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Construction and co-expression of a polycistronic plasmid encoding carbonyl reductase and glucose dehydrogenase for production of ethyl (*S*)-4-chloro-3-hydroxybutanoate

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ABSTRACT

Biocatalysis of ethyl 4-chloro-3-oxobutanoate (COBE) to ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] was carried out using *Escherichia coli* co-expressing a carbonyl reductase gene from *Pichia stipitis* and a glucose dehydrogenase gene from *Bacillus megaterium*. An efficient polycistronic plasmid with a high-level of enzyme co-expression was constructed by changing the order of the genes, altering the Shine–Dalgarno (SD) regions, and aligned spacing (AS) between the SD sequence and the translation initiation codon. The optimal SD sequence was 5-TAAGGAGG-3, and the optimal AS distance was eight nucleotides. Asymmetric reduction of COBE to (*S*)-CHBE with more than 99% enantiomeric excess was demonstrated by transformants, using a water/ethyl caprylate system. The recombinant cells produced 1260 mM product in the organic phase, and the total turnover number, defined as moles (*S*)-CHBE formed per mole $NADP^+$, was 12,600, which was more than 10-fold higher than in aqueous systems.

1. Introduction

(*S*)-4-chloro-3-hydroxybutanoate ethyl [(*S*)-CHBE] is a chiral intermediate in the synthesis of pharmacologically active compounds such as hydroxymethylglutaryl-CoA reductase inhibitors (Karanewsky et al., 1990; Lee and Park, 2009). Asymmetric reduction of 4-chloro-3-oxobutanoate ethyl (COBE) to (*S*)-CHBE by reductases has several positive attributes, including low cost, mild reaction conditions, high yield, and remarkable enantioselectivity (Mateo et al., 2007; Goldberg et al., 2007). Biocatalysis of COBE to (*S*)-CHBE by reductases, however, can be quite challenging, since they often require expensive cofactors such as NADH or NADPH (Nakamura et al., 2003). We previously purified a novel carbonyl reductase (PsCR) from *Pichia stipitis* that requires only NADPH as a cofactor for catalysis (Ye et al., 2009b). Because of the high cost of the pyridine cofactors, *in situ* cofactor regeneration is critical to the economic viability of industrial-scale biotransformations using PsCR, and other reductases (van der Donk and Zhao, 2003; Liu and Wang, 2007). The most convenient and useful method for regenerating pyridine cofactors is to employ dehydrogenases that recycle nicotinamide coenzymes (Hummel, 1999). For example, NAD can be recycled from NADH for production of (*S*)-CHBE using *Escherichia coli* co-expression of a carbonyl reductase and a formate dehydrogenase (Yamamoto et al., 2004). Although many co-expression systems have been proposed to create this type of recycling process (Kataoka et al., 1999; Kizaki et al., 2001; Gómez et al., 2007; Liu et al., 2008), few have experimentally manipulated the configuration of polycistronic plasmids to obtain high-level co-expression of the enzymes. In addition, not all genes are expressed efficiently in this organism because of unique and subtle structural features of gene sequences and expression elements, among other factors (Makrides, 1996; Hannig and Makrides, 1998).

In our previous work, we described a yield of greater than 99% enantiomeric excess (e.e.) using PsCR from *P. stipitis* for the production of (*S*)-CHBE (Ye et al., 2009a). For industrial applications, we wished to co-express the PsCR gene and a glucose dehydrogenase (BmGDH) gene from *Bacillus megaterium* to regenerate the cofactor. To achieve high-level expression of both PsCR and BmGDH, several important factors were experimentally investigated, including the order of the genes on the plasmid, the sequence of the Shine–Dalgarno (SD) regions, and the aligned spacing (AS) between the SD sequence and the translation initiation codon. Furthermore, we analyzed the biocatalysis of COBE to (*S*)-CHBE by recombinant cells using a water/ethyl caprylate system.

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2. Methods

2.1. Cloning and expression of BmGDH gene in E. coli

Genomic DNA was isolated from *B. megaterium* (DSM 2894) using a TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit Ver. 2.0 (TaKaRa, China). Oligonucleotide primers specific for the full-length BmGDH gene were designed using GenBank Accession number 142974. The forward primer was BmGDHs, 5′-GGAATTCCA TATGTATACAGATTAAAAGAT-3′ (*Nde*I site is underlined). The reverse primer was BmGDHr, ATATGGATCCCTATTAGCCTCTTC (*Bam*HI site is underlined). The amplified DNA fragment was double-digested with *Nde*I and *Bam*HI and then inserted into the expression vector pET-22b. The resulting plasmid, pET-22b–BmGDH, was transformed into *E. coli* Rosetta (DE3) pLysS from Merck. Expression was induced in *E. coli* transformant cultures with 1 mM IPTG at OD600 = 0.6. The culture was shifted to 25 °C to induce protein expression and cells were harvested after 10 h. Protein purity was determined by SDS–PAGE analysis using standard procedures (Bradford, 1976).

2.2. Construction of a polycistronic co-expressing plasmid

Details on cloning and expression of the PsCR gene were described previously (Ye et al., 2009a). Plasmids containing PsCR gene and BmGDH gene were constructed (Fig. 1). The plasmids differed in the order of the two genes, the SD regions (SD1 and SD2) and the AS sequences (AS1 and AS2) (Table 1). To construct plasmid pET-P–SD1-AS1-B, two oligonucleotide primers were used for PCR with the BmGDH gene as the template. The PCR product was named SD1-AS1-B and was double-digested with *Bam*HI and *Xho*I. The resulting fragment SD1-AS1-B was cloned into the *Bam*HI/*Xho*I site of pET-22b–PsCR. The resulting plasmid (pET-P–SD1-AS1-B) with the two enzyme genes was transformed into *E. coli* Rosetta (DE3). Other plasmids were similarly constructed. The primers for each clone are shown in Table S1.

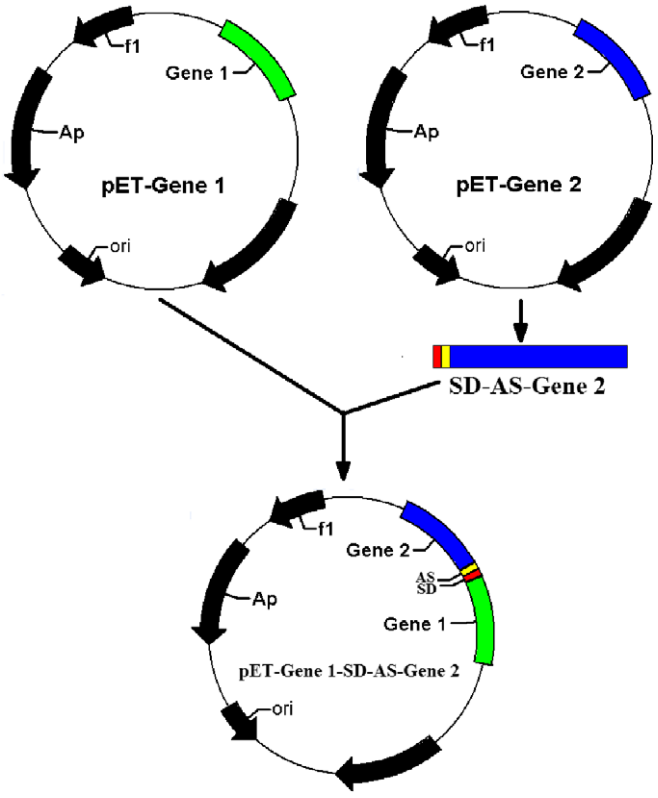

Fig. 1. Schematic presentation of polycistronic plasmids. As shown in Table 1, in plasmids pET-P-B, pET-P–SD1-AS1-B, pET-P–SD2-AS1-B, pET-P–SD1-AS2-B and pET-P–SD2-AS2-B, gene 1 represents the PsCR gene, and gene 2 represents the BmGDH gene. In pET-B-P, pET-B-SD1-AS1-P, pET-B-SD2-AS1-P, pET-B-SD1-AS2-P, and pET-B-SD2-AS2-P, upstream represents the BmGDH gene and gene 2 represents the PsCR gene. The fragment SD-AS-Gene 2 was amplified using the pET-gene 2 as the template and the primers listed in Table S1. Construction of ten polycistronic plasmids was performed as described in Section 2.

2.3. Enzyme stability

A mixture of 100 mM potassium phosphate buffer (pH 6.0), PsCR (15 U/ml), and BmGDH (10 U/ml), with various concentrations of COBE or CHBE, was incubated at 30 °C for 24 h to study enzyme stability in the presence of COBE or CHBE. Enzyme activities were assayed as described below. Equal volumes of 1 ml of 100 mM potassium phosphate buffer (pH 6.0) containing cell-free extracts of *E. coli* Rosetta carrying pET-B-SD2-AS1-P (PsCR: 15 U/ml, BmGDH: 10 U/ml) and 1 ml of test organic solvent were mixed in a closed vessel and shaken at 30 °C for 48 h, and the enzyme activity remaining in the aqueous phase was assayed as described below.

2.4. Biocatalysis of COBE to (S)-CHBE

For aqueous reactions, a 100-ml reaction mixture of 100 mM potassium phosphate buffer (pH 6.0), COBE (30 mM), $NADP^+$ (0.1 mM), glucose (200 mM), and 3 g of dry cells was incubated at 30 °C, 220 rpm for 24 h, in a 250-ml reacting vessel. The pH was adjusted to 6.0 with 2 M $Na(OH)_2$. At 0.5, 2, 4 and 11 h, 30 mM of COBE was added. For the diphasic system, a 100-ml reaction mixture of 100 mM potassium phosphate buffer (pH 6.0), COBE (200 mM), $NADP^+$ (0.1 mM), glucose (1.5 M), 3 g of dry cells and an equal volume of ethyl caprylate was incubated at 30 °C, 220 rpm for 24 h, in a 250-ml reacting vessel. The pH was adjusted to 6.0 with 2 M $Na(OH)_2$. At 0.5, 1, 2, 4, 7 and 11 h, 200 mM of COBE was added to reaction.

2.5. Enzyme assay and analysis of COBE and CHBE

Transformed *E. coli* cells were harvested by centrifugation and disrupted with an ultrasonic oscillator. Cell debris was removed by centrifugation, and the supernatant used as a crude enzyme extract (Ye et al., 2009b). PsCR activity was determined spectrophotometrically with 100 mM potassium phosphate buffer (pH 6.0), 0.1 mM NADPH, and 1 mM COBE. The assay mixture for GDH activity was 100 mM potassium phosphate buffer (pH 6.0), 2 mM NADP, and 100 mM glucose. The reaction was monitored at 30 °C by absorbance at 340 nm. One unit of enzyme was the amount of PsCR catalyzing the reduction of 1 μmol $NADP^+$ per minute, or the amount of GDH catalyzing the oxidation of 1 μmol NADPH per minute. For aqueous reactions, an equal volume of ethyl acetate was added to the reaction mixture, which was vigorously mixed and centrifuged. The top phase (organic layer) was assayed to determine yield and optical purity. For two-phase reactions, the organic layer obtained after centrifugation of the reaction mixture was assayed. The concentrations of COBE and CHBE were determined by gas chromatography, and the optical purity of (*S*)-CHBE was determined by HPLC (Ye et al., 2010).

3. Results

3.1. Gene cloning, expression and activity analysis of BmGDH

The BmGDH gene was amplified from *B. megaterium* by PCR (Fig. 2A, lane 2). Sequence analysis indicated that the BmGDH gene

Table 1
Structure of polycistronic plasmids with different gene order, SD regions and AS for PsCR and BmGDH genes.

Plasmids	Upstream	Shine–Dalgarno (SD)[a]	Aligned spacing (AS)[b]	Downstream
pET-P-B	PsCR	–	–	BmGDH
pET-B-P	BmGDH	–	–	PsCR
pET-P–SD1-AS1-B	PsCR	SD1	AS1	BmGDH
pET-B-SD1-AS1-P	BmGDH	SD1	AS1	PsCR
pET-P–SD2-AS1-B	PsCR	SD2	AS1	BmGDH
pET-B-SD2-AS1-P	BmGDH	SD2	AS1	PsCR
pET-P–SD1-AS2-B	PsCR	SD1	AS2	BmGDH
pET-B-SD1-AS2-P	BmGDH	SD1	AS2	PsCR
pET-P–SD2-AS2-B	PsCR	SD2	AS2	BmGDH
pET-B-SD2-AS2-P	BmGDH	SD2	AS2	PsCR

[a] Shine–Dalgarno (SD) SD1 GAAGGAGA, SD2 TAAGGAGG.
[b] Aligned spacing (AS) AS1ATATACAT, AS2 TACAT.

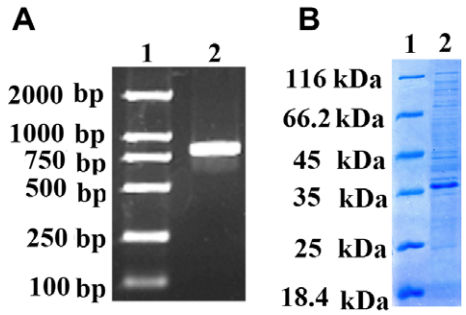

Fig. 2. (A) Cloning of the BmGDH gene from *Bacillus megaterium* by PCR. Lane 1: DL2000 marker. Lane 2: BmGDH gene. (B) SDS–PAGE analysis of recombinant protein of BmGDH. Lane 1: protein molecular weight marker. Lane 2: supernatant (soluble proteins) after sonication of *E. coli* Rosetta (DE3) with pET-22b–BmGDH.

(783 bp) encoded 261 amino acids. The calculated molecular mass of BmGDH was 28.1 kDa and the pI was 4.6. Amino acid sequence alignments of BmGDH and other GDHs from *B. megaterium* are shown in Fig. S1. The results showed highest similarity with GDH from *B. megaterium* IAM1030 (98% identity). The expression of BmGDH is shown in Fig. 2B, lane 2. *E. coli* Rosetta (DE3) with the pET-22b–BmGDH plasmid exhibited an activity of 12 U/mg.

3.2. Construction of a polycistronic co-expression system

Fig 3A shows PsCR and BmGDH activities achieved with plasmid constructs, and SDS–PAGE analysis of protein extracts from *E. coli* Rosetta (DE3) harboring the different plasmids is shown in Fig. 3B. *E. coli* Rosetta (DE3) carrying plasmid pET-P-B exhibited an activity of 25 U/mg using COBE as a substrate, while *E. coli* Rosetta (DE3) harboring plasmid pET-B-P showed an activity of 12 U/mg using glucose as a substrate. When the two genes had the same SD and AS sequences, the expression level from the upstream position was higher than that from the downstream position. *E. coli* Rosetta (DE3) carrying pET-22b–PsCR exhibited an activity of 27 U/mg with COBE as a substrate, but *E. coli* Rosetta (DE3) with pET-22b–BmGDH showed only 12 U/mg activity using glucose. Since the BmGDH activity was lower than the activity of PsCR expressed alone, the BmGDH gene was placed in the upstream position. Without SD sequences, the activity of the second enzyme encoded in the downstream position of plasmids pET-P-B and pET-B-P was low, demonstrating that SD regions are not essential for gene expression, as also observed by Fargo et al. (1998). Due to this reason, we used the native SD region of pET-22b (SD1, GAAGGAGA) to express PsCR. The PsCR activity from plasmid pET-B-SD1-AS1-P was 10 U/mg, less than half of the activity from pET-22b–PsCR. We substituted SD1 of pET-22b with SD2 (TAAGGAGG) to improve the expression level (Gold, 1988), resulting in PsCR activities from pET-B-SD2-AS1-P of approximately 16 U/mg. An optimal AS is 5 nucleotides (nt), according to some reports (Chen et al., 1994), but the AS of pET-22b is 8 nt. Therefore, the effects of AS1 (5 nt) and AS2 (8 nt) were investigated in the polycistronic plasmid. Recombinant PsCR in *E. coli* Rosetta (DE3) harboring plasmid pET-B-SD2-AS2-P exhibited an activity of 11 U/mg, which was lower than for *E. coli* Rosetta (DE3) carrying pET-B-SD2-AS1-P. Ringquist et al. observed that natural mRNAs of *E. coli* have an optimal AS of 5 nt (Ringquist et al., 1992). Nevertheless, our enzyme assays showed that AS 1 (8 nt) of the pET-22b system was more efficient for co-expression of PsCR.

3.3. Effects of COBE and CHBE on PsCR and BmGDH stability

Fig. 4A shows the stability of PsCR and BmGDH in the presence of COBE. The results suggested that PsCR and BmGDH were stable at 10 mM at 30 °C for 24 h. The stabilities of the two recombinant enzymes decreased sharply at greater than 30 mM COBE. At 40 mM COBE, the activities of PsCR and BmGDH decreased significantly, losing almost 80% of the original activity. In contrast, PsCR and BmGDH were relative stable in CHBE, with both enzymes showing stability at 60 mM CHBE, which decreased at 100 mM CHBE. BmGDH was less stable than PsCR. At 360 mM, the activity of PsCR decreased significantly, losing almost 70% of its original activity in 100 mM CHBE, and no BmGDH activity was observed after 24 h at this CHBE concentration.

3.4. Effects of diphasic systems on PsCR and BmGDH stability

The stabilities of PsCR and BmGDH were measured in various water/organic solvent systems. Generally, PsCR was more stable than BmGDH at 30 °C for 48 h. Table 2 shows that PsCR showed 85% of its original activity in an aqueous system, which was more than twofold higher than the activity of BmGDH. PsCR was stable in most organic solvents, including hexane, octane and heptane. Both PsCR and BmGDH, however, were strongly inactivated in ethyl acetate, *n*-butyl acetate and chloroform, which cause partition of substrate and the product. Furthermore, PsCR and BmGDH were stable in water/ethyl caprylate, water/1-octanol, water/dibutylphthalate systems. Because of the partition of COBE and CHBE in diphasic systems, ethyl caprylate was selected as the organic phase for asymmetric reduction of COBE to (*S*)-CHBE by *E. coli* Rosetta (DE3) carrying pET-B-SD2-AS1-P.

3.5. Biocatalysis of COBE to (S)-CHBE in aqueous systems

Fig. 5 shows the biocatalysis of COBE to (*S*)-CHBE in an aqueous monophase reaction by *E. coli* Rosetta (DE3) with pET-B-SD2-AS1-P. The maximum reaction rate was achieved from 0 to 2 h. Because CHBE was inhibitory, the velocity of the reaction decreased sharply

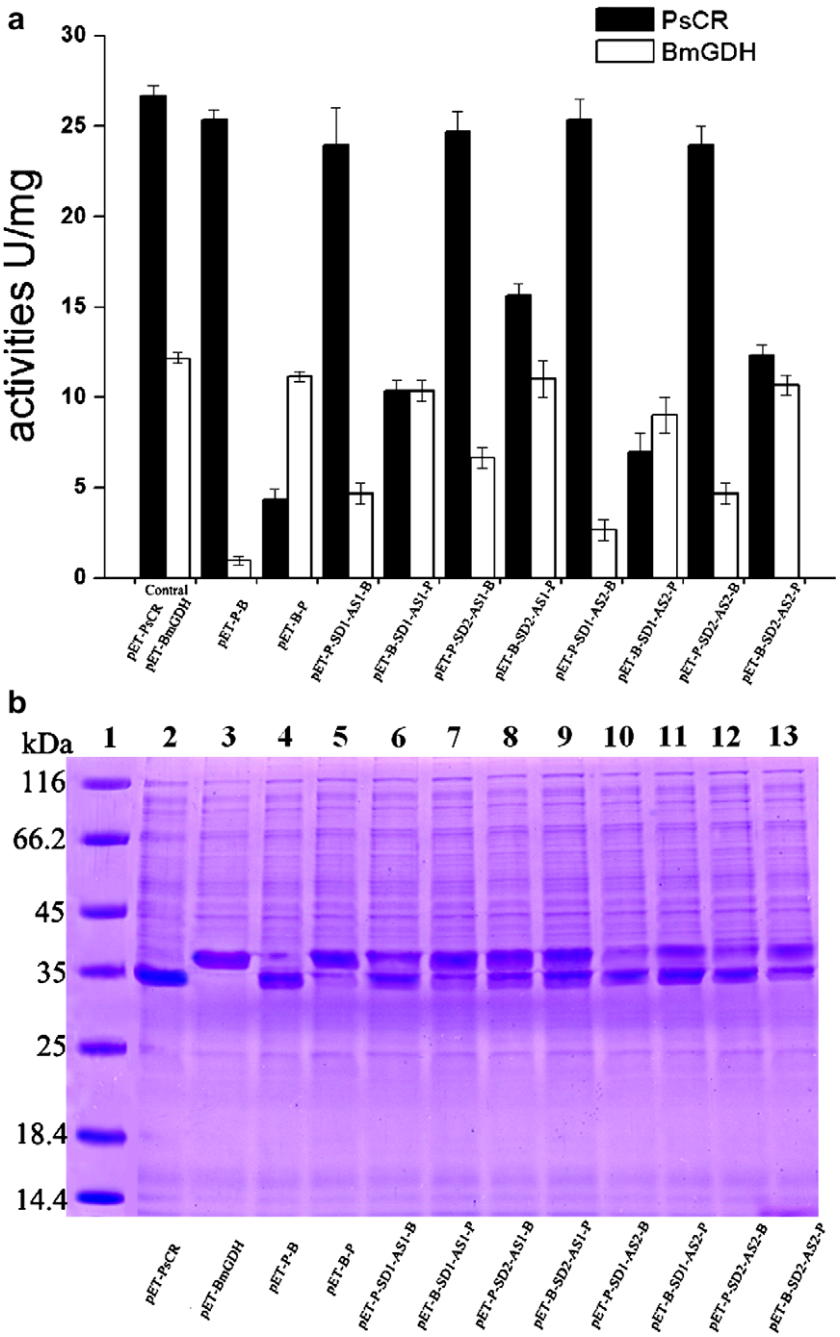

Fig. 3. (A) Effects of different gene order, SD regions and AS on PsCR and BmGDH activity. The black columns represent PsCR activity and the white columns represent BmGDH activity. Reactions were performed in triplicate, and error bars represent the standard error of the mean (SEM). (B) SDS–PAGE analysis of recombinant protein of co-expression. Each lane represents soluble proteins in supernatant after sonication of recombinant *E. coli* except marker.

when the concentrations of the products reached approximately 120 mM. Recombinant cells produced 133 mM CHBE with a molar yield of 95% and an optical purity of the (*S*)-isomer of greater than 99% e.e., respectively. In this system, the total turnover number (TTN), defined as moles (*S*)-CHBE formed per mole $NADP^+$, was 1330 mol/mol.

3.6. Biocatalysis of COBE to (S)-CHBE in a diphasic system

COBE to (*S*)-CHBE biocatalysis was conducted in water/ethyl caprylate (Fig. 6). Because of substrate partition, and stabilities of PsCR and BmGDH in the diphasic system, 200 mM COBE was used as the initial concentration. At this concentration, after 0.5 h, more than 150 mM CHBE and less than 40 mM COBE were observed. The maximum reaction rate was achieved from 0 to 2 h, when 500 mM CHBE was achieved. From 4 to 24 h, reaction velocities decreased slowly because of CHBE accumulation. At 24 h, transformants strains showed 1260 mM CHBE in the organic phase with a molar yield of 90%. The optical purity of the (*S*)-CHBE was more than 99% e.e. (Fig. S2) and the TTN was 12,600.

4. Discussion

(*S*)-CHBE is a precursor for enantiopure intermediates for chiral drugs, including cholesterol-lowering hydroxymethylglutaryl-CoA reductase inhibitors like Pfizer's Lipitor (atorvastatincalcium). This

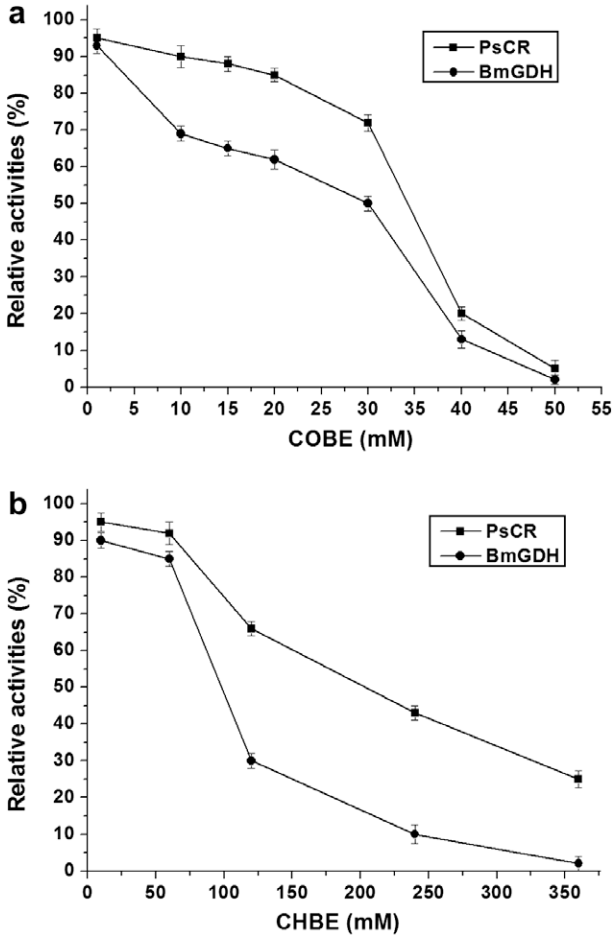

Fig. 4. Effects of COBE and CHBE on stabilities of PsCR and BmGDH. (A) Effect of COBE on the stabilities of the PsCR and BmGDH. (B) Effect of CHBE on the stabilities of the PsCR and BmGDH. The enzyme activities were measured as described in the text. Reactions were performed in triplicate, and error bars represent the standard deviations (StDev).

Table 2
Partition efficiencies of organic solvents for COBE and CHBE and stabilities of PsCR and BmGDH in various organic solvent–water diphasic systems.

Organic solvent	Partition efficiency[a]		Remaining activity (%)	
	COBE	CHBE	PsCR	BmGDH
None	–	–	85.5 ± 0.7	48.5 ± 0.4
Ethyl acetate	16.2[b]	23.5[b]	0	0
n-Butyl acetate	10.6[b]	15.8[b]	22.1 ± 0.4	11.3 ± 0.5
Ethyl caprylate	6.7[d]	9.3[d]	65.7 ± 0.9	39.8 ± 0.4
1-Octanol	5.3[c]	3.8[c]	72.7 ± 0.4	43.5 ± 0.3
Chloroform	17.7[b]	7.7[b]	5.5 ± 0.4	0
Octane	0.9[c]	0.2[c]	77.5 ± 0.6	20.7 ± 0.7
Hexane	1.5[c]	0.3[c]	62.6 ± 0.5	13.4 ± 0.5
Dibutylphthalate	6.2[c]	4.2[c]	60.2 ± 0.8	41.3 ± 0.6
Heptane	1.0 [b]	0.25 [b]	75.4 ± 0.6	15.5 ± 0.8
Isopropyl ether	2.2[b]	3.9[b]	24.4 ± 0.6	7.2 ± 0.5

[a] Partition efficiency is the molar ratio of each compound found in the organic phase to the same compound in the aqueous phase.
[b] Data from Shimizu et al. (1990).
[c] Data from He et al. (2006).
[d] COBE and (*S*)-CHBE (100 μmol each) in 10.0 ml of 100 mM potassium phosphate buffer (pH 6.0) were shaken with ethyl caprylate (10.0 ml) at 30 °C in a closed vessel. The concentrations of COBE and CHBE in each phase were determined by GC as described in Section 2.

is the largest-selling prescription medicine, with worldwide annual sales exceeding US $10 billion (Asako et al., 2009; Lee and Park, 2009). Previously, we described PsCR, a novel NADPH-dependent

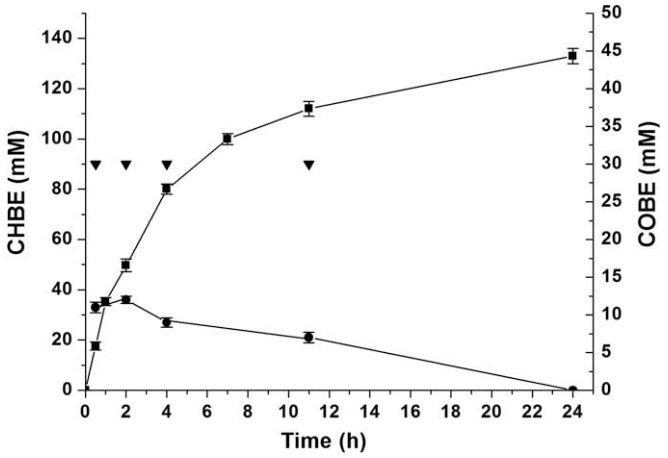

Fig. 5. Biosynthesis of ethyl (*S*)-4-chloro-3-hydroxybutanoate using recombinant *E. coli* Rosetta pET-B-SD2-AS1-P in an aqueous monophase system. Symbols, ■: concentration of CHBE; ●: concentration of COBE; ▼: 30 mM COBE was added to the system at 0.5, 2, 4, and 11 h. Reactions were performed in triplicate, and error bars represent the standard deviations (StDev).

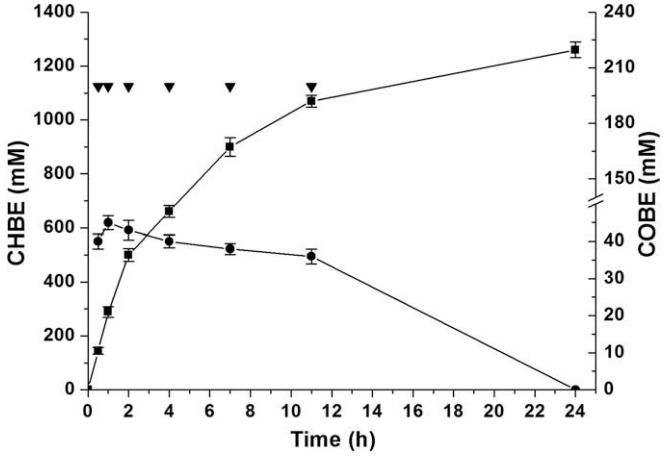

Fig. 6. Biosynthesis of ethyl (*S*)-4-chloro-3-hydroxybutanoate using recombinant *E. coli* Rosetta pET-B-SD2-AS1-P in a water/ethyl caprylate system. Symbols, ■: concentration of CHBE in organic phase; ●: concentration of COBE in organic phase; ▼: 200 mM COBE was added to the system at 0.5, 1, 2, 4, 7 and 11 h. Reactions were performed in triplicate, and error bars represent the standard deviations (StDev).

carbonyl reductase from *P. stipitis* that exhibits high activity and high enantioselectivity for several prochiral ketones. Analysis of the amino acid sequence of PsCR revealed less than 60% identity to known reductases that produce (*S*)-CHBE with greater than 99% e.e. (Ye et al., 2009b). However, PsCR required reduced nicotinamide coenzymes as an electron donor, which are too expensive to be used as stoichiometric reagents. To lower cost, we used glucose, NADP and commercially available GDH to create a regeneration system for converting NADPH from NADP (Ye et al., 2009a). In this study, we constructed a polycistronic plasmid to express PsCR and BmGDH in *E. coli*, and investigated the enzymatic reduction of COBE to (*S*)-CHBE.

We cloned and expressed a glucose dehydrogenase (GDH) gene, since the GDH substrate is inexpensive, and GDH can reduce NADP to NADPH. The activity of BmGDH in our research was tested in 100 mM potassium phosphate buffer (pH 6.0), which is different from studies in the literature that used Tris–HCl buffer (pH 8.0). COBE is unstable under alkaline conditions, and the reduced nicotinamide cofactor readily decomposes under acidic conditions (Shimizu et al., 1990), and purified PsCR exhibited maximum

activity at pH 6.0 (Ye et al., 2009b). Therefore, we chose a potassium phosphate buffer with a pH of 6.0 for all our enzymatic assays. Our results indicated that the activity of BmGDH from *B. megaterium* was less than half the activity of PsCR. To obtain high-levels of both two enzymes, the two genes were co-expressed in *E. coli*. This addressed the problem of the imbalance in the enzyme expression levels, which needed to be minimized to prevent different enzyme activity levels. We also investigated ways to maximize expression levels for both enzymes by altering the order of the genes on the plasmid, the SD sequence, and the AS sequence. All these factors substantially influence gene expression (Makrides, 1996). Fig. 3 suggested that the expression level of the first enzyme gene on the plasmid (upstream) showed little difference whether it was co-expressed or expressed alone, consistent with several reports (Itoh et al., 2004; Kizaki et al., 2001). To balance the activities of the two enzymes, the BmGDH was moved to closer to the promoter, for a higher expression level. To improve PsCR expression, optimal SD2 and AS1 sequences were used in the polycistronic plasmid. As a result, *E. coli* Rosetta (DE3) with pET-B-SD2-AS1-P exhibited an activity of 16 U/mg with COBE as a substrate, and 11 U/mg with glucose as a substrate. In the co-expression system, the activity of PsCR is 60% of the original activity. On the other hand, BmGDH is 90%.

Biocatalysis of COBE to (*S*)-CHBE was first investigated using an aqueous system. Fig. 4A demonstrates that both PsCR and BmGDH were unstable in more than 30 mM COBE, so this was used as an initial concentration. COBE at 30 mM was fed to the reaction at 0.5, 2, 4 and 11 h. In this reaction system, NADPH was regenerated by coupling glucose oxidation to BmGDH. The resulting gluconate was neutralized with NaOH to maintain pH 6.0, at which purified PsCR exhibited maximum activity (Ye et al., 2009b). Fig. 4B indicates that PsCR was less stable than another carbonyl reductase, S1 from *Candida magnoliae*, which produces (*S*)-CHBE at a concentration of 100 mM (Kizaki et al., 2001). At a CHBE concentration of 100 mM in our system, the reaction rate decreased significantly because of inhibition of PsCR and BmGDH. In our system recombinant cells produced a maximum of 133 mM CHBE with a molar yield of 95%. The remaining COBE substrate was not detectable because of incomplete extraction. To minimize inhibition of PsCR and BmGDH by COBE and CHBE, an asymmetric reduction of COBE to (*S*)-CHBE in a diphasic system was developed. Table 2 demonstrates that PsCR was unstable in a water/butyl acetate system, which is different from other reductases that produce CHBE, which are stable under these conditions (Kataoka et al., 1997; Kizaki et al., 2001; Yamamoto et al., 2002). Furthermore, although dibutylphthalate is biocompatible with both PsCR and BmGDH, and has been used as an organic phase for production of (*S*)-CHBE (He et al., 2006), COBE and CHBE showed poor partition in a water/dibutylphthalate system. Ethyl caprylate was both biocompatible for PsCR and BmGDH, and gave favorable partition of substrate and product, and was used as the organic phase for biocatalytic synthesis of (*S*)-CHBE. Our strain yielded 1260 mM CHBE in a water/ethyl caprylate system, which is 10-fold higher than observed for the aqueous system. The molar yield of CHBE was 90% because of the partitioning of CHBE in the water/ethyl caprylate system. Several previous reports concluded that TTNs of 10^3–10^5 may be sufficient to make a process economically viable (Zhao and van der Donk, 2003). In our diphasic system, the TTN of CHBE forming $NADP^+$ was 12,600, higher than in our previous experiments with an aqueous systems (1400), suggesting that the process could be economically feasible.

5. Conclusions

In summary, we constructed a polycistronic plasmid for efficient co-expression of PsCR and BmGDH. The optimal SD sequence was 5-TAAGGAGG-3, and the optimal AS distance was eight nucleotides. *E. coli* Rosetta (DE3) containing the polycistronic plasmid pET-B-SD2-AS1-P exhibited an activity of 16 U/mg with COBE as a substrate, and 11 U/mg with glucose as a substrate. The recombinant cells produced 1260 mM product in the organic phase, and the total turnover number, defined as moles (*S*)-CHBE formed per mole $NADP^+$, was 12,600, more than 10-fold higher than comparable aqueous systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.03.099.

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