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

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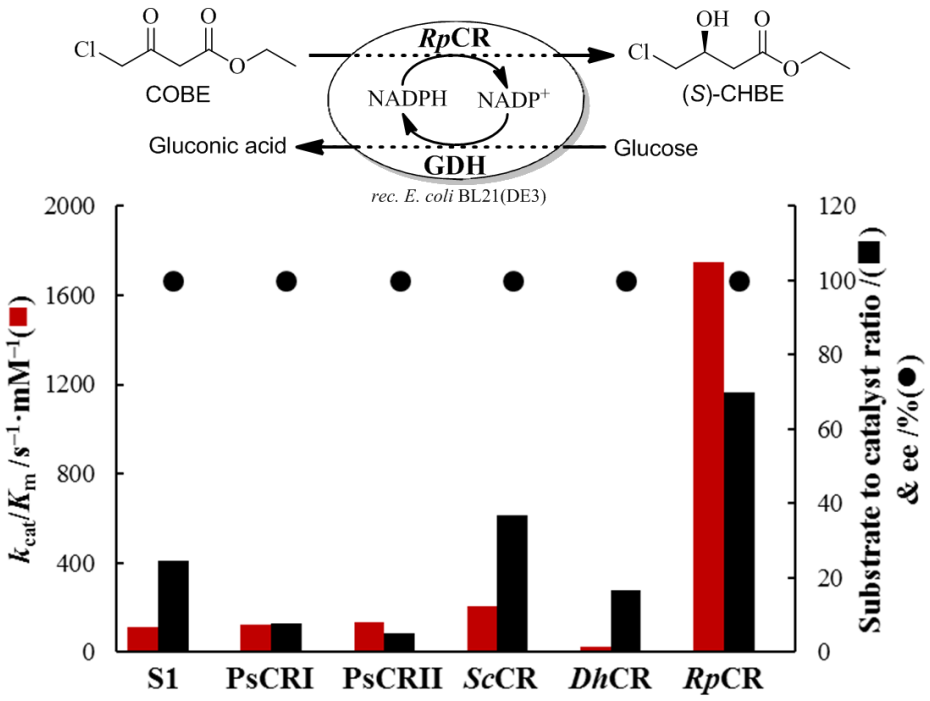
Graphical Abstract



Highlights

- *Rp*CR shares low sequence identity with known COBE carbonyl reductases.
- *Rp*CR exhibits a high k_{cat}/K_m of 1747 $s^{-1} \cdot mM^{-1}$.
- The substrate to catalyst ratio and space-time yield of *Rp*CR reached 70 and 1480 $g \cdot L^{-1} \cdot d^{-1}$.

Abstract

An NADPH-dependent carbonyl reductase (*Rp*CR) from *Rhodococcus pyridinivorans* was discovered by genome mining for the asymmetric reduction of ethyl 4-chloro-3-oxo-butanoate (COBE). *Rp*CR has been soluble expressed in *Escherichia coli* BL21(DE3). The highest activity is determined at pH 5.0 and 50 °C towards COBE. The apparent K_m and k_{cat}/K_m are 0.39 mM and 1747 $s^{-1}\cdot mM^{-1}$, endowed *Rp*CR with high catalytic efficiency in reduction of COBE. Employing merely 0.1 g recombinant *Rp*CR-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 $g\cdot L^{-1}\cdot d^{-1}$. Our results indicate the robust *Rp*CR could be potentially applied in the preparation of optically pure (*S*)-CHBE.

Keywords: Asymmetric reduction; Carbonyl reductase; Ethyl (*S*)-4-chloro-3-hydroxybutanoate; Substrate to catalyst ratio

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)$^+$ [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 interests 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-hydeoxy-3-methyl-glutaryl CoA (HMG-CoA) reductase inhibitors (namely statins) [10,11], which rank the class I best-selling drugs due to its excellent therapeutic effect and low side effect. The asymmetric reduction of ethyl 3-oxo-4-chlorobutanoate (COBE) into (*S*)-CHBE was 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 *Dh*CR from *Debaryomyces hansenii* [21]. The highest catalytic efficiency (V_{max} = 349 μmol·min^{-1}·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 and coworkers displayed 99% *ee* and a low K_m (0.19 mM), while its V_{max} (200 μmol·min^{-1}·mg^{-1}) was not as high as ARII [19].

Besides high stability and enantioslelectivity, 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* (*Rp*CR) was identified by genome mining, and soluble expressed in *Escherichia coli* BL21(DE3). *Rp*CR 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 *Rp*CR 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 Escherichia coli BL21(DE3)*

Genomic DNA was extracted from *Rhodococcus pyridinivorans* using a TIANamp Bacteria

DNA Kit from Tiangen (Shanghai). Primers with *Hin*dIII and *Xho*I restriction sites were designed according to the *Rp*CR coding gene (*rpcr*) sequence (GenBank accession No.: **EHK80525.1**). The PCR product of *rpcr* was double digested with *Hin*dIII and *Xho*I 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_{600} 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 (10000 × *g*, 30 min) at 4 °C. Afterwards, the supernatant was loaded onto a Histrap 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 *Rp*CR 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 *Rp*CR 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 *Rp*CR was determined under above mentioned standard condition at various temperatures (25–70 °C). The pH-profile of purified *Rp*CR 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^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Ag^{+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Al^{3+}, EDTA, DTT, imidazole, β-mercaptoethanol, SDS, Tris, Triton-X100 and Tween 20) on *Rp*CR 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 *Rp*CR activity was performed by added with 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 $mg \cdot mL^{-1}$), 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 *Rp*CR 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 *Rp*CR were calculated according to Lineweaver-Burk plot [23].

2.4.5 *Substrate specificity*

Substrate profile of purified *Rp*CR 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 *Rp*CR 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 *Rp*CR 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 *Rp*CR and glucose dehydrogenase (GDH) in *Escherichia 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* (*Rp*CR-GDH) was cultivated in LB medium supplemented with 50 μg/mL kanamycin and chloramphenicol at 37 °C and 180 rpm. When OD_{600} 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 *Rp*CR was investigated with 1.65 g COBE, 3.0 g glucose and 0.05 g dry cells (*Rp*CR-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 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 fed-up*

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_2CO_3. About 20 μL sample was taken intermittently , diluted with 480 μL PBS buffer (0.1 M, pH 7.0), and then extracted with 500 μL 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 × *g*, 10 min). The organic phase was combined and dried over anhydrous Na_2SO_4. 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 heterogeneous expressed in *E. coli*. The carbonyl reductase (GenBank accession No.: **EHK80525.1**) from *Rhodococcus pyridinivorans* displayed the highest activity and

enantioselectivity towards COBE, which was designated as *Rp*CR and chosen for further studies (**Table S1**). As shown in **Fig. 1**, majority of *Rp*CR was expressed in soluble form.

3.2 Multiple sequence alignment and sequence analysis

BLAST analysis in protein database revealed that *Rp*CR 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 *Rp*CR 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 *Rp*CR belongs to cD2 subfamily of SDR [25]. Moreover, the secondary structure elements of *Rp*CR was predicted by the online program psipred (http://bioinf.cs.ucl.uk/psipred). Conserved patterns of *Rp*CR 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 *Rp*CR. In all the homogenous SDR proteins, there is a conserved aspartic acid residue in the loop region between β3 and α3, which is essential for the stabilization of adenine ring of cofactor. The conserved NNAG motif of *Rp*CR is vital for the stabilization of central β-sheets [27]. The active sites in α4-β5-α5 are also conserved in *Rp*CR. Furthermore, a proline residue in the β6, involving in structural and reaction direction, is also found in region 183–195 of *Rp*CR. Consequently, *Rp*CR is a member of classic SDR family.

3.3 Protein purification

*Rp*CR with an *N*-terminal His-tag was purified to homogeneity by nickel affinity chromatography. The specific activity of the purified *Rp*CR is 223 $U \cdot mg^{-1}$, 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 *Rp*CR is 118 kDa, suggesting *Rp*CR is a homotetramer, with subunit of 29.5 kDa.

3.4 Temperature and pH profiles of purified RpCR

The optimum temperature for *Rp*CR was determined by measuring activity at 25–70 °C as illustrate in **Fig. 2(A)**. The *Rp*CR exhibits the highest activity at 50 °C. Remarkably, *Rp*CR retained 60% of activity at temperature as high as 65 °C. The pH profiles of *Rp*CR was measured at various pH ranging from 3.0 to 10.0. According to **Fig. 2(B)**, the optimum pH of *Rp*CR is observed at pH 5.0. And the activity of purified *Rp*CR 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 *Rp*CR. The existence of Ag^{+}, Fe^{3+}, and Al^{3+} show inhibitory effect on the activity of *Rp*CR. The effect of some additives was also examine. SDS is extremely toxic to *Rp*CR. Addition of EDTA has no influence on the activity, indicating *Rp*CR is not a metal ion dependent enzyme,

which is similar to most of other SDR members.

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,** *Rp*CR retains higher residual activity in aliphatic alkane and toluene after incubation for 12 h. Although pentane, hexane, heptane and nonane are more biocompatible with *Rp*CR, their partition coefficients for COBE are lower. Among all the organic solvent test, 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 *Rp*CR is decreased to less than 10% in most of the solvents. Although *Rp*CR 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**, *Rp*CR 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 *Rp*CR. The activity towards 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 *Rp*CR towards ethyl 4-phenyl-2-oxobutanoate (8) is 92.1 U·mg^{-1}. While the highest activity is found with COBE (117 U·mg^{-1}), a β-ketoester. No activity is detected towards 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 alphatic diketones [28]. Enantioselectivity analysis reveals that *Rp*CR 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 *Rp*CR displays no activity with NADH and full activity with NADPH, suggesting that *Rp*CR is NADPH-dependent. The kinetic parameters of purified *Rp*CR were also determined (**Table 5)**. The K_m and V_{max} of *Rp*CR towards COBE are 0.39±0.03 mM and 335±6 μmol·min^{-1}·mg^{-1}, while the K_m and V_{max} to NADPH were 0.17±0.01 mM and 398±7 μmol·min^{-1}·mg^{-1}. As summarized in **Table 5**, the K_m and V_{max} of *Rp*CR are comparable with the K_m of CPE and V_{max} of PsCRI respectively [16,17]. However, the V_{max} of CPE (200 μmol·min^{-1}·mg^{-1}) and K_m of PsCRI (4.9 mM) are not as beneficial as those of *Rp*CR. Consequently, *Rp*CR displays the highest k_{cat}/K_m value (1747 s^{-1}·mM^{-1}), 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 *Rp*CR and GDH were co-expressed in recombinant *E. coli* BL21(DE3), with specific activities of 52 and 18 U·mg^{-1} crude extract at pH 5.0 and 8.0 respectively. However the

recombinant *Rp*CR and GDH display different pH-profiles. As shown in **Fig. 2(B)**, *Rp*CR 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 *Rp*CR and GDH. To achieve the balance of *Rp*CR 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 is observed at pH 7.0, when *Rp*CR retained 70% activity and GDH also displayed high activity. Besides, pH does not affect the enantioselectivity of *Rp*CR (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_2CO_3 to neutralize the gluconic acid generated in glucose oxidation. To avoid the extra addition of expensive $NADP^+$, the amount of biocatalyst loading was optimized. And the results show that 10 $g \cdot L^{-1}$ DCW is sufficient for the complete conversion of 100 $g \cdot L^{-1}$ 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 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 7^{th} 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 *Rp*CR-GDH dry cells within 11 h without addition of external $NADP^+$. 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 $g \cdot L^{-1} \cdot d^{-1}$, which was attributed to the high catalytic efficiency of *Rp*CR 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 $g \cdot L^{-1}$) (*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 $NADP^+$. Although PsCRI and PsCRII display higher V_{max} values, the substrate to biocatalyst ratios are 7.7 and 5.1 respectively, which are a result of the higher K_m towards 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 $g \cdot L^{-1}$ COBE under assistance of 0.3 mM NAD^+ [34]. The *Rp*CR displays better performance in the preparation of (*S*)-CHBE with regard to the cofactor addition, k_{cat}/K_m value and substrate to biocatalyst ratio. As much as 7.0 g COBE can be completed reduced into (*S*)-CHBE by merely 0.1 g recombinant whole cells without addition of external cofactor. The substrate to biocatalyst ratio of *Rp*CR (70) in this study ranks the highest record due to its high k_{cat}/K_m value of 1747 compared with other COBE reductases (**Table 5**). To further decrease the addition of biocatalyst and improve the productivity, protein engineering of *Rp*CR is undergoing in our laboratory for lowering the K_m value towards $NADP^+$.

4. Conclusions

The COBE reductase *Rp*CR from *Rhodococcus pyridinivorans* was discovered by genome mining. *Rp*CR displays high substrate affinity and catalytic efficiency towards COBE. *Rp*CR 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 *Rp*CR in asymmetric reduction of COBE are about 70 and 1480 $g \cdot L^{-1} \cdot d^{-1}$, respectively. To our best knowledge, this is the first report on newly identified *Rp*CR serving as an efficient reductase for asymmetric preparation of (*S*)-CHBE.

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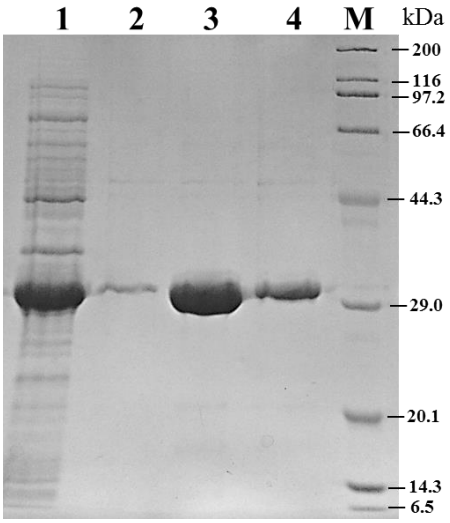


Figure 1. SDS-PAGE analysis of the purified *Rp*CR. Lane 1, supernatant; Lane 2, precipitant; Lanes 3 & 4, purified *Rp*CR with different dilution fold; Lane M, protein molecular mass markers.

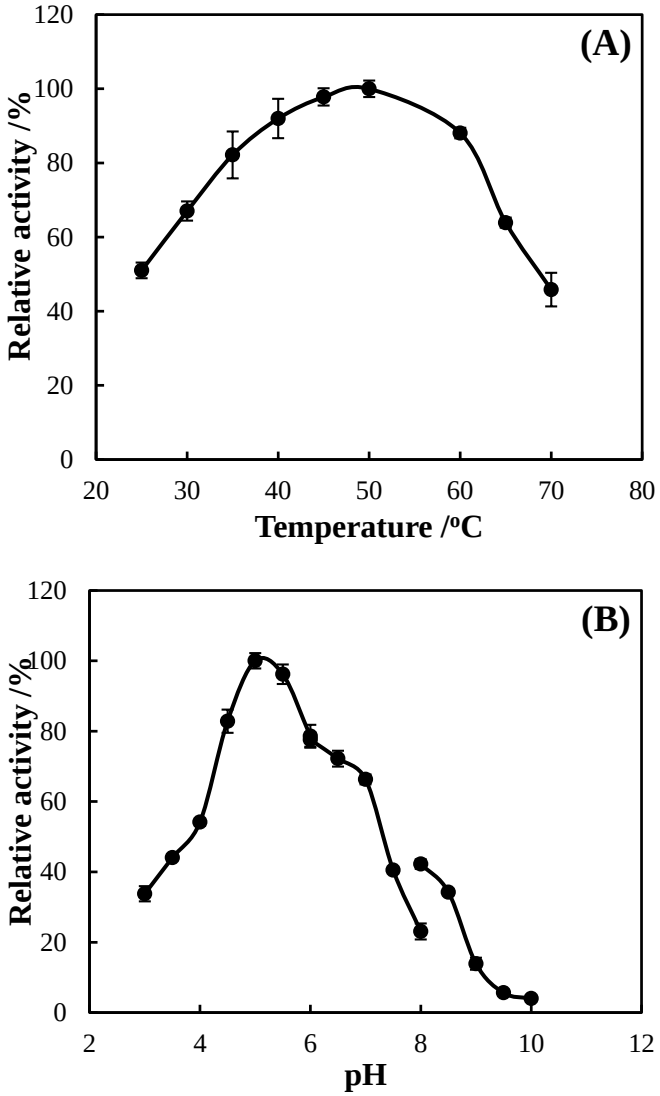


Figure 2. Effect of temperature and pH on the activities of purified *Rp*CR. (A) Activity-temperature profile was estimated at various temperatures (25–70 °C) in PBS buffer (pH 7.0, 100 mM). (B) Activity-pH profile was determined using standard protocol in the following 100 mM buffers: (i) citrate (pH 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.

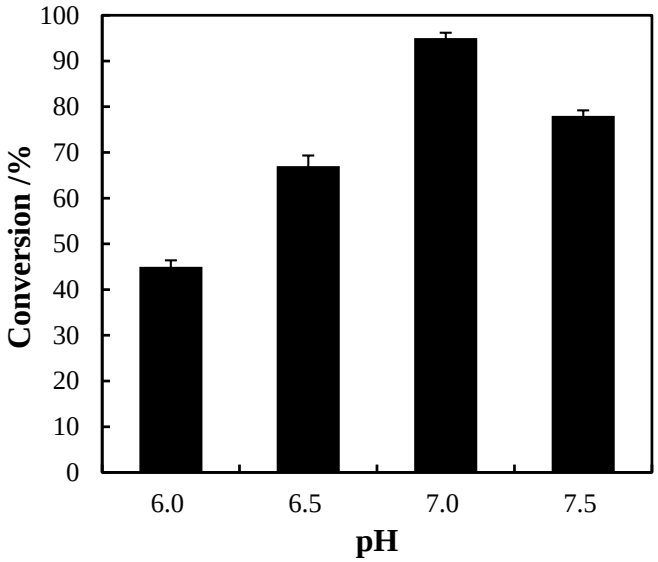


Figure 3. Effect of pH on the asymmetric reduction of COBE employing recombinant *E. coli* BL21 coexpressing *Rp*CR-GDH. Reactions were carried out with 10 mmol COBE, 15 mmol glucose, and 0.1 g dry cells in 10 mL PBS buffer (pH 6.0, 6.5, 7.0, 7.5; 100 mM) at 30 °C and 180 rpm for 12 h.

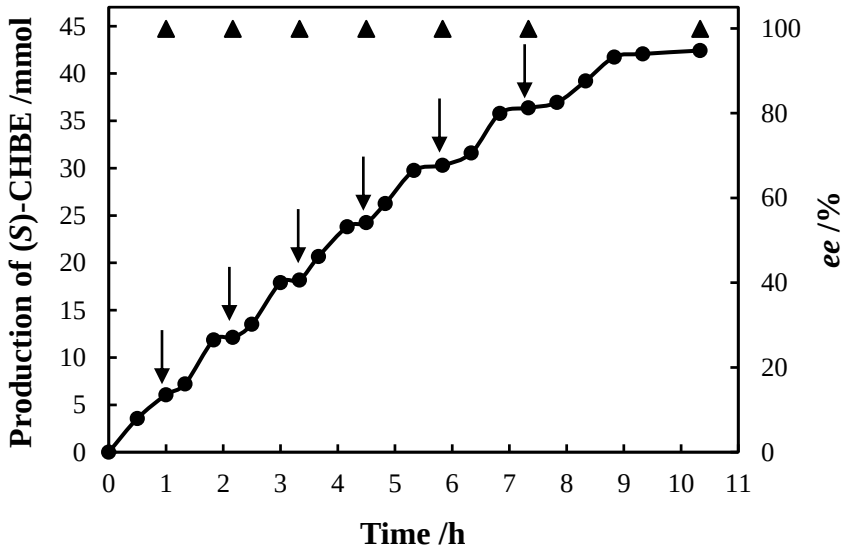


Figure 4. Time course of (*S*)-CHBE production catalyzed by recombinant *E. coli* coexpressing *Rp*CR-GDH in a toluene/aqueous biphasic system with substrate feeding. (●) production of (*S*)-CHBE; (▲) *ee*. Reduction of COBE using 0.1 g dry cells was conducted at 30 °C in 5 mL PBS buffer (pH 7.0, 100 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.

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

Secondary structure element	Conserved motifs of classical SDR	Function	Motifs of *Rp*CR	Position in *Rp*CR
β1-α1	TGxxxGhG	Coenzyme binding region	TGASSGLG	17–24
β3-α3	Dhx[cp]	Adenine ring binding of coenzyme	DIAD	68–71
β4	GxhDhhhNNAGh	Structural role in stabilizing central β-sheet	GRVDILINNAGI	87–98
α4	hNhxG	Part of active site	INLNG	118–122
β5	Gxhhxhx SSh	Part of active site	GRVMQPGSSI	131–140
α5	Yx[A/S][S/T]K	Part of active site	YSASK	159–163
β6	h[K/R]h[N/S]xhxPGxxxT	Structural role, reaction direction	IRVNAIAPGFFRT	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.

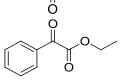
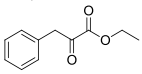
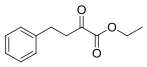
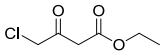
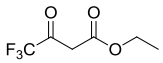
Table 2. Effect of metal ions and additives on the activity of *Rp*CR.

Metal ion	Relative activity /%	Additive	Relative activity /%
Control	100	EDTA	98.8 ± 1.7
Mn^{2+}	88.7 ± 4.7	DTT	79.8 ± 1.7
Mg^{2+}	81.3 ± 1.8	Imidazole	69.1 ± 0.8
Zn^{2+}	63.0 ± 3.3	β-mercaptoethanol	85.0 ± 3.4
Ca^{2+}	91.7± 3.2	SDS	1.1 ± 0.8
Co^{2+}	83.0 ± 0.8	Tris base	83.0 ± 0.8
Ag^{+}	14.4 ± 0.5	Triton-X100	91.1 ± 4.6
Fe^{3+}	17.1 ± 1.6	Tween 20	106.7 ± 1.9
Ni^{2+}	74.1 ± 1.4		
Cu^{2+}	96.9 ± 0.6		
Al^{3+}	28.2 ± 1.5		

Table 3. Effect of organic solvents, ionic liquids and PEG on the activities of *Rp*CR.

Organic solvent	Residual activity /%	Organic solvent	Residual activity /%
Control	73.2 ± 2.9	PEG400	10.0 ± 0.9
n-Pentane	102 ± 3.2	PEG600	5.62 ± 0.52
Cyclohexane	93.8 ± 8.8	PEG1000	15.1 ± 0.3
n-Hexane	101 ± 4	PEG2000	4.02 ± 0.06
n-Heptane	103 ± 3	PEG4000	0.87 ± 0.15
n-Decane	85.9 ± 0.5	ChCl/glycerol	135 ± 1
n-Nonane	103 ± 4	ChCl/Urea	85.9 ± 2.9
iso-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		

Table 4. Substrate specificity of *Rp*CR towards various prochiral ketones.

	Substrate	Specific activity /U·mg^{-1}
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.* [a]
12		*n. d.*

[a] n. d.: no activity was detected.

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

Enzyme [a]	S1	ARII	CPE	PsCRI	PsCRII	ScCR	SOU1	*Dh*CR	*Rp*CR
Accession No. [b]	Q9C4B3	Q9UUN9	G8B541	A3GF07	A3GF05	Q9EX28	P87219	Q6BQ25	H0JYI9
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
Vmax for COBE ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	270	349	200	337	224	43.7	–	16.6	335
k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	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