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Scalable biocatalytic synthesis of optically pure ethyl (*R*)-2-hydroxy-4-phenylbutyrate using a recombinant *E. coli* with high catalyst yield

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ABSTRACT

Ethyl (*R*)-2-hydroxy-4-phenylbutanoate [(*R*)-HPBE] is a versatile and important chiral intermediate for the synthesis of angiotensin-converting enzyme (ACE) inhibitors. Recombinant E. coli strain coexpressing a novel NADPH-dependent carbonyl reductase gene iolS and glucose dehydrogenase gene gdh from *Bacillus subtilis* showed excellent catalytic activity in (*R*)-HPBE production by asymmetric reduction. IolS exhibited high stereoselectivity (>98.5% ee) toward α-ketoesters substrates, whereas fluctuant ee values (53.2–99.5%) for β-ketoesters with different halogen substitution groups. Strategies including aqueous/organic biphasic system and substrate fed-batch were adopted to improve the biocatalytic process. In a 1-L aqueous/octanol biphasic reaction system, (*R*)-HPBE was produced in 99.5% ee with an exceptional catalyst yield (gproduct/gcatalyst) of 31.7 via bioreduction of ethyl 2-oxo-4-phenylbutyrate (OPBE) at 330 g/L.

1. Introduction

Chiral alcohols are useful and important starting materials for the synthesis of various pharmaceuticals and other fine chemicals (Kataoka et al., 2003). In comparison with chemical methods, asymmetric reduction of prochiral carbonyl compounds using isolated enzymes or whole-cell systems is a promising method for the production of chiral alcohols, which has several advantages such as low cost, high yield, environmentally benign, and remarkable chemo-, regio-, and stereoselectivity (Xie et al., 2010; Ni et al., 2011; Jakoblinnert et al., 2013). One major challenge to chiral alcohols synthesis through bioreduction is expensive cofactors such as NAD(P)H/NAD(P)$^+$ are usually required. Several convenient and useful methods have been developed to solve this issue, including enzyme-coupled and substrate-coupled systems (Nakamura et al., 2003; Kroutil et al., 2004; Goldberg et al., 2007; Schroer et al., 2007; Hollmann et al., 2010; Savile et al., 2010; Wang et al., 2011).

Ethyl (*R*)-2-hydroxy-4-phenylbutanoate [(*R*)-HPBE] is a versatile and important chiral intermediate for the synthesis of angiotensin-converting enzyme (ACE) inhibitors, which are useful for the treatment of hypertension, such as enalapril, benazepril, and lisinopril etc. (Oda et al., 1998; Iwasaki et al., 1989). Various chemical and biological approaches have been explored for the synthesis of (*R*)-HPBE. Zhu et al. presented an effective strategy for the conversion of inexpensive OPBE directly into optically (*R*)-HPBE by asymmetric hydrogenation. And up to 92.6% enantiomeric excess (*ee*) was achieved with [RuCl(benzene)(S)-SunPhos]Cl as the catalyst and 1 M aq HBr as the additive (Zhu et al., 2011). A lipase from *Pseudomonas cepacia* was employed for the kinetic resolution of (*R*)-HPBE, resulting (*R*)-2-hydroxy ester with 99.5% *ee* and a space-time yield of 275 g/L d^{-1} (Liese et al., 2002). In our previous study, a highly potent carbonyl reductase-producing strain *Candida krusei* SW2026 was isolated for the enantioselective reduction of OPBE, giving (*R*)-HPBE in 97.4% *ee* and 82.0% yield at 20 g/L of OPBE in water/dibutyl phthalate biphasic system. Furthermore, the carbonyl reductase from *C. krusei* was purified to homogeneity through three chromatography columns and its enzymatic properties were investigated (Zhang et al., 2009; Li et al., 2010). In recent years, asymmetric synthesis of (*R*)-HPBE catalyzed by recombinant reductases has shown significant advantages. A recombinant *E. coli* BL21 overexpressing YiaE from *E. coli* and glucose dehydrogenase (GDH) from *Bacillus subtilis* was constructed, resulting (*R*)-HPBE in 98% *ee* (Yun et al., 2005). A recombinant *E. coli* strain harboring both CgKR2 and GDH encoding genes was successfully applied in preparing (*R*)-HPBE with desirable *ee* and yield (99% and 100%, respectively) at 1 M (approximately 206 g/L) OPBE, and the space-time yield of (*R*)-HPBE production reached 700 g/L d^{-1} (Shen et al., 2012).

In preliminary study, a NADPH-dependent carbonyl reductase gene *iolS* was cloned from *B. subtilis* and co-expressed with *gdh* gene for cofactor regeneration, and the recombinant *E. coli* strain was

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applied for (*R*)-HPBE synthesis in aqueous phase (Su et al., 2012). In this study, the kinetics, substrate specificity and stereoselectivity of purified IolS were characterized. To solve the substrate-tolerance obstacle and enhance the process productivity, substrate feeding and aqueous/organic biphasic system were successfully adopted, giving an unprecedented catalyst yield of 31.7 and a space-time yield of 660 g/L d^{-1}.

2. Material and methods

2.1. Protein expression

For the recombinant expression of carbonyl reductase *iolS*, *E. coli*/pET20b-*iolS* (Su et al., 2012) was cultivated in LB medium and induced with 0.4 mM IPTG at 25 °C when the OD_{600} reached around 0.6. To achieve high-level expression of both *iolS* and *gdh*, two-enzyme coexpressed strain *E. coli* BL21(DE3)/pET-G-T7-I (Su et al., 2012) was grown in LB medium containing ampicillin and kanamycin and induced with 0.8 mM IPTG at 25 °C when the OD_{600} reached around 0.6.

2.2. Enzymatic activity and stereoselectivity assays

Carbonyl reductase IolS and GDH activities were determined spectrophotometrically by monitoring the increase or decrease in the absorption of NADPH at 340 nm under 30 °C. The reaction mixture for IolS was composed of 100 mM phosphate buffer (pH 6.0), 1.0 mM OPBE, 5.0 mM NADPH, and an appropriate amount of enzyme in a final volume of 0.25 mL. The reaction mixture for GDH comprised 75 mM Tris-HCl (pH 8.0), 2.0 mM $NADP^+$, 0.1 mM glucose, and an appropriate amount of enzyme in a final volume of 0.25 mL. One unit of activity was defined as the amount of enzyme required for catalyzing the oxidation of 1 μmol of NADPH per minute (IolS) or the reduction of 1 μmol $NADP^+$ per minute (GDH). The protein concentration was measured by Bradford method, using bovine serum albumin as the standard.

The *ee* values of (*R*)-HPBE and other chiral products were determined using Varian CP 3900 gas chromatograph (USA) equipped with Chirasil-Dex CB column (CP 7502, 25 m × 0.25 mm × 0.25 m, VARIAN, USA).

2.3. Protein purification

Briefly, the recombinant cells were harvested after 4 h induction, and resuspended in buffer A (20 mM sodium phosphate, pH 7.4, containing 500 mM NaCl and 5 mM imidazole). The cells were disrupted by ultrasonication (285 W, pulse 1 s, pause 3 s) for 10 min. Cell debris was removed by centrifugation at 8000 × *g* for 15 min at 4 °C, and the supernatant was applied to a HisTrap-FF crude chelating affinity column equilibrated with buffer A. The bound enzyme was eluted by applying a stepwise gradient of imidazole concentration, using buffer A containing 5 mM imidazole to 1 M imidazole. The protein samples were analyzed by SDS–PAGE.

2.4. Substrate specificity of IolS and optimization of OPBE bioreduction

Substrate specificity and stereoselectivity of IolS were determined using various α- and β-ketoesters, as well as aromatic ketones. Reaction mixture containing 1 g wet cells (ca. 0.18 g dry cells), 50 g/L glucose, 0.1 mM $NADP^+$, 1 g/L of each substrate in 20 mL potassium phosphate buffer (0.1 mol/L, pH 6.0) was carried out at 30 °C for 24 h. The supernatant was separated by centrifugation (12,000 × *g*, 15 min) and extracted using ethyl acetate. The organic layer was dried using anhydrous magnesium sulfate, and the supernatant was subjected to chiral GC analysis to determine the product yield and *ee* value as described in Section 2.2.

Glucose concentration was optimized in the asymmetric reduction of OPBE by recombinant *E. coli* cells. Reaction mixture containing 1 g wet cells (ca. 0.18 g dry cells), 20–400 g/L glucose, 0.1 mM $NADP^+$, 20 g/L of OPBE in 20 mL potassium phosphate buffer (0.1 mol/L, pH 6.0) and 20 mL octanol was carried out at 30 °C for 15 h.

Various amount of $NADP^+$ was supplemented in the reaction mixture to evaluate its effect on the asymmetric reduction of OPBE by recombinant *E. coli* cells. The reaction mixture containing 1 g wet cells (ca. 0.18 g dry cells), 200 g/L glucose, 20 g/L of OPBE, $NADP^+$ (0, 0.05, 0.1 mM) in 20 mL potassium phosphate buffer (0.1 mol/L, pH 6.0) and 20 mL octanol, and was carried out at 30 °C and 15 h.

2.5. Bioreduction of OPBE to (R)-HPBE in a 1 L biphasic system

The reaction mixture (1 L) contained 60 g wet cells (ca. 10.4 g dry cells), 200 g/L glucose, and 0.05 mM $NADP^+$ in a biphasic system of 500 mL potassium phosphate buffer (0.1 mol/L, pH 6.0) and 500 mL octanol. Substrate OPBE (30 g) was fed once per hour. The biphasic bioreduction was performed with magnetic agitation at 30 °C for 12 h. After the reaction, the mixture was centrifuged (8000 rpm for 10 min) for the separation of two phases. The organic layer was analyzed directly by GC and evaporated under reduced pressure in an oil bath to afford 263.9 g of optically pure (*R*)-HPBE with >98% chemical purity, 99.5% *ee* and 79.2% overall yield. The structure of (*R*)-HPBE was also confirmed by ^1H and ^{13}C NMR analysis as follows: ^1H NMR ($CDCl_3$ 400 MHz): δ1.29 (3H, t, OCH_2CH_3), 1.90–1.99 (1H, m, $ArCH_2CHH$), 2.07–2.16 (1H, m, $ArCH_2CHH$), 2.73–2.79 (2H, m, $ArCH_2$), 3.14 (1H, s, O*H*), 4.16–4.22 (3H, m, OCH_2CH_3,*CH*OH), 7.16–7.29 (5H, m, Ar-*H*); ^{13}C NMR ($CDCl_3$ 100 MHz): δ14.11, 30.96, 35.90, 61.64, 69.63, 125.94, 128.37, 141.13, 175.14.

3. Results and discussion

3.1. Kinetic parameters of IolS

In preliminary study, a 933-bp *iolS* gene (GenBank accession No. JQ782389) from *B. subtilis* was cloned and expressed in *E. coli*. It encodes 310 amino acids with a calculated molecular mass of 36 kDa and an estimated pI of 5.50 (Su et al., 2012). Based on amino acid sequence analysis, IolS belongs to aldo-keto reductases (AKRs) superfamily. His-tagged recombinant protein IolS was purified to electrophoretic homogeneity by HisTrap-FF crude chelating affinity column as confirmed by SDS-PAGE. The specific activity of purified IolS was 5.1 U mg^{-1}, corresponding to a 3.4-fold increase in activity compared with crude extract.

As the maximum reaction rate of carbonyl reductase IolS was attained at 5.0 mM OPBE and 2.0 mM NADPH, its kinetic parameters were estimated over concentration ranges of OPBE (0.1–5.0 mM) and NADPH (0.04–2.0 mM) at 30 °C in phosphate buffer. The V_{max} and K_m were obtained from Lineweaver–Burk plot, specifically, 2.61 mM and 4.18 μmol/min·mg for OPBE, 0.69 mM and 5.26 μmol/min mg for NADPH. Compared with other carbonyl reductases (Table 1), IolS exhibited higher K_m (2.61 mM) toward substrate OPBE than that from *Candida glabrate* (0.1 mM), while lower K_m than those from *Trichosporon fermentans* (20.1 mM) and *Candida magnoliae* (7.9 mM). The results indicate that IolS has a moderate K_m and reaction rate compared with previous reported reductases. Also, K_{cat} and K_{cat}/K_m for OPBE and NADPH were calculated to be 0.409 s^{-1} and 0.157 mM^{-1} s^{-1}, 0.515 s^{-1} and 0.746 mM^{-1} s^{-1}, respectively.