



# Asymmetric synthesis of lipitor chiral intermediate using a robust carbonyl reductase at high substrate to catalyst ratio



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## ARTICLE INFO

### Article history:

Received 24 October 2015

Accepted 1 November 2015

Available online 7 November 2015

### Keywords:

Asymmetric reduction

Carbonyl reductase

Ethyl (S)-4-chloro-3-hydroxybutanoate

Substrate to catalyst ratio

## ABSTRACT

An NADPH-dependent carbonyl reductase (RpCR) from *Rhodococcus pyridinivorans* was discovered by genome mining for the asymmetric reduction of ethyl 4-chloro-3-oxo-butanoate (COBE). RpCR has been soluble expressed in *Escherichia coli* BL21(DE3). The highest activity is determined at pH 5.0 and 50 °C toward COBE. The apparent  $K_m$  and  $k_{cat}/K_m$  are 0.39 mM and 1747 s<sup>-1</sup> mM<sup>-1</sup>, endowing RpCR with high catalytic efficiency in reduction of COBE. Employing merely 0.1 g recombinant RpCR-GDH in a toluene-aqueous biphasic system, as much as 7.0 g COBE could be asymmetrically reduced into ethyl (S)-4-chloro-3-hydroxybutanoate [(S)-CHBE] (>99% ee) without addition of external cofactor, achieving molar isolation yield of 91%, substrate to biocatalyst ratio of 70 and space-time yield of 1480 g L<sup>-1</sup> d<sup>-1</sup>. Our results indicate the robust RpCR could be potentially applied in the preparation of optically pure (S)-CHBE.

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## 1. Introduction

Asymmetric reduction of ketones is one of the most important and practical approaches for the production of chiral secondary alcohols, which is essential for the synthesis of industrially important chemicals such as pharmaceuticals, agrochemicals and natural products [1–3]. As an alternative to chemical methodologies, bioreductive preparation of (R) or (S)-enantiomers of alcohols using isolated enzymes or whole-cell systems has been extensively investigated due to its high enantio-, regio- and chemoselectivities, mild conditions, reproducibility and easy operation [4,5]. However, two major challenges for the scale-up application of bioreduction systems are the lack of efficient biocatalysts and the necessity of expensive cofactors such as NAD(P)H/NAD(P)<sup>+</sup> [6,7]. The employment of robust biocatalysts with high cofactor utilization efficiency could reduce or even avoid the addition of external cofactors. Consequently, exploration of robust biocatalysts is of special interest to solve above issues [8,9].

Ethyl (S)-3-hydroxyl-4-chlorobutanoate [(S)-CHBE] is a versatile and important chiral intermediate for the production of chiral drugs, including the cholesterol lowering 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors (namely statins) [10,11], which rank the class I best-selling drugs due to their excellent therapeutic effect and low side effect. The asymmetric

reduction of ethyl 3-oxo-4-chlorobutanoate (COBE) into (S)-CHBE is the most promising approach. In recent years, various microorganisms have been identified for the efficient synthesis of (S)-CHBE and well-reviewed by Ye et al. [11]. However the application of wild-type strains is often hindered by their complicated dehydrogenases/reductases systems with variable stereospecificity and low expression level of key reductases [11,12]. An effective solution is discovering novel reductases by genome mining and their heterogeneous overexpression [11,13]. A number of enantioselective carbonyl reductases have been identified with S-selectivity in the asymmetric reduction of COBE, including S1 from *Candida magnolia* [14], ARII from *Sporobolomyces salmonicolor* [15], CPE from *Candida parapsilosis* [16], PsCRI and PsCRII from *Pichia stipitis* [17,18], ScCR from *Streptomyces coelicolor* [19], SOU1 from *Candida albicans* [20] and DhCR from *Debaryomyces hansenii* [21]. The highest catalytic efficiency ( $V_{max} = 349 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ) was reported for ARII, however at a high substrate concentration ( $K_m = 1.49$ ), and the ee was relatively lower for application [15]. A carbonyl reductase CPE identified by Wang et al. displayed 99% ee and a low  $K_m$  (0.19 mM), while its  $V_{max}$  ( $200 \mu\text{mol min}^{-1} \text{mg}^{-1}$ ) was not as high as ARII [19].

Besides high stability and enantioselectivity, the lower  $K_m$  and higher  $V_{max}$  (or higher  $k_{cat}/K_m$ ) is one of the crucial parameters of biocatalysts in large-scale application. Biocatalysts with high  $k_{cat}/K_m$  value could often reach the maximum velocity and maintain the activity for a longer time even at a low substrate concentration [22]. In current study, a novel carbonyl reductase from *Rhodococcus pyridinivorans* (RpCR) was identified by genome

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mining, and soluble expressed in *Escherichia coli* BL21(DE3). RpCR exhibited a high enantioselectivity, lower  $K_m$  than CPE and higher catalytic efficiency in the asymmetric reduction of COBE. Furthermore, the synthesis of (S)-CHBE employing RpCR at high substrate loading was achieved in an organic solvent-aqueous biphasic system to evaluate its potential for industrial applications.

## 2. Experimental procedures

### 2.1. Cloning and expression of RpCR coding gene in *E. coli* BL21(DE3)

Genomic DNA was extracted from *R. pyridinivorans* using a TIANamp Bacteria DNA Kit from Tiangen (Shanghai). Primers with HindIII and XhoI restriction sites were designed according to the RpCR coding gene (*rPCR*) sequence (GenBank accession No.: EHk80525.1). The PCR product of *rPCR* was double digested with HindIII and XhoI and then inserted into the expression vector pET28a. The resultant plasmid, pET28-*rPCR*, was transformed into *E. coli* BL21(DE3). The cells were cultivated at 37 °C in LB medium supplemented with 50 µg/mL kanamycin. When OD<sub>600</sub> of the culture reached 0.6, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM, and the culture was further cultivated at 25 °C for 12 h.

### 2.2. Purification of RpCR

Cells were harvested by centrifugation (8000 × g, 10 min), washed twice with saline and resuspended in buffer A (20 mM PBS, 500 mM NaCl, 10 mM imidazole, pH 7.4), followed by disruption by ultrasonication (400 W, work 3 s and stop 2 s for 15 min). The cell lysate was centrifuged (10,000 × g, 30 min) at 4 °C. Afterwards, the supernatant was loaded onto a HiTrap column (1 mL, GE Corp.) pre-equilibrated with buffer A, and the proteins were eluted with an increasing imidazole gradient from 10 to 500 mM with buffer B (20 mM PBS, 500 mM NaCl, 500 mM imidazole, pH 7.4) at a flow rate of 1 mL/min. Then the collected eluents were desalted and concentrated at 4 °C. The purity of fractions was determined by SDS-PAGE. The purified RpCR was stored at –80 °C with 20% glycerol for further use.

### 2.3. Enzyme activity assay protocol

Standard enzyme activity assay was performed spectrophotometrically by monitoring the changes in absorbance of NADPH at 340 nm and 30 °C. The reaction mixture for RpCR consisted of 2 mM COBE, 0.5 mM NADPH in 190 µL PBS buffer (pH 7.0, 100 mM) and 10 µL enzyme solution with appropriated concentration. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol NADPH per minute under standard condition.

### 2.4. Characterization of purified RpCR

#### 2.4.1. Optimum pH and temperature

The optimum temperature of RpCR was determined under above mentioned standard condition at various temperatures (25–70 °C). The pH-profile of purified RpCR was determined in the following buffers (final concentration 100 mM): sodium citrate (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0), Glycine-NaOH (pH 8.0–10.0). All the activities were assayed in triplicates.

#### 2.4.2. Effect of metal ions and additives on RpCR activity

The effect of various metal ions and additives (including Mn<sup>2+</sup>, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Ca<sup>2+</sup>, Co<sup>2+</sup>, Ag<sup>+</sup>, Fe<sup>3+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Al<sup>3+</sup>, EDTA, DTT, imidazole, β-mercaptoethanol, SDS, Tris, Triton-X100 and Tween 20)

on RpCR activity was examined by adding each compound (final concentration of 1 mM) in the reaction mixture for 60 min at 30 °C, and the residual enzyme activity was measured under the standard condition. Control was performed in the absence of any test compound. All the activities were assayed in triplicates.

#### 2.4.3. Effect of organic solvents on RpCR activity

The influence of organic solvents on RpCR activity was performed by adding 50% v/v organic solvents (*n*-pentane, cyclohexane, *n*-hexane, *n*-heptane, *n*-decane, *n*-nonane, isooctane, toluene, ethyl lactate, ethyl acetate and glycerol), or 10% m/v PEG derivatives (PEG400, 600, 1000, 2000, and 4000), or 20% ionic liquid v/v (glycerol, choline chloride (ChCl), ChCl/glycerol, ChCl/urea, ChCl/formic acid, ChCl/acetic acid, ChCl/oxalic acid, ChCl/malonic acid and ChCl/citric acid) in the enzyme solutions (0.1 mg mL<sup>-1</sup>), and incubated at 30 °C for 12 h. Then the mixtures were centrifuged at 8000 × g and 4 °C for 5 min. The activity of the enzyme aqueous phase was determined using standard activity protocol. Protein concentration was measured using Bradford method with BSA as standard protein.

#### 2.4.4. Kinetic analysis

The kinetic parameters of the purified RpCR were determined by determining the activity at different COBE concentrations (0.25–5.0 mM) and a fixed NADPH concentration of 1.0 mM. Similarly, the apparent  $K_m$  value for NADPH was determined at different NADPH concentrations (0.1–1 mM) and a fixed COBE concentration of 2 mM. All the activity was assayed in triplicates. The apparent  $K_m$  and  $V_{max}$  values of the purified RpCR were calculated according to Lineweaver–Burk plot [23].

#### 2.4.5. Substrate specificity

Substrate profile of purified RpCR was determined using standard activity assay method, except different prochiral ketone substrates including diketone, aromatic ketone, α-ketoester and β-ketoester (final concentration, 2 mM) were used. All the activities were assayed in triplicates.

#### 2.4.6. Enantioselectivity

Enantioselectivity of RpCR in the asymmetric reduction of COBE was performed using chiral GC analysis as previously described [21].

### 2.5. Asymmetric reduction of COBE to (S)-CHBE employing RpCR

#### 2.5.1. Construction of RpCR and GDH coexpression strain

Glucose dehydrogenase coding gene (*gdh*) was cloned from *Bacillus megaterium* and inserted into pACYDuet for expression as previously described [21]. The coexpression of RpCR and glucose dehydrogenase (GDH) in *E. coli* was developed simply by transformation of pET28-*rPCR* and pACYDuet-*gdh* into *E. coli* BL21(DE3), and screening for double resistance against kanamycin and chloramphenicol. Recombinant strain *E. coli* BL21(DE3) harboring pET28-*rPCR* and pACYDuet-*gdh* (RpCR-GDH) was cultivated in LB medium supplemented with 50 µg/mL kanamycin and chloramphenicol at 37 °C and 180 rpm. When OD<sub>600</sub> reached 0.6, 0.5 mM IPTG was added and further cultivated at 25 °C and 180 rpm for 12 h. The cells were harvested by centrifugation followed by lyophilization. The dry cells of recombinant *E. coli* were stored at 4 °C and used as biocatalyst.

#### 2.5.2. Optimization of (S)-CHBE production by RpCR

The effect of reaction pH on the performance of RpCR was investigated with 1.65 g COBE, 3.0 g glucose and 0.05 g dry cells (RpCR-GDH), in 5 mL PBS buffer (pH 6.0, 6.5, 7.0 and 7.5, 100 mM) and 5 mL toluene at 30 °C and 180 rpm for 6 h. Then the reaction

**Table 1**  
The secondary structure element motifs in classic SDR and RpCR.

Secondary structure element	Conserved motifs of classical SDR	Function	Motifs of RpCR	Position in RpCR
$\beta$ 1- $\alpha$ 1	<u>TGxxxGhG</u>	Coenzyme binding region	<u>TGASSGLG</u>	17–24
$\beta$ 3- $\alpha$ 3	<u>Dhx[cp]</u>	Adenine ring binding of coenzyme	<u>DIAD</u>	68–71
$\beta$ 4	<u>CxhDhhhNNAGh</u>	Structural role in stabilizing central $\beta$ -sheet	<u>GRVDILINNAGI</u>	87–98
$\alpha$ 4	<u>hNhxC</u>	Part of active site	<u>INLNG</u>	118–122
$\beta$ 5	<u>CxhhxhSSh</u>	Part of active site	<u>GRVMQPGSSI</u>	131–140
$\alpha$ 5	<u>Yx[A/S][S/T]K</u>	Part of active site	<u>YSASK</u>	159–163
$\beta$ 6	<u>h[K/R]h[N/S]xhxPGxxxT</u>	Structural role, reaction direction	<u>IRVNAIAPGFFRT</u>	183–195

Note: in the motifs: 'h', a hydrophobic residue; 'c', a charged residue; 'p', a polar residue; 'x', any residue. Conserved amino acids are underlined. Alternative ones are given within brackets.

was stopped and extracted with equal volume of ethyl acetate and the conversion and *ee* were analyzed by chiral GC analysis.

### 2.5.3. Bioreductive preparation of (*S*)-CHBE with substrate feeding

A 10 mL reaction was performed by adding 0.1 g dry cells and 5 mL PBS buffer (pH 7.0, 0.1 M), 1.8 g glucose (9.2 mmol) in 5 mL PBS buffer (0.1 M, pH 7.0) and pre-incubated at 30 °C for 10 min. Then the reaction was started by addition of 1.0 g COBE (6.1 mmol) dissolved in 5 mL toluene into the reaction mixture, and then conducted at 30 °C and 180 rpm. The reaction pH was maintained at pH 7.0 by titration with 0.5 M K<sub>2</sub>CO<sub>3</sub>. About 20  $\mu$ L sample was taken intermittently, diluted with 480  $\mu$ L PBS buffer (0.1 M, pH 7.0), and then extracted with 500  $\mu$ L ethyl acetate supplemented with 10 mM dodecane as internal standard. When the reaction was completed, 1.0 g COBE (6.1 mmol) and 1.2 g glucose were fed each time for six times. Then the mixture was extracted with equal volume of ethyl acetate and centrifuged (8000  $\times$  g, 10 min). The organic phase was combined and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Afterwards, the extract was evaporated and further dried in drying cabinet for 48 h.

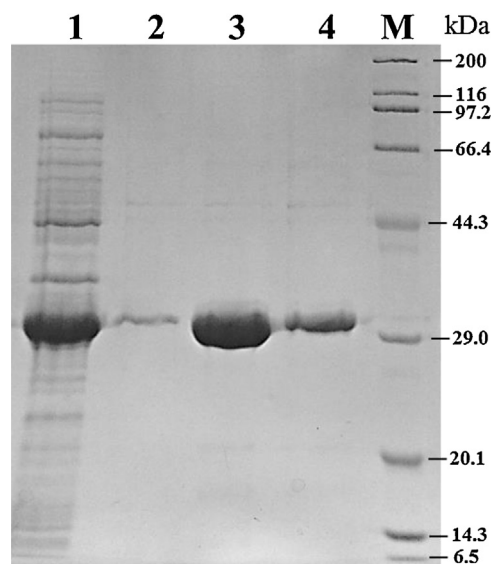
## 3. Results and discussion

### 3.1. Screening of recombinant reductases

Genome mining strategy was adopted to explore novel enzymes capable of reducing COBE. Twenty oxidoreductases sharing 40–60% amino acid sequence identities with known COBE reductases were selected from NCBI databases. All the potential COBE reductases were heterogeneously expressed in *E. coli*. The carbonyl reductase (GenBank accession No.: EHK80525.1) from *R. pyridinivorans* displayed the highest activity and enantioselectivity toward COBE, which was designated as RpCR and chosen for further studies (Table S1). As shown in Fig. 1, majority of RpCR was expressed in soluble form.

### 3.2. Multiple sequence alignment and sequence analysis

BLAST analysis in protein database reveals that RpCR shares high identity (over 55%) with several hypothetical proteins. The highest identity of 43% is found with FabG from *Bacillus subtilis*, which is reported to be a robust reductase in the preparation of ethyl (*S*)-2-hydroxy-4-phenylbutanoate [(*S*)-HPBE] [24]. Key motif analysis reveals that RpCR is a potential member of short-chain dehydrogenases/reductases (SDR). Sequence-search against Hidden Markov Model library of SDR database (<http://www.sdr-enzymes.org/>) returns that RpCR belongs to cD2 subfamily of SDR [25]. Moreover, the secondary structure elements of RpCR was predicted by the online program psipred (<http://bioinf.cs.ucl.ac.uk/psipred>). Conserved patterns of RpCR were identified with classic SDR as shown in Table 1. Compared with the Rossmann fold for NADPH binding of TGxxxGhG in classic SDRs [26], a highly conserved TGASSGLG was found in RpCR. In all the homogenous



**Fig. 1.** SDS-PAGE analysis of the purified RpCR. Lane 1, supernatant; Lane 2, precipitant; Lanes 3 and 4, purified RpCR with different dilution folds; Lane M, protein molecular mass markers.

SDR proteins, there is a conserved aspartic acid residue in the loop region between  $\beta$ 3 and  $\alpha$ 3, which is essential for the stabilization of adenine ring of cofactor. The conserved NNAG motif of RpCR is vital for the stabilization of central  $\beta$ -sheets [27]. The active sites in  $\alpha$ 4- $\beta$ 5- $\alpha$ 5 are also conserved in RpCR. Furthermore, a proline residue in the  $\beta$ 6, involving in structural and reaction direction, is also found in region 183–195 of RpCR. Consequently, RpCR is a member of classic SDR family.

### 3.3. Protein purification

RpCR with an N-terminal His-tag was purified to homogeneity by nickel affinity chromatography. The specific activity of the purified RpCR is 223 U mg<sup>-1</sup>, representing a purification fold of 4.6 compared with crude extract. The purified enzyme was confirmed by SDS-PAGE as a single band of about 29 kDa (Fig. 1), which is in accordance with the calculated molecular mass based on gene sequence. According to gel-exclusion chromatography, the molecular weight of RpCR is 118 kDa, suggesting RpCR is a homotetramer, with subunit of 29.5 kDa.

### 3.4. Temperature and pH profiles of purified RpCR

The optimum temperature for RpCR was determined by measuring activity at 25–70 °C as illustrate in Fig. 2(A). The RpCR exhibits the highest activity at 50 °C. Remarkably, RpCR retained 60% of activity at temperature as high as 65 °C. The pH profiles of RpCR was measured at various pH ranging from 3.0 to 10.0. According to Fig. 2(B), the optimum pH of RpCR is observed at pH 5.0. And the

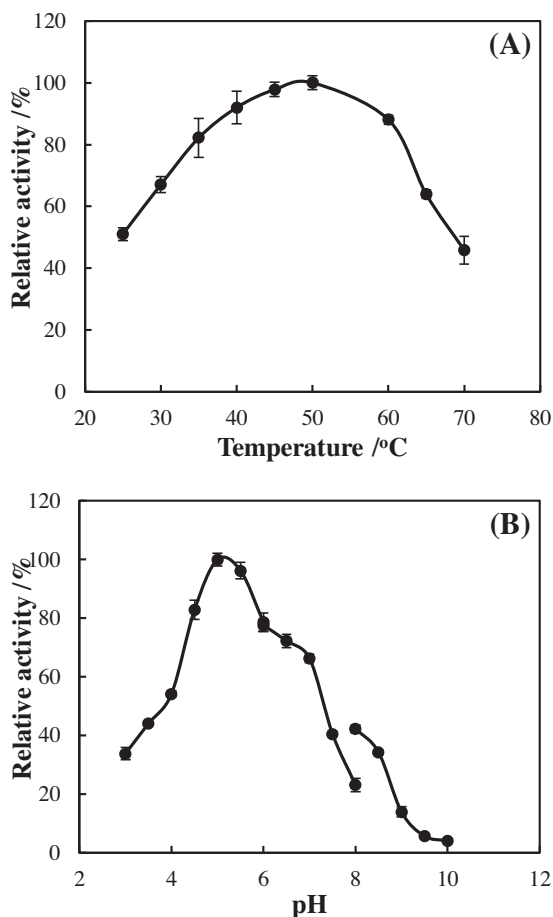
**Table 2**  
Effect of metal ions and additives on the activity of RpCR.

Metal ion	Relative activity/%	Additive	Relative activity/%
Control	100	EDTA	98.8 ± 1.7
Mn <sup>2+</sup>	88.7 ± 4.7	DTT	79.8 ± 1.7
Mg <sup>2+</sup>	81.3 ± 1.8	Imidazole	69.1 ± 0.8
Zn <sup>2+</sup>	63.0 ± 3.3	β-Mercaptoethanol	85.0 ± 3.4
Ca <sup>2+</sup>	±3.2	SDS	1.1 ± 0.8
Co <sup>2+</sup>	83.0 ± 0.8	Tris base	83.0 ± 0.8
Ag <sup>+</sup>	14.4 ± 0.5	Triton-X100	91.1 ± 4.6
Fe <sup>3+</sup>	17.1 ± 1.6	Tween 20	106.7 ± 1.9
Ni <sup>2+</sup>	74.1 ± 1.4		
Cu <sup>2+</sup>	96.9 ± 0.6		
Al <sup>3+</sup>	28.2 ± 1.5		

activity of purified RpCR decreases sharply at pHs higher or lower than 5.0.

### 3.5. Effect of various metal ions and additives on RpCR

Various metal ions were evaluated as shown in Table 2. No metal ions is found with ability in activating RpCR. The existence of Ag<sup>+</sup>, Fe<sup>3+</sup>, and Al<sup>3+</sup> show inhibitory effect on the activity of RpCR. The effect of some additives was also examined. SDS is extremely toxic to RpCR. Addition of EDTA has no influence on the activity, indicating RpCR is not a metal ion dependent enzyme, which is similar to most of other SDR members.



**Fig. 2.** Effect of temperature and pH on the activity of purified RpCR. (A) Activity-temperature profile was estimated at various temperatures (25–70 °C) in PBS buffer (pH 7.0, 100 mM). (B) Activity-pH profile was determined using standard protocol in the following 100 mM buffers: (i) citrate (pH 3.0–6.0), (ii) PBS buffer (pH 6.0–8.0) and (iii) Gly-NaOH (pH 8.0–10.0). Relative activity was expressed as a percentage of the maximum activity under experimental conditions.

**Table 3**  
Effect of organic solvents, ionic liquids and PEG on the activity of RpCR.

Organic solvent	Residual activity/%	Organic solvent	Residual activity/%
Control	73.2 ± 2.9	PEG400	10.0 ± 0.9
<i>n</i> -Pentane	102 ± 3.2	PEG600	5.62 ± 0.52
Cyclohexane	93.8 ± 8.8	PEG1000	15.1 ± 0.3
<i>n</i> -Hexane	101 ± 4	PEG2000	4.02 ± 0.06
<i>n</i> -Heptane	103 ± 3	PEG4000	0.87 ± 0.15
<i>n</i> -Decane	85.9 ± 0.5	ChCl/glycerol	135 ± 1
<i>n</i> -Nonane	103 ± 4	ChCl/urea	85.9 ± 2.9
<i>iso</i> -Octane	91.1 ± 7	ChCl/formic acid	2.79 ± 0.11
Toluene	98.3 ± 3.6	ChCl/acetic acid	1.50 ± 0.11
Ethyl lactate	0.76 ± 0.53	ChCl/oxalic acid	3.87 ± 0.46
Ethyl acetate	73.2 ± 8.8	ChCl/malonic acid	1.80 ± 0.20
Glycerol	122 ± 3	ChCl/citric acid	1.45 ± 0.06
ChCl	65.6 ± 5.4		

### 3.6. Effect of organic solvents and ionic liquids on RpCR

Since COBE is unstable in aqueous system and could be toxic to reductases, employing organic solvents or ionic liquids in the reaction system could eliminate undesired side reactions [19]. Ten organic solvents with different Log *P* values were examined. As shown in Table 3, RpCR retains higher residual activity in aliphatic alkane and toluene after incubation for 12 h. Although pentane, hexane, heptane and nonane are more biocompatible with RpCR, their partition coefficients for COBE are lower. Among all the organic solvents tested, toluene is regarded as the preferred one as other reports [19].

Some PEG derivatives and deep eutectic solvents were also attempted to reduce the spontaneous hydrolysis of COBE. However, after incubation for 12 h, the residual activity of RpCR is decreased to less than 10% in most of the solvents. Although RpCR could retain higher activity in ChCl/glycerol (135%) and glycerol (122%), the low solubility of COBE is not advantageous for the bioreduction.













### 3.7. Substrate specificity

As shown in Table 4, RpCR could catalyze the reduction of all the tested diketones. Along the chain length (1–3) increase, the specific activity also increases. The location of the carbonyl group in the substrates is also important for the activity of RpCR. The activity toward 2,3-hexanedione (4) is higher than that of 3,4-hexanedione (5). With regard to the four tested α-ketoesters, the existence of aromatic side-chain seems to be beneficial to the activity, except for ethyl phenyl pyruvate (7). The specific activity of RpCR toward ethyl 4-phenyl-2-oxobutanoate (8) is 92.1 U mg<sup>-1</sup>. While the highest activity is found with COBE (117 U mg<sup>-1</sup>), a β-ketoester. No activity is detected toward aromatic ketones. A possible explanation is that the native substrates of 3-ketoacyl-(acyl carrier protein) reductases (FabG) contain ester bonds, and therefore the activity is also dependent on the recognition of ester bonds or aliphatic diketones [28]. Enantioselectivity analysis reveals that RpCR follows anti-Prelog's rule in the asymmetric reduction of prochiral ketones and could reduce COBE into (*S*)-CHBE.

### 3.8. Kinetic properties

The purified RpCR displays no activity with NADH and full activity with NADPH, suggesting that RpCR is NADPH-dependent. The kinetic parameters of purified RpCR were also determined (Table 5). The *K*<sub>m</sub> and *V*<sub>max</sub> of RpCR toward COBE are 0.39 ± 0.03 mM and 335 ± 6 μmol min<sup>-1</sup> mg<sup>-1</sup>, while the *K*<sub>m</sub> and *V*<sub>max</sub> to NADPH were 0.17 ± 0.01 mM and 398 ± 7 μmol min<sup>-1</sup> mg<sup>-1</sup>. As summarized in Table 5, the *K*<sub>m</sub> and *V*<sub>max</sub> of RpCR are comparable with the *K*<sub>m</sub> of CPE and *V*<sub>max</sub> of PsCRI, respectively [16,17]. However, the *V*<sub>max</sub> of CPE (200 μmol min<sup>-1</sup> mg<sup>-1</sup>) and *K*<sub>m</sub> of PsCRI (4.9 mM) are not as

**Table 4**  
Substrate specificity of RpCR toward various prochiral ketones.

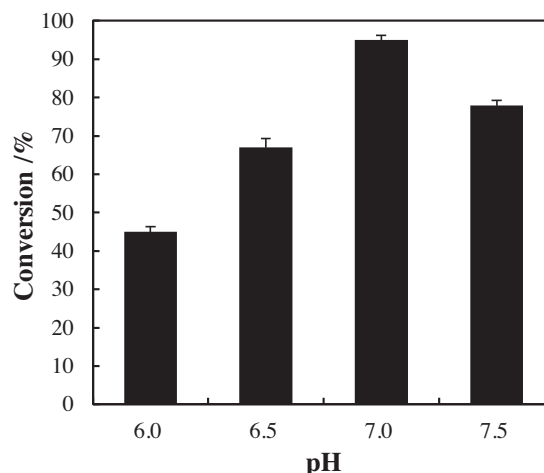
	Substrate	Specific activity/U mg <sup>-1</sup>
1		8.37 ± 0.22
2		11.0 ± 0.4
3		26.0 ± 0.5
4		9.81 ± 0.22
5		4.59 ± 0.14
6		5.69 ± 0.03
7		0.51 ± 0.01
8		92.1 ± 0.6
9		117 ± 1
10		0.99 ± 0.02
11		n. d. <sup>a</sup>
12		n. d.

<sup>a</sup> n. d.: no activity was detected.

beneficial as those of RpCR. Consequently, RpCR displays the highest  $k_{cat}/K_m$  value (1747 s<sup>-1</sup> mM<sup>-1</sup>), and is supposed to be a highly efficient enzyme in the asymmetric reduction of COBE.

### 3.9. Effect of pH on the asymmetric reduction COBE

The RpCR and GDH were co-expressed in recombinant *E. coli* BL21(DE3), with specific activities of 52 and 18 U mg<sup>-1</sup> crude extract at pH 5.0 and 8.0 respectively. However the recombinant RpCR and GDH display different pH-profiles. As shown in Fig. 2(B),



**Fig. 3.** Effect of pH on the asymmetric reduction of COBE employing recombinant *E. coli* BL21 coexpressing RpCR-GDH. Reactions were carried out with 10 mmol COBE, 15 mmol glucose, and 0.1 g dry cells in 10 mL PBS buffer (pH 6.0, 6.5, 7.0, 7.5; 100 mM) at 30 °C and 180 rpm for 12 h.

RpCR exhibits the highest activity at an acidic pH of around 5.0. However, glucose dehydrogenase (GDH) displays a basic pH optimum of 8.0–8.5 [29], and only 34% activity was remained at pH 5.0. Consequently, the pH of reaction system is critical for the asymmetric reduction catalyzed by *E. coli* coexpressing RpCR and GDH. To achieve the balance of RpCR and GDH activity in the coexpression strain, the pH of the reaction mixture was optimized over a pH range from 6.0 to 7.5 (Fig. 3). After 6 h of reaction, the highest conversion was observed at pH 7.0, when RpCR retained 70% activity and GDH also displayed high activity. Besides, pH does not affect the enantioselectivity of RpCR (data not shown). Therefore, the optimum pH of the reaction is 7.0.

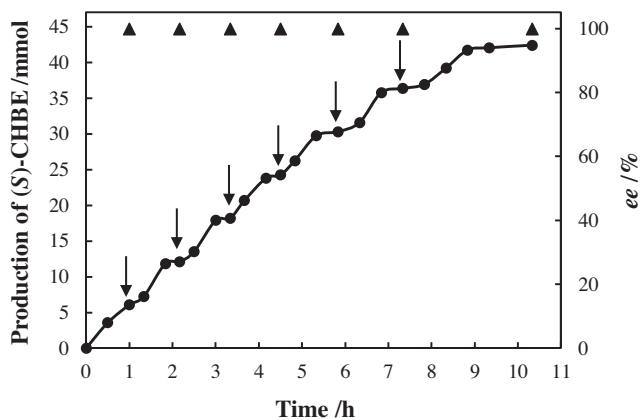
### 3.10. (S)-CHBE production with substrate feeding

To eliminate the toxicity and spontaneous hydrolysis of COBE and improve the productivity, a substrate feeding strategy in a 10-mL toluene/aqueous (1:1, v/v) biphasic system was employed (Fig. 4). Substrate COBE (total 7.0 g) was fed at 1.0 g for each batch. At the same time, the reaction system was maintained at pH 7.0 by titration with 0.5 M K<sub>2</sub>CO<sub>3</sub> to neutralize the gluconic acid generated in glucose oxidation. To avoid the extra addition of expensive NADP<sup>+</sup>, the amount of biocatalyst loading was optimized. And the results show that 10 g L<sup>-1</sup> DCW is sufficient for the complete conversion of 100 g L<sup>-1</sup> COBE within 3 h (data no shown).

Within 1 h, 1.0 g COBE (6.1 mmol) was entirely reduced into (S)-CHBE with >99% ee. Then 1.0 g COBE (6.1 mmol) and 1.2 g glucose

**Table 5**  
Comparison of various enzymes capable of reducing COBE into (S)-CHBE.

Enzyme	S1	ARI1	CPE	PsCRI	PsCRII	ScCR	SOU1	DhCR	RpCR
Accession No.	Q9C4B3	Q9UUN9	G8B541	A3GF07	A3GF05	Q9EX28	P87219	Q6BQ25	H0JY19
MW of subunit (kDa)	32	37	37.7	33	30.8	26	31	33.6	29.5
Protein family	SDR	AKR	SDR	SDR	SDR	SDR	SDR	SDR	SDR
Cofactor	NADPH	NADPH	NADPH	NADPH	NAD(P)H	NADH	NADPH	NADPH	NADPH
Optimum pH	5.5	5.5	5.5	6	6	6.5	6.2	6.5	5
Optimum Temp.	55	40	40	30	30	45	–	55	50
$K_m$ for COBE (mM)	4.6	1.49	0.19	4.9	3.3	0.49	–	1.3	0.39
$V_{max}$ for COBE (μmol min <sup>-1</sup> mg <sup>-1</sup> )	270	349	200	337	224	43.7	–	16.6	335
$k_{cat}/K_m$ (s <sup>-1</sup> mM <sup>-1</sup> )	110	380	1675	125	132	206	–	22.8	1747
Stereoselectivity (%)	100	>93	>99	>99	>99	>99	>99	>99	>99
Gene size (bp)	849	1032	1029	849	855	798	846	840	762
Molar yield (%)	89	–	91.1	90	91	93	82.6	92.5	91
Substrate/catalyst	24.7	–	–	7.7	5.1	36.8	30.9	16.5	70
References	[14,30]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	This work



**Fig. 4.** Time course of (S)-CHBE production catalyzed by recombinant *E. coli* co-expressing RpCR-GDH in a toluene/aqueous biphasic system with substrate feeding. (●) production of (S)-CHBE; (▲) ee. Reduction of COBE using 0.1 g dry cells was conducted at 30 °C in 5 mL PBS buffer (pH 7.0, 100 mM) and 5 mL toluene containing 1.0 g COBE and 1.8 g glucose; 1.0 g COBE and 1.2 g glucose were fed at the end of each batch as shown by arrows.

was added. The second batch was completed within 70 min. Afterwards, another 1.0 g COBE (6.1 mmol) and 1.2 g glucose was fed when COBE was converted thoroughly. At the 7th batch, at least 3 h was required for a complete reaction. All 7.0 g COBE was fully reduced into (S)-CHBE by merely 0.1 g RpCR-GDH dry cells within 11 h without addition of external NADP<sup>+</sup>. After extraction, about 6.29 g (S)-CHBE (>99% ee) was recovered, with a molar yield of about 91%. The substrate to catalyst ratio and the space-time yield were about 70 and 1480 g L<sup>-1</sup> d<sup>-1</sup>, which was attributed to the high catalytic efficiency of RpCR in the biphasic system with substrate-feeding. Table 4 shows a comparison of the asymmetric reduction of COBE to (S)-CHBE by various recombinant *E. coli* strains. It has been reported that S1 could accumulate 2580 mM (430 g L<sup>-1</sup>) (S)-CHBE in the organic phase with a molar isolation yield of 89% and substrate to catalyst ratio of 27.7 [30,31], however under the addition of 0.13 mM expensive NADP<sup>+</sup>. Although PsCRI and PsCRII display higher V<sub>max</sub> values, the substrate to biocatalyst ratios are 7.7 and 5.1 respectively, which are a result of the higher K<sub>m</sub> toward COBE [32,33]. The substrate to biocatalyst ratios of ScCR and SOU1 are higher than 30. Notably, the application potential of ScCR has been successfully scaled up at a 40-L volume loaded with 100 g L<sup>-1</sup> COBE under assistance of 0.3 mM NADP<sup>+</sup> [34]. The RpCR displays better performance in the preparation of (S)-CHBE with regard to the cofactor addition, k<sub>cat</sub>/K<sub>m</sub> value and substrate to biocatalyst ratio. As much as 7.0 g COBE was completely reduced into (S)-CHBE by merely 0.1 g recombinant whole cells without addition of external cofactor. The substrate to biocatalyst ratio of RpCR (70) in this study ranks the highest record due to its high k<sub>cat</sub>/K<sub>m</sub> value of 1747 compared with other COBE reductases (Table 5). To further decrease the addition of biocatalyst and improve the productivity, protein engineering of RpCR is undergoing in our laboratory for lowering the K<sub>m</sub> value toward NADP<sup>+</sup>.

#### 4. Conclusions

The COBE reductase RpCR from *R. pyridinivorans* was discovered by genome mining. RpCR displays high substrate affinity and catalytic efficiency toward COBE. RpCR is a promising biocatalyst for the synthesis of (S)-CHBE considering its high stereoselectivity, yield and final product concentration. The substrate to catalyst ratio and the space-time yield of RpCR in asymmetric reduction of COBE are about 70 and 1480 g L<sup>-1</sup> d<sup>-1</sup>, respectively. To our best knowledge, this is the first report on newly identified RpCR serving as an efficient reductase for asymmetric preparation of (S)-CHBE.

#### Acknowledgements

We are grateful to National Natural Science Foundation of China (21276112, 21506073), Natural Science Foundation of Jiangsu Province (BK20150003), the Fundamental Research Funds for the Central Universities (JUSRP51409B), the Program of Introducing Talents of Discipline to Universities (111-2-06), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions for the financial support of this research.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molcatb.2015.11.001>.

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