(*S*-enantiomer)]/[(*R*-enantiomer) + (*S*-enantiomer)] × 100% [4,5].

2.3. Cloning and expression of SM_SDR in S. marcescens BCRC10948

The pQE-30 and pET30a-*sdr10* [4] plasmids were used as templates for PCR amplification. Specific primers were designed for the amplification of the T5 promoter and the *SM_SDR* gene. The following primer pair was used for amplification of the T5 promoter: pUC-T5-EcoRVF′ (forward) 5′-CACGAGGATATCTCGTCTTCACGTCGAG-3′ and pUC-T5-*Xho*IR′ (reverse) 5′-CCTCTCATCTCGAGTTTCTCCTCTTTAATGAA-3′. The following primer pair was designed for amplification of the *SM_SDR* gene: pUC-*sdr*10*Xho*IF (forward) 5′-TATACTCGAGATGCATCACCATCA CCATCACACCACAGCACATCCTCTGCAAGGCAAAG-3‘ and pUC-*sdr*10NdeIR (reverse) 5′-AAACATATGTTATTACGCCGAGAAACCGC CGT-3′. The DNA fragment containing the T5 promoter was PCR amplified from the pQE-30 plasmid with a *Xho*I restriction site at the 3′ end, and the *SM_SDR* gene was PCR amplified from pET30a-*sdr10* containing a N-terminal His_6-tag and a 5′ *Xho*I site. The amplified DNA fragments were digested by *Xho*I, recovered with a clean/gel extraction kit (Favorgen, Taipei, Taiwan) and ligated by T4 DNA ligase. The ligated DNA fragment containing the *SM_SDR* gene under the control of the T5 promoter was used as a template for PCR amplification using pUC-T5-EcoRVF′ and pUC-*sdr*10NdeIR primer pair. The PCR products were recovered and digested by *Eco*RV and *Nde*I and cloned into *Pvu*II/*Nde*I digested pUC57-Kan (Fig. S2). The resulting pUC-T5-*sdr*10 plasmid was then transformed into *S. marcescens* BCRC10948 cells by electroporation (BIO-RAD Gene Pulser™, CA, USA) [10].

For high-level expression of the SM_SDR, *S. marcescens* BCRC10948 (pUC-T5-*sdr*10) was grown in YP broth containing kanamycin (50 μg/ml) at 30 °C for 16 h with shaking. The culture was diluted (1:100) with 100 ml of YP and grown at 30 °C for 21 h. The bacterial cells were collected by centrifugation at 9000 × *g* and 4 °C for 10 min. The 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 cells were disrupted by sonication, and the cell debris was 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 was carried out in accordance with the manufacturer's instructions. The expression level of the SM_SDR protein was analyzed by SDS-PAGE. The concentration of purified protein was measured with a Bradford protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

2.4. Conversion of HPMAE to PE by recombinant S. marcescens BCRC10948 and E. coli cells

Recombinant *S. marcescens* BCRC10948 (pUC-T5-*sdr*10) cells were cultivated in YP medium at 30 °C with shaking at 150 rpm for 21 h and collected by centrifugation at 9000 × *g* for 5 min. Recombinant *E. coli* BL21 (DE3) harboring plasmids containing *sdr* genes was cultivated in Luria-Bertani medium at 37 °C until the OD_{600} reached 0.8. Gene expression was induced by the addition of 20 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at 28 °C. After induction for six hours, the cells were collected by centrifugation at 9000 × *g* for 10 min. Then, 5% (wet weight**)** of the cells were used to convert HPMAE to PE. The reaction mixture (10 ml) containing 10–70 mM HPMAE, 100 mM sodium phosphate buffer (pH 7.0) and a 2% carbon source was incubated at 30 °C with shaking. The cells were removed by centrifugation, and the supernatant was subjected to HPLC analysis. The substrate consumption and product formation were then determined.

2.5. Toxicity of HPMAE and PE to S. marcescens BCRC10948

S. marcescens BCRC10948 (pUC57-kan or pUC-T5-*sdr*10) cells were cultivated in YP at 30 °C for 21 h. The cells were collected by centrifugation at 9000 × *g* for 5 min. To maintain the osmotic equilibrium [11] of the cells and to stabilize the cell proteins [12], the cell pellet was washed with 10% glycerol and then resuspended in the reaction