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Efficient production of ethyl (*R*)-4-chloro-3-hydroxybutanoate by a novel alcohol dehydrogenase from *Lactobacillus curieae* S1L19

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ABSTRACT

Ethyl (*R*)-4-chloro-3-hydroxybutanoate ester [(*R*)-CHBE] is an important chiral intermediate for the synthesis of chiral drugs. In this study, a novel short-chain, NADH-dependent dehydrogenase (LCRIII) from *Lactobacillus curieae* S1L19 was discovered to exhibit high activity and enantioselectivity in the production of (*R*)-CHBE by reduction of ethyl 4-chloroacetoacetate (COBE). LCRIII was heterologously overexpressed in *Escherichia coli* and the protein was purified to homogeneity. Characterization of LCRIII showed broad substrate specificity towards a variety of ketones. In addition, an efficient cofactor regeneration system was constructed by co-expressing LCRIII and glucose dehydrogenase (GDH) in *E. coli* cells. Up to 1.5 M (246.8 g/L) COBE could be completely reduced to (*R*)-CHBE with excellent enantiomeric excess (>99% *ee*) in a monophasic aqueous system. Moreover, the process could be performed even without external addition of cofactors. These results demonstrate the great potential of this process in industrial applications.

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1. Introduction

Optically active alcohols are the most important chiral building blocks for the synthesis of pharmaceuticals and agricultural chemicals [1]. Of these, (*R*)-ethyl-4-chloro-3-hydroxybutanoate [(*R*)-CHBE] is a valuable intermediate in the synthesis of afatinib, (*R*)-carnitine, and macrolide A [2–5].

To date, two main enzymatic routes have been used to obtain optically active alcohols: resolution of racemic alcohols catalyzed by lipase and enantioselective reduction of prochiral ketones catalyzed by alcohol dehydrogenase (ADH) [6,7]. However, kinetic resolution approaches are naturally limited to a maximum of approximately 50%; biocatalytic asymmetric reduction offers a more promising route for the production of optically pure alcohols, owing to a theoretical yield of 100%, high enantioselectivity, and mild reaction conditions [8]. Various ADHs have been successfully used for the synthesis of (*S*)-CHBE (the intermediate for Lipitor synthesis [9]), the opposite enantiomer of (*R*)-CHBE, with excellent enantioselectivity and high yields [10]. However, few alcohol dehydrogenases that could synthesize (*R*)-CHBE with high enantioselectivity have been reported [11,12]. A robust biocatalyst with high enantioselectivity and specific activity is crucial for improving manufacturing processes and reducing industrial production costs. Therefore, the discovery of novel alcohol dehydrogenases for the synthesis of (*R*)-CHBE with high enantioselectivity and catalytic activity is of great interest.

The biocatalytic asymmetric reduction of prochiral ketones by ADHs often requires NADH or NADPH as an electron donor [13,14]. The high cost of cofactors is a restrictive factor for industrial biosynthesis of optically active alcohols. To solve this problem, two cofactor regeneration systems, enzyme-coupled and substrate-coupled, have been developed and successfully applied in industrial-scale biocatalysis [15]. Substrate-coupled reactions employ ADH to catalyze two separate reactions simultaneously: oxidation and reduction. ADH is used to catalyze the asymmetric reduction of prochiral ketones with the concomitant oxidation of NAD(P)H to $NAD(P)^+$; at the same time, ADH also oxidizes cheap alcohols to ketones as a byproduct of the concomitant reduction of $NAD(P)^+$ to NAD(P)H. However, the equilibrium of the reaction needs to be taken into account and the ADH used should have high oxidative activity and tolerance to high concentrations of co-substrate alcohols [16]. The enzyme-coupled system employs a second enzyme to catalyze the cofactor regeneration reaction; a low-cost substrate, D-glucose or formate, is often used for the reduction of NAD^+ to NADH [17]. The most commonly used enzymes for these reactions are glucose dehydrogenases (GDHs)

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Table 1
Primers for gene amplification of putative ADHs.

Gene	Primer sequence[a]	Restriction site
lcrI	F:5′-GGAATTCCATATGGATAAATTTAATGCAATTGTG-3′	*Nde*I
	R:5′ −CGCGGATCCTTAAATTTTTACAATCGTTCGG −3′	*Bam*HI
lcrII	F:5′ −CGCGGATCCATGACAGAATTAGCGATTG- 3′	*Bam*HI
	R:5′ −CCCAAGCTTAACGATATTCCATTCCGCCA-3′	*Hind*III
lcrIII	F:5′ −CGCGGATCCATGGCAAAAGTTGCGATGA-3′	*Bam*HI
	R:5′ −CCCAAGCTTAAAGCAATCCGCCGCCATC-3′	*Hind*III
lcrIV	F:5′ −GGAATTCCATATGAAAGCCATCGGTTATAC-3′	*Nde*I
	R:5′ —CGCGGATCCTTAATCGTTCGTGATTACTAC-3′	*Bam*HI
lcrV	F:5′-GGAATTCCATATGAAAGCAGCAGTTTTAAC-3′	*Nde*I
	R:5′-CGCGGATCCTTATATAAGAATTACTGGCAGAC-3′	*Bam*HI
Bsgdh	F:5′-CGCCATATGTATCCGGATTTAAAAGG-3′	*Nde*I
	R:5′-CCCAAGCTTTTAACCGCGGCCTGC-3′	*Hind*III
sd-as -*bsgdh*	F:5′ −CCCAAGCTTAAGGAGATATACATATGTATCCGGAT-3′	*Hind*III
	R:5′-CCGCTCGAGTTAACCGCGGCCTGCCTGGA-3′	*Xho*I

[a] Restriction sites are underlined.

or formate dehydrogenases (FDHs) [18]. The advantage of the enzyme-coupled cofactor regeneration system is that the alcohol products can be easily extracted from the reaction mixture using a solvent, because of the high ionic strength caused by the high concentration of water-soluble byproducts such as D-gluconate. However, FDHs usually exhibit low activity and stability compared with GDHs; this issue could be resolved by either finding new enzymes or using protein engineering to enhance the performance of existing FDHs [19,20]. In general, these systems mentioned above can afford efficient regeneration of cofactors, thus minimizing cofactor loading and greatly reducing the cost of the biocatalytic process.

The biocatalytic asymmetric reduction routes used to date for the production of chiral alcohols have been successfully operated with minimal or no external addition of cofactors, using whole *E. coli* cells co-expressing ADH and GDH [21]. In this system, the internal cofactors present in the *E. coli* cells are sufficient to initiate the reaction and complete reduction via cofactor regeneration [22]. However, external addition of a reduced cofactor would probably result in accelerated reactions. To perform the reaction successfully, it is essential to co-express both of the ADH and GDH effectively. The co-expression systems usually employ recombinant *E. coli* strains harboring two plasmids containing the individual genes or one plasmid with both genes [23]. However, expression of both genes in one cell is not always satisfactory. To achieve high-level expression of ADH and GDH with one plasmid, several factors must be taken into account including transcription and translation efficiency [24].

In this study, a novel NADH-dependent, short-chain dehydrogenase (LCRIII) from *Lactobacillus curieae* S1L19 was identified. This enzyme exhibits excellent enantioselectivity and high activity in the production of (*R*)-CHBE. LCRIII and glucose dehydrogenase were successfully co-expressed in *E. coli*. Moreover, COBE was effectively converted into (*R*)-CHBE at concentrations of up to 1.5 M (246.8 g/L) with excellent enantioselectivity (>99% *ee*), using co-expressing cells without cofactor addition.

2. Experimental

2.1. Strains and materials

Lactobacillus curieae S1L19 was deposited at the China Center for Type Culture Collection (CCTCC M 2011381). The Phanta super-fidelity DNA polymerase (Vazyme Biotech, Nanjing, China) was used for DNA amplification. The expression vector pET-28a (+) and *E. coli* DH5α and BL21 (DE3) cells were used to clone and express target genes. The Bacteria DNA Kit was purchased from TIANGEN (Tiangen Biotech, Beijing, China). Ketones and chiral alcohol standard samples were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

2.2. Cloning and expression of five ADH genes in E. coli

L. curieae S1L19 is a novel species of the genus *Lactobacillus* isolated by our laboratory [25]. Due to the novelty of this strain, a search for new ADHs was conducted in *L. curieae* S1L19 based on its genomic sequence (GenBank Accession No.NZ_JTAL01000001) and five putative ADHs [GenBank Accession No. WP_035165759.1 (LCRI), WP_035165771.1 (LCRII), WP_035165810.1 (LCRIII), WP_035166587.1 (LCRIV), and WP_035167176.1 (LCRV)] were identified [26]. Primers and restriction enzymes are listed in Table 1. DNA fragments containing the different ADH genes (*lcrI, lcrII, lcrIII, lcrIV, lcrV*) were amplified by polymerase chain reaction (PCR) and the purified fragments were cloned into the pET-28a (+) expression vector, resulting N-terminally His-tagged recombinant proteins. Finally, recombinant plasmids were individually transformed into *E. coli* BL21 (DE3) competent cells. Positive clones were grown in 5 mL of Luria-Bertani (LB) liquid medium containing 50 μg/mL kanamycin at 37 °C for 12 h. Cultures were subsequently transferred into 250-mL Erlenmeyer flasks containing 50 mL of LB medium supplemented with 50 μg/mL kanamycin. When cultures reached OD_{600} = 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce protein expression. Cells were shaken at 20 °C for 20 h prior to harvesting.

2.3. Screening of recombinant ADHs by asymmetric reduction of COBE

A small-scale reduction of COBE was performed with the concomitant regeneration of NADH, using D-glucose and glucose dehydrogenase (BsGDH) from *Bacillus subtilis* (GenBank Accession No. M 1.1398) in a monophasic aqueous system. The reaction mixture (1 mL) contained D-glucose (1.5 equivalents of COBE), BsGDH (4 U), $NAD(P)^+$ (0.25 mM), ADH (2 g/L dried cells), and 10 mM COBE in phosphate buffer (100 mM, pH 7.0). Mixtures were shaken at 200 rpm and 30 °C for 24 h. Each sample was extracted with two volumes of ethyl acetate (EtOAc). Samples were split into two equal fractions; one was used to analyze the conversion ratio by gas chromatography (GC) and the other was dried and solubilized in isopropanol to analyze the enantiomeric excess (*ee*) by chiral HPLC.

2.4. Purification of LCRIII

Cells were harvested by centrifugation (8500*g*, 5 min) at 4 °C and washed twice with saline solution. Cells were subsequently resus-

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