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ARTICLE

Two-Enzyme Coexpressed Recombinant Strain for Asymmetric Synthesis of Ethyl (*R*)-2-Hydroxy-4-phenylbutyrate

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Abstract: (*R*)-2-Hydroxy-4-phenylbutyrate (HPBE) is an important chiral intermediate for the synthesis of angiotensin-converting enzyme (ACE) inhibitors. Asymmetric reduction of ethyl 2-oxo-4-phenyl-butyrate (OPBE) to (*R*)-HPBE using a recombinant strain can provide high enantioselectivity. Cofactor regeneration is a critical issue in the application of a recombinant strain. A carbonyl reductase gene (*iolS*) and a glucose dehydrogenase (GDH) gene from *Bacillus subtilis* were cloned. Recombinant IolS was purified using a Ni-NTA column and its enzyme activity properties were investigated. The purified IolS exhibited maximum activity at pH 6.0 and 30 °C, and the enzyme showed good thermostability below 40 °C. It retained over 75% of its activity in the acidic pH range of 5.5–7.0. Three coexpression strategies were used for the recombinant vectors. The recombinant *E. coli* strain containing polycistronic plasmid pET-G-T7-I showed excellent carbonyl reductase activity, and the specific activity of both IolS and GDH in the crude cell extract reached 1.5 U/mg. In the asymmetric reduction of OPBE by recombinant *E. coli* cells in aqueous system, the yield of (*R*)-HPBE reached over 99% with an enantiomeric excess of 99.5% at 10 g/L of OPBE within 15 h.

Key words: carbonyl reductase; glucose dehydrogenase; coexpression; (*R*)-2-hydroxy-4-phenylbutyrate; asymmetric reduction

Angiotensin-converting enzyme (ACE) inhibitors are widely used for the treatment of hypertension, congestive heart failure, and other cardiovascular diseases because they can reduce the Angiotensin Ⅱ receptor (AngII) to control blood pressure and protect target organs [1]. (*R*)-2-hydroxy-4-phenylbutyrate (HPBE) is an important chiral intermediate for the synthesis of ACE inhibitors such as benazepril, enalapril, and lisinopril [2]. Various approaches for the preparation of (*R*)-HPBE have been reported in recent years, including chemical and biological methods. Both of these can be carried out in two ways: resolution and synthesis. The chemical method usually involves multiple steps and a large amount of organic reagents [3]. In the case of resolution, the theoretical maximum yield is only 50% [4–6]. The microbial reduction of ethyl 2-oxo-4-phenyl -butyrate (OPBE) to (*R*)-HPBE has been intensively studied in the last decade. As compared with a recombinant whole-cell catalyst, wild strains produce (*R*)-HPBE in relatively lower enantiomeric excess (ee) due to their multiple intracellular carbonyl reductases that have variable or opposite stereospecificity [7–9]. Previously, a highly potent carbonyl reductase-producing strain *Candida krusei* SW 2026 was isolated in our laboratory, which catalyzed the enantioselective reduction of OPBE to (*R*)-HPBE. At 20 g/L of OPBE, the *ee* and yield were 97.4% and 82.0%, respectively. The carbonyl reductase was purified to homogeneity using three chromatograph columns and its enzyme activity properties were investigated [10, 11]. There have been few reports on the asymmetric reduction of OPBE to (*R*)-HPBE catalyzed by recombinant strains so far. Shen et al. [12] were successful in getting (*R*)-HPBE with good *ee* and yield (99% and 100%, respectively) at 1 mol/L (ap-

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proximately 206 g/L) of OPBE using recombinant *E. coli* strain with both CgKR2 and GDH encoding genes. Since the expensive cofactor NAD(P)H was required for the oxidoreduction process, in situ cofactor regeneration is critical for the economics of the industrial scale biotransformation using the recombinant strain.

The use of a coexpression system solves the problem of cofactor regeneration. The technology enables the expression of a number of heterologous proteins within the same strain. Glucose dehydrogenase (GDH) has been widely used for co-enzyme regeneration to recycle the nicotinamide coenzyme NAD(P)H from $NAD(P)^+$ [13–15]. In this study, three methods were used for the construction of a two-enzyme coexpression system comprising the carbonyl reductase gene (*iolS*) and glucose dehydrogenase gene (*gdh*) from *Bacillus subtilis*. The bioconversion catalyzed by the recombinant whole cells were achieved in aqueous system

1 Experimental

1.1 Strains, plasmids, and chemicals

The strains and plasmids used in this study are listed in Table 1. Restriction enzymes and T4 ligase were purchased from TaKaRa Biotechnology Co. PCR related products were purchased from Dongsheng Biotech Co., China. (*R*)-HPBE and OPBE were purchased from Sigma-Aldrich Chemical Co. $NADP^+$ and NADPH were purchased from Sangon Biotech Co., China. All other reagents and solvents were from local commercial suppliers and were of analytical grade or biochemical reagents.

1.2 Cloning and expression of glucose dehydrogenase

Genomic DNA was isolated from *Bacillus subtilis* using a bacterial genomic DNA extraction kit. Two oligonucleotide primers for the amplification of the full length *gdh* were designed based on its nucleotide sequence (GenBank No. M12276). These are listed in Table 2. The amplified DNA fragment was ligated into the pMD18-T to create the recombinant plasmid pMD18-T-*gdh*. DNA sequencing was performed by Sangon Biotech Co. The pMD18-T-*gdh* was double digested with *Nde* I and *Hind* III and then inserted into the expression vector pET-24a. The resulting plasmid, pET24a-*gdh*, was transformed into *E. coli* BL21(DE3), and positive clones were screened and confirmed by colony PCR and restriction enzyme digestion. The expression of the GDH was induced with 0.4 mmol/L IPTG when the OD_{600} reached 0.6, and the incubation temperature was changed to 25 °C for the induction of protein expression. Then the cells were harvested after 4 h. Protein concentration was determined by SDS-PAGE analysis performed according to standard procedures [16].

Table 1 Strains and plasmids in this study

Strain and plasmid	Application	Source
Bacillus subtilis	source of CR and GDH	our laboratory
E. coli K12	source of CR	our laboratory
E. coli JM109	cloning host	our laboratory
E. coli BL21 (DE3)	expression host	our laboratory
pMD18-T	cloning vector	Takara
pET-24a	expression vector	Novagen
pET-20b	expression vector	Novagen

Table 2 Primers used in this study

Name	Primer sequences (5'→3')	Restriction site
GDHf	GGAATTCCATATGTATCCGGATTTAAAAGC	*Nde*I
GDHr	CCCAAGCTTTTAACCGCGGCCTGC	*Hind*III
YcsNf	GGGAATTCCATATGCAGCGTATTCAAT	*Nde*I
YcsNr	CCGCTCGAGTGGAATATCGTAACCCTGTAC	*Xho*I
IolSf	GGAATTCCATATGAAAAAAGCGAAGCTCGG	*Nde*I
IolSr	CCGCTCGAGTGCGAACAGCTTATCAAT	*Xho*I
YccKf	GGGAATTCCATATGGATCAAACACGTACAC	*Nde*I
YccKr	CCGCTCGAGTTTGAAAATGTCGCTGATG	*Xho*I
YdhFf	GGGAATTCCATATGGTTCAGCGTATTACT	*Nde*I
YdhFr	TTCCGCTCGAGCGGTACGTCGTACCCCAGT	*Xho*I
YajOf	GGGAATTCCATATGCAATACAACCCCTTAG	*Nde*I
YajOr	CCGCTCGAGTTTAAATCCTACGACAGG	*Xho*I
SDf	AAGGAAAAAAGCGGCCGCTAAGGAGGATA-TACATATGAAAAAAGCGAAGCTCGG	*Not*I
T7f	AAGGAAAAAAGCGGCCGCTAATACGACTCAC-TATAG	*Not*I

1.3 Screening, cloning, and expression of carbonyl reductases

The genomic DNA of *Bacillus subtilis* and *E. coli* K12 was isolated as described above. The primers designed based on the nucleotide sequences of the various carbonyl reductases (CRs) are listed in Table 2. The CR encoding genes *ycsN* (NP_388296.1), *iolS* (AL009126.3), and *yccK* (NC_000964) were from *Bacillus subtilis*, while *ydhF* (P76187.2) and *yajO* (P77735.2) were from *E. coli* K12. The cloning, expression, and analysis methods were the same as described for the *gdh* gene in section 1.2.

1.4 Enzyme activity assays

Dehydrogenase and carbonyl reductase activities were determined spectrophotometrically by monitoring the increase or decrease in the absorption of NADPH at 340 nm and 30 °C. The reaction mixture for GDH comprised 75 mmol/L Tris-HCl (pH 8.0), 2.0 mmol/L $NADP^+$, 0.1 mmol/L glucose, and an appropriate amount of enzyme in a final volume of 0.25 ml. The reaction mixture for CR was composed of 100 mmol/L

phosphate buffer (pH 6.0), 1.0 mmol/L OPBE, 5.0 mmol/L NADPH, and an appropriate amount of enzyme in a final volume of 0.25 ml. One unit of activity was defined as the amount of enzyme required for catalyzing the oxidation of 1 μmol of NADPH per minute (CR) or reduction of 1 μmol $NADP^+$ per minute (GDH). The protein concentration was measured by the Bradford method using bovine serum albumin as the standard.

1.5 Bioreduction of OPBE to (*R*)-HPBE

A 2.5 ml reaction mixture containing 100 mmol/L potassium phosphate buffer (pH 6.0), OPBE (20 mmol/L), NAD(P)H (10 mmol/L), and an appropriate amount of enzyme was incubated at 30 °C and 220 r/min for 12 h. Then the mixture was centrifuged and the supernatant was extracted three times with ethyl acetate, which was followed by drying over anhydrous $MgSO_4$ for further GC analysis.

1.6 Protein purification

The induced recombinant cells were harvested and suspended in buffer A (20 mmol/L sodium phosphate, pH 7.4, containing 500 mmol/L NaCl, and 5 mmol/L imidazole). The cells were disrupted by ultrasonication (285 W, pulse 1 s, pause 3 s) for 10 min. Cell debris was removed by centrifugation at 8000 g for 15 min at 4 °C, and the supernatant was sent to a HisTrap-FF crude chelating affinity column equilibrated with buffer A. The bound enzyme was eluted by a stepwise gradient of imidazole concentration, from buffer A containing 5 mmol/L imidazole to the same buffer containing 1000 mmol/L imidazole. The protein samples were analyzed by SDS-PAGE.

1.7 Effects of pH and temperature on purified enzyme

The optimum temperature was determined by incubation in the range of 10 to 70 °C. To determine the optimum pH, activities were assayed in 100 mmol/L buffers with various pH (4.0–10): sodium citrate buffer (pH 4.0–6.0), sodium phosphate buffer (pH 6.0–8.5), and glycine-NaOH buffer (pH 8.5–10). For thermal stability, aliquots of the purified enzyme were incubated at temperatures in the range of 10–60 °C in 100 mmol/L phosphate buffer (pH 6.0) for 1 h followed by cooling on ice before activity assay. The pH stability was determined after incubating the purified enzyme in the same buffers with different pH values (4.0–9.0) for 24 h at 4 °C. All activities were measured under the standard conditions described in section 1.4.

1.8 Coexpression of IolS and GDH

To achieve a high level expression of both IolS and GDH, three coexpression strategies were attempted in this study. The first method was to construct recombinant plasmid pET-G-SD-I (Fig. 1). The forward primer (SDf) and reverse primer (IolSr) were designed for PCR using pET20b-*iolS* as template. The resulting fragment, *iolS* gene with SD sequence at 5' end, was cloned into pET24a-*gdh* to give a two-enzyme coexpression plasmid pET-G-SD-I. A second method was to construct recombinant plasmid pET-G-T7-I (Fig. 2). The forward primer (T7f) and the reverse primer (IolSr) were designed for PCR using pET20b-*iolS* as the template. The resulting fragment, *iolS* gene with T7 promoter and rbs sequence at 5' end, was cloned into the pET24a-*gdh* to give a two-enzyme coexpression plasmid pET-G-T7-I. In the third method, both pET-20b-*iolS* and pET-24a-*gdh* were transformed into *E. coli* BL21(DE3) to result in a two-plasmid system. The strain was grown on a LB plate containing ampicillin and kanamycin.

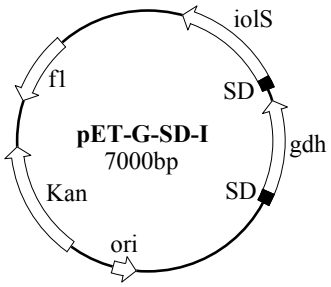

Fig. 1. Structure of coexpression plasmid pET-G-SD-I.

1.9 Optimization of expression conditions

E. coli BL21(DE3)/pET24a-*gdh* and *E. coli* BL21(DE3)/pET20b-*iolS* were induced at different temperatures (15, 20, 25, 30, 35, and 40 °C) and different concentrations of IPTG (final concentration of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/L).

1.10 Bioreduction of OPBE to (*R*)-HPBE catalyzed by recombinant *E. coli*

The reaction mixture (20 ml) containing 100 mmol/L potassium phosphate buffer (pH 6.0), NAD^+ (0.05 mmol/L), glucose (200 mmol/L), OPBE (50 mmol/L), and 2 g of wet cells was incubated at 30 °C and 220 r/min for 15 h. After the reaction, the supernatant was extracted three times with ethyl

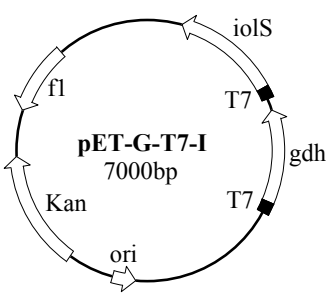

Fig. 2. Structure of coexpression plasmid pET-G-T7-I.

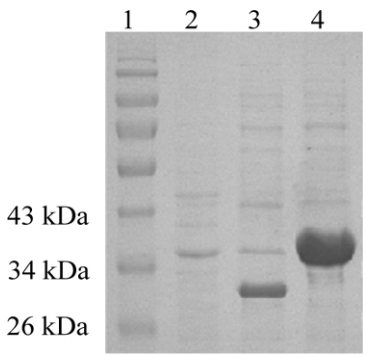

Fig. 3. SDS-PAGE analysis of recombinant expression of GDH and IolS. Lane 1: protein molecular weight marker; Lane 2: cell-free extract of *E. coli* BL21(DE3); Lane 3: cell-free extract of *E. coli* BL21(DE3)/pET24a-*gdh*; Lane 4: cell-free extract of *E. coli* BL21(DE3)/pET20b-*iolS*.

acetate and dried over anhydrous $MgSO_4$ for further GC analysis.

2 Results and discussion

2.1 Cloning and expression of GDH and optimization of inducing conditions

The sequence analysis indicated that the *gdh* (783 bp) encoded 260 amino acids. The calculated molecular mass of GDH was 28 kDa. The cloned *gdh* showed 98% similarity with the nucleotide sequence of *B. subtilis gdh* (GenBank No. M12276), and 100% similarity with its amino acid sequence. Figure 3 shows the SDS-PAGE of the cell-free extract of *E. coli* BL21(DE3)/pET24a-*gdh*. The recombinant GDH product was observed at 28 kDa, which was consistent with our prediction based on the gene sequence.

The effects of IPTG concentration and temperature on enzyme activity of GDH are shown in Fig. 4. The optimal activity was reached at the IPTG concentration of 0.8 mmol/L and 30 °C, and the enzyme activity of GDH was 9.5 U/mg.

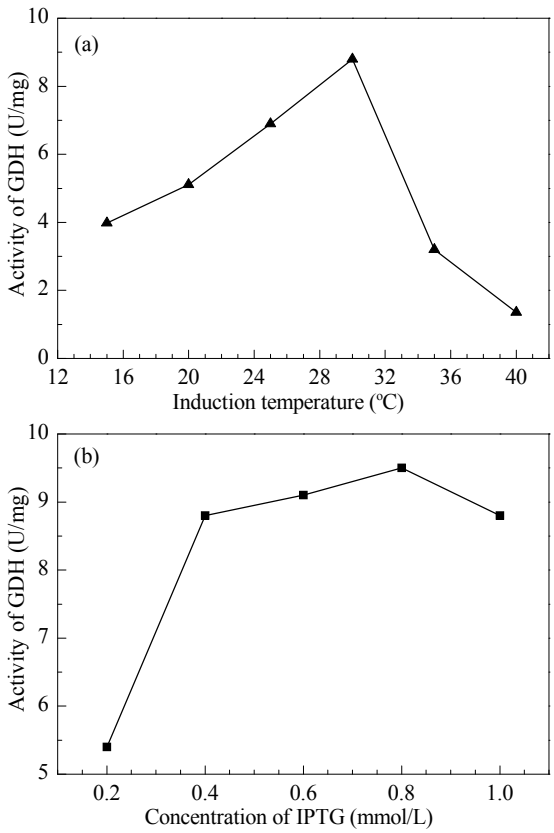

Fig. 4. Effects of inducing temperature (a) and IPTG concentration (b) on recombinant GDH activity.

2.2 Screening, cloning, and expression of CR genes and optimization of induction conditions

To obtain new reductases capable of reducing OPBE to (*R*)-HPBE with a high product *ee* and yield, five kinds of carbonyl reductases of different origins were cloned and their enzyme activities were investigated (Table 3). The IolS from *Bacillus subtilis* exhibited the highest enzyme activity and enantioselectivity. The sequence analysis indicated that the *iolS* (933 bp) encoded 310 amino acids, and the calculated molecular mass of IolS was 36 kDa. IolS belongs to the Aldo-keto reductases (AKRs) superfamily. The nucleotide sequences of *iolS* have been deposited in GenBank with the accession No. JQ782389. Figure 3 shows the SDS-PAGE of the cell-free extract of *E. coli* BL21(DE3)/pET20b-*iolS*. The expressed recombinant IolS product was observed at about 36 kDa, which agreed with the prediction from the gene sequence.

The effects of IPTG concentration and temperature on the enzyme activity of IolS are shown in Fig. 5. The optimal activity was achieved at the IPTG concentration of 0.4 mmol/L and 25 °C, and the enzyme activities of GDH were 1.5 U/mg.

Table 3 Comparison of carbonyl reductases of different origins

Strain	Gene	Activity (U/mg)	Cofactor-dependency	Yield (%)	ee (%)
Bacillus Subtilis	*ycsN*	0.41	NADH	25.2%	92.2%
	iolS	1.45	NADPH	89.6%	99.5%
	yccK	0	—	ND	ND
E. coli K12	*ydhF*	0.012	NADH	ND	ND
	yajO	0.027	NADPH	ND	ND

ND means not detected.

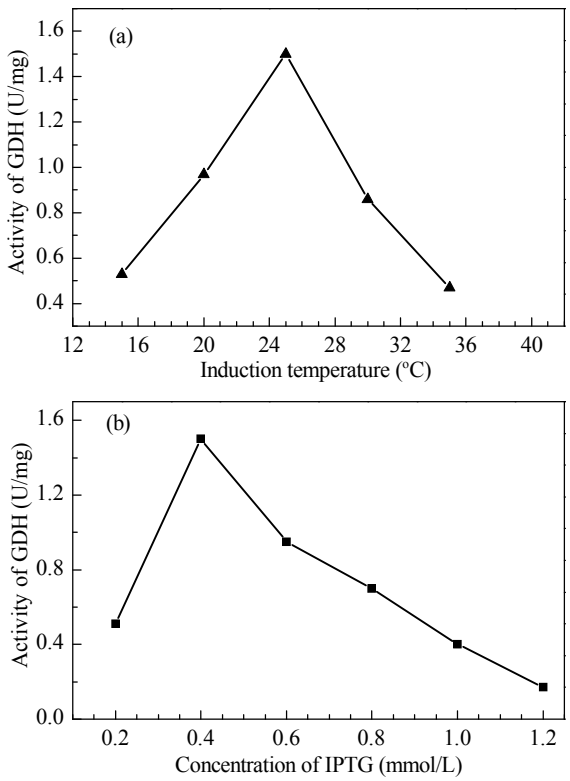

Fig. 5. Effects of inducing temperature (a) and IPTG concentration (b) on recombinant IolS activity.

2.3 Protein purification

The *N*-terminal His-tagged recombinant enzyme IolS was purified to electrophoretic homogeneity with a HisTrap-FF crude chelating affinity column. The specific activity of purified IolS was 5.1 U/mg, which corresponded to a 3.4 fold increase in purity as compared with the crude extract. Figure 6 shows the SDS-PAGE of the crude extract and target fraction after purification.

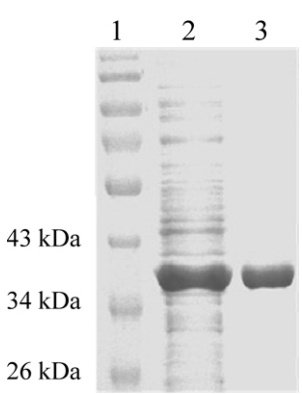

Fig. 6. SDS-PAGE analysis of purified IolS. Lane 1: protein molecular weight marker; Lane 2: cell-free extract of *E. coli* BL21(DE3)/pET20b-*iolS*; Lane 3: purified enzyme.

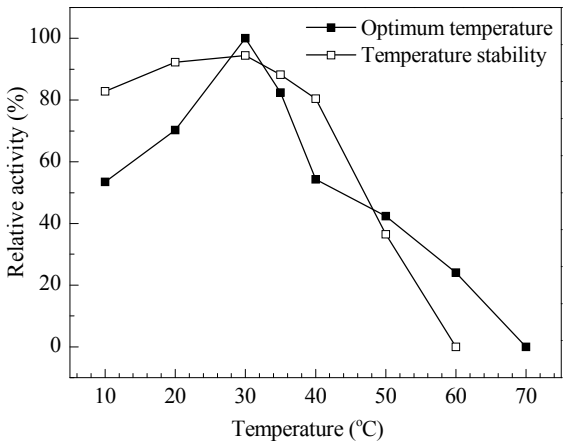

Fig. 7. Optimum temperature and thermostability of purified IolS. Enzyme assay was performed using standard assay procedure: 1 mmol/L OPBE and 5 mmol/L NADPH at various temperature conditions. Thermostability of IolS was measured after pre-incubation at various temperatures for 1 h. Activity at pH 6.0 and 4 °C without pre-incubation was taken as 100%.

2.4 Enzyme activity characterization of carbonyl reductase

The carbonyl reductase showed maximum activity at 30 °C. The activity dropped rapidly when the temperature was increased. The enzyme was extremely weak at temperatures higher than 40 °C, which is demonstrated in Fig. 7. The effect of pH on the enzyme activity was also studied. The results are shown in Fig. 8. The maximum enzyme activity was observed at pH 6.0 (sodium phosphate buffer) and > 75% of the activity

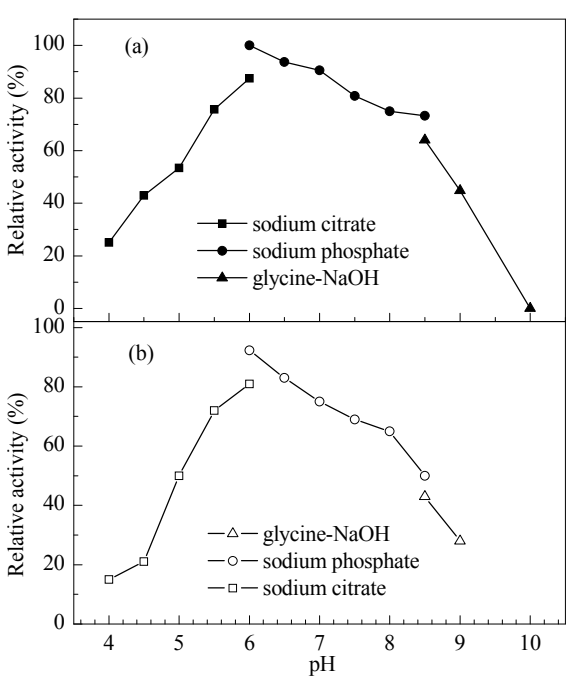

Fig. 8. Optimum pH (a) and pH stability (b) of purified IolS.

Table 4 Effect of different coexpression strategies on GDH and IolS activity

Coexpression	GDH activity (U/mg)	IolS activity (U/mg)
pET-G-T7-I	1.5	1.5
pET-G-SD-I	4.8	0.5
Two plasmids	3.4	0.9

Induction conditions: recombinant *E. coli* cells were induced in LB medium with 0.8 mmol/L IPTG for 4 h at 25 °C and 220 r/min.

was retained between pH 5.5 and 7.0 after 24 h.

2.5 Coexpression of IolS and GDH

The IolS and GDH activities of *E. coli* BL21(DE3) harboring different plasmids are shown in Table 4. The highest GDH activity was observed with *E. coli* BL21(DE3) harboring pET-G-SD-I, where the GDH activity was 4.8 U/mg. However, a much lower IolS activity (0.5 U/mg) was observed. *E. coli* BL21(DE3) with two separate plasmids (pET20b-*iolS* and pET24a-gdh) exhibited IolS and GDH activities of 0.9 and 3.4 U/mg, respectively. Of the three strategies used, the highest IolS activity (1.5 U/mg) was attained with *E. coli* BL21(DE3) carrying pET-G-T7-I, where GDH showed a comparable activity (1.5 U/mg) to IolS. The induction condition for *E. coli* BL21(DE3)/pET-G-T7-I was optimized, and the results suggested that the IPTG concentration of 0.8 mmol/L and induction temperature of 25 °C gave a high level expression of both GDH and IolS (data not shown). Because IolS is the key enzyme for the reduction of OPBE, and a similar GDH activity was achieved, *E. coli* BL21(DE3)/pET-G-T7-I was selected as the biocatalyst for the subsequent experiments. From the perspective of transcription and translation, with the use of an independent T7 promoter and a rbs sequence located upstream of *iolS*, pET-G-T7-I can effectively transcript two mRNA: one of which contained the coding information of GDH and IolS and the other contained the coding information of IolS. Therefore, *iolS* can be transcribed and translated twice, to lead to an enhanced enzyme expression level. This was also consistent with our experimental results. The expressions of GDH and IolS are shown in Fig. 9, which indicated that IolS and GDH were highly expressed in the recombinant *E. coli* strain carring pET-G-T7-I.

2.6 Bioreduction of OPBE to (*R*)-HPBE

The time course of the bioreduction of OPBE to (*R*)-HPBE in an aqueous system catalyzed by *E. coli* BL21(DE3)/pET-G-T7-I is shown in Fig. 10. After 13 h of reaction, over 99% yield and 99.5% *ee* were achieved at 10 g/L of OPBE.

2.7 Discussion

A carbonyl reductase (IolS) was identified in *Bacillus subtilis*, which exhibited high enantioselectivity (> 99.5%) in the reduction of OPBE to (*R*)-HPBE. The recombinant IolS was purified to homogeneity and characterized. The coexpression of *iolS* and *gdh* was adopted to address the cofactor regeneration issue in the oxidoreductase catalyzed reaction. Two coexpression methods were pursued: the tandem expression of the *gdh*/*cr* in one expression plasmid, in which the SD sequence, T7 promoter, and other special sequences were inserted between two genes [17,18] and a second method where two separate recombinant plasmids were transformed into *E. coli* cells to construct a two-plasmid system [19,20]. The results suggested the insertion of the T7 promoter and rbs sequence was beneficial for the expression of *iolS*. Only 0.05 mmol/L $NADP^+$ was necessary for the bioreduction of OPBE to (*R*)-HPBE catalyzed by *E. coli* BL21(DE3)/pET-G-T7-I in aqueous system, and this resulted in 99% yield and 99.5% *ee* at 10 g/L OPBE. To further enhance the reaction efficiency and substrate concentration, studies on the optimization of the reaction process including substrate feed batch, aqueous/organic biphasic system, and pH regulation were carried out.

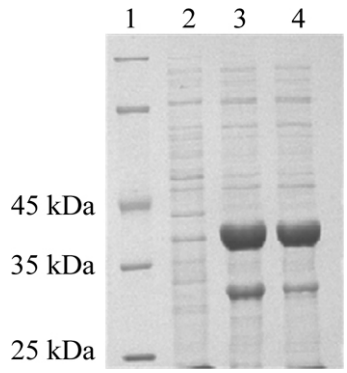

Fig. 9. SDS-PAGE analysis of recombinant protein expression of *E. coli* BL21(DE3)/pET-G-T7-I. Lane 1: protein molecular weight marker; Lane 2: soluble proteins of *E. coli* BL21(DE3); Lane 3: whole cell extract of *E. coli* BL21(DE3)/pET-G-T7-I; Lane 4: soluble proteins of *E. coli* BL21(DE3)/pET-G-T7-I.

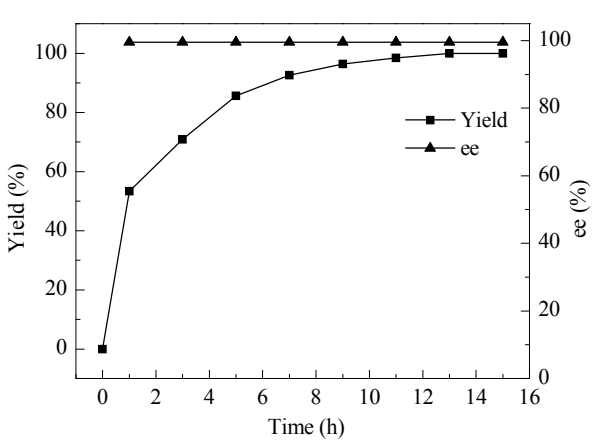

Fig. 10. Time course of the asymmetric reduction of OPBE by recombinant *E. coli* BL21(DE3)/pET-G-T7-I.

3 Conclusions

In summary, a carbonyl reductase gene (*iolS*) and a glucose dehydrogenase (GDH) gene from *Bacillus subtilis* were cloned. A recombinant *E. coli* capable of the highly stereoselective reduction of OPBE to (*R*)-HPBE have been constructed and internal coenzyme regeneration to give a feasible approach for the industrial production of (*R*)-HPBE by biocatalysis.

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