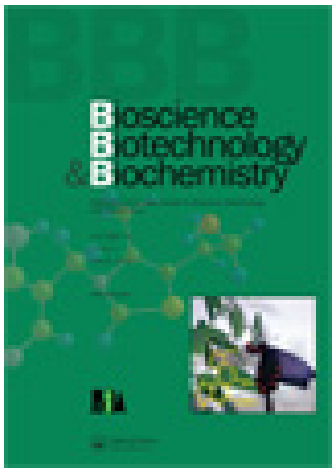

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Asymmetric synthesis of (S)-4-chloro-3-hydroxybutanoate by sorbose reductase from Candida albicans with two co-existing recombinant Escherichia coli strains

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Published online: 13 Mar 2015.

Bioscience, Biotechnology, and Biochemistry, 2015

Asymmetric synthesis of (*S*)-4-chloro-3-hydroxybutanoate by sorbose reductase from *Candida albicans* with two co-existing recombinant *Escherichia coli* strains

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Received October 20, 2014; accepted January 9, 2015
http://dx.doi.org/10.1080/09168451.2015.1012145

An NADPH-dependent sorbose reductase from *Candida albicans* was identified to catalyze the asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (COBE). The activity of the recombinant enzyme toward COBE was 6.2 U/mg. The asymmetric reduction of COBE was performed with two coexisting recombinant *Escherichia coli* strains, in which the recombinant *E. coli* expressing glucose dehydrogenase was used as an NADPH regenerator. An optical purity of 99% (*e.e.*) and a maximum yield of 1240 mM (*S*)-4-chloro-3-hydroxybutanoate were obtained under an optimal biomass ratio of 1:2. A highest turnover number of 53,900 was achieved without adding extra $NADP^+$/NADPH compared with those known COBE-catalytic systems.

Key words: NADPH-dependent sorbose reductase; *Candida albicans*; (*S*)- 4-chloro-3-hydroxybutanoate; coenzyme regeneration

Ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] is a promising chiral intermediate in the synthesis of pharmacologically active compounds, such as hydroxymethylglutaryl-CoA reductase inhibitors and 4-hydroxypyrrolidone.[1)] The asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (COBE) by enantioselective oxidoreductases has been proved one of the most efficient methods for the production of (*S*)-CHBE.[2)] However, two major limitations apply to the biotransformation process, namely, the preparation of high-performance oxidoreductase for COBE reduction and the efficient recycling of coenzymes $NAD(P)^+$/NAD(P)H.

Several reductases have been reported to perform the (*S*)-CHBE synthesis, including CaCR from *Candida albicans,*[3)] KaCR from *Kluyveromyces aestuarii*,[4)] PsCR[5)] and PsCRII[6)] from *Pichia stipitis*, S1 from *Candida magnoliae,*[7)] and CpCR from *Candida parapsilosis*.[8)] Glucose dehydrogenase was coupled with all of these reductases for coenzyme regeneration because of its high stability and cheap substrate. The biocatalysts were usually obtained by coexpressing the reductases and glucose dehydrogenase in one strain, but the order of the genes, Shine-Dalgarno (SD) regions, or aligned spacing between the SD sequence and the translation initiation codon may affect the expression level of either enzyme.[9)] In addition, the velocities of synthesis reaction and coenzyme regeneration may be difficult to regulate in a coexpression system.[10)] Thus, a two-strain coexisting system was proposed based on the principle that the coenzyme regeneration system in one strain can supply the biosynthesis in another different strain.[11,12)] The two-strain system is more flexible than the coexpression system because the balance between synthesis reaction and coenzyme regeneration can be easily achieved by adjusting the biomass of the two strains.

In this study, we reported an NADPH-dependent sorbose reductase (SOU1) from *C. albicans* to catalyze the reduction of COBE. Greenberg et al. [13)] first reported SOU1 as an important reductase involved in sugar metabolism. However, the amino acid sequence analysis revealed that SOU1 shared high sequence homology with known COBE catalytic-enzymes. SOU1 exhibited a reduction activity toward COBE. Thus, a two-strain coexisting system for (S)-CHBE production was constructed using SOU1 and glucose dehydrogenase. The recombinant strain *Escherichia coli* Rosseta (pET-22b-SOU1) was mixed with *E. coli* Rosseta (pET-22b-GDH) expressing glucose dehydrogenase for both COBE reduction and coenzyme regeneration (Fig. 1).

Materials and methods

Enzymes and chemicals. COBE, (*R*)-CHBE, and (*S*)-CHBE were obtained from Acros Organics. NADPH was supplied by Sigma-Aldrich Chemie GmbH. All restriction endonucleases, Taq polymerase, and T4 ligase were purchased from TaKaRa (Dalian) Co. All other chemicals used were of analytical grade purity and commercially available.

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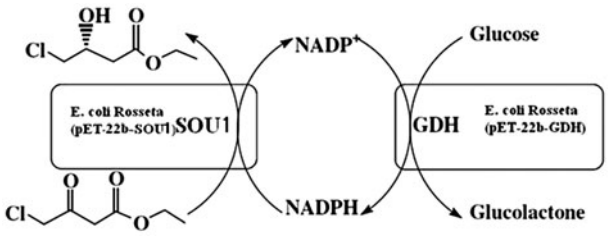

Fig. 1. Two-strain coexisting system for the production of (*S*)-CHBE.

Strains and vectors. The *gdh* gene was cloned from *Bacillus megaterium* DSM 2894. The *E. coli* Rosseta (pET-22b-GDH) constructed earlier to produce recombinant GDH was used for the cofactor regeneration in (*S*)-CHBE biosynthesis.[3] *C. albicans* was preserved in our laboratory. *E. coli* Rosseta (DE3) and pET-22b were used for the overexpression of the recombinant SOU1.

Cloning and expression of the SOU1 gene. The genome of *C. albicans* was extracted using a TIANamp yeast DNA kit (TIANGEN, China). The SOU1 gene from *C. albicans* was amplified by PCR with designed primers of 5′-GGAATTCCATATGATGAGTGAAGAAATCATTTCA-3′ (*Nde*I site is underlined) and 5′-CCGGAATTCTTATGGACATGTATAACCCCCAT-3′ (*Eco*RI site is underlined). The PCR conditions were as follows: 35 cycles of 60 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C. The 846 bp DNA fragment was inserted into pET-22b, resulting in recombinant plasmid pET-22b-SOU1, with its structure verified by double-enzyme cleavage and gene sequence analysis. The recombinant *E. coli* Rosseta (DE3)/pET-22b-SOU1 was obtained by heat shock transformation.

The recombinant strains were grown to an optical density (OD 600 nm) of 0.6 at 37 °C and 200 rpm in LB medium containing 100 mg/L ampicillin and 20 mg/L chloramphenicol. The temperature was then changed to 30 °C, and expression was induced by 0.8 mM IPTG. After 10 h, the cells were harvested by centrifugation (5000 *g*, 5 min, 4 °C), washed with 100 mM potassium phosphate buffer (pH 6.2), and used for biotransformation.

Enzyme activity assay. Cells were disrupted ultrasonically in 100 mM potassium phosphate buffer (pH 6.2). The debris was removed by centrifugation at 13,000 *g* for 10 min. The supernatant was used as the cell-free extract. SOU1 activity was determined using 10 mM COBE with 1 mM NADPH, following changes in absorbance at 340 nm. The extinction coefficient of NADPH was 6.22 $mM^{-1}\,cm^{-1}$. One unit of SOU1 was defined as the disappearance of 1 μmol NADPH per min. Protein concentration was measured by the Bradford method using bovine serum albumin as the standard. The repeatability was determined by three time measurements.

Bioconversion of COBE to (S)-CHBE. Cells were harvested by centrifugation (10,000 *g*, 20 min, 4 °C) and washed with 100 mM potassium phosphate buffer (pH 6.2). The bioconversion of COBE to (S)-CHBE was performed in an aqueous–butyl acetate (4:1, v/v) biphase system containing 100 mM potassium phosphate buffer (pH 6.2), 1500 mM COBE, 1550 mM glucose, 1550 mM Na_2CO_3, Triton X-100 (1‰, v/v), 0.1 g of dry cell weight (DCW) of *E. coli* Rosseta (pET-22b-SOU1), and *E. coli* Rosseta (pET-22b-GDH), respectively, at a total volume of 25 mL. The reaction was performed at 30 °C and 220 rpm for 5 h. The organic layer was isolated to determine the product concentration and optical purity.

To study the effect of cell mass ratio between *E. coli* Rosseta (pET-22b-SOU1) and *E. coli* Rosseta (pET-22b-GDH) on (S)-CHBE yield, we adjusted the mass ratio of the two strains under the same amount of total biomass, which was 200 mg of the gross dry cell weight in the same reaction system.

Analytical methods. The amount of COBE and CHBE was measured by gas chromatography and the optical purity of (*S*)-CHBE was determined by HPLC on a Chiralcel OB-H column (4.6 mm × 250 mm; Daicel Chemical Industries, Japan) as described previously.[9] The intracellular level of $NADP^+$/NADPH was measured after extracting the reaction strain sample following a previously described method.[14]

Results

SOU1 activity toward COBE

SOU1 activity was determined using COBE as the substrate and NADPH as the electron donor. SOU1 exhibited an activity of 6.2 U mg^{-1} toward COBE. Meanwhile, the crude enzyme extract from control cells *E. coli* Rosseta (pET-22b) showed no activity on the substrate.

Bioconversion of COBE to (S)-CHBE with two coexisting E. coli *strains*

The SOU1 expressed in *E. coli* Rosseta (pET-22b-SOU1) functioned as main catalyst for the synthesis of COBE to (*S*)-CHBE, whereas the GDH expressed in *E. coli* Rosseta (pET-22b-GDH) catalyzed the reaction from $NADP^+$ to NADPH to satisfy the needs of NADPH for the former reaction. Their DCW ratio was 1:1, and no $NADP^+$ or NADPH was added in the mixture. After 5 h of bioconversion, the coexisting system produced 1050 mM of (*S*)-CHBE from 1500 mM COBE. The enantiomeric excess was over 99%.

Effect of cell mass ratio on (S)-CHBE yield

Given the differences between SOU1 activity toward COBE in *E. coli* Rosseta (pET-22b-SOU1) and the ability of NADPH regeneration catalyzed by *E. coli* Rosseta (pET-22b-GDH), the two-strain mass ratio was adjusted to establish favorable conditions for two enzymes functioning corporately in the reaction.

The bioconversion protocol was applied under the same amount of total biomass, except that different cell mass ratios of two strains were used. As shown in Fig. 2, the maximum yield of (*S*)-CHBE (1240 mM)

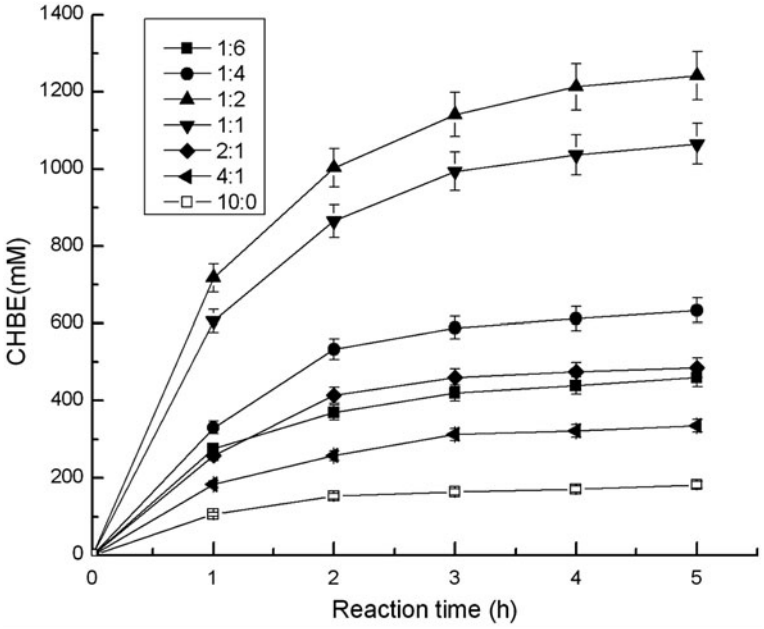

Fig. 2. Effects of different biomass ratios between *E. coli* Rosseta (pET-22b-SOU1) and *E. coli* Rosseta (pET-22b-GDH) on the yield of (*S*)-CHBE.

Table 1. Comparison of several biocatalytic processes for the synthesis of (*S*)-CHBE.

Catalyst	S1	MER	PsCRII	SCR2	CaCR	CAR	SOU1
Coupling strategy	Coexpression	Coexpression	Coexpression	Crude enzymes	Coexpression	Two-strain coexisting	Two-strain coexisting
Reaction time (h)	34	8	30	6	25	10	5
Biomass ($g\,L^{-1}$ DCW)	–	–	50	6	12	10	8
Space-time yield ($mM\,L^{-1}h^{-1}g^{-1}$ DCW)	–	–	1	26.5	15	0.6	31
Optical purity(%)	99	92	99	99	99	99	99
Add coenzyme or not	Yes	Yes	Yes	Yes	No	No	No
TTN	21,600	12,900	13,980	40,000	38,200	–	53,900
Reference	10)	16)	17)	15)	3)	12)	This work

was achieved when *E. coli* Rosseta (pET-22b-SOU1) and *E. coli* Rosseta (pET-22b-GDH) were added at a ratio of 1:2 after 5 h of biotransformation. The enantiomeric excess was more than 99%. The system performed poorly because of the low regeneration of NADPH without adding *E. coli* Rosseta (pET-22b-GDH).

Discussion

An NADPH-dependent SOU1 in *C. albicans* was identified to catalyze the reduction of COBE. SOU1 shared a high degree of similarity in three conserved segments with other COBE-catalytic reductases from SDR family. The recombinant SOU1 can catalyze the asymmetric reduction of COBE to (*S*)-CHBE with high activity and enantioselectivity.

The present work adopted a two-strain coexisting system for the production of (*S*)-CHBE. The SOU1 expressed in *E. coli* Rosseta(pET-22b-SOU1) catalyzed the biosynthesis of (*S*)-CHBE, whereas the GDH expressed in *E. coli* Rosseta(pET-22b-GDH) provided NADPH recycling. The system produced 1240 mM (*S*)-CHBE without adding extra $NADP^+$/NADPH, and the *e.e.* value of (*S*)-CHBE was higher than 99%. The best production of (*S*)-CHBE was achieved under the mass ratio of 1:2 between SOU1 and GDH. A large amount of *E. coli* Rosseta (pET-22b-GDH) was required to maintain sufficient NADPH for the whole system, suggesting that the efficiency of NADPH regeneration is the major limiting factor for the production of (*S*)-CHBE.

Several reported biocatalytic processes for the synthesis of (*S*)-CHBE using GDH as cofactor regenerator were compared with this system (Table 1). One of the advantages of our system is that $NADP^+$/NADPH addition can be omitted. The internal cofactor of *E.coli* host cells is sufficient to start and maintain the reaction recycle. The turnover number of intracellular coenzyme to (*S*)-CHBE formed was 53,900 (mol/mol), which is higher than those known (*S*)-CHBE production systems. The space-time yield (31 $mmol\,L^{-1}h^{-1}\,g^{-1}$DCW) is the highest one and can compete against the one with crude enzyme reaction system (26.5 $mmol\,L^{-1}h^{-1}\,g^{-1}$DCW).[15] Moreover, the two strains would be cultivated individually at optimal culture conditions and mixed in an appropriate ratio to maximize the yield of (*S*)-CHBE production, making the process easy and flexible for practical operations.

Funding

This work was supported by the National Basic Research Program of China [grant number 2011CBA0807]; National High Technology Research and Development Program of China [grant number 2012AA022101]; Priority Academic Program Development of Jiangsu Higher Education Institutions [grant number 1001020B].

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