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Effective biosynthesis of ethyl (*R*)-4-chloro-3-hydroxybutanoate by supplementation of L-glutamine, D-xylose and β-cyclodextrin in *n*-butyl acetate–water media

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ABSTRACT

To avoid adding NAD^+ and effectively transform ethyl 4-chloro-3-oxobutanoate, the mixture of L-glutamine (200 mM) and D-xylose (250 mM) was added into in *n*-butyl acetate–water (10:90, v/v) biphasic system instead of NAD^+ for increasing the biocatalytic efficiency. To further improve the synthesis of optically pure ethyl (*R*)-4-chloro-3-hydroxybutanoate (>99% *ee*), β-cyclodextrin was also added into this reaction media, and ethyl (*R*)-4-chloro-3-hydroxybutanoate (>99% *ee*) could be effectively synthesized from 800 mM ethyl 4-chloro-3-oxobutanoate in the yield of 100% by whole-cells of recombinant *E. coli* CCZU-A13. Finally, the possible mechanism for improving the reductase activity by supplementation of L-glutamine, D-xylose and β-CD was proposed. In conclusion, this strategy has high potential for the effective biosynthesis of ethyl (*R*)-4-chloro-3-hydroxybutanoate (>99% *ee*).

1. Introduction

Chiral chemicals are one kind of important chiral intermediates for synthesizing a series of pharmaceuticals, agrochemicals, and fine chemicals (Ema et al., 2006, 2008; He et al., 2014a, 2014c; Houng et al., 2003; Ni et al., 2010; Ning et al., 2014). Optically active ethyl (*R*)-4-chloro-3-hydroxybutanoate ester [(*R*)-CHBE] is a key chiral synthon for the production of (*R*)-4-amino-3-hydroxybutyric acid, L-carnitine, (*R*)-4-hydroxy-2-pyrrolidone, etc. (Shimizu et al., 1990; Xie et al., 2010; He et al., 2014c). Compared with conventional chemical approach, biosynthesis method is of current interest due to its mild reaction condition and substrate specificity. In recent years, asymmetric bioreduction of ethyl 4-chloro-3-oxobutanoate (COBE) into optically active CHBE has been of great interest due to its mild reaction condition and high stereoselectivity (He et al., 2014a; Wang et al., 2011), which can provide theoretically 100% yield of (*R*)-CHBE. An NADPH-dependent reductase B Yue D from *Bacillus* sp. could reduce COBE to (*R*)-CHBE (yield 91.7%; *ee* >99%) in a toluene–water biphasic media (Shimizu et al., 1990). An NADH-dependent reductase gox2036 from *Gluconobacter oxydans* could transform COBE to (*R*)-CHBE (>99% *ee*) with the yield of 96.9% (Liu et al., 2014). In our previous study, an NADH-dependent reductase (SsCR) discovered by genome data mining could convert COBE into (*R*)-CHBE (>99% *ee*) from 600 mM COBE with the yield of 100% (He et al., 2014c).

Notably, asymmetric biotransformation of COBE to optically active CHBE (>99% *ee*) by reductases is quite challenging due to the requirement of expensive cofactors NADH or NADPH (He et al., 2014b; Kizaki et al., 2001). Thus, it is necessary to regenerate these cofactors. It is well-known that in situ cofactor regeneration is a cost-effective route to the economic viability of industrial-scale biotransformation process (Breuer et al., 2004; Hummel and Gröger, 2014). However, supplementation of $NAD(P)^+$ into reaction system for in situ cofactor regeneration will cause the increase the production cost. Various precursors (e.g., D-xylose, L-arabinose, etc.) have been used to increase the intracellular NAD(P)H concentrations (Nie et al., 2009).

However, the main problem for the biotransformation of carbonyl compounds remains to be their poor water solubility or water insolubility, which causes poor availability of substrates. To improve the solubility of these substrates in the aqueous media, cyclodextrins (CDs), which can form inclusion complexes with lipophilic substrates in their cavities, have been used for the biotransformations (Manosroi et al., 2008; Mosinger et al., 2001). During the asymmetric biotransformation of COBE, the poor water solubility of substrate COBE is used. Recently,

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β-cyclodextrin (β-CD), one kind of cyclic oligosaccharides, is water-soluable and non-toxic with low price. Its structure is rigid and well defined. Most importantly, β-CD possesses a hydrophobic cavity that entraps various molecules to form an inclusion complex (Manosroi et al., 2008; Zidovetzki and Levitan, 2007). It has been used to increase the utility of biocatalysts by increasing the availability of insoluble substrates, improving the biocatalysis efficiency, reducing substrate and/or product inhibition (Zhang et al., 2013).

In this study, various precursors instead of NAD^+ were attempted to enhance the biosynthesis of (*R*)-CHBE by whole-cells of recombinant *E. coli* CCZU-A13 in *n*-butyl acetate–water (10:90, v/v) biphasic media. Moreover, β-CD was used for improving the availability of insoluble substrate COBE, permeabilizing the whole cells and increasing the COBE-reducing activity. Finally, an effective biosynthesis of (*R*)-CHBE from COBE by whole-cells of *E. coli* CCZU-A13 was successfully demonstrated by some precursors and β-CD in *n*-butyl acetate–water (10:90, v/v) biphasic media.

2. Materials and methods

2.1. Materials

n-Butyl acetate, β-CD, glycine, and D-xylose were purchased from Sinopharm Chemical Reagent Co., Ltd (China). L-asparagic acid, D-asparagic acid, COBE, L-glutamine, D-glutamine, and nicotinic acid were obtained from Aladdin Chemistry Co. Ltd (Shanghai, China). All other chemicals were also from commercial sources and of analytical grade.

2.2. Microorganism

In this study, recombinant *E. coli* CCZU-A13 with reductase activity was used (He et al., 2014c). The culture was conducted as the previous method (He et al., 2014c). The resting cells were harvested by centrifugation (10,000 × *g*) for 15 min at 4 °C.

2.3. Asymmetric reduction of COBE by supplementation of various precursors and/or β-CD in n-butyl acetate–water (10:90, v/v) biphasic media

In a 50 mL Erlenmeyer flask capped with a septum, l mL *n*-butyl acetate, 9 mL KPB (100 mM, pH 6.5), glucose (1 mol glucose/mol COBE), and 0.50 g of resting cells were mixed well. To investigate the effects of precursors on the initial reaction rate, various potential precursors (L-asparagic acid, D-asparagic acid, nicotinic acid, L-glutamine, D-glutamine, glycine, and D-xylose) (200 mM) or NAD^+ (0.1 mmol NAD^+/mol COBE) were added into this biphasic media, respectively. To investigate the effects of β-CD on the initial reaction rate, l mL *n*-butyl acetate, 9 mL KPB (100 mM, pH 6.5), glucose (1 mol glucose/mol COBE), 0.50 g of resting cells, and β-CD (0–0.1 mol β-CD/mol COBE) were mixed well in a 50 mL Erlenmeyer flask capped with a septum, and then the mixture of L-glutamine (200 mM) plus D-xylose (250 mM) was added into this biphasic media. After pre-incubated in the thermostatic shaker for 5 min at 30 °C and 180 rpm, COBE (100–1200 mM) was added and then the incubation was continued at 30 °C and 180 rpm. During biotransformation, the reaction media was controlled at pH 6.5 with 4 M NaOH. Samples were taken periodically for the following assay of (*R*)-CHBE concentration.

2.4. Enzyme activity assay

The mixture contained 100 mM of KPB (pH 6.5), 0.2 mM of NADH, and 20 mM of COBE. The reaction was monitored at 30 °C by absorbance at 340 nm. The enzyme activity was determined with a spectrophotometry. One unit (U) of reductase activity was defined as the amount catalyst catalyzing the reduction of 1 μmol of NADH per minute. All experiments were performed in triplicate. Error bars represent standard deviation from the mean.

2.5. Analytical methods

Resting cells of recombinant *E. coli* CCZU-A13 were resuspended in the solution containing 200 μL KPB (100 mM, pH 6.5) and 10 mM EDTA in an Eppendorf centrifuge tube. The cell suspension was further sonicated with a JY9Z-II ultrasonic cell disruptor (Ningbo Scientz Biotechnology, China) in cold ice-water bath at 400 W for 20 times (working 3 s and intervals 7 s as one cycle). After this performance, the cell debris was removed by centrifugation (20,000 × *g*) for 30 min at 4 °C. Immediately, the NADH concentration of supernatant was further assayed by RP-HPLC (Li et al., 2012b).

Assays by Fourier transformed IR (FTIR) and scanning electron microscopy (SEM), were conducted as the previous methods (He et al., 2015). The transmission electron microscopy (TEM) were carried out as follows: After the β-CD-treated and untreated cells were fixed overnight in 250 mM glutaraldehyde in KPB (100 mM, pH 6.5), the cells were washed, dried, embedded in epoxy resin, sliced and stained. Furthermore, they were observed with a HT7700 TEM instrument (Hitachi, Japan) at an accelerating voltage of 80 kV.

In the *n*-butyl acetate–water (10:90, v/v) biphasic system, the concentrations of COBE and (*R*)-CHBE refer only to the concentrations in the total reaction media. The concentrations of COBE and CHBE were assayed by GC (Ni et al., 2013). The *ee* value of (*R*)-CHBE was assayed by HPLC (He et al., 2014c).

3. Results and discussion

3.1. Asymmetric reduction of COBE by supplementation of precursors in the n-butyl acetate–water (10:90, v/v) biphasic media

To improve the synthesis of (*R*)-CHBE, various precursors (e.g., L-asparagic acid, D-asparagic acid, nicotinic acid, L-glutamine, D-glutamine, glycine, and D-xylose) (200 mM), which may directly or

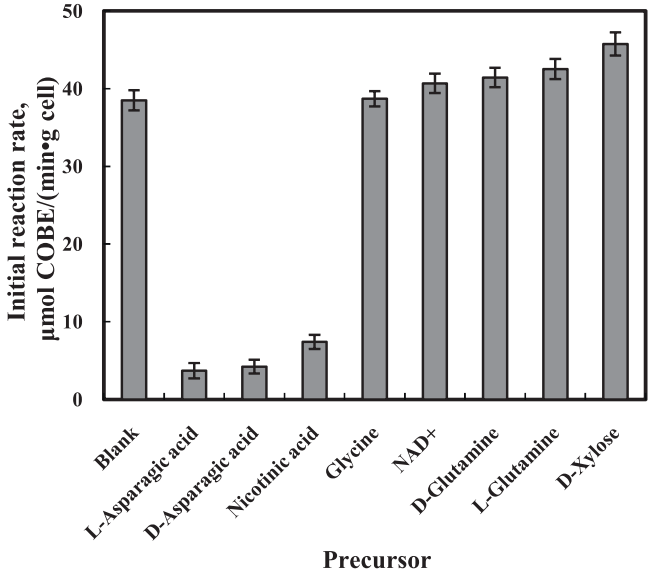

Fig. 1. Effects of potential precursor on the initial reaction rate. Biotransformations were conducted at 30 °C by adding 8.0 mmol COBE, 8.0 mmol glucose, 0.50 g wet cells, various precursor (e.g., L-asparagic acid, D-asparagic acid, nicotinic acid, L-glutamine, D-glutamine, glycine, or D-xylose; 200 mM) or NAD^+ (0.1 mmol NAD^+/mol COBE) into 10 mL the biphasic media (pH 6.5). All experiments were performed in triplicate. Error bars represent standard deviation from the mean.