An Alkali-tolerant Carbonyl Reductase from *Bacillus subtilis* by Gene Mining: Identification and Application

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Abstract

Enantiopure alcohols have received much attention due to their widespread use as pharmaceutical intermediates. In the asymmetric biosynthesis of enantiopure alcohols, the excellent performance of carbonyl reductase makes it be the best choice as the biocatalysts. In this work, an alkali-tolerant carbonyl reductase (*Bs*CR, encoded by *yueD*) from *Bacillus subtilis* (strain 168) was obtained through gene mining, and successfully heterologously expressed in *Escherichia coli* with pET-32a. *Bs*CR showed excellent alkali resistance and even can keep more than 70% of its peak activity after incubation in Tris–HCl buffer at pH 9.0 for 40 h. The Michaelis constants and maximal velocity of the *Bs*CR to NADPH (A) and ethyl 4-chloroacetoacetate (B) are $K_m^A = 5.390 \times 10^{-2}$ mmol/L, $K_m^B = 1.855$ mmol/L, and $V_{max} = 147.3$ µmol·min^{-1}·mg^{-1}, respectively. Applying the *E. coli* BL21(DE3)/pET-32a-yueD to catalyze asymmetric reduction of ethyl 4-chloroacetoacetate and acetophenone, the yield of *S*-CHBE reached 89.9% and *S*-1-phenyl ethanol reached 66.7%, and *e.e.* of both products reached more than 99%. This work provides a novel CR for asymmetric reduction.

Graphic Abstract

A carbonyl reductase (*Bs*CR) and its gene were identified through gene mining, and overexpressed in *Escherichia coli* BL21(DE3) for whole-cell biocatalytic asymmetric reduction
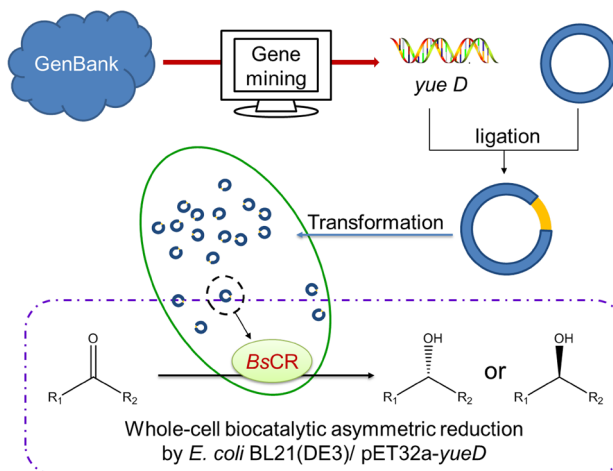
Keywords Gene mining · Carbonyl reductase · *Bacillus subtilis* · Alkali-tolerant · Asymmetric reduction · Chiral alcohols

Extended author information available on the last page of the article

1 Introduction

Chiral alcohols are important pharmaceutical intermediates in various multi-step asymmetric synthesis due to their unique structural properties [1–3]. In general, the two enantiomers of the chiral compound tend to have different biological activities and opposite pharmacological activities. It is particularly important to obtain enantiomerically pure chiral alcohols [4]. Chiral alcohols can be synthesized from ketones by chemical or biological catalysis routes. In order to obtain highly stereoselective alcohols, chemical methods often require toxic metals, expensive hydrides, and stringent conditions compared to biological methods [5–7]. Thanks to the advantages of low pollution, remarkable enantioselectivity, regioselectivity and mild reaction conditions, carbonyl reductases (CRs) are widely applied to catalyze the reduction of various ketones to the corresponding chiral alcohols making considerable contributions to the industrial production of valuable chiral pharmaceutical intermediates including anticancer drugs, β-lactam antibiotics and other important drugs [8–12]. Because of the irreplaceable application prospect of CRs in asymmetric synthesis, they have attracted more and more attention and favor in the field of biocatalysts. To obtain enantiopure chiral alcohols, Wei et al. isolated a strain *Acetobacter* sp. CCTCC M209061 which can specifically catalyze ethyl 4-chloroacetoacetate (COBE) to chiral alcohols with anti-prelog stereospecificity [13]. Liu et al. reported a newly cloned CR expressed in recombinant *Escherichia coli* that catalyzes the asymmetric reduction of ET-4 to (*S*)-ET-5 [14]. Wang et al. cloned an NADH-dependent *Kl*AKR and an NADPH-dependent *Km*AKR to synthesize the *t*–butyl 6–cyano–(3*R*,5*R*)–dihydroxyhexanoate [15, 16]. These developed enzymes can effectively reduce the cost for the chiral building blocks production. Nevertheless, due to its high specificity, many enzymes are difficult to adapt to the practical conditions, such as high-temperature environments [17], high substrate tolerance [18] and highly stereoselective product [19]. Therefore, there is a great interest to develop new CRs with high enantioselectivity and excellent application properties.

Bacillus subtilis is generally regarded as safe (GRAS) bacterium because of its free endotoxins, and it's genetic, physiology are well characterized [20]. The enzymes from *B. subtilis* such as YqiG [21], BDH/AR [20] and P5C reductase [22] perform excellent catalytic ability. Especially, the GDHs (glucose dehydrogenase) from *B. subtilis* are often used for coenzyme regeneration in recombinant cells [20, 23, 24]. However, as laboratory strains, only a few CRs from *B. subtilis* were discovered. In recent years, with the rapid development of high-throughput gene sequencing technology, the whole DNA genome sequence

of *B. subtilis* has been sequenced. On this basis, it is very economical and convenient to discover potential industrial enzymes in this bacterium. In the current study, we mined an NADPH-dependent CR from *B. subtilis* (strain 168), which has not been reported before. The corresponding gene *yueD* was cloned, and expressed in *E. coli* BL21 (DE3) cells with pET-32a vector. Its enzymatic properties were detailly investigated. Furthermore, its application as a catalyst for the asymmetric synthesis of chiral alcohols was preliminarily studied.

2 Materials and Methods

2.1 Chemicals, Plasmids and Strains

The enzymes for gene manipulation were purchased from New England Biolabs (Beijing) Ltd. The DNA markers were purchased from Vazyme Biotech (Nanjing) Co., Ltd. 2×Taq PCR Master Mix, Plasmid Miniprep Kit, Gel Extraction Kit and PCR Purification Kit were purchased from TIANGEN Biotech (Beijing) Co., Ltd. The primers were synthesized by Quintiles Biosciences (Wuhan) Co., Ltd. Other chemicals were purchased from Sigma-Aldrich and Aladdin.

All strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α and *E. coli* BL21(DE3) were used for plasmid amplification and expression of a host cell, respectively. The pET-32a plasmid with ampicillin resistance was used as the cloning and expression vector. All strains were cultured in LB medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 1 L deionized H_2O), and the antibiotics ampicillin, if needed, were added at a concentration of 100 µg/mL.

2.2 Sequence Analysis and Molecular Docking

Potential CRs were screened from the UniProt knowledgebase and BLAST tool at NCBI was used to check the gene of

Table 1 Strains and plasmids used in this study

Strains and plasmids	Stress	Source
pET-32a	Amp	Preserved in our lab
pET-32a-*yueD*	Amp	This study
E. coli DH5α	none	Preserved in our lab
E. coli BL21(DE3)	none	Preserved in our lab
E. coli DH5α/pET-32a	Amp	Preserved in our lab
Bacillus subtilis 168	none	Preserved in our lab
E. coli BL21(DE3)/pET-32a	Amp	This study
E. coli DH5α/pET-32a-*yueD*	Amp	This study
E. coli BL21(DE3)/pET-32a-*yueD*	Amp	This study

Amp ampicillin

the CRs. The amino acid sequence and structure alignment of CR were analyzed on the website of SWISS-MODEL (https://www.swissmodel.expasy.org/), CLUSTALW (https://www.genome.jp/tools-bin/clustalw) and Espript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/index.php). Molecular docking between substrate and CR was conducted by the AutoDock 4.2.

2.3 Construction of the Plasmid pET-32a-*yueD* and *E. coli* BL21(DE3)/pET-32a-*yueD*

The gene *yueD* was obtained by PCR amplification using *B. subtilis* 168 chromosomal DNA as the template [reaction conditions: initial denaturation: 95 °C, 2 min; 34×(denaturation: 95 °C, 10 s; annealing: 63 °C, 20 s; extension: 72 °C, 30 s) final extension: 72 °C, 10 min]. The forward primer is: 5′-CGCG/GATCCATGGAACTTTATATCATCACC GGAG-3′ (with BamHI site, G/GATCC), and the reverse primer is: 5′-CCGC/TCGAGCAAAAACTCTTTAATATC ATAAATGCG-3′ (with XhoI site, C/TCGAG). As shown in Supplementary Scheme 1, the pET-32a-*yueD* vector was constructed by ligation reaction with T4 ligase between the digestion product of PCR and pET-32a digested by BamHI and XhoI, which was the expression vector for the target protein *Bs*CR. After the recombinant plasmids were transformed *E. coli* DH5α by electroporation for plasmid amplification, the positive transformant was identified with colony PCR and verified with double restriction endonuclease digestion. Further, the recombinant plasmid was sequenced to confirm that the *yueD* gene was correctly ligated into the plasmid vector. To express the *yueD* gene, the vector pET-32a-*yueD* was anew transformed *E. coli* BL21(DE3) by electroporation. The transformant *E. coli* BL21(DE3)/ pET-32a-*yueD* was selected with LB medium agar plate with ampicillin, then the recombinant *E. coli* BL21(DE3)/pET-32a-*yueD* was obtained.

2.4 Expression and Purification of *Bs*CR

E. coli BL21(DE3)/pET-32a-*yueD* was cultivated in LB medium containing 100 µg/mL ampicillin at 37 °C with 220 rpm in a constant temperature shaker. When the optical density at 600 nm (OD_{600}) of the culture reached 0.6–0.8, IPTG (Isopropyl β-D-Thiogalactoside, 0.4 mM) was added to induce *Bs*CR expression at 24 °C for 12 h. The cells were collected by centrifuge (10,000×*g* for 10 min) at 4 °C and then washed twice with 0.1 mol/L phosphate buffer (PBS, pH 7.0). To release the expression product, the cells were lysed with sonication. The supernatant was collected with centrifuge (10,000×*g* for 10 min) at 4 °C, which was BsCR crude enzyme solution. *Bs*CR was further purified by affinity chromatography with Ni^{2+} affinity resin (Profinity IMAC

Resins, Bio-rad Shanghai, China). The expressed and purified *Bs*CR were detected by SDS-PAGE.

2.5 Enzymatic Properties of *Bs*CR

The following enzymatic properties of *Bs*CR expressed by the *E. coli* BL21(DE3)/pET-32a-*yueD* were evaluated: the optimum pH, temperature and effects of metal ions (include EDTA, a metal chelating agent). The temperature range was from 25 to 65 °C. The pH range was from 5.0 to 9.0, the buffering systems at different pH were: citric acid buffer for pH 5.0–6.0, sodium phosphate buffer for pH 6.0–8.0, Tris–HCl buffer for pH 8.0–9.0. The concentration of metal ions was 1 mmol/L. The thermal and pH stability were also evaluated. Furthermore, its kinetic parameters were assessed. All the above experiments were completed in triplicate, the average values were calculated based on the three independent experiments, and the standard deviation was calculated.

2.6 Asymmetric Reduction Reactions of Prochiral Ketones by Whole-cell

To assess the application prospects of *Bs*CR, the *E. coli* BL21(DE3)/pET-32a-*yueD* was applied to catalyze asymmetric reduction reactions of COBE and acetophenone to the corresponding chiral alcohols (CHBE and 1-phenyl ethanol). The general procedure as follows: 10 mL PBS buffer (100 mM, pH 6.5) containing 40 mM substrate, 500 mM glucose and 100 mg/mL wet-cells was added to a 25 mL stoppered flask capped with a septum. The reaction mixture was shaken in an air-bath shaker at 250 rpm, 37 °C and samples (800 µL) were taken regularly. The product and the residual substrate were extracted from the sample with 2×800 µL of ethyl acetate containing 40 mM benzaldehyde (as an internal standard) and dried over anhydrous Na_2SO_4. Finally, the concentrations of the product and substrate were analyzed by GC (Gas chromatography) and HPLC (High-performance liquid chromatography), the chemical yield and enantioselectivity were then evaluated.

2.7 Analytical Methods

2.7.1 Enzyme Activity Assay

The molecular mass of *Bs*CR was measured by SDS-PAGE with 12% separating gel. According to the catalytic reaction Eq. (1), the activity was assayed at 30 °C by monitoring the decrease in absorbance of NADPH at 340 nm using prochiral ketones as the substrate ([S] represents the substrates: COBE, ethyl acetoacetate, acetone, 2-octanone, acetophenone, α-chloroacetophenone, 2′-fluoroacetophenone, 3′-fluoroacetophenone, 4′-fluoroacetophenone and

2,2,2-trifluoroacetophenone. [P] represents the corresponding catalytic products).

$$[S] + NADPH \overset{BsCR}{\Rightarrow} [P] + NADP^+ \tag{1}$$

The reaction mixture consisted of 100 mM of sodium phosphate buffer (pH 6.5), 5 mM of substrate, 0.5 mM of NADPH, and 50 µL of *Bs*CR solution in a total volume of 200 µL. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of NADPH per min. The potential of NADH and NADPH to act as electron donors was tested by replacing NADPH with NADH during the reaction.

2.7.2 Kinetic Analysis

A double substrate Michaelis–Menten equation was applied as BsCR kinetic equation. The kinetic parameters of *Bs*CR were assessed according to the initial-velocity double-reciprocal plot by using COBE as the substrate at varying concentrations (2.0, 3.0, 4.0, and 5.0 mM), and the NADPH concentrations for 0.10, 0.15, 0.20, 0.25 and 0.3 mM. *Bs*CR catalyzed-reaction followed the ordered sequential reaction mechanism.

The double substrate Michaelis–Menten equation is presented as follows:

$$v = \frac{V_{max}[A][B]}{K_m^A[B] + K_m^B[A] + K_s^A K_m^B + [A][B]} \tag{2}$$

where [A] represents the concentration of NADPH, [B] represents the concentration of COBE, V_{max} is the maximal velocity, K_m^A and K_m^B are the Michaelis constants of the purified *Bs*CR for NADPH and COBE. K_s^A is the dissociation constant for the binding of *Bs*CR and NADPH.

The slopes of a series of 1/v versus 1/[A] are calculated to be:

$$\frac{K_m^A}{V_{max}} + \frac{K_s^A K_m^B}{V_{max}[B]} \tag{3}$$

The vertical intercepts of straight lines are calculated to be:

$$\frac{1}{V_{max}} + \frac{K_m^B}{V_{max}[B]} \tag{4}$$

2.7.3 Asymmetric Reduction Reaction Assay

The concentrations of product and substrate were quantitatively analyzed by GC (GC-2010 plus, Shimadzu) equipped with an RT-βDEXm chiral column (Restek, America, 30 m × 0.32 mmID × 0.25 µm df) and FID detector. The configurations of phenyl ethanol were determined by GC

and the configurations of ethyl *S*-4-chloro-3-hydroxybutyrate (CHBE) were determined by HPLC (UltiMate3000, Thermo Fisher Scientific). The HPLC equipped with a CHIRALCEL OB column (4.6 × 250 mm) (Daicel Chemical Industries, Japan), and conditions were hexane/2-propanol (85/15, v/v) as mobile phase, flow rate of 0.8 mL/min, ambient column temperature, and ultraviolet (UV) detection set at 220 nm. The analysis method was the same as our previous work [25, 26].

The extent of reaction and enantioselectivity are indicated by yield (chemical yield, %) and *e.e.* (enantiomeric excess, %), respectively, defined as follows:

$$Yield = \frac{C_{[p]}}{C_{[s]}} \times 100\% \tag{5}$$

$$e.e. = \frac{C_S - C_R}{C_S + C_R} \times 100\% \tag{6}$$

where $C_{[S]}$ is the initial substrate concentration, $C_{[P]}$ is the final product concentration, C_S is the final *S*-configuration product concentration, and C_R is the final *R*-configuration product concentration.

3 Results and Discussion

3.1 Gene Mining Result

Previously, a carbonyl reductase (BYueD) from *Bacillus* sp. ECU0013 has been reported. As a versatile reductase, the enzyme has a broad substrate and exhibits excellent diastereoselectivity [27]. In this work, the *B. subtilis* (strain 168) was chosen as the source strain for mining the target CR. A CR was obtained by the similarity of the amino acid sequences between *B. subtilis* (strain 168) and BYueD. This CR was encoded by the gene *yueD* (Gene ID: 936558) and named *Bs*CR. The sequence of *Bs*CR contains 243 amino acids and has 40.24% identity to the *yueD* protein isolated from *Bacillus cereus* by Maruyama et al. [28]. The amino acid sequence analysis results show that *Bs*CR contains the active center conserved region Tyr-X-X-X-Lys (position 147–151) and the cofactor binding site conserved region Thr-Gly-X-X-X-Gly-X-Gly (position 7–14), which belongs to the short-chain dehydrogenases/reductases family (SDRs) [27]. The alignment of multiple amino acid sequences of *Bs*CR with other three SDRs is shown in Fig. 1. The structure of *Bs*CR was built based on the protein PDB: 4H15 as the model and the substrate molecule COBE which was docked into the *Bs*CR with program AutoDock 4.2. The protein was set to be rigid and the small molecule to be a flexible
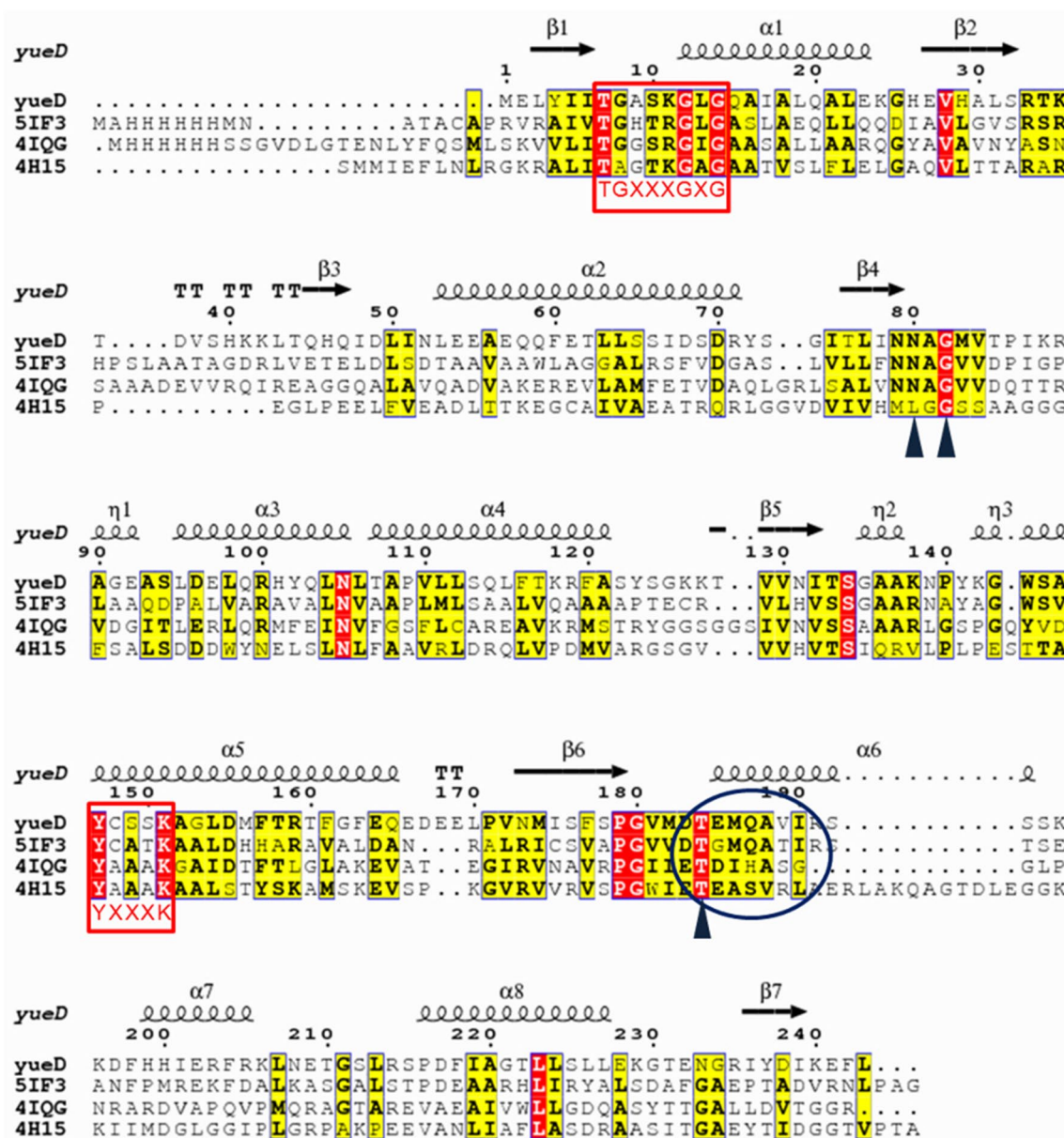
Fig. 1 Alignment of multiple amino acid sequences of *Bs*CR and other three SDRs. A conserved coenzyme-binding motif Thr-Gly-X-X-X-Gly-X-Gly (position 7–14) was found in four sequences, which means both of them preferred NADPH to NADH. The conserved motif Tyr-X-X-X-Lys (position 147–151) was regarded as the active center. The Asn (position 80), Gly (position 82) and Thr (position 184) were regarded as the substrate binding site

residue during the docking process. As shown in Fig. 2a, the protein structure of *Bs*CR has four subunits. Combining with the results of the sequence analysis, the cofactor binding site, the active center conserved region and the substrate binding site have been labeled in the figure. Further, the molecular docking results are shown in Fig. 2b. The results indicate that this CR has the potential ability to reduce carbonyl compounds.

3.2 Construction of the Plasmid pET-32a-*yueD* and *E. coli* BL21(DE3)/pET-32a-*yueD*

In order to explore the properties of *Bs*CR, an efficient expression vector pET-32a-*yueD* containing the *yueD* gene was constructed to express the *Bs*CR in *E. coli*. Supplementary Figs. 1 and 2 were the agarose gel electrophoresis results of *yueD* PCR product and double enzymes (BamHI

Fig. 2 Molecular Simulation of *Bs*CR. **a** The structure of the *Bs*CR, **b** Molecular docking results
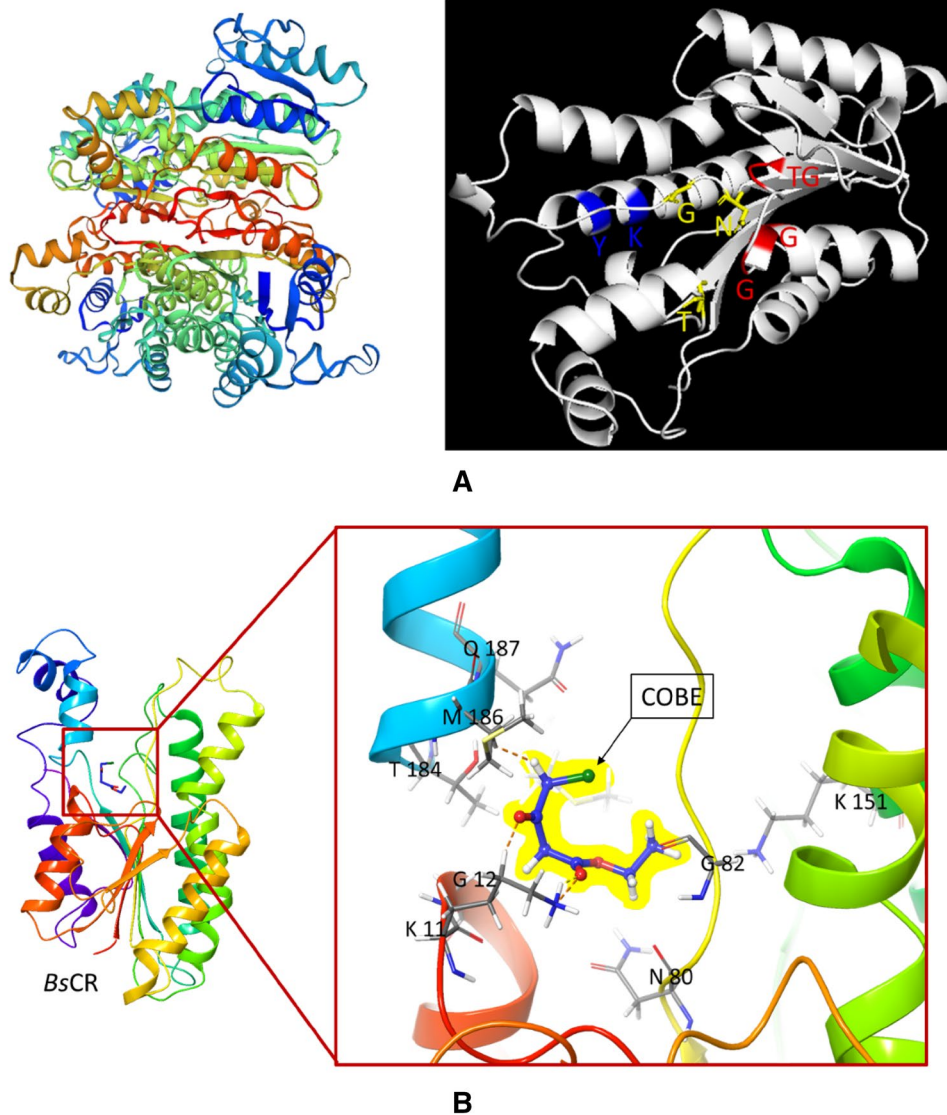
and XhoI) digestion product of pET-32a-*yueD*. First, the size of the PCR product was about 730 bp, which was consistent with the 723 bp of the gene shown in NCBI. Second, the double enzymes digestion product shows that there were two bands: one was about 5900 bp for the plasmid pET-32a, and the other was about 723 bp for *yueD* gene. Also, the cloned *yueD* was sequenced and aligned with the DNA sequence of Gene ID: 936558. The result was presented in Supplementary Fig. 3. All of the results evidenced that the expression vector pET-32a-*yueD* was successfully constructed.

3.3 Expression and Purification of *Bs*CR

For expression of *yueD* gene, the vector pET-32a-*yueD* was transformed into *E. coli* BL21(DE3) to obtain the heterologous overexpression system, *E. coli* BL2 1 (DE3)/pET-32a-*yueD*. The *yueD* was expressed by *E. coli* BL21(DE3)/

pET-32a-*yueD* in LB medium with low-temperature induction after IPTG was added. The proteins were extracted by the afore mentioned method and detected by SDS-PAGE. The results, shown in Fig. 3a, indicated that the *Bs*CR was successfully expressed with a molecular weight of 31 kDa, which is consistent with the value predicted based on the amino acid sequence. Based on the presence of a 6×His tag at the C terminus, the affinity chromatography with Ni^{2+} affinity resin was used to successfully purify the target protein. The *Bs*CR was collected from the eluent. Figure 3b is the SDS-PAGE for the purified product.

3.4 Enzymatic Properties of *Bs*CR

*Bs*CR showed activity only when NADPH was used as the coenzyme, this indicated that it is an NADPH depend CR, which is consistent with amino acid sequence analysis

Fig. 3 SDS-PAGE for the *Bs*CR expressed in *E. coli* BL21(DE3). **a** Total proteins in *E. coli* BL21(DE3), M: Protein Marker; Lane 1: Proteins from *E. coli* BL21(DE3)/pET-32a; Lane 2: Proteins from *E. coli* BL21(DE3)/pET-32a-*yueD*. **b** Purified *Bs*CR overexpressed in *E. coli* BL21(DE3), M: Protein Marker; Lane 1: flow-through of the crude extract; Lane 2: purified *Bs*CR
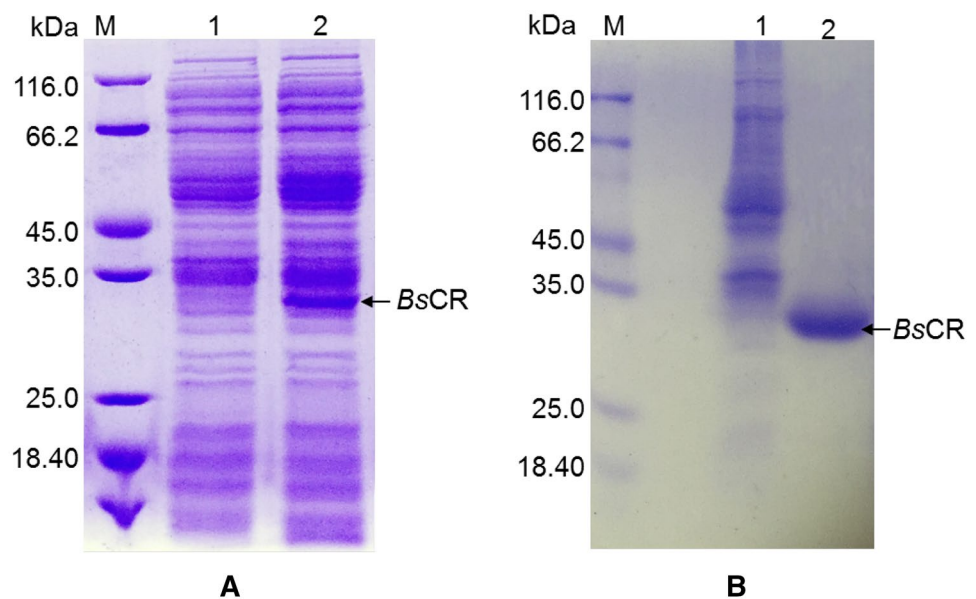
Table 2 Substrate specificity of *Bs*CR

Substrate	Concentration (mM)	Relative activity (%)
COBE	4.0	100.0 ± 0^{N}
Ethyl acetoacetate	4.0	23.7 ± 0.1
Acetone	4.0	20.2 ± 0.3
2-Octanone	4.0	64.6 ± 0.7
Acetophenone	4.0	25.7 ± 0.9
α-Chloroacetophenone	4.0	40.6 ± 0.4
2′-Fluoroacetophenone	4.0	20.2 ± 0.8
3′-Fluoroacetophenone	4.0	18.8 ± 0.2
4′-Fluoroacetophenone	4.0	12.4 ± 0.6
2,2,2-trifluoroacetophenone	4.0	126.2 ± 0.5

N to calculate the relative activity, the activity with COBE was taken as 100%

Table 3 Effects of metal ions and EDTA on *Bs*CR activity

Chemicals	Concentration	Relative activity (%)
Control	–	100 ± 0
Mn^{2+}	1 mM	114.16 ± 0.61
Fe^{2+}	1 mM	108.52 ± 4.72
Ca^{2+}	1 mM	115.98 ± 2.44
Cu^{2+}	1 mM	114.16 ± 0.91
Zn^{2+}	1 mM	115.83 ± 0.76
Co^{2+}	1 mM	115.68 ± 0.61
Fe^{3+}	1 mM	101.98 ± 8.83
Al^{3+}	1 mM	102.74 ± 2.59
Mg^{2+}	1 mM	94.22 ± 1.37
Ba^{2+}	1 mM	92.24 ± 2.74
Li^{+}	1 mM	100.76 ± 2.74
Ni^{+}	1 mM	106.09 ± 3.50
EDTA	1 mM	101.37 ± 1.98

Control enzyme activity was assayed as 100% without addition of any chemical agents

results. To investigate the substrate specificity of *Bs*CR, various ketones were examined as the substrates for this CR. The results shown in Table 2 demonstrated that *Bs*CR exhibit wide substrate spectrum. Specially, it shows superior catalytic ability to halogenated carbonyl compounds and long-chain carbonyl compounds.

The effect of metal ions and EDTA on *Bs*CR activity was shown in Table 3. The activity of BsCR was slightly inhibited by 1 mM Mg^{2+} and Ba^{2+} while enhanced by the other metal ions to a varying extent. EDTA had a negligible effect on *Bs*CR activity, this indicated that *Bs*CR was a nonmetallic dependent enzyme, which followed the SDRs family feature.

The effect of pH on *Bs*CR activity was studied at 30 °C, in different buffer systems (200 mM). As shown in Fig. 4a, the optimum reaction pH for the *Bs*CR is about

6.3. Compared to the activity in sodium phosphate buffer, *Bs*CR showed better activity in citric acid buffer at the same pH values. The pH stability of *Bs*CR was assessed by detecting the residual activity in various buffers with pH from 5.0 to 9.0 at 4 °C for up to 40 h. Samples were withdrawn at an appropriate time and the residual activity was detected under the standard assay conditions with the unincubated *Bs*CR as the control. The results, as shown in Fig. 4b, demonstrated that *Bs*CR was quite stable at pH 7.0, and can keep more than 70% of its peak activity after being incubated in pH 9.0 buffer for 40 h. This means that *Bs*CR is more advantageous for storage in alkaline
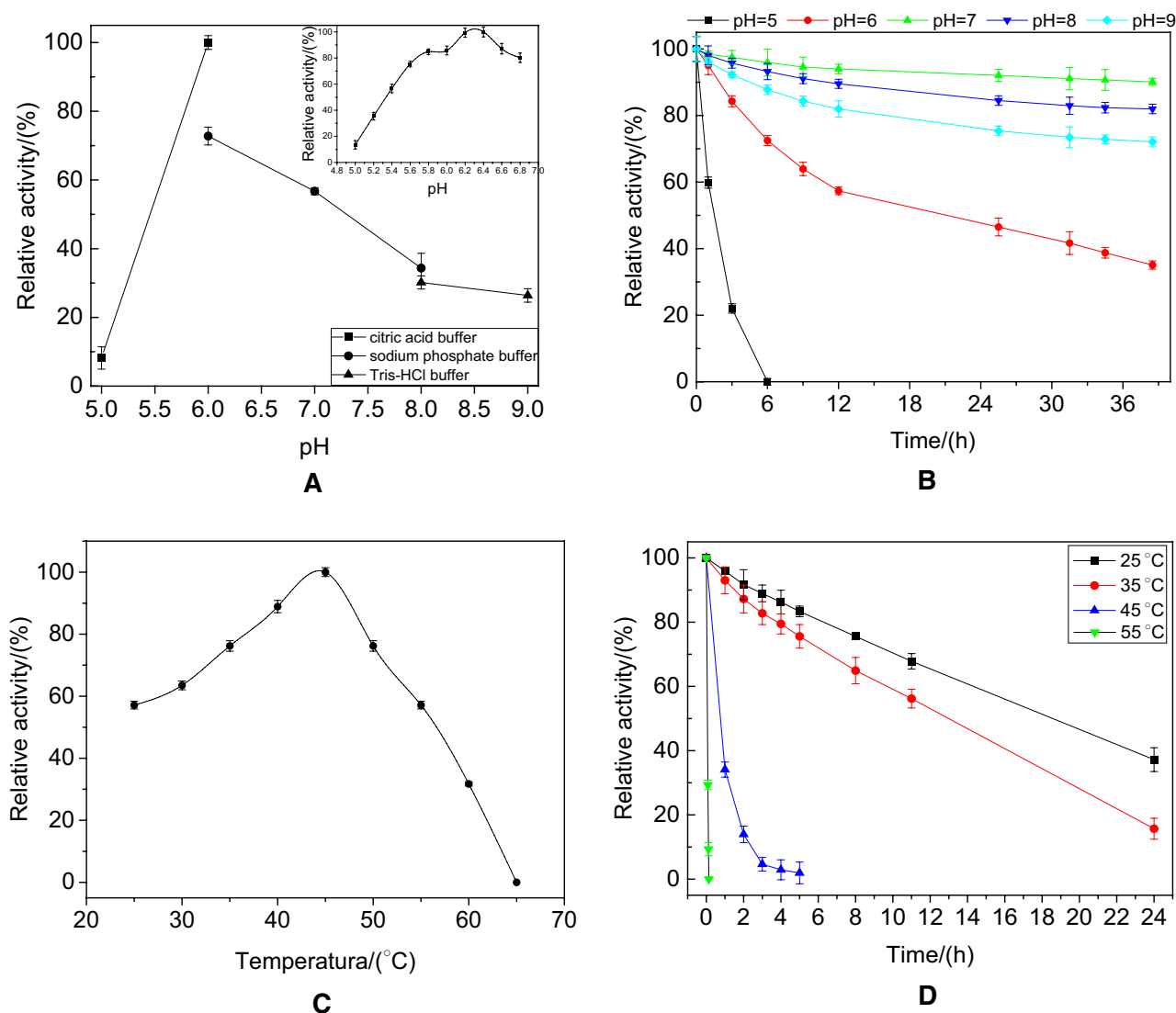
Fig. 4 Effects of pH and temperature on *Bs*CR activity and stability. **a** Effects of pH on activity: pH 5.0–9.0, **b** pH stability: various buffers of pH 5.0–9.0 at 4 °C for up to 40 h, **c** Effects of temperature on activity: 25–65 °C, **d** Temperature stability: 25, 35, 45 and 55 °C, pH 7.0 for 24 h

environments than most CRs such as *Ps*CRII [30], *Lc*SDR [31], and *Sc*CR [32].

The effect of temperature on *Bs*CR activity was investigated at temperatures ranging from 25 to 65 °C. As shown in Fig. 4c, within a temperature range of 25–45 °C, *Bs*CR activity increased with temperature and peaked at 45 °C. When the temperature was above 45 °C, *Bs*CR activity decreased rapidly due to the thermal inactivation. Therefore, we can deduce that the optimum temperature is around 45 °C. To evaluate its thermo-stability, *Bs*CR was incubated at 25, 35, 45 and 55 °C. As presented in Fig. 4d, its half-life ($t_{1/2}$) is 18.57, 12.99, 0.76 and 0.06 h at 25, 35, 45 and 55 °C respectively.

3.5 Kinetic Analysis

The enzymatic properties are the fundamental enzymology data to enzyme research and its application. The kinetic parameters of the *Bs*CR expressed by *E. coli* BL21(DE3)/ pET-32a-*yueD* were evaluated based on the double substrate Michaelis–Menten model. From the data in Fig. 5a, it is apparent that these straight lines intersected at the same point (− 1.1195, 0.0078). In addition, the slope and intercept of each line were plotted as shown in Fig. 5b, c. From the results of the correlational analysis, the values of V_{max}, K_m^A, and K_m^B were 147.3 μmol·min^{-1}·mg^{-1}, 5.390×10^{-2} mM and 1.855 mM, respectively. Compared with the reported SDR,
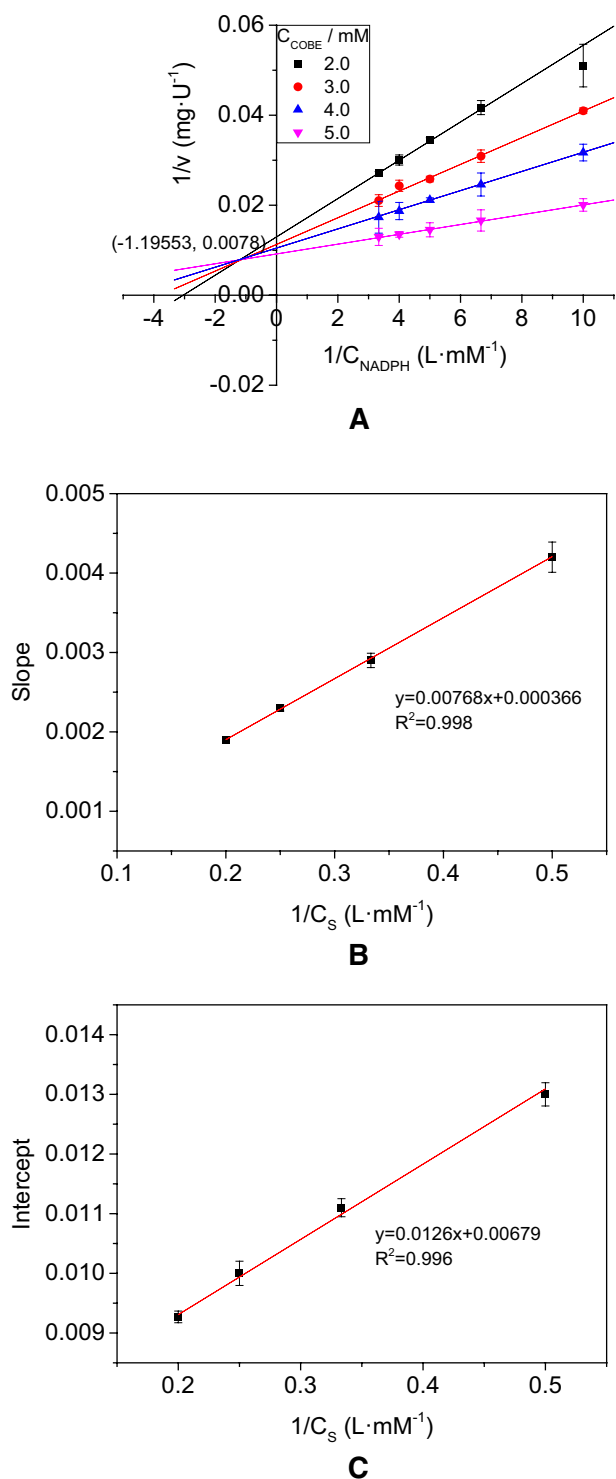
Fig. 5 Kinetics analysis. **a** Sequence reaction double-reciprocal plot, **b** Plot of the slopes versus the reciprocal of substrate concentration, **c** Plot of the intercepts versus the reciprocal of substrate concentration
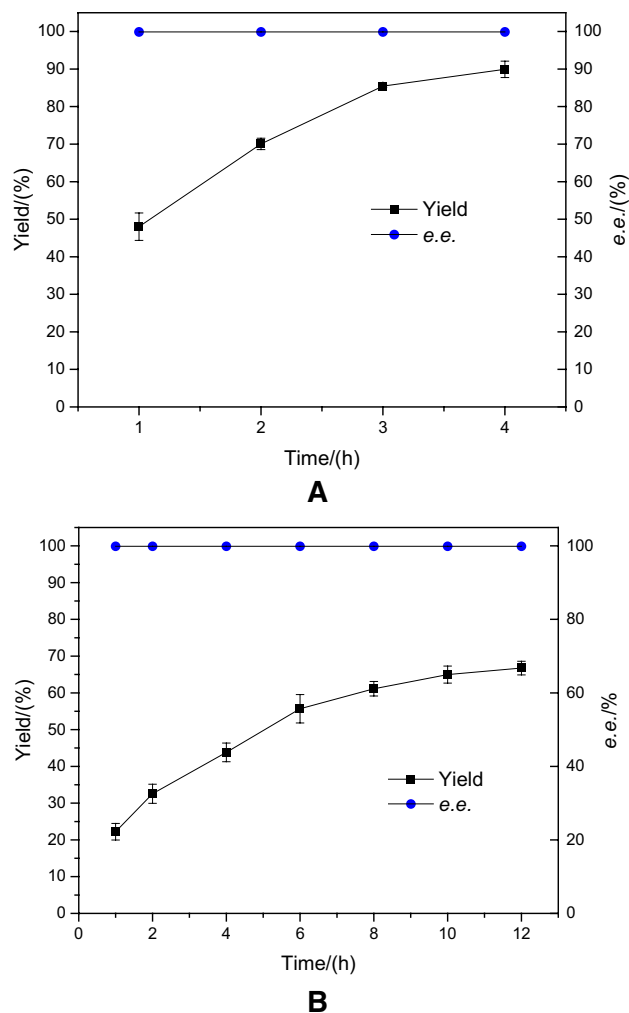

Fig. 6 Asymmetric reduction reactions of prochiral ketones by whole-cell. **a** Asymmetric reduction of COBE with *E. coli* BL21(DE3)/pET-32a-*yueD* resting cell, **b** Asymmetric reduction of acetophenone with *E. coli* BL21(DE3)/pET-32a-*yueD* resting cell

3.6 Asymmetric Reduction Reactions of Prochiral Ketones by Whole-cell

To investigate the catalysis capability of the recombinant *E. coli* BL21(DE3)/pET-32a-*yueD*, the asymmetric reduction reactions of prochiral ketones are carried out with whole-cell. Due to the product, chiralCHBE and 1-phenyl ethanol are key important chiral building blocks and which were widely researched, COBE and acetophenone were chosen as the model substrates for β-oxo ester and aromatic ketone respectively. Results were given in Fig. 6. After investigated, the optical purity of CHBE and phenyl ethanol were over 99% *e.e.* for *S*-configuration. As shown in Fig. 6a, when the reaction was carried out for 4 h, the yield of *S*-CHBE reached 89.9%. In Fig. 6b, with the increase of reaction time, the yield of *S*-1-phenyl ethanol

such as *Bc*CR (0.22 μmol·min^{-1}·mg^{-1}) [29] and *Lc*SDR (133.9 μmol·min^{-1}·mg^{-1}) [31], the *Bs*CR has a higher V_{max}, which means greater prospects in industrial applications.

increased from 22.2% at 0.5 h to 66.7% at 12 h. These indicate that *E. coli* BL21(DE3)/pET-32a-*yueD* show good catalysis capability to β-oxo ester and aromatic ketone and display potential application prospects. For example, the CR from *Streptomyces coelicolor* can only catalyze the conversion of acetophenone in 55% yield but get a great application in water/toluene biphasic system [32]. This also provides a direction for our further researches.

4 Conclusion

With the increasing availability of public genome information, many putative CRs can be obtained from GenBank. Compared to traditional CR screening, the use of gene mining technology can not only improve efficiency but also save costs. In this work, an alkali-tolerant CR (*Bs*CR) was discovered from *B. subtilis* (strain 168) through gene mining. The gene of *Bs*CR was cloned and successfully expressed in *E. coli* BL21 (DE3) with pET-32a-*yueD* vector. Further, the enzymatic properties of *Bs*CR were studied. The results showed that the optimal reaction pH and temperature for the CR are 6.3 and 45 °C respectively. The most unexpected result to emerge from the data is that *Bs*CR exhibits excellent alkali resistance, and there are literature reported describing the characteristics of an alkali-resistant CR. The Michaelis constants and maximal velocity of *Bs*CR to NADPH and COBE are $K_m^A = 5.390 \times 10^{-2}$ mM, $K_m^B = 1.855$ mM, and $V_{max} = 147.3$ µmol·min^{-1}·mg^{-1}. The asymmetric reduction reactions of prochiral ketones catalyzed by the *E. coli* BL21(DE3)/pET32a-*yueD* was investigated. Without additional NADPH addition, the yield of *S*-CHBE reached 89.9% at 4 h, and the yield of *S*-1-phenyl ethanol reached 66.7% at 12 h. The reaction show excellent enantioselectivity. This work not only indicates a detailed understanding of the properties and applications of *Bs*CR, but further demonstrates that gene mining technology provides an efficient route to discover new CRs from existing strains.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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