ISSN 1330-9862
(FTB-3086)

*original scientific paper*

# A Novel *Microbacterium* sp. for Asymmetric Reduction of Simple Aromatic Ketone and Its Key Reductase

*Li Luo[1], Zhong-Hua Yang[1]*, Rong Zeng[2]*, Wei Zhou[1], Yan Zhao[1], Geng-Hua Chen[1] and Hao Huang[1]*

[1]College of Chemical Engineering and Technology, Wuhan University of Science and Technology, Wuhan 430081, PR China

[2]College of Chemistry and Chemical Engineering, Hubei University, Wuhan 430062, PR China

Received: August 8, 2012
Accepted: November 6, 2012

**Summary**

A novel strain, *Microbacterium* sp., which can be used as a catalyst in the asymmetric reduction of simple aromatic ketones to produce chiral aromatic alcohols, was isolated from soil by microbial screening. Using acetophenone as a model substrate for asymmetric reduction, 82 % reaction yield with more than 99 % enantiomeric excess of (*R*)-1-phenylethanol were achieved under the optimal conditions. Furthermore, the key reductase responsible for the asymmetric reduction was purified from the newly isolated *Microbacterium* sp. through $(NH_4)_2SO_4$ fractional precipitation, anion exchange chromatography and gel filtration chromatography. The purified reductase is a NADH-dependent aromatic ketone reductase. Gel filtration chromatography and SDS-PAGE results reveal that it is a homodimer with two 28-kDa subunits. The enzymatic properties of the reductase indicate that it is a highly efficient catalyst for the asymmetric reduction of simple aromatic ketone.

*Key words*: asymmetric reduction, *Microbacterium* sp., aromatic ketone reductase, acetophenone

## Introduction

Because of safety, therapeutical and regulatory concerns, there is an increasing interest in the development of a process capable of producing single-enantiomer pharmaceuticals (*1*). Usually, chiral building blocks, such as single-enantiomer alcohols and amines, are important intermediates in the construction of target chiral products. Due to the structural superiority, enantiopure aromatic alcohols are the key chiral building blocks for many important chiral pharmaceuticals, such as L-chlorpheniramine, *S*-fluoxetine, *R*-tomoxetine, *R*-denopamine, *etc.* (*2–6*). Asymmetric reduction of the corresponding prochiral simple aromatic ketones with chemical catalyst or biocatalyst is one of the most promising routes to the enantiopure aromatic alcohol production (*7,8*). Since 1980s, both biological and chemical methods have been developed to produce the chiral aromatic alcohols by this route (*9–11*). Compared with the chemical process, biological approach has many advantages, such as high enantioselectivity, mild reaction conditions and environmental compatibility (*9,12*).

Both whole-cell biocatalyst and isolated oxidoreductase from a microorganism are applied in this kind of biocatalytic asymmetric reduction. In recent research, more than 200 microbes, plants and microalgae have been reported for the production of various enantiopure alcohols through asymmetric reduction of the corresponding ketones (*13–15*). To discover the bioreduction reaction mechanism and evolve the key enzyme, many kinds of reductases have been purified from these microorganisms (*16–21*).

In our previous work, we devoted our efforts to exploring the asymmetric reduction of aromatic ketones and β-ketoesters by microalgae, plant tissue and yeast

*Corresponding author; Phone: ++86 27 8656 3448, ++86 27 6392 7637; E-mail: yangzh@wust.edu.cn, rongzengce@163.com

(*14,15,22*). We also developed techniques to improve the asymmetric reduction processes by introducing resin adsorption and organic solvent-aqueous biphasic system (*23–25*). Furthermore, screening and obtaining an excellent microbe strain is always the most important and essential way to improve the asymmetric reduction. In this paper, we will devote our efforts to isolation of a novel microbe which can reduce prochiral simple aromatic ketones to produce enantiopure aromatic alcohols with high stereospecificity. Acetophenone was used as the model substrate for the asymmetric reduction. To determine the mechanism of this asymmetric reduction catalyzed by the microbe, the key reductase which is responsible for the asymmetric reduction was purified from the newly isolated microbe. Furthermore, the enzymatic properties of this reductase were also evaluated.

## Materials and Methods

### *Materials and media*

Acetophenone (ACP) and benzaldehyde were purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, PR China), both of analytical grade. (*R*)-1-phenylethanol (*R*-PEA) and (*S*)-1-phenylethanol (*S*-PEA) were purchased from ACROS Organics (Thermo Fisher Scientific, Hoboken, NJ, USA), both of laboratory reagent grade. NADH disodium salt, dithiothreitol (DTT) and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade and commercially available. Materials for enzyme protein separation were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA).

The following media were used for microbe screening and cultivation: the medium used to enrich the microbe was composed of (in g/L): glucose 20.0, peptone 20.0, yeast extract 10.0, beef extract 30.0, $KH_2PO_4$ 2.0 and NaCl 1.0. Mineral salt medium (MSM) was used to screen the target microbe, and it was composed of (in g/L): $(NH_4)_2SO_4$ 2.0, $KH_2PO_4$ 2.0, NaCl 1.0 and $MgSO_4{\cdot}7H_2O$ 0.2. Fermentation medium was used to cultivate the isolated microbe, and it was composed of (in g/L): glucose 15.0, yeast extract 5.0, peptone 5.0, $KH_2PO_4$ 1.0, $K_2HPO_4$ 1.0, NaCl 1.0, $MgSO_4{\cdot}7H_2O$ 0.5 and $(NH_4)_2SO_4$ 2.0. All media were adjusted to pH=7.0 and autoclaved at 121 °C for 30 min. The corresponding solid media were prepared by supplementation with 2 % agar.

### *Screening the microbe for asymmetric reduction of ACP and microbe identification*

The microorganisms for asymmetric reduction of ACP were isolated from a soil sample collected from Wuhan Botanical Garden (30°32'52'' N, and 114°24'02'' E, Wuhan, PR China). Soil sample (2.0 g) was suspended in 20 mL of sterile distilled water. Then, 0.2 mL of the supernatant were inoculated into 50 mL of enrichment medium and incubated in an orbital shaker operating at 150 rpm and 30 °C for 24 h. The enrichment broth was inoculated into the MSM plate (containing 0–5 g/L of glucose and 10–15 mmol/L of ACP) for the isolation of target microbe. The plates and a 25-mL glass beaker containing 10 mL of ACP were sealed in a plastic bag and incubated at 30 °C for 2 days. Colonies that appeared on the plate were inoculated individually into slant culture media (with fermentation medium) and cultured in an incubator for 1 day at 30 °C. Then different ACP-utilizing microorganisms were obtained and their ability as catalysts in the asymmetric reduction of simple aromatic ketones was assayed.

Morphological and physiological analyses of the isolated microbe were done according to the Bergey's Manual of Determinative Bacteriology (*26*). A light microscope was used to observe its morphological features. Genomic DNA for molecular identification of the strain was extracted using the modified benzyl chloride method (*27*). In order to identify the isolated microbe, the 16S rDNA was cloned by PCR. The PCR amplification of 16S rDNA was carried out in 25 μL of reaction mixture containing 10× *Pfu* reaction buffer (with $Mg^{2+}$), 10 mmol/L of each deoxynucleoside triphosphate, 1.25 U of *Pfu* DNA polymerase (Tiangen Biotech, Beijing, PR China), 1.25 μmol/L of each forward primer (5'-GAGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-AAGGAGGTGATCCAGCC-3') and 50–100 ng of template DNA. The PCR reaction conditions were as follows: 5 min at 95 °C for initial denaturation, 1 min at 94 °C for denaturation, 1 min at 67 °C for annealing of DNA, 2 min at 72 °C for extension with total of 30 cycles of amplification and 10 min at 72 °C of final extension. The PCR-amplified products were purified by Gel Extraction kit (Omega Bio-Tek, Norcross, GA, USA) and sequenced by Sangon Biotech (Shanghai, PR China). The 16S rDNA was compared with sequences in nucleotide database (NCBI) using the BLAST algorithm (*28*). Multiple sequence alignment was carried out with CLUSTALW (Conway Institute UCD, Dublin, Ireland) multiple sequence alignment. The neighbour-joining phylogenetic analysis was constructed with MEGA v. 4.0 software (Center for Evolutionary Medicine and Informatics, The Biodesign Institute, Tempe, AZ, USA).

### *Microbial culture and asymmetric reduction*

To obtain plenty of cells for the biocatalysis of the asymmetric reduction and enzyme separation and purification, the microbe was aerobically cultured in a 7-litre fermentor (BIOSTAT®A plus, Sartorius AG, Goettingen, Germany) with 5 L of fermentation medium for 24 h. The fermentor was operated at 30 °C, 300 rpm and pH= 7.0. The cells were harvested by centrifugation (10 000×*g* for 10 min, at 4 °C), and were washed with normal saline. Finally, the resting cells were conserved at –86 °C for enzyme separation and bioreduction.

Properties of this isolated microorganism as a biocatalyst for asymmetric reduction of ACP were also studied. The resting cells were used to investigate the effects of reduction conditions, such as temperature, pH, substrate concentration, cosubstrate and reaction time on the asymmetric reduction of ACP.

### *Key ketone reductase separation and purification procedure*

The key ketone reductase, used for the asymmetric reduction of aromatic ketones, was separated and purified by cell-free extract, fractionation salting out, anion

exchange chromatography and gel filtration chromatography. The anion exchange chromatography and gel filtration were performed with an AKTA prime chromatography system (GE Healthcare Life Sciences). All separation and purification steps were carried out at 4 °C. Detailed information for the separation and purification procedure is given in the Supplementary material S1.

### *Determination of the enzyme molecular mass*

The molecular mass of the native enzyme was evaluated by gel filtration column chromatography using a Sephadex G-75 column with phosphate buffer containing 0.14 mol/L of NaCl as the mobile phase at a flow rate of 0.5 mL/min. The column was precalibrated with a standard protein ladder: bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), protease K (28.5 kDa) and lysozyme (14.4 kDa).

The molecular mass of the subunit of the purified enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with an unstained SDS-PAGE molecular mass ladder (composed of seven standard proteins from 14.4 to 116 kDa) as the standard.

### *Evaluation of enzymatic properties*

The following enzymatic properties of the purified ketone reductase were evaluated: cofactor requirement, the optimum pH and pH stability, the optimum temperature and thermal stability, effect of metal ions, and storage stability. The standard experimental procedure for the general evaluation of enzymatic properties was used.

### *Analysis of the reaction products*

The concentrations of ACP, *R*-PEA and *S*-PEA were determined by a gas chromatography (GC) with a β-cyclodextrin (Cyclodex B) chiral capillary chromatographic column. The GC analytical approach was the same as in our previous work (*14,15*). The reaction degree and the enantioselectivity were indicated by chemical yield and enantiomeric excess (e.e.), respectively, which were calculated as follows:

$$\text{Yield} = \frac{c_P}{c_O} \cdot 100 \qquad /1/$$

$$\text{e.e.} = \frac{c_R - c_S}{c_R + c_S} \cdot 100 \qquad /2/$$

where $c_O$ is initial substrate concentration, $c_P$ is final product concentration, $c_R$ is final *R*-PEA concentration, and $c_S$ is final *S*-PEA concentration.

### *Enzyme activity assay*

The aromatic ketone reductase activity of ACP was assayed using spectrophotometrical method (*29*) based on the reduction of one molecule of ACP coupled with oxidation of one molecule of NADH to $NAD^+$. NADH has a characteristic absorbance at 340 nm, therefore the decrease of the absorbance at 340 nm can represent the ketone reductase activity. The standard assay mixture contained 700 μL of phosphate buffer (0.1 mol/L, pH= 7.0), 5 mmol/L of ACP and 0.25 mmol/L of NADH. The mixture was preheated to 30 °C, and then the enzyme solution was added. The reaction mixture was monitored by absorbance decrease at 340 nm for 2 min with a spectrophotometer (Ultrospec 3300 Pro UV/VIS Spectrophotometer, GE Healthcare Life Sciences) using reaction kinetics application modules of SWIFT II software (GE Healthcare Life Sciences). One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADH to $NAD^+$ per minute, accompanied by the reduction of ACP. Specific activity was expressed as units per milligram of protein.

### *Protein content assay*

Protein content was estimated by Bradford protein--dye binding method using bovine serum albumin as the protein standard (*30*).

## Results and Discussion

### *Microbial screening and identification*

The purpose of screening procedure was to obtain a microbial strain that possessed satisfactory catalytic activity and excellent stereoselectivity. Also, it should tolerate high concentrations of the substrate. With the novel screening procedure, a total of 18 microbial strains were obtained, which were able to reduce ACP to the corresponding chiral alcohol. In terms of yield and e.e., strain no. 1–2 was the best for the asymmetric reduction of simple aromatic ketone. The reaction yield and e.e. were more than 72 and 90 %, respectively. The superior enantiomer of the product is *R*-PEA, which demonstrates that the asymmetric reaction catalyzed by the newly isolated strain follows anti-Prelog rule.

To identify strain no. 1–2, its 16S rDNA region was cloned by PCR and sequenced. The nucleotide blast analysis was conducted in NCBI (*28*) to find homologous sequences. Fourteen corresponding sequences of representative species were selected and used to construct an unrooted phylogenetic tree (using MEGA v. 4.0 software). The 16S rDNA sequence and the unrooted phylogenetic tree are given in Supplementary material S2 and S3. The results show that strain no. 1–2 is a *Microbacterium* sp. Furthermore, the morphological and physiological identification experiments also confirm that the strain no. 1–2 is a *Microbacterium* sp. according to Bergey's Manual of Determinative Bacteriology (*26*). The morphological, physiological and biochemical data are given in Supplementary material S4. The newly isolated strain, *Microbacterium* sp., was deposited in the China Center for Type Culture Collection (CCTCC) with CCTCC Accession No. M 2011449. The 16S rDNA sequence of the newly isolated *Microbacterium* sp. was deposited at the GenBank database with the Accession No. JQ080914.

### *Asymmetric reduction of ACP with Microbacterium sp. resting cells*

The reaction characteristics of asymmetric reduction of ACP catalyzed by the newly isolated *Microbacterium*

sp. were determined. The effects of substrate concentration, reaction time, pH, temperature and cosubstrate on the asymmetric reduction of ACP were investigated.

The results in Fig. 1 show that these factors markedly affected the reaction yield. In all cases, the stereoselectivity was outstanding, and the e.e. was almost 100 %. The optimal reduction conditions were: 30 °C, pH=5.0, 15 mmol/L of ACP, reaction time of 30 h and 2-propanol as the cosubstrate. With the optimal reduction conditions, 82 % reaction yield and >99 % e.e. of *R*-PEA were achieved. The reaction followed anti-Prelog rule, which indicated that the novel *Microbacterium* sp. was an efficient biocatalyst for the asymmetric reduction of prochiral simple aromatic ketones. In previous report, *Microbacterium* sp. MA 5614 was able to produce MK-0476 (*S*)-hydroxy ester by asymmetric reduction of the corresponding keto ester (*31*). This indicates that *Microbacterium* sp. has a broad substrate range.

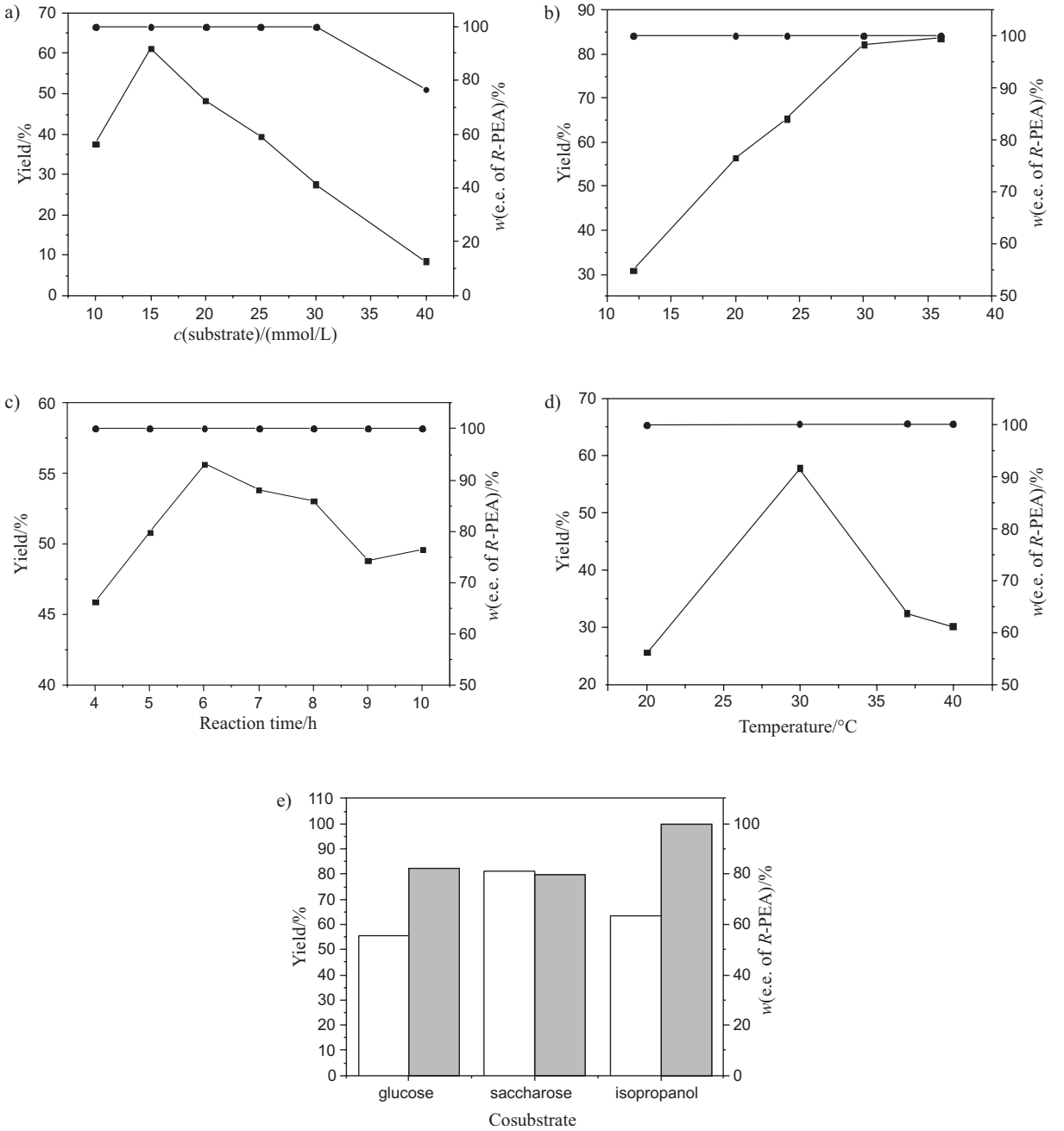



**Fig. 1.** Reaction characteristics (yield and enantiomeric excess (e.e.) of (*R*)-1-phenylethanol (*R*-PEA)) of asymmetric reduction of acetophenone (ACP) catalyzed by the newly isolated *Microbacterium* sp.: a) the effect of substrate concentration on the asymmetric reduction (reaction conditions: 30 °C, potassium phosphate buffer (KPB) 20 mL, pH=7.0, resting cells 2 g, reaction time 24 h); b) the effect of reaction time on the asymmetric reduction (reaction conditions: 30 °C, KPB 20 mL, pH=7.0, resting cells 2 g, ACP 15 mmol/L); c) the effect of pH on the asymmetric reduction (reaction conditions: 30 °C, resting cells 2 g, reaction time 24 h, ACP 15 mmol/L); d) the effect of temperature on the asymmetric reduction (reaction conditions: KPB 20 mL, pH=7.0, resting cells 2 g, reaction 24 h, ACP 15 mmol/L); e) the efect of cosubstrate on the asymmetric reduction (reaction conditions: 30 °C, KPB buffer 20 mL, pH=7.0, resting cells 2 g, ACP 15 mmol/L, cosubstrate 10 g/L, reaction time 24 h). ■: yield, ● e.e. for a, b, c, d and □: yield, ▨ : e.e. for e

### *Purification of the ketone reductase from Microbacterium sp.*

The reductase responsible for the asymmetric reduction of simple aromatic ketones was purified from the newly isolated *Microbacterium* sp. With the three complementary separation steps (fractional precipitation, anion exchange chromatography and gel filtration chromatography), it was purified 87.5-fold to a SDS-PAGE purity with a 29.9 % yield. The results of each separation step are given in Table 1. The SDS-PAGE of the purified ketone reductase is shown in Fig. 2.

### *Molecular mass of the enzyme*

The molecular mass of the whole native aromatic ketone reductase is about 61.9 kDa as determined by gel filtration chromatography in comparison with the elution volume of standard proteins. The gel filtration chromatography data are given in Supplementary material S5. The molecular mass of the subunit is 28 kDa as determined by SDS-PAGE and presented in Fig. 2. According to the results of gel filtration chromatography and SDS-PAGE, it could be deduced that the *Microbacterium* sp. aromatic ketone reductase is a homodimer with two 28-kDa subunits. This result coincides with the ACP reductase from *Geotrichum candidum* NBRC 4597 (*17*), which consists of two 36-kDa identical subunits. In general, dehydrogenases and reductases are low-molecular-mass monomeric enzymes (*32*). However, many reported reductases consist of two subunits, such as carbonyl reductase from *C. parapsilosis* DSM 70125, which comprises two 67-kDa identical subunits (*33*), and heterodimeric carbonyl reductase from *Geotrichum capitatum* JCM 3908, which comprises a 39- and 41-kDa subunits (*20*).

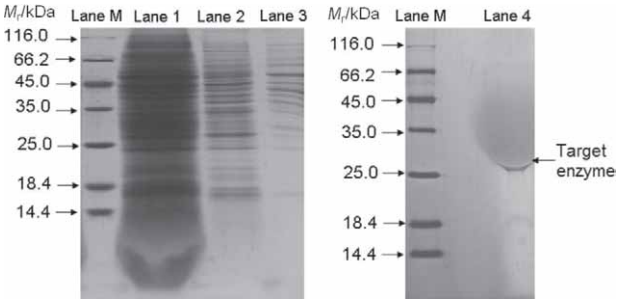



**Fig. 2.** SDS-PAGE of the purified aromatic ketone reductase from the newly isolated *Microbacterium* sp. Lane M: standard protein ladder ($M_r$/kDa), β-galactosidase (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase (35.0), REase Bsp 981 (25.0), β-lactoglobulin (18.4 kDa), lysozyme (14.4); lane 1: cell-free extract; lane 2: $(NH_4)_2SO_4$ fractional precipitation; lane 3: DEAE Sepharose FF ion exchange chromatography; lane 4: Sephadex G-75 gel filtration chromatography. Gel was stained with 0.25 % Coomassie Brilliant Blue R-250

### *Enzymatic properties*

#### Coenzyme requirement

The coenzyme requirement for the purified reductase showed that the specific enzyme activity was about 16.3 U/mg with NADH as the coenzyme, whereas its activity was null with NADPH as the coenzyme. These results clearly indicate that the aromatic ketone reductase from *Microbacterium* sp. is NADH-dependent. The coenzyme requirement of other reported ketone reductases from various microorganisms were compared and listed in Table 2 (*16*,*17*,*32*,*34*–*38*). The results clearly show that the specific activity of the purified ketone reduc-

Table 1. Summary of the purification steps of the aromatic ketone reductase from *Microbacterium* sp.

| Purification step | *m*(protein) | Activity | Specific activity | Yield | Purification |
|---|---|---|---|---|---|
| | mg | U | U/mg | % | fold |
| Cell-free extract | 56.9 | 11.7 | 0.2 | 100 | 1.00 |
| $(NH_4)_2SO_4$ fractional precipitation | 3.2 | 10.5 | 3.3 | 89.7 | 16.5 |
| DEAE Sepharose FF ion exchange chromatography | 2.0 | 6.9 | 3.5 | 59.0 | 17.5 |
| Sephadex G-75 gel filtration chromatography | 0.2 | 3.5 | 17.5 | 29.9 | 87.5 |

Table 2. Specific activities of ketone reductases from different microorganisms

| Microorganism | Substrate | Coenzyme | Specific activity | Reference |
|---|---|---|---|---|
| | | | U/mg | |
| *Microbacterium* sp. | ACP | NADH | 16.23 | this study |
| *Candida krusei* SW 2026 | OPBE | NADPH | 12.6 | (*16*) |
| *Geotrichum candidum* | 1-acetonaphthone | NADH | 590 | (*32*) |
| *Geotrichum candidum* NBRC 4597 | ACP | NADH | 40.1 | (*17*) |
| *Candida viswanathii* MTCC 5158 | ACP | NADH | 358.58 | (*34*) |
| *Williopsis saturnus* var. *mrakii* AJ-5620 | HPPO | NADPH | 0.89 | (*35*) |
| *Sporobolomyces salmonicolor* AKU 4429 | COBE | NADPH | 10.4 | (*36*) |
| *C. parapsilosis* ATCC 28474 | β-hydroxyacetophenone | NADH | 14 | (*37*) |
| *C. macedoniensis* AKU 4588 | quinones | NADPH | 131 | (*38*) |

ACP=acetophenone, OPBE=ethyl 2-oxo-4-phenylbutyrate, HPPO=3-hydroxy-1-phenylpropane-1-one, COBE=ethyl 4-chloroacetoacetate

tases from different microorganisms lies in a broad range from 0.89–590 U/mg. The disparity in the activity is due to their different microorganism source or protein structure.

Effect of pH

The effect of pH on the purified reductase activity is given in Fig. 3a with pH ranges from 4.0–9.0. In a broad pH range, from 6.0 to 9.0, this reductase showed good activity. The optimum pH was 7.0. However, the reductase activity rapidly decreased with pH decrease from 6.0 to 4.0. The optimum pH zone of the enzyme in a neutral pH was also observed for other carbonyl reductases from *Geotrichum capitatum* JCM 3908 (*20*) and *Candida magnolia* KFCC 11023 (*39*).

The pH stability of purified reductase was determined in a pH range from 3.0 to 9.0. The results are presented in Fig. 3b. It shows that the reductase was stable in pH= 6.0–8.0. The residual activity was able to keep more than 90 % in this pH range. The reductase stability sharply decreased when pH was lower than 6.0 or higher than 8.0, which proves that the reductase was sensitive to pH. A similar trend of the stability profile of reductases was observed with other reported carbonyl reductases such as those from *Candida viswanathii* MTCC 5158 (*34*) and *Rhodococcus ruber* DSM 44541 (*40*), which showed good stability at pH=6.5–7.0 and pH=6.0–7.0, respectively.

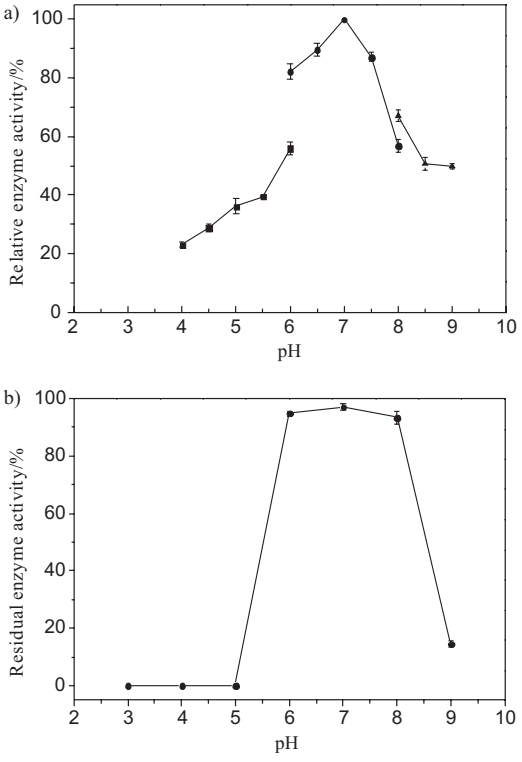



**Fig. 3.** The effect of pH on the aromatic ketone reductase isolated from the *Microbacterium* sp.: a) the effect of pH on the enzyme activity, ■ *c*(acetate buffer)=0.1 mmol/L, ● *c*(phosphate buffer)=0.1 mmol/L, ▲ *c*(Tris–HCl)=0.1 mmol/L (the maximum activity in the tested buffers was taken as 100 %, and relative activities of other pH values were expressed as its percentage); b) the effect of pH on the enzyme stability (the activity without preincubation in different pH buffers was taken as 100 %, and the residual activities under corresponding pH were expressed as its percentage)

Effect of temperature

The effect of temperature on the reductase activity was investigated by measuring the enzyme activity at different temperatures (from 20 to 60 °C). The results are given in Fig. 4a. The optimum temperature was 40 °C, at which the reductase showed the highest enzymatic activity. Its activity rapidly increased with the increase of the reaction temperature from 20 to 40 °C. Further increase of the reaction temperature resulted in the decrease of reductase activity. Regarding the optimum temperature of the purified aromatic ketone reductase, it was similar to the carbonyl reductase from *Rhodococcus erythropolis* DSM 743 (*41*). However, it was different from the carbonyl reductases from *Geotrichum candidum* NCIM 980 (*32*), *Candida viswanathii* MTCC 5158 (*34*) and *Rhodococcus ruber* DSM 44541 (*40*); their optimum temperatures are about 50 to 65 °C. On the other hand, carbonyl reductases from *Candida krusei* SW 2026 (*16*) and *Geotrichum capitatum* JCM 3908 (*20*) showed lower optimum temperature, about 30 °C.

The thermal stability of the reductase was also evaluated. The preincubation temperature range was from 10 to 60 °C. The results in Fig. 4b demonstrate that this reductase is thermally stable. Although it was preincubated at 60 °C for 1 h, it still retained about 80 % residual activity. The good thermal stability indicated that this reductase possessed a good prospect for practical application.

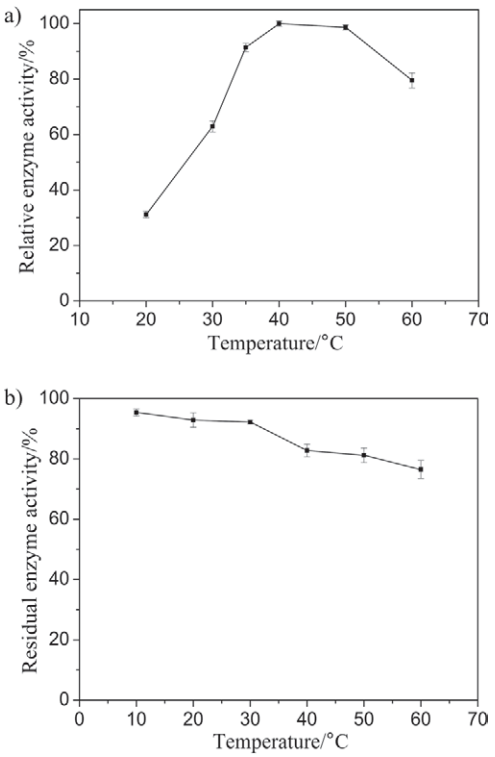



**Fig. 4.** The effect of temperature on the aromatic ketone reductase isolated from the *Microbacterium* sp.: a) the effect of temperature on the enzyme activity (the maximum activity at the tested temperatures was taken as 100 %, and relative activities of other temperatures were expressed as its percentage); b) the effect of temperature on the enzyme stability (the activity without heat preincubation was taken as 100 %, and residual activities under corresponding temperature were expressed as its percentage)

Effect of metal ions

$Cu^{2+}$, $Ca^{2+}$, $Fe^{2+}$, $Zn^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ were used to evaluate the effect of metal ions on the aromatic ketone reductase from *Microbacterium* sp. Also, EDTA was used to chelate the possible metal ion in the solution in order to find the effect of the absence of trace metal ions.

The experimental results (Table 3) show that $Fe^{2+}$ could significantly improve the activity of the reductase, more than 30 %, while $Cu^{2+}$ could remarkably inhibit the reductase activity, even cause a total loss of its activity. Generally, ketone reductases have thiol group at the catalytic site. $Cu^{2+}$ is a thiol-specific ion, and it can bind with the group to deactivate its activity (*32*). With the addition of EDTA, the relative activity was just about 85 %, which proved that the reductase from *Microbacterium* sp. needed the metal ions to perform its highest activity.

Table 3. Effect of metal ions and EDTA on the aromatic ketone reductase activity

| Metal ion | Relative activity/% |
|---|---|
| blank | 100 |
| $Cu^{2+}$ | 0 |
| $Ca^{2+}$ | 108 |
| $Fe^{2+}$ | 132 |
| $Zn^{2+}$ | 99.2 |
| $Mg^{2+}$ | 106 |
| $Mn^{2+}$ | 94.7 |
| EDTA | 85.9 |

*c*(metal ion)=1 mmol/L. The activity without metal ions was taken as 100 %. Relative activity was expressed as its percentage

Storage stability

Stability of the reductase from *Microbacterium* sp. was also measured. The results in Fig. 5 show that the enzyme possessed good storage stability. After 13.5 days at 4 °C, it still retained more than 80 % residual activity.

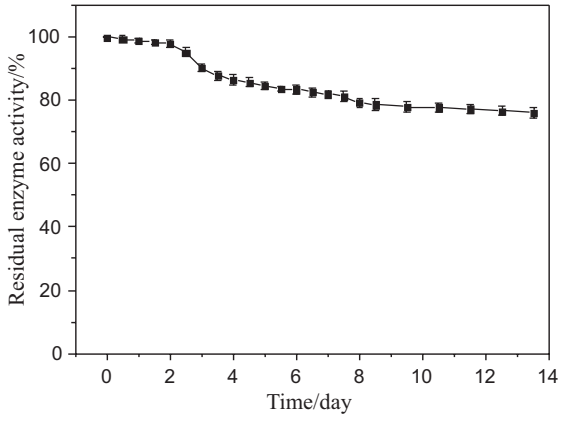



**Fig. 5.** Storage stability of the aromatic ketone reductase enzyme isolated from the *Microbacterium* sp. (the activity without storage was taken as 100 %, and residual activity was expressed as its percentage)

## Conclusion

A novel *Microbacterium* sp. was isolated that can catalyze the asymmetric reduction of prochiral simple aromatic ketones with high catalytic activity and excellent enantioselectivity. Using acetophenone as a model substrate for asymmetric reduction, the reaction yield of 82 % and e.e. of 99 % were obtained under the optimal reaction conditions. The key reductase, which was responsible for the reduction, was purified from the newly isolated *Microbacterium* sp. It is a 61.9-kDa NADH-dependent dimeric reductase. The enzymatic properties show that it is an excellent reductase for the asymmetric reduction of simple aromatic ketones. Based on these findings, it can be concluded that this newly obtained microbe has a good prospect for practical application.

*Acknowledgements*

This work was partially supported by the Foundation of Educational Commission of Hubei Province of PR China (grant no. D20121108), the National Natural Science Foundation of China (grant no. 21376184), the open fund of Research Center of Green Manufacturing and Energy-Saving & Emission Reduction Technology at Wuhan University of Science and Technology (grant no. B1013) and the Key Youth Researcher fund of Wuhan University of Science and Technology (grant no. 2012XZ012), PR China. We are grateful to Ms Yu Wang, Mr Xiao-Hu Wang and Mr Xu Chang for their technical assistance.

## References

1. A.M. Rouhi, Chiral business, *Chem. Eng. News*, *81* (2003) 45–56.
2. J. Mangas-Sánchez, M. Rodrígez-Mata, E. Busto, V. Gotor-Fernández, V. Gotor, Chemoenzymatic synthesis of rivastigmine based on lipase-catalyzed processes, *J. Org. Chem. 15* (2009) 5304–5310.
3. J.A.R. Rodrigues, P.J.S. Moran, G.J.A. Conceição, L.C. Fardelone, Recent advances in the biocatalytic asymmetric reduction of acetophenones and α,β-unsaturated carbonyl compounds, *Food Technol. Biotechnol. 42* (2004) 295–303.
4. T. Rosen, C.H. Heathcock, Synthetic and biological studies of compactin and related compounds. 4. Total synthesis of (+)-compactin, *J. Am. Chem. Soc. 107* (1985) 3731–3733.
5. S. Shimizu, M. Kataoka, K. Kita, Chiral alcohol synthesis with yeast carbonyl reductases, *J. Mol. Catal. B: Enzym. 5* (1998) 321–325.
6. Y.L. Zhang, J. Liao, D.M. Chen, H.T. Shun, Asymmetric synthesis of phenylethanolamines, *Chem. Res. Appl. 5* (1999) 480–483 (in Chinese).
7. W. Kroutil, H. Mang, K. Edegger, K. Faber, Recent advances in the biocatalytic reduction of ketones and oxidation of *sec*-alcohols, *Curr. Opin. Chem. Biol. 8* (2004) 120–126.
8. T. Matsuda, R. Yamanaka, K. Nakamura, Recent progress in biocatalysis for asymmetric oxidation and reduction, *Tetrahedron: Asymmetry, 20* (2009) 513–557.
9. Y. Ni, J.H. Xu, Asymmetric reduction of aryl ketones with a new isolate *Rhodotorula* sp. AS2.2241, *J. Mol. Catal. B: Enzym. 18* (2002) 233–241.
10. R.V. Malyala, C.V. Rode, M. Arai, S.G. Hegde, R.V. Chaudhari, Activity, selectivity and stability of Ni and bimetallic Ni–Pt supported on zeolite Y catalysts for hydrogenation of acetophenone and its substituted derivatives, *Appl. Catal. A: Gen. 193* (2000) 71–86.

*11.* K. Molvinger, M. Lopez, J. Court, Asymmetric reduction and hydrogenation over heterogenous catalysts prepared by reacting nickel-boride with norephedrine, *J. Mol. Catal. A: Chem. 150* (1999) 267–273.

*12.* S. Zeror, J. Collin, J.C. Fiaud, L.A. Zouioueche, Enantioselective ketoester reductions in water: A comparison between microorganism- and ruthenium-catalyzed reactions, *Tetrahedron: Asymmetry, 21* (2010) 1211–1215.

*13.* K. Nakamura, R. Yamanaka, T. Matsuda, T. Harada, Recent developments in asymmetric reduction of ketones with biocatalysts, *Tetrahedron: Asymmetry, 14* (2003) 2659–2681.

*14.* Z.H. Yang, L. Luo, X. Chang, W. Zhou, G.H. Chen, Y. Zhao, Y.J. Wang, Production of chiral alcohols from prochiral ketones by microalgal photo-biocatalysis asymmetric reduction reaction, *J. Ind. Microbiol. Biotechnol. 39* (2012) 835–841.

*15.* Z.H. Yang, R. Zeng, G. Yang, Y. Wang, L.Z. Li, Z.S. Lv *et al.*, Asymmetric reduction of prochiral ketones to chiral alcohols catalyzed by plants tissue, *J. Ind. Microbiol. Biotechnol. 35* (2008) 1047–1051.

*16.* N. Li, Y. Ni, Z. Sun, Purification and characterization of carbonyl reductase from *Candida krusei* SW 2026 involved in enantioselective reduction of ethyl 2-oxo-4-phenylbutyrate, *J. Mol. Catal. B: Enzym. 66* (2010) 190–197.

*17.* Y. Nakata, T. Fukae, R. Kanamori, S. Kanamaru, T. Matsuda, Purification and characterization of acetophenone reductase with excellent enantioselectivity from *Geotrichum candidum* NBRC 4597, *Appl. Microbiol. Biotechnol. 86* (2010) 625–631.

*18.* N. Itoh, R. Morihama, J. Wang, K. Okada, N. Mizuguchi, Purification and characterization of phenylacetaldehyde reductase from a styrene-assimilating *Corynebacterium* strain, ST-10, *Appl. Environ. Microbiol. 63* (1997) 3783–3788.

*19.* K. Inoue, Y. Makino, N. Itoh, Purification and characterization of a novel alcohol dehydrogenase from *Leifsonia* sp. strain S749: A promising biocatalyst for an asymmetric hydrogen transfer bioreduction, *Appl. Environ. Microbiol. 71* (2005) 3633–3641.

*20.* K. Yamada-Onodera, M. Fukui, Y. Tani, Purification and characterization of alcohol dehydrogenase reducing *N*-benzyl-3-pyrrolidinone from *Geotrichum capitatum*, *J. Biosci. Bioeng. 103* (2007) 174–178.

*21.* K. Yamada-Onodera, A. Nakajima, Y. Tani, Purification, characterization, and gene cloning of glycerol dehydrogenase from *Hansenula ofunaensis*, and its expression for production of optically active diol, *J. Biosci. Bioeng. 102* (2006) 545–551.

*22.* Z.H. Yang, S.J. Yao, D.Q. Lin, Improving the stereoselectivity of asymmetric reduction of 3-oxo ester to 3-hydroxy ester with pretreatments on bakers' yeast, *Ind. Eng. Chem. Res. 43* (2004) 4871–4875.

*23.* Z.H. Yang, S.J. Yao, Y.X. Guan, A complex process of asymmetric synthesis of β-hydroxy ester by baker's yeast accompanied by resin adsorption, *Ind. Eng. Chem. Res. 44* (2005) 5411–5416.

*24.* Z.H. Yang, R. Zeng, X. Chang, X.K. Li, G.H. Wang, Toxicity of aromatic ketone to yeast cell and improvement of the asymmetric reduction of aromatic ketone catalyzed by yeast cell with the introduction of resin adsorption, *Food Technol. Biotechnol. 46* (2008) 322–327.

*25.* Z.H. Yang, R. Zeng, Y. Wang, W. Li, Z.S. Lv, A complex process of the asymmetric reduction of prochiral aromatic ketone by yeast cell with the introduction of an organic solvent as the separation medium, *Asia-Pacif. J. Chem. Eng. 3* (2008) 217–222.

*26.* R.E. Buchanan, N.E. Gibbons: *Bergey's Manual of Determinative Bacteriology*, Science Press, Beijing, PR China (1984) (in Chinese).

*27.* S.J. Xue, T.L. Yue, J. Guan, Y.H. Yuan, Z.P. Gao, An improved method for extracting fungal DNA, *Food Res. Dev. 27* (2006) 39–40.

*28.* BLAST®, National Center for Biotechnology Information (NCBI), Bethesda, MD, USA (*http://www.ncbi.nlm.nih.gov/*).

*29.* C.S. Badal, Purification and characterization of novel mannitol dehydrogenases from *Lactobacillus intermedius*, *Biotechnol. Progr. 20* (2004) 537–542.

*30.* M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72* (1976) 248–254.

*31.* C. Roberge, A. King, V. Pecore, R. Greasham, M. Chartrain, Asymmetric bioreduction of a keto ester to its corresponding (*S*)-hydroxy ester by *Microbacterium* sp. MB 5614, *J. Ferment. Bioeng. 81* (1996) 530–533.

*32.* A. Singh, M.S. Bhattacharyya, U.C. Banerjee, Purification and characterization of carbonyl reductase from *Geotrichum candidum*, *Process Biochem. 44* (2009) 986–991.

*33.* J. Peters, T. Minuth, M.R. Kula, A novel NADH-dependent carbonyl reductase with an extremely broad substrate range from *Candida parapsilosis*: Purification and characterization, *Enzyme Microb. Technol. 15* (1993) 950–958.

*34.* P. Soni, H. Kansal, U.C. Banerjee, Purification and characterization of an enantioselective carbonyl reductase from *Candida viswanathii* MTCC 5158, *Process Biochem. 42* (2007) 1632–1640.

*35.* I. Kira, N. Onishi, Purification and characterization of a (*S*)-1-phenyl-1,3-propanediol-producing enzyme from *Williopsis saturnus* var. *mrakii* AJ-5620, *J. Biosci. Bioeng. 107* (2009) 116–118.

*36.* K. Kita, K.I. Nakase, H. Yanase, M. Kataoka, S. Shimizu, Purification and characterization of new aldehyde reductases from *Sporobolomyces salmonicolor* AKU4429, *J. Mol. Catal. B: Enzym. 6* (1999) 305–313.

*37.* Y. Nie, Y. Xu, M. Yang, X.Q. Mu, A novel NADH-dependent carbonyl reductase with unusual stereoselectivity for (*R*)-specific reduction from an (*S*)-1-phenyl-1,2-etanediol--producing micro-organism: Purification and characterization, *Lett. Appl. Microbiol. 44* (2007) 555–562.

*38.* N. Kataoka, Y. Doi, T. S. Sim, S. Shimizu, H. Yamada, A novel NADPH-dependent carbonyl reductase of *Candida macedoniensis*: Purification and characterization, *Arch. Biochem. Biophys. 294* (1992) 469–474.

*39.* J.K. Lee, S.Y. Kim, Y.W. Ryu, J.H. Seo, J.H. Kim, Purification and characterization of a novel erythrose reductase from *Candida magnolia*, *Appl. Environ. Microbiol. 7* (2003) 3710–3718.

*40.* B. Kosjek, W. Stampfer, M. Pogorevc, W. Goessler, K. Faber, W. Kroutil, Purification and characterization of a chemotolerant alcohol dehydrogenase applicable to coupled redox reactions, *Biotechnol. Bioeng. 86* (2004) 55–62.

*41.* T. Zelinski, J. Peters, M.R. Kula, Purification and characterization of a novel carbonyl reductase isolated from *Rhodococcus erythropolis*, *J. Biotechnol. 33* (1994) 283–292.

# Supplementary material

## S1. Aromatic ketone reductase separation and purification procedure

Step 1. *Preparation of cell-free extract*. Washed cells (6.0 g) were suspended in 10-mL buffer A (20 mM, pH=7.0, phosphate buffer) with 1 mM DTT and 1 mM PMSF. The cells were disrupted by ultrasonication (200–240 W, pulse 4 s, pause 7 s) for 30 min. Cell debris was removed by centrifugation (10 000×*g* for 25 min) at 4 °C. Then the clear supernatant, crude enzyme solution designated as cell-free extract (CFE) was obtained.

Step 2. *Ammonium sulphate fractional precipitation*. Initially, ammonium sulphate was added to the CFE to a final content of 50 % saturation and the mixture was settled at 4 °C for 1 h. The precipitate was removed by centrifugation (10 000×*g* for 25 min). The clear supernatant was subjected to increased concentration of ammonium sulphate to 70 % saturation. The solution was further settled for 1 h at 4 °C. The resulting precipitate was collected by centrifugation (10 000×*g* for 25 min). Then it was resuspended in buffer A and dialyzed against the same buffer containing 1 mM DTT at 4 °C overnight.

Step 3. *Anion exchange chromatography*. The dialyzed fraction was filtered through a 0.45-μm micropore membrane, and loaded on a DEAE Sepharose Fast Flow column (1.6×20 cm, GE Healthcare Life Sciences) prebalanced with buffer A. The column was washed with buffer A. Then the enzyme was eluted with a linear gradient of NaCl (from 0 to 0.8 M) in the same buffer with a flow rate of 1 mL/min. Fractions containing aromatic ketone reductase activity were collected and concentrated with polyethylene glycol. The concentrated protein solution was dialyzed against buffer A containing 1 mM DTT at 4 °C overnight.

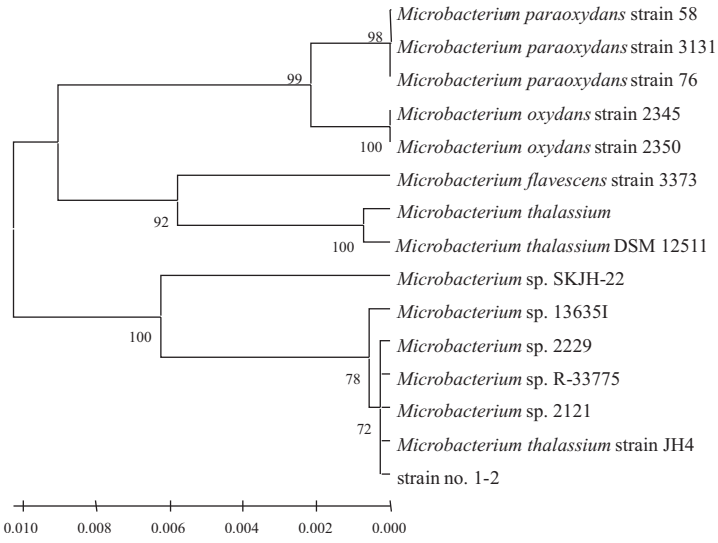
Step 4. *Gel filtration chromatography*. The dialyzed solution was then loaded on Sephadex G-75 column (1.6×70 cm). The column was balanced and eluted with buffer A mixed in 0.14 M NaCl until the basic line was plain. The active fraction was collected. It was a purified enzyme solution, which was used for the following enzyme characterization experiment.

## S2. 16S rDNA sequence of strain no. 1–2

```
1    CCGAAAGGGG GCGTGCCTA C CATGCAAGTC GAACGGT GAA GCAGAGCTTG    50
51   CTCTGTGGAT CAGTG GCGAA CGGGTGAGGA ACA CGTGAGC AACCTGCCCT   100
101  GGACTCTGGG ATAAGCGCTG GAAACGGCGT CTAATACTGG ATACGAGACG   150
151  TGGCCGCATG GTCAACGTTT GGAAAGATTT TTTGGTTCAG GATGGGCTCG   200
201  CGGCCTATCA GCTTGTTGGT GAGGTAATGG CTCACCAAGG CGTCGACGGG   250
251  TAGCCGGCCT GAGAGGGTGA CCGGCCACAC TGGGACTGAG ACACGGCCCA   300
301  GACTCCTACG GGAGGCAGCA GTGGGGAATA TTGCACAATG GGCGAAAGCC   350
351  TGATGCAGCA ACGCCGCGTG AGGGATGACG GCCTTCGGGT TGTAAACCTC   400
401  TTTTAGCAAG GAAGAAGCGA AAGTGACGGT ACTTGCAGAA AAAGCGCCGG   450
451  CTAACTACGT GCCAGCAGCC GCGGTAATAC GTAGGGCGCA AGCGTTATCC   500
501  GGAATTATTG GGCGTAAAGA GCTCGTAGGC GGTTTGTCGC GTCTGCTGTG   550
551  AAAACTGGAG GCTCAACCTC CAGCCTGCAG TGGGTACGGG CAGACTAGAG   600
601  TGCGGTAGGG GAGATTGGAA TTCCTGGTGT AGCGGTGGAA TGCGCAGATA   650
651  TCAGGAGGAA CACCGATGGC GAAGGCAGAT CTCTGGGCCG TAACTGACGC   700
701  TGAGGAGCGA AAGGGTGGGG AGCAAACAGG CTTAGATACC CTGGTAGTCC   750
751  ACCCCGTAAA CGTTGGGAAC TAGTTGTGGG GACCATTCCA CGGTTTCCGT   800
801  GACGCAGCTA ACGCATTAAG TTCCCCGCCT GGGGAGTACG GCCGCAAGGC   850
851  TAAAACTCAA AGGAATTGAC GGGGACCCGC ACAAGCGGCG GAGCATGCGG   900
901  ATTAATTCGA TGCAACGCGA AGAACCTTAC CAAGGCTTGA CATATACGAG   950
951  AACGCTGCAG AAATGTAGAA CTCTTTGGAC ACTCGTATAC AGGTGGTGCA 1000
1001 TGGTTGTCGT CAGCTCGTGT CGTGAGATGT TGGGTTAAGT CCCGCAACGA 1050
1051 GCGCAACCCT CGTTCTATGT TGCCAGCACG TAATGGTGGG AACTCATGGG 1100
1101 ATACTGCCGG GGTCAACTCG GAGGAAGGTG GGGATGACGT CAAATCATCA 1150
1151 TGCCCCTTAT GTCTTGGGCT TCACGCATGC TACAATGGCC GGTACAAAGG 1200
1201 GCTGCAATAC CGTGAGGTGG AGCGAATCCC AAAAAGCCGG TCCCAGTTCG 1250
1251 GATTGAGGTC TGCAACTCGA CCTCATGAAG TCGGAGTCGC TAGTAATCGC 1300
1301 AGATCAGCAA CGCTGCGGTG AATACGTTCC CGGGTCTTGT ACACACCGCC 1350
1351 CGTCAAGTCA TGAAAGTCGG TAACACCTGA AGCCGGTGGC CTAACCCTTG 1400
1401 TGGAGGGAGC CGTCGAAGGT GGGATCGGTA ATTAGGACTA AGTCGAACAA 1450
1451 GTGCCCCCC
```

### S3. Unrooted phylogenetic tree

The unrooted phylogenetic tree was constructed based on the BLAST results from NCBI with the 16S rDNA sequences (*28*)




### S4. Morphological, physiological and biochemical analyses of strain no. 1–2

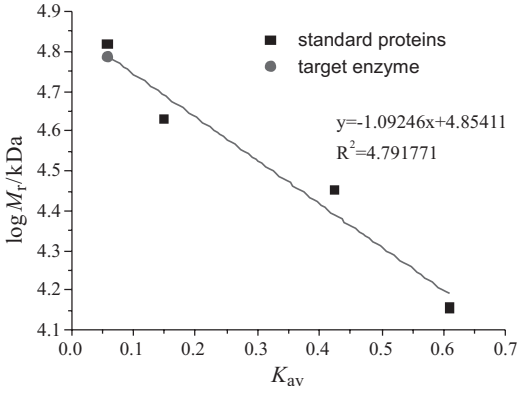
Single colony of strain no. 1–2

Physiological and biochemical characteristics of the strain no. 1–2

| Test | Result | Test | Result |
|---|---|---|---|
| gelatin liquefaction | – | D-glucose | ++ |
| milk solidification | + | D-fructose | + |
| milk peptonization | – | sucrose | + |
| starch hydrolysis | – | D-xylose | ++ |
| cellulose hydrolysis | – | D-mannitol | – |
| nitrate reduced | – | L-inositol | – |
| hydrogen sulphide produced | – | L-arabinose | – |
| pigment production using tyrosine | – | L- rhamnose | – |

The morphological and physiological analyses were conducted according to the Bergey's Manual of Determinative Bacteriology (*26*)
–=negative reaction, + and ++=positive reaction; the more +, the stronger the reaction degree

**S5. Standard curve of gel filtration chromatography to determine the ketone reductase molecular mass**




$M_r$=molecular mass, $K_{av}=(V_e-V_0)/(V_t-V_0)$, $V_e$=elution volume, $V_0$=void volume, $V_t$=total liquid volume
standard proteins: bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), protease K (28.5 kDa), lysozyme (14.4 kDa)