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Enzymatic synthesis of an ezetimibe intermediate using carbonyl reductase coupled with glucose dehydrogenase in an aqueous-organic solvent system

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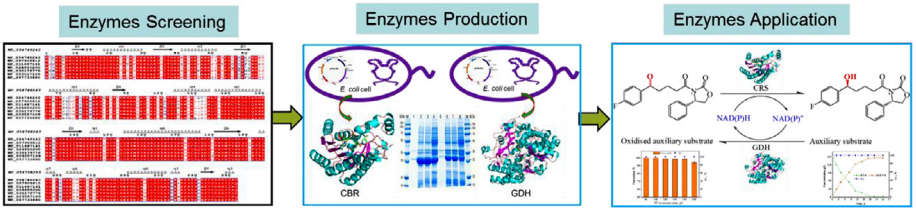
HIGHLIGHTS

- A newly synthesized SCR gene was expressed in *E. coli* for (*S*)-ET-5 production.
- The bioproduction of (*S*)-ET-5 in a biphasic solvent was first constructed.
- The (*S*)-ET-5 was achieved by bioconversion without extra addition of cofactor.
- The conversion of 99.1%, *d.e.* > 99.9% was achieved at 150 g L^{-1} ET-4.

GRAPHICAL ABSTRACT



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ABSTRACT

(4*S*)-3-[(5*S*)-5-(4-Fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one ((*S*)-ET-5) is an important chiral intermediate in the synthesis of chiral side chain of ezetimibe. Recombinant *Escherichia coli* expressing carbonyl reductase (CBR) was successfully constructed in this study. The total *E. coli* biomass and the specific activity of recombinant CBR in 5 L fermenter culture were 10.9 g DCW L^{-1} and 14900.3 U g^{-1} DCW, respectively. The dual-enzyme coupled biocatalytic process in an aqueous-organic biphasic solvent system was first constructed using *p*-xylene as the optimal organic phase under optimized reaction conditions, and 150 g L^{-1} (4*S*)-3-[5-(4-fluorophenyl)-1,5-dioxophentyl]-4-phenyl-1,3-oxazolidin-2-one (ET-4) was successfully converted to (*S*)-ET-5 with a conversion of 99.1% and diastereomeric excess of 99% after 24-h, which are the highest values reported to date for the production of (*S*)-ET-5.

1. Introduction

(4*S*)-3-[(5*S*)-5-(4-Fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one, (*S*)-(ET-5), is a key chiral intermediate mainly used in the synthesis of the chiral side chain of ezetimibe, which is the first selective cholesterol absorption inhibitor that reduces plasma LDL cholesterol levels and increases plasma HDL levels (Fu et al., 2003; Sudhop et al., 2002). Therefore, synthesis of (*S*)-ET-5 has attracted more and more attention (Clader, 2004). High-yield chemical synthesis of (*S*)-ET-5 has been extensively studied. The chiral catalyst oxazaborolidine has been used in the synthesis of (*S*)-ET-5 (Corey et al., 1987; Fu et al., 2003; Thiruvengadam et al., 2001). However, chemically synthesized (*S*)-ET-5 is not chirally pure (diastereomeric excess, *d.e.* < 98%), and the boron compounds used in these reactions results in environmental pollution (Bertrand et al., 2007; Singh et al., 2012). In

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addition, this process requires special equipment and should be performed at low temperature (Singh et al., 2012).

Currently, due to high enantioselectivity and relatively low cost, the application of biocatalysts for asymmetric reduction of ketones to the corresponding chiral alcohols has become an important alternative to the chemical method (Caner et al., 2004; Goldberg et al., 2007; Mueller, 2005), favoring large-scale pharmaceutical manufacturing of important chiral intermediates (Nakamura et al., 2003; He et al., 2016a,b; Kosjek et al., 2004; Liu et al., 2015; Ye et al., 2011). It has been reported that (*S*)-ET-5 was successfully synthesized using whole cells of *Schizosaccharomyces octosporus* (ATCC 2479) and *S. octosporus* (ATCC 4206) with yields of 33% and 34%, respectively, and enantiometric excess (*e.e.*) > 99% when the concentration of ET-4 was 1 g L^{-1} (Homann and Previte, 1997). Singh et al. has reported the synthesis of (*S*)-ET-5 using whole cells of *Burkholderia cenocepacia*, where the yield and *d.e.* achieved at 54% and >99%, respectively (Singh et al., 2009). Mundorff and Vries (2012) constructed a recombinant *E. coli* line expressing carbonyl reductase from *Lactobacillus kefir*, which uses isopropyl alcohol as cosubstrate. The resulting yield and *d.e.* were both >99% at an ET-4 concentration of 12.5 g/L (Mundorff and Vries, 2012). However, these few reports revealed several deficiencies in the biocatalytic synthesis of the chiral intermediate of ezetimibe (e.g., low substrate concentration and conversion). Compared with chemical synthesis, bioproduction of (*S*)-ET-5 under mild conditions has excellent merits including low cost, high yield, and stereoselectivity, without environmental concerns (Fu et al., 2003; Bertrand et al., 2007; Huisman et al., 2010; Patel, 2008). Although asymmetric reduction by biocatalysts has become a main route for the preparation of chiral intermediates, the efficient asymmetric reduction of ET-4 to (*S*)-ET-5 has not been reported due to the low solubility of ET-4 in aqueous solutions, lack of a carbonyl reductase with high activity, and the issue of co-factor regeneration (Singh et al., 2012; Homann and Previte, 1997). Therefore, it is important and urgent to develop an efficient enzymatic approach to prepare chirally pure (*S*)-ET-5 for the synthesis of ezetimibe.

Carbonyl reductases (CBRs, EC 1.1.1.148) are nicotinamide cofactor-dependent enzymes capable of catalyzing ketones to their corresponding alcohols (Hollmann et al., 2011; Shimizu et al., 1998). CBRs have been wildly used in the asymmetric reduction of ketones to the corresponding chiral alcohols (Kosjek et al., 2004; Goldberg et al., 2007; Liu et al., 2015; He et al., 2016a,b). However, applying CBR requires the addition of a large amount of nicotinamide cofactor ($NAD(P)^+$), resulting in an costly production process. The dual-enzyme coupled system and substrate-coupled system are two promising strategies for efficient regeneration of cofactors (Donk and Zhao, 2003). The former is frequently applied in redox catalytic systems with CBR coupled with glucose dehydrogenase (GDH) (Chen et al., 2016; Kosjek et al., 2004; Pennacchio et al., 2011; Yamamoto et al., 2004). The substrate-coupled system contains only CBR but requires simultaneous oxidation and reduction activities because the substrate and auxiliary substrate are bound to different active sites of the enzyme (Findrik et al., 2005), making the construction and control of the substrate-coupled system challenging.

In this study, a newly cloned CBR expressed in recombinant *E. coli* that catalyzes the asymmetric reduction of ET-4 to (*S*)-ET-5 is reported. The optimal aqueous-organic solvent system was constructed using *p*-xylene as the organic phase, and the distribution of ET-4 in the reaction system was greatly improved. The conversion of ET-4 reached 99.1% with *d.e.* > 99.9% at ET-4 concentration of 150 g L^{-1} without extra addition of cofactor, which is more efficient and lower cost than any previous reports. The results revealed that the CBR obtained in this study had excellent biocatalytic activity and stereoselectivity in the asymmetric reduction of ET-4, thus a foundation for the industrial bioproduction of (*S*)-ET-5 was established.

2. Materials and methods

2.1. Strains, mediums and chemicals

E. coli BL21(DE3) (Novagen, Darmstadt, Germany) was selected as the host organism, and plasmids pGEM-T (Promega, Beijing, China) and pET28b (Novagen) were used for cloning and expression, respectively. Recombinant *E. coli* BL21 (DE3) harboring GDH was stored in our lab and used to construct dual-enzyme coupled system with *E. coli* BL21 (DE3) harboring CBR. Luria–Bertani (LB) medium containing 5 g L^{-1} of yeast extract, 10 g L^{-1} of tryptone, and 10 g L^{-1} of NaCl was used for cultivation of recombinant *E. coli*. Fermentation medium consisting of peptone 20 g L^{-1}, yeast extract 12 g L^{-1}, NaCl 10 g L^{-1}, glycerol 10 g L^{-1}, KH_2PO_4 1.5 g L^{-1}, $K_2HPO_4{\cdot}3H_2O$ 3.0 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.25 g L^{-1}, and defoamer 0.1% (v/v) was used for 5 L fermenter cultures. Kanamycin sulfate (Kan, 50 μg mL^{-1}) was added to provide selective pressure during cultivation of both recombinant *E. coli* strains. Isopropyl-*β*-D-thiogalactopyranoside (IPTG) was purchased from Sangon Biotech (Shanghai, China). The 5 L fermenter was purchased from Shanghai BaoXing Bio-engineering Equipment Co., LTD (Shanghai, China). ET-4 and (*S*)-ET-5 were provided by Neo-Dankong Pharmaceutical Co., Ltd (Zhejiang, China). All other chemicals were of analytical-grade purity and were commercially available.

2.2. Cloning of SCR in E. coli cells

The CBR gene encoding a 253-amino acid protein was designed and codon-optimized by Gene Designer using *E. coli* as host, based on the amino acid sequence with GenBank accession No. WP_054748243. The CBR gene was artificially synthesized by the PCR assembly method after optimization of the codons (Liu et al., 2009; Rydzanicz et al., 2005). The synthesized gene was subcloned into the vector pGEM-T to construct the recombinant plasmid pGEM-T-CBR for cloning and sequencing. The CBR gene was removed from pGEM-T-CBR with *Nde* I and *Xho* I restriction enzymes and subcloned into the *Nde* I/*Xho* I sites of pET28b to construct pET28b-CBR. The recombinant plasmid pET28b-CBR was transformed into *E. coli* BL21 (DE3) competent cells by the heat-shock method (Chung et al., 1989).

2.3. Expression and SDS-PAGE analysis of CBR

Recombinant *E. coli* cells harboring the CBR and GDH genes were inoculated in LB medium containing 50 μg mL^{-1} Kan, and the cultures were grown at 37 °C and 150 rpm. When the optical density of the culture reached 0.8 at 600 nm (OD_{600}), the temperature was lowered to 28 °C, and IPTG was added to a final concentration of 0.4 mM. After induction for 11 h, cells were harvested by centrifugation at 4 °C, 8000 rpm for 10 min and were stored at −20 °C for further use. The cells were suspended in physiological saline solution and disrupted by sonication. The supernatant resulting from centrifugation at 15,000 rpm for 10 min was used as a crude enzyme preparation. The protein size and expression of the recombinant CBR and GDH were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, which was performed with a 5% acrylamide stacking gel (pH 6.8) and 12% separating gel (pH 8.8) (Laemmli, 1970).