Bioresource Technology 172 (2014) 342–348

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Discovery of a reductase-producing strain recombinant *E. coli* CCZU-A13 using colorimetric screening and its whole cell-catalyzed biosynthesis of ethyl (*R*)-4-chloro-3-hydroxybutanoate

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HIGHLIGHTS

- One high-throughput screening strategy for COBE-reducing enzymes was built.
- An NADH-dependent reductase (SsCR) was discovered by genome data mining.
- Highly stereoselective bioreduction of COBE was demonstrated.
- Effective biotransformation of COBE was in butyl acetate–water (10:90, v/v) media.
- Broad substrate specificity was shown.

ARTICLE INFO

Article history:
Received 8 August 2014
Received in revised form 11 September 2014
Accepted 14 September 2014
Available online 22 September 2014

Keywords:
Asymmetric bioreduction
Recombinant *E. coli* CCZU-A13
Ethyl 4-chloro-3-oxobutanoate
Ethyl (*R*)-4-chloro-3-hydroxybutanoate
Biphasic system

ABSTRACT

An NADH-dependent reductase (SsCR) was discovered by genome data mining. After SsCR was overexpressed in *E. coli* BL21, recombinant *E. coli* CCZU-A13 with high reductase activity and excellent stereoselectivity for the reduction of ethyl 4-chloro-3-oxobutanoate (COBE) into ethyl (*R*)-4-chloro-3-hydroxybutanoate ((*R*)-CHBE) was screened using one high-throughput colorimetric screening strategy. After the reaction optimization, a highly stereoselective bioreduction of COBE into (*R*)-CHBE (>99% *ee*) with the resting cells of *E. coli* CCZU-A13 was successfully demonstrated in *n*-butyl acetate–water (10:90, v/v) biphasic system. Biotransformation of 600 mM COBE for 8 h in the biphasic system, (*R*)-CHBE (>99% *ee*) could be obtained in the high yield of 100%. Moreover, the broad substrate specificity in the reduction of aliphatic and aromatic carbonyl compounds was also found. Significantly, *E. coli* CCZU-A13 shows high potential in the industrial production of (*R*)-CHBE (>99% *ee*) and its derivatives.

1. Introduction

Optically active ethyl (*R*)-4-chloro-3-hydroxybutanoate ester (CHBE) is an important precursor for the production of (*R*)-carnitine, (*R*)-4-amino-3-hydroxybutyric acid, (*R*)-4-hydroxy-2-pyrrolidone, and other fine chemicals (Ema et al., 2006; Kita et al., 1996; Liu et al., 2014; Yamamoto et al., 2002; Yu et al., 2007). Compared with conventional chemical synthesis, asymmetric bioreduction of prochiral ketone has been used as an economical and practical way for synthesizing highly optically active alcohols (Asako et al., 2009; Breuer et al., 2004; Cao et al., 2011; Ema et al., 2008, 2006; Goldberg et al., 2007; Gröger et al., 2006; Ni et al., 2010; Yamamoto et al., 2004; Ye et al., 2011). Although several carbonyl reduction enzymes for synthesizing (*R*)-CHBE from ethyl 4-chloro-3-oxobutanoate (COBE) have been found that required inexpensive NADPH for biocatalysis, few studies have addressed reductases that require the relative cheap cofactor NADH as an electron donor (Kita et al., 1996; Liu et al., 2014; Yu et al., 2007). Thus, discovery for new carbonyl reduction enzymes and improving their application potential for the synthesis of (*R*)-CHBE (>99% *ee*) are of great interest (He et al., 2014a; Kizaki et al., 2001; Ye et al., 2010b).

It is well-known that laborious traditional biocatalyst discovery is based on the screening from soil samples for searching the microbes with desired enzyme activity (He et al., 2011; Ye et al., 2011). However, this kind of screening strategy is always time consuming. Notably, only less than 1% of microbes in the environment can be culturable (He et al., 2014b). In post-genomic era, genome

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http://dx.doi.org/10.1016/j.biortech.2014.09.062
0960-8524/

data mining offers an unprecedented opportunity for searching novel and useful biocatalysts with industrial application potential due to the abundant gene resources in the gene database (Hohne et al., 2010; Wang et al., 2011). Recently, genome data mining has been effectively used to search gene data bases for sequences similar to those of known reductases (He et al., 2014a; Yamamoto et al., 2003; Wang et al., 2011). However, there are a limited data about its application in the biotransformations of COBE into (*R*)-CHBE (>99% *ee*).

To obtain more COBE-reducing enzymes, it is important to establish a convenient, rapid and accuracy method for screening the desired biocatalysts. Many high-throughput methods have been employed to screen biocatalysts (He et al., 2011). The use of the conventional chromatographic method is usually time-consuming and inefficient (Yasohara et al., 1999; Liu et al., 2014). In this study, it is the first report that a high-throughput assay strategy for the screening of β-carbonyl carboxylic ester reductase based on ferric perchlorate spectrophotometry at room temperature was built. Moreover, an NADH-dependent reductase (SsCR) from *Sporidiobolus salmonicolor* was discovered by genome data mining. After SsCR was overexpressed in *E. coli* BL21, a high activity of reductase-producing strain, recombinant *E. coli* CCZU-A13, was employed for the efficient synthesis of ethyl (*R*)-CHBE (>99% *ee*) from the reduction of COBE. To increase the yield of (*R*)-CHBE, various parameters (cosubstrate, organic solvent, volumetric phase ratio, reaction temperature, reaction pH, metal ion additive, cell dosage, and substrate loading) on the reductase activity were investigated in the aqueous-organic biphasic media. Subsequently, highly efficient synthesis of ethyl (*R*)-CHBE and its derivatives by recombinant *E. coli* CCZU-A13 was successfully demonstrated.

2. Methods

2.1. Chemicals

COBE (95% purity) was obtained from Aladdin Chemistry Co. Ltd (Shanghai, China). All other chemicals were also from local commercial sources and of analytical grade.

2.2. Microorganism

E. coli CCZU-K14 (He et al., 2014a) with COBE-reducing activity was employed to build a high-throughput assay strategy for the screening of β-carbonyl carboxylic ester reductase, and it was also used as probe for screening high activity of reductases.

Cloning and expression of SsCR gene in *Escherichia coli* was carried out as followings: genomic DNA was extracted from *S. salmonicolor* provided by Dr. Xian Zhang at Rice University (USA), using a TIANamp Bacteria DNA Kit from Tiangen (Shanghai). Oligonucleotide primers (SsCR-F: 5′-GCGCATATGAAGCTTAATAATTTTGTTTA ACTTTAAGAAGGAGATATAATGGTCGGCACTACTACC-3′ and SsCR-R: 5′-CCGGGATCCCTACTTGATCTTCACGGCGTTCTTG-3′) with *Nde*I and *BamH*I restriction sites were designed according to the SsCR gene sequence (GenBank accession No. U26463.1) (Kita et al., 1996). The DNA fragment of SsCR gene was amplified and double-digested with *Nde*I and *BamH*I and then inserted into the expression vector pET-28a (Novagen, Shanghai). The resulting plasmid, pET-28a-SsCR, was transformed into *E. coli* BL21 (DE3) cells. The cells were cultivated at 37 °C in LB medium containing 50 μg/mL kanamycin. When the OD_{600} of the culture reached 0.60, IPTG as inducer was added to the culture media at a final concentration of 1.0 mM, and cultivation was continued at 37 °C for a further 6 h. The cells were harvested by centrifugation (8000*g*, 6 min) at 4 °C, and washed twice with potassium phosphate buffer (100 mM, pH 6.5). One high throughput screening strategy was used for screening the high COBE-reducing activity of recombinant *E. coli*.

2.3. Optimization of a high-throughput screening strategy for β-carbonyl carboxylic ester reductase

Ferric perchlorate spectrophotometric determination of COBE in aqueous solutions was performed with a UV–Vis spectrophotometer (Gold Spectrumlab 53, Shanghai Lengguang Technology Co., Ltd, China). After the COBE in the potassium phosphate buffer (100 mM, pH 6.5) was extracted by ethyl acetate, 390 μL acidic ferric perchlorate solution (0.005–0.1 M ferric perchlorate, 0.7 M perchloric acid) and 10 μL COBE samples were added into a 24-well plate. After the mixture was incubated for 5–30 min at 20–45 °C, 3.6 mL ethanol was added into the mixture. To validate the accuracy of the spectrophotometry, the samples were assayed by analytical GC (Chirasil-DEX CB, Varian, USA) (He et al., 2014a).

2.4. Optimization of bioconversion process

To efficiently synthesize (*R*)-CHBE, various parameters (e.g., cosubstrate, organic solvent, reaction temperature, reaction pH, metal ion additive, cell dosage and substrate concentration) on the effects of catalytic activity were investigated at 30 °C and 180 rpm. The effects of cosubstrate on the reductase activity were carried out by adding 0.75 g *E. coli* CCZU-A13 wet cells into 10 mL potassium phosphate buffer (100 mM, pH 6.5) containing 6.0 mmol COBE, 0.60 μmol NAD^+ and various potential cosubstrate (glucose, glycerol, mannitol, mannose, and isopropanol; 12.0 mmol). To test the effect of glucose concentration, biotransformation was performed with 6.0 mmol COBE, 0.60 μmol NAD^+ and certain concentration of glucose (0.25–3.0 mmol glucose/mmol COBE). To test the effects of organic solvent on the catalytic activity, biotransformation was performed by adding 0.75 g wet cells into the biphasic system containing 9 mL potassium phosphate buffer (100 mM, pH 6.5), 1 mL organic solvent (*n*-butyl acetate, dibutyl phthalate, ethyl caprylate, *n*-heptane, *n*-hexane, *n*-octane, or toluene), 6.0 mmol COBE, 6.0 mmol glucose and 0.60 μmol NAD^+. To test the effect of phase ratio on the catalytic activity, biotransformation of 6.0 mmol COBE was performed by adding 0.75 g wet cells, 6.0 mmol glucose and 0.60 μmol NAD^+ into 10 mL biphasic system containing a certain volume (1–10 mL) of potassium phosphate buffer (100 mM, pH 6.5) and a certain volume (1–9 mL) of *n*-butyl acetate. To test the effect of reaction temperature and pH on the reduction, biotransformations were performed at different temperature (25–50 °C) and pH (citrate buffer solution, pH 4.0–6.0; phosphate buffer solution, pH 6.5–8.0; carbonate buffer solution, pH 8.0–8.5) by adding 0.75 g wet cells, 6.0 mmol COBE, 6.0 mmol glucose and 0.60 μmol NAD^+ into the biphasic media containing 9 mL potassium phosphate buffer and 1 mL *n*-butyl acetate. To test the metal ion on the catalytic activity, Al^{3+},Ca^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Ni^{2+}, or Zn^{2+} (0.2 mM) was added into the biphasic media media, respectively. To investigate Zn^{2+} concentration on the catalytic activity, various concentrations of Zn^{2+} (0–0.40 mM) were added into the biphasic media. To test the effects of cell dosage on catalytic activity, biotransformation was performed by adding wet cells (0.025–0.20 g/mL), 6.0 mmol COBE, 6.0 mmol glucose and 0.60 μmol NAD^+ into the biphasic media. The 10 mL reaction mixture without COBE was added into a 50 mL Erlenmeyer flask capped with a septum. After pre-incubated in a 180 rpm rotary shaker at selected reaction temperature and pH for 15 min, a certain amount of COBE containing 20 μL dodecane (internal standard) were added and then the incubation was continued. Samples were taken periodically for the assay.