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PAPER

Highly stereoselective reduction of prochiral ketones by a bacterial reductase coupled with cofactor regeneration†

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A carbonyl reductase gene (*yueD*) from *Bacillus* sp. ECU0013 was heterologously overexpressed in *Escherichia coli*, and the encoded protein (BYueD) was purified to homogeneity and characterized. The NADPH-dependent reductase showed a broad substrate spectrum towards different aromatic ketones, and α- and β-ketoesters. Although the enantioselectivity was high to moderate for the reduction of α-ketoesters, all the tested β-ketoesters and aromatic ketones were reduced to the corresponding chiral alcohols in enantiomerically pure forms. Furthermore, the practical applicability of this enzyme was evaluated for the reduction of ethyl 4-chloro-3-oxobutanoate (**1a**). Using *Escherichia coli* cells coexpressing BYueD and glucose dehydrogenase, 215 g L^{-1} (1.3 M) of **1a** was stoichiometrically converted to ethyl (*R*)-4-chloro-3-hydroxybutanoate ((*R*)-**1b**) in an aqueous-toluene biphasic system by using a substrate fed-batch strategy, resulting in an overall hydroxyl product yield of 91.7% with enantiomeric purity of 99.6% ee.

Introduction

Chiral secondary alcohols with additional functional groups are frequently required as important and valuable intermediates for pharmaceuticals and other fine chemicals. Asymmetric bioreduction of prochiral ketones to (*S*) or (*R*)-enantiomers of alcohols using isolated enzymes or whole cell systems has attracted great attention and has been extensively investigated due to its high stereoselectivity, mild reaction conditions and environmental compatibility.[1] Although the requirement of expensive cofactors represents an inherent disadvantage of redox enzymes, several remarkable achievements have been made in the development of efficient and cost-effective cofactor recycling systems, such as the enzyme-coupled approach and substrate-coupled system.[2–5]

Optically active ethyl 4-chloro-3-hydroxybutyrate (**1b**) is a useful chiral building block in the synthesis of biologically and pharmacologically important compounds. For example, (*R*)-**1b** is a precursor for L-carnitine[6] and (*R*)-4-amino-3-hydroxybutyric acid (GABOB),[7] while (*S*)-**1b** is a chiral intermediate for HMG-CoA reductase inhibitors[8,9] and 4-hydroxypyrrolidone.[10] Several enzymes from various microorganisms have been found to be able to produce (*S*)-**1b**,[11–17] whereas (*R*)-**1b** is in great demand yet less readily available.[18,19] A recombinant aldehyde reductase from *Sporobolomyces salmonicolor* has been utilized to produce (*R*)-**1b** at an impressive substrate concentration of 300 g L^{-1}, however, with only 92% enantiomeric excess.[20] Several other synthetic procedures have been developed to obtain (*R*)-**1b**;[21–23] nevertheless, the relatively low substrate loadings do limit their potential for practical application. The discovery of oxidoreductases with the capability of producing optically pure (*R*)-**1b** efficiently is therefore of great interest. Apart from conventional screening approaches, increasing availability of genomic information seems to offer an enormous potential to provide biocatalysts with great applicability.[24,25]

Previously, we reported the isolation and identification of a ketone reductase-producing *Bacillus* sp., strain no. ECU0013.[26] This paper deals with the cloning and heterologous expression of a *yueD* gene encoding reductase (BYueD) from the strain. The YueD protein from *Bacillus subtilis*, which shows 41% amino acid identity to *Bacillus cereus* benzil reductase, has been identified to reduce benzil to benzoin.[27] Since then, however, there has been no report about any further characterization of this reductase, which renders it an interesting candidate for us to explore, especially regarding its substrate spectrum. In this paper, the substrate-specificities and stereo-specificities of BYueD were evaluated for the reduction of a variety of ketones. Furthermore, we showed its applicability in the production of (*R*)-**1b** coupled with a NADPH-regenerating system by glucose dehydrogenase (GDH).

Results & discussion

Protein over-expression and purification

BYueD was successfully expressed in *E. coli* cells with standard cloning techniques and the genomic DNA of *Bacillus* sp. ECU0013

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as template. A BLAST search revealed that the protein showed 98% amino acid identity to that from *Bacillus subtilis* 168 (Genbank accession no. AAC44308) and 41% identity to *Bacillus cereus* benzil reductase (Genbank accession no. BAB86009). The His-tagged recombinant protein was mainly present in the soluble fraction and was purified through affinity chromatography. The specific activity of BYueD after purification was 0.86 U mg^{-1} corresponding to a 1.6 fold improvement in activity compared to the crude extract. Protein separation by SDS-PAGE resulted in a single band corresponding to a molecular mass of 30.7 kDa (Fig. 1), in agreement with its theoretical value. Additionally, the purified protein eluted from the TSK G2000 SWxl column as a single peak with an elution volume corresponding to an apparent molecular mass of 59.3 kDa, which indicated that BYueD was a homodimeric enzyme consisting of two identical subunits.

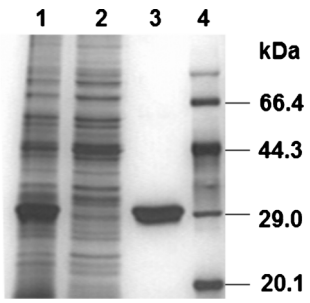


Fig. 1 SDS-PAGE analysis of the purified reductase. Lane 1, crude extract; Lane 2, flow through; Lane 3, purified enzyme; Lane 4, protein markers. Protein bands were visualized by silver staining.

Dependence of enzyme activity on pH and temperature

The effects of temperature and pH on the reductase were investigated by monitoring the change in enzyme activity over a range of 20–60 °C (Fig. 2a), and a pH range of 5.0–11.0 (Fig. 2b). According to the activity–temperature profile, the maximum enzyme activity was observed at a temperature of about 45–50 °C and then reaction rate decreased rapidly over 50 °C due to thermal inactivation. The activity–pH profile showed that the enzyme activity exhibited a pH optimum at 7.5–8.0. An approximate thirty percent decrease in activity was obtained using Gly-NaOH buffer instead of phosphate buffer at the same pH values (pH 8.5). These results are in agreement with benzil reductase from *B. cereus* which showed an optimum temperature of 50 °C and an optimum pH of 6.0–8.0.[27]

Studies concerning the thermostability were performed at three different temperature points of 30, 40 and 50 °C. The enzyme was quite labile at higher temperature (50 °C) but much more stable at 30 °C and 40 °C, giving half-lives of 330, 107 and 0.7 h at 30, 40 and 50 °C, respectively. It suggests that although higher temperatures resulted in a rapid loss of activity, BYueD was stable in mild reaction conditions.

Substrate spectrum

The enzyme showed no activity with NADH and full activity with NADPH. To find a reductase with a broad substrate spectrum, specific activity and enantioselectivity of the purified enzyme were assayed in the reduction of various ketoesters and aromatic

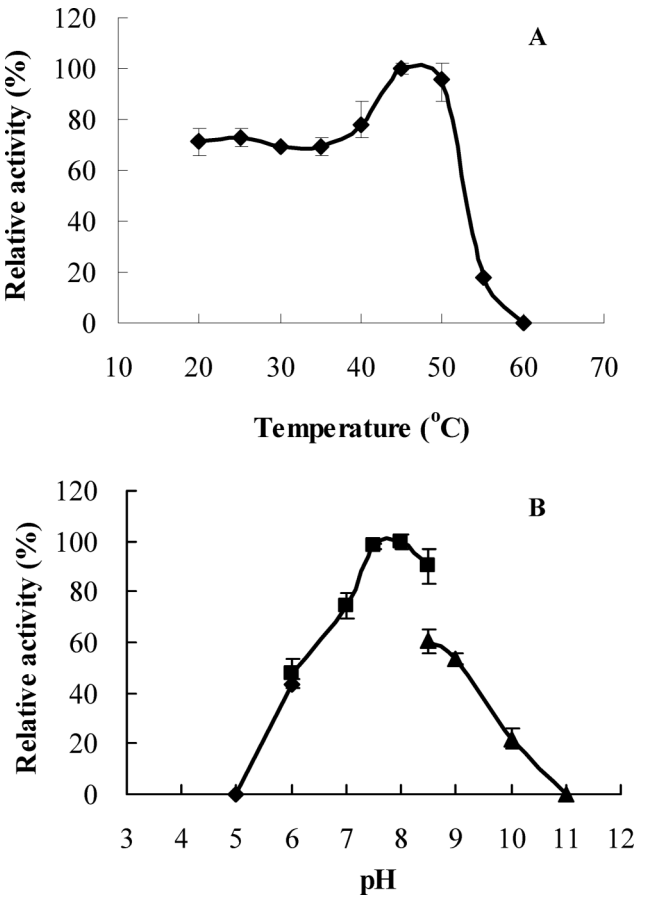
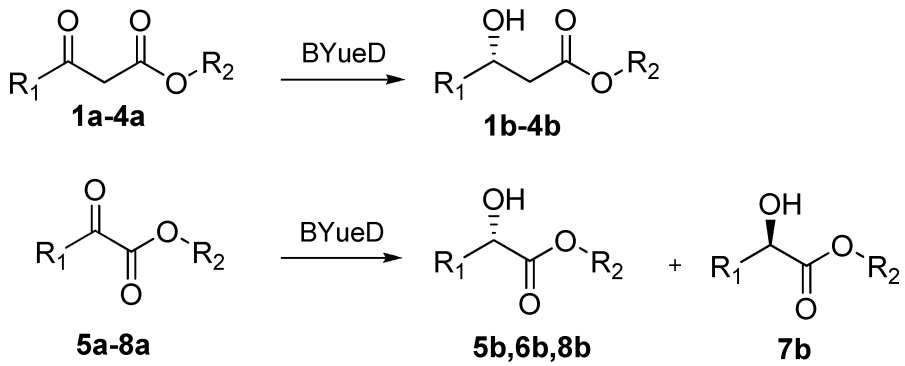


Fig. 2 Effects of temperature and pH on activity of the purified enzyme. (A) Activity–temperature profile was estimated at various temperatures (20–60 °C) in phosphate buffer (50 mM, pH 7.0) using standard assay. (B) Activity–pH profile was determined using standard assay in the following 50 mM buffers: (i) citrate (pH 5.0–6.0); (ii) phosphate (pH 6.0–8.5) and (iii) Gly-NaOH (pH 8.5–11.0). Relative activity was expressed as a percentage of the maximum activity under experimental conditions.

ketones. Substrate specificity of the enzyme was assessed rapidly by using spectrophotometric assays and enantioselectivity was analyzed in aqueous phosphate buffer using an external NADPH regeneration system consisting of glucose dehydrogenase (GDH) and glucose. As shown in Table 1, the reductase efficiently catalyzed the reduction of α- and β-ketoesters, with a dramatically high activity towards ethyl pyruvate. Interestingly, the reductase exhibited excellent prochiral selectivity in the reduction of β-ketoesters (>99% ee), while α-ketoesters were reduced with high to moderate enantioselectivity. It seems that the size of the R group had an impact on determining enzyme activity and selectivity for the reduction of α-ketoesters. As shown in Table 2, the reductase was also active for the reduction of acetophenone derivatives and acetyl pyridines, giving corresponding alcohols in excellent enantiomeric purity (>99% ee). R groups of acetophenone derivatives exerted a relatively significant effect on the enzyme activity, with 2,2,2-trifluoroacetophenone being the most active substrate. On the whole, the enantioselectivity data indicates that the enantiopreference of this enzyme generally follows Prelog's rule. Some of the chiral products have been used as important building blocks for pharmacologically active compounds or fine

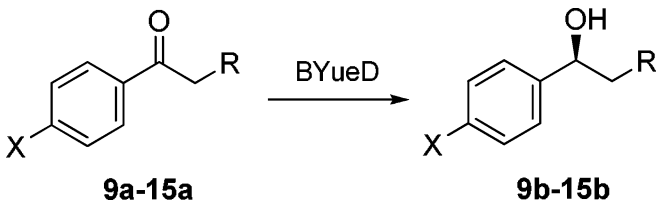
Table 1 Reduction of α- and β-ketoesters



Entry	R_1	R_2	Specific activity[a]	ee (%)[b]
1	CH_2Cl	C_2H_5	0.86	>99 (*R*)[c]
2	CH_2Br	C_2H_5	0.28	>99 (*R*)[c]
3	CH_3	C_2H_5	0.11	>99 (*S*)
4	CF_3	C_2H_5	0.012	>99 (*R*)
5	CH_3	C_2H_5	58	>99 (*S*)
6	$(CH_3)_2CH$	C_2H_5	2.0	82 (*S*)[d]
7	C_6H_5	CH_3	1.6	75 (*R*)[d]
8	$C_6H_5(CH_2)_2$	C_2H_5	0.20	26 (*S*)

[a] The unit of specific activity was U mg^{-1} protein. [b] The ee value was measured by chiral GC analysis unless indicated otherwise. [c] Determined by chiral GC analysis after acetylation of the product. [d] Determined by chiral HPLC analysis.

Table 2 Reduction of aromatic ketones



Entry	*R*	X	Specific activity[a]	ee (%)[b]
9	H	H	0.023	>99 (*S*)
10	Cl	H	0.014	>99 (*R*)
11	Br	H	0.12	>99 (*R*)
12	CF_3	H	1.0	>99 (*R*)
13	CH_3	H	0.014	>99 (*S*)
14	H	Cl	0.010	>99 (*S*)
15	H	Br	0.026	>99 (*S*)
16	2-Acetylpyridine		0.067	>99 (*S*)
17	3-Acetylpyridine		0.018	>99 (*S*)
18	4-Acetylpyridine		0.049	>99 (*S*)

[a] The unit of specific activity was U mg^{-1} protein. [b] The ee value was measured by chiral GC analysis.

chemicals, for example, (*R*)-**1b** for nutrient L-carnitine;[6] (*R*)-**4b** for antidepressant befloxatone;[28] (*R*)-**10b** for antidepressants fluoxetine, tomoxetine, and nisoxetine;[29] and (*R*)-**12b** for liquid crystals.[30]

Kinetic constants

The kinetic constants of the reductase determined for several of the most active substrates are shown in Table 3. Based on the specificity constant (k_{cat}/K_m), this enzyme shows the greatest preference for ethyl pyruvate as compared with other ketones tested. However, the natural substrate and function of the reductase are unknown. It is worth mentioning that the enzyme also exhibited a relatively superior preference for ethyl 4-chloro-3-oxobutanoate (**1a**), which can be reduced by the enzyme to optically pure (*R*)-**1b**. Therefore, the asymmetric reduction of **1a** with BYueD was further investigated as a case study for evaluating the practical applicability of this recombinant reductase.

Coexpression of YueD and GDH genes

In order to establish the efficient asymmetric bioreduction of **1a**, a glucose dehydrogenase (GDH) from *Bacillus subtilis* was introduced for the regeneration of the oxidized cofactor ($NADP^+$).

Table 3 Steady-state kinetic constants

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Ethyl 4-chloro-3-oxobutanoate	0.70 ± 0.10	1.08 ± 0.04	1.56
Ethyl pyruvate	0.094 ± 0.002	48 ± 3	513
Ethyl 3-methyl-2-oxobutanoate	8.5 ± 1.5	9.0 ± 0.6	1.07
Ethyl 2-oxo-4-phenylbutanoate	1.7 ± 0.1	0.53 ± 0.03	0.32
Methyl benzoylformate	2.6 ± 0.1	3.4 ± 0.1	1.31
2-Chloroacetophenone	4.3 ± 1.3	0.29 ± 0.05	0.07
2,2,2-Trifluoacetophenone	2.3 ± 0.2	1.9 ± 0.1	0.83

Two *E. coli* transformant strains were constructed based on different coexpression styles. On one hand, an expression plasmid (pET-Y-G) with both BYueD and GDH genes were created and transformed into the *E. coli* cells (Fig. 3); on the other hand, two separate plasmids with each of the GDH and BYueD genes (pET20b-GDH and pET28a-BYueD) were introduced sequentially into the cells. When each of the two designer cells was subjected to reduction of **1a** in the presence of glucose and $NADP^+$, the higher production of (*R*)-**1b** was observed with *E. coli* cells possessing pET-Y-G, in which BYueD and GDH activities were 200 U and 1490 U per gram of dry cells, respectively. In contrast, relatively lower BYueD activity (50 U g^{-1} dry cells) but higher GDH activity (1630 U g^{-1} dry cells) were observed in the *E. coli* cells containing two plasmids, which probably resulted in the moderate reducing power. Obviously, recombinant cells with higher BYueD activity would be an ideal choice, since BYueD activity tended to be the limiting factor of the reaction. Consequently, *E. coli* cells harboring pET-Y-G were used as catalysts for the subsequent experiments.

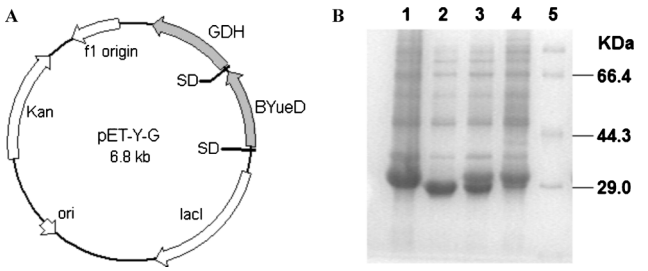


Fig. 3 Schematic presentation of the plasmid containing BYueD and GDH genes and analysis of expression level by SDS-PAGE. (A) Structure of the coexpression plasmid pET-Y-G. (B) SDS-PAGE analysis of the expression. Lane 1, soluble fraction of cell-free extract from *E. coli* with pET20b-GDH; Lane 2, soluble fraction of cell-free extract from *E. coli* with pET28a-BYueD; Lane 3, soluble fraction of cell-free extract from *E. coli* with pET-Y-G; Lane 4, soluble fraction of cell-free extract from *E. coli* with pET20b-GDH and pET28a-BYueD; Lane 5, protein markers.

Bioconversion of 1a to (*R*)-1b in a biphasic system

As **1a** is unstable in an aqueous solution, an water/organic solvent biphasic system was applied for the bioreduction of **1a**. The stabilities of BYueD and GDH in the presence of various organic solvents were first determined. From Table 4, it can be seen that both BYueD and GDH were strongly inactivated in butyl acetate which had been frequently introduced as the non-aqueous phase in the reduction of **1a**.[20,32,35–38] Although the enzymes were stable in

Table 4 Partition coefficients of organic solvents for **1a** and **1b** and stabilities of BYueD and GDH in various water/organic solvent biphasic systems

		Partition coefficient[b]		Remaining activity (%)[c]	
Organic solvent	Log *P*[a]	**1a**	**1b**	BYueD	GDH
Control	—	—	—	95.1 ± 1.0	90.7 ± 0.5
Butyl acetate	1.7	10.6	15.8	0.6 ± 0.3	0.1 ± 0.0
Toluene	2.5	21.3	2.8	4.2 ± 0.0	52.6 ± 1.8
n-Hexane	3.5	1.5	0.3	102.5 ± 0.5	94.9 ± 0.9
Ethyl caprylate	3.8	6.7	9.3	90.6 ± 1.5	1.4 ± 0.3
n-Heptane	4.0	1.0	0.25	97.2 ± 2.0	95.2 ± 4.2
Isooctane	4.5	0.9	0.2	98.2 ± 2.5	94.6 ± 1.4
Dibutylphthalate	5.4	6.2	4.2	62.0 ± 1.0	95.8 ± 0.5

[a] Ref. 31. [b] Partition coefficient is the molar ratio of each compound found in the organic phase to the same compound in the aqueous phase (data from ref. 32, 33 and 34). [c] 0.1 M phosphate buffer (pH 7.0) containing BYueD (2 U mL^{-1}) and GDH (16 U mL^{-1}) was combined with an equal volume of each test organic solvent in a closed vessel. The mixture was shaken at 30 °C for 12 h and the residual enzyme activities in the aqueous solution were assayed.

n-hexane, *n*-heptane and isooctane, **1a** and **1b** could not be efficiently extracted into the organic layer because of the poor partition efficiencies. Due to the too high boiling point of ethyl caprylate and dibutylphthalate which would increase the difficulty of product separation, toluene was eventually selected as the organic phase throughout the experiments.

Subsequently, a substrate fed-batch strategy was carried out to mitigate its toxicity and deactivation effect on the enzymes, since preliminary experiments showed that the reductase activity was repressed in the presence of high substrate concentration. The reaction was performed in a water–toluene biphasic system with a total **1a** addition of 1.3 M (215 g L^{-1}) that was fed in three steps, during which the resulting gluconic acid was neutralized with Na_2CO_3 to maintain an initial pH of 6.8. As shown in Fig. 4, at 5 h, 1.1 M **1b** was produced in the organic phase with a satisfactory enantiomeric purity of 99.6% ee for the (*R*)-isomer and the remaining **1a** substrate was not detectable. The aqueous solution was extracted because of the moderate partition efficiency of **1b** in the water–toluene system. After normal work-up, the desired hydroxyl product was obtained with an isolated yield of 91.7%. As far as we know, this is the first report of BYueD used as a biocatalyst for highly stereoselective reduction of **1a** to (*R*)-**1b**. This asymmetric reduction is promising from a practical viewpoint, since only one example of a bioreduction system has been reported that can produce (*R*)-**1b** with a concentration higher than 200 g L^{-1} but with enantiopurity of 92% ee.[20]

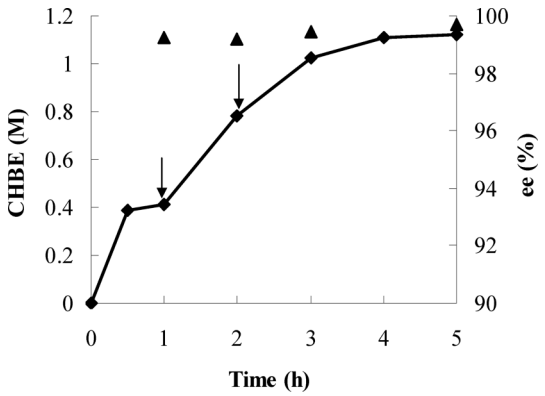


Fig. 4 Production of (*R*)-**1b** using *E. coli* BL21 harboring pET-Y-G in a water–toluene system with a substrate fed-batch strategy. Reduction of **1a** using 0.5 g dry cells was conducted at 30 °C in 10 mL 0.1 M phosphate buffer (pH 6.8) and 10 mL toluene containing 10 μmol $NADP^+$, 10.0 mmol glucose and 5.0 mmol **1a**. 5.0 mmol and 3.0 mmol **1a** together with 2 equiv. glucose were added to the system at 1 h and 2 h, respectively. (◆) Concentration of **1b** in organic phase; (▲) ee.

Conclusions

In this work, a carbonyl reductase (BYueD) from *Bacillus* sp. ECU0013 was overexpressed in *E. coli* and purified to homogeneity, and its enzymatic properties were characterized, especially with respect to its substrate specificity and stereospecificity. BYueD served as a versatile reductase with a broad substrate spectrum accepting a variety of aromatic ketones, and α- and β-ketoesters. Although the enantioselectivity was high to moderate for the reduction of α-ketoesters, all the tested β-ketoesters and aromatic ketones were reduced to the corresponding chiral alcohols with excellent enantiomeric purity. Furthermore, a tailor-made whole-cell biocatalyst containing BYueD and GDH was successfully created and practically used to catalyze the reduction of **1a**. Strategies such as an aqueous/organic biphasic system as well as substrate fed-batch mode were adopted to alleviate the decomposition of **1a** and its inhibition of the biocatalyst. Consequently, the designer cells gave a substrate conversion efficiency of 100% and an enantiomeric purity of the (*R*)-isomer of 99.6% ee when subjected to the reduction of **1a** at a total concentration of 1.3 M (215 g L^{-1}). This study not only provides useful guidance for further application of BYueD in biocatalytic production of chiral alcohols but also establishes an efficient process for the direct synthesis of (*R*)-**1b** with excellent enantiopurity.

Experimental section

Cloning, expression and purification

Bacillus sp. ECU0013, currently deposited in China General Microbiological Cultures Center with an accession number of CGMCC No. 2549, was used as the DNA donor. The strain was cultivated as described previously.[26] The genomic DNA of *Bacillus* sp. ECU0013 was extracted and purified using the TIANamp Bacteria DNA Kit (Tiangen, Shanghai, China). DNA fragments containing *yueD* were amplified by polymerase chain reaction (PCR) using primers based on the *yueD* sequence of *Bacillus subtilis* 168 (5′-ATGGAACTTTATATCATCACCGGAG-3′ and 5′-CTACAAAAACTCTTTAATATCATAAATGCG-3′). The amplified DNA was directly cloned into the TA cloning site of pMD-18T and the desired gene was subcloned into expression plasmid pET28a (+) between the *BamH*I and *Sal*I restriction sites using standard methods, which was subsequently transformed into *Escherichia coli* BL21(DE3) cells. The *yueD* nucleotide sequences identified in this study have been submitted to GenBank (accession no. HM590487). The recombinant protein was expressed and purified as described.[39]

Enzyme assays

Reductase and dehydrogenase activities were assayed spectrophotometrically at 30 °C, by monitoring the decrease or increase in the absorption of NADPH at 340 nm. The standard assay mixture (1 mL) for reductase consisted of 50 mM phosphate buffer (pH 7.0), 2.0 mM carbonyl substrate and 0.05 mM NADPH. The assay mixture (1 mL) for GDH activity was 50 mM phosphate buffer (pH 7.0), 2.0 mM glucose and 0.05 mM $NADP^+$. One unit of activity was defined as the amount of enzyme which catalyzes the oxidation of 1 μmol NADPH per minute (BYueD) or reduction of 1 μmol $NADP^+$ per minute (GDH). Kinetic parameters were determined by altering the concentration of various substrates at a constant NADPH concentration (0.05 mM) and calculated by Lineweaver–Burk plots.

Effect of pH and temperature on activity

The enzyme activity was estimated using a standard assay protocol with **1a** as substrate. The optimum pH value was determined within a pH range of 5.0–11.0, using citrate for a pH range from 5.0 to 6.0, phosphate for a pH range from 6.0 to 8.5 and Gly-NaOH for a pH range from 8.5 to 11.0. The optimum temperature was determined at different temperatures in the range of 20–60 °C. The stability was examined by incubating the purified enzymes in the same phosphate buffer (pH 7.0) at 30, 40 or 50 °C for a varied period of time and the residual activities were assayed as described above.

Enantioselectivity

The enantioselectivity was determined by examining the reduction of carbonyl substrates using an NADPH regeneration system consisting of the purified reductase and externally added glucose dehydrogenase (GDH). The reaction was carried out in a reaction mixture (0.4 mL) comprising 50 mM potassium phosphate buffer (pH 7.0), 10 mM keto-substrate, 0.2 U of the purified reductase, 0.4 U of GDH, 20 mM glucose and 0.5 mM $NADP^+$ with shaking for 12 h at 30 °C. After the reaction, each reaction mixture was extracted twice with ethyl acetate. The substrate conversion and product ee value were determined by GC analysis using a CP-Chirasil-DEX CB (Varian, USA) or HPLC analysis using a Chiralcel OD-H column (Daicel Co., Japan; 4.6 × 250 mm).

Coexpression of reductase and GDH genes

Construction of the expression vector pET20b-GDH was described by Zhang *et al.*[40] To construct plasmid pET-Y-G, the primers with 15 bases of sequence homology to linearized vectors were used for PCR with the GDH gene as the template. The

resulting fragment GDH gene was then cloned into the SalI/XhoI site of pET28a-BYueD using CloneEZ™ PCR Cloning Kit (GenScript, Nanjin, China). The resulting plasmids with reductase and GDH genes were transformed into *E. coli* BL21(DE3). To construct the two-plasmid system, *E. coli* BL21 (DE3) harboring pET20b-GDH was transformed with pET28a-BYueD and grown on an LB plate containing ampicillin (100 mg L^{-1}) and kanamycin (50 μg mL^{-1}). The transformation was confirmed by agarose gel electrophoresis and enzymatic activity assay.

Bioreduction of 1a to (*R*)-1b

Bioconversion of **1a** to (*R*)-**1b** was carried out by whole-cell reaction of *E. coli* harboring pET-Y-G. The reaction mixture consisted of 1.0 mmol potassium phosphate buffer (pH 7.0), 10.0 mmol of glucose, 10 μmol $NADP^+$, 0.5 g of dry cells in 10 mL of aqueous solution, to which **1a** (830 mg, 5.0 mmol; dissolved in 10 mL of toluene) was added. The two phase bioreaction was performed by magnetic agitation at 30 °C for 5 h. **1a** (5.0 and 3.0 mmol) and glucose (10.0 and 6.0 mmol) were supplemented to the reaction system at 1 h and 2 h. The pH was automatically adjusted to 6.8 by titrating 2 M Na_2CO_3. The reaction mixture was centrifuged (10 000 × *g* for 5 min) to promote the phase separation; the aqueous phase was saturated with NaCl and then extracted three times with ethyl acetate. The organic solution was combined, dried over anhydrous Na_2SO_4 and evaporated under vacuum. Chemical yield of the product was determined by GC analysis and the enantiomeric purity of (*R*)-**1b** was determined by chiral GC analysis after acetylation. (*R*)-**1b** was afforded as a colorless liquid, with a yield of 1.97 g (91.7%). $[\alpha]_D^{25}$: +22.3 (*c* 5.0, $CHCl_3$), 99.6% ee, (*R*) {lit.[41] $[\alpha]_D^{25}$: +20.1 (*c* 8.24, $CHCl_3$), 96% ee, (*R*)}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.29 (t, 3H, J = 7.1 Hz), 2.59–2.67 (m, 2H), 3.20 (br s, 1H), 3.59–3.64 (m, 2H), 4.19 (q, 2H, J = 7.1 Hz), 4.24–4.29 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 38.6, 48.2, 60.5, 67.9, 171.8.

Acknowledgements

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