BIOCATALYSIS

Isolation and characterization of a novel *Rhodococcus* strain with switchable carbonyl reductase and *para*-acetylphenol hydroxylase activities

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Abstract In the search for an effective biocatalyst for the reduction of acetophenones with unprotected hydroxy group on the benzene ring, a microorganism, which reduced *para*-acetylphenol to *S*-(−)-1-(*para*-hydroxyphenyl)ethanol under anaerobic conditions, was isolated from soil samples and the 16S rDNA study showed that it was phylogenetically affiliated with species of the genus *Rhodococcus* and was most similar to *Rhodococcus pyridinivorans*. Unexpectedly, this strain also hydroxylated *para*-acetylphenol to give 4-acetylcatechol in presence of oxygen, possessing *para*-acetylphenol hydroxylase activity. While the reduction of *para*-acetylphenol had an optimal reaction pH at 7 and a broad optimal temperature range (35–45 °C), the hydroxylation reached the maximum conversion at the pH range of 7–8 and 35 °C. This study identified for the first time a *Rhodococcus* strain with *para*-acetylphenol hydroxylase activity, which also contains highly enantioselective carbonyl reductase activity with potential applications for the asymmetric reduction of these less-explored but important ketones such as α-aminoacetophenone, 3′-hydroxyacetophenone and 4′-hydroxyacetophenone. The *para*-acetylphenol hydroxylase and carbonyl reductase activity are switchable by the reaction conditions.

Keywords Phenol hydroxylase · Carbonyl reductase · *Rhodococcus* · Hydroxylation · Bioreduction

Abbreviations

Denopamine	[(±)-α,α-(3,4-dimethoxyphenethylaminomethyl)-4-hydroxybenzyl alcohol]
Salmeterol	[(±)-1,3-benzenedimethanol-4-hydroxy-α-[((6-(4-phenylbutoxy)hexyl)-amino)methyl]
MSM	Mineral salt medium
NRM	Nutrient-rich medium
TLC	Thin-layer chromatography
e.e.	Enantiomeric excess
HPLC	High-performance liquid chromatography

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Introduction

Denopamine [(±)-α,α-(3,4-dimethoxyphenethylaminomethyl)-4-hydroxybenzyl alcohol] and Salmeterol [(±)-1,3-benzenedimethanol-4-hydroxy-α-[((6-(4-phenylbutoxy)hexyl)-amino)methyl] [1, 7, 16] are very important β-adrenoceptor agonists marketed as racemates, but the (*R*)-enantiomers are the active components and more desired products [28]. As such, efficient methods have been sought for the synthesis of optically pure (*R*)-Denopamine and

(R)-salmeterol [2–4, 11, 24, 35]. In all these studies, the key step is the synthesis of chiral (4′-hydroxyphenyl)ethanol or (4′-hydroxy-3′-hydroxymethylphenyl)ethanol. The straightforward approach to the synthesis of these chiral alcohol building blocks is the reduction of the corresponding ketones such as *para*-haloacetylphenol and 3′-haloacetylsalicylaldehyde. Although biocatalytic reduction offers advantages such as high enantioselectivity and environmentally benign reaction conditions [44], the efficiency of bioreduction of acetophenones with unprotected hydroxy group on the benzene ring is still far from the requirement for practical use [15, 25, 30, 37]. Furthermore, we are aiming to develop a concise approach to synthesize (R)-salmeterol by converting 3′-haloacetylsalicylaldehyde to (R)-(−)-2-bromo-1-(4′-hydroxy-3′- hydroxymethylphenyl)ethanol, in which both the aldehyde and ketone groups are reduced (Scheme 1). Therefore, we initiated a project to search for both aldehyde reductase and ketoreductase from natural resources. Microorganisms are believed to be rich in all kinds of enzymes with diverse activities [8, 10]. For this purpose, salicylaldehyde and *para*-acetylphenol were used as substrates in the initial screening study. A *Rhodococcus* sp. 1-0130 strain, which could reduce *para*-acetylphenol to S-(−)-1-(*para*-hydroxyphenyl)ethanol, was isolated. Interestingly, this strain also could transform *para*-acetylphenol to give a hydroxylated product under different conditions, thus offering an opportunity to introduce a hydroxyl group on the aromatic ring and a chiral alcohol functionality in the same molecule just by switching the reaction conditions. Herein we report the isolation and characterization of this strain and the studies on its hydroxylase activity and carbonyl reductase activity.

Materials and methods

Media and chemicals

Mineral salt medium (MSM, pH 7.0) contains 1.0 g of $K_2HPO_4 \cdot 3H_2O$, 1.0 g of $Na_2HPO_4 \cdot 3H_2O$, 2.0 g of $NH_4H_2PO_4$, 2.0 g of $NaNO_3$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 1.0 mg of $FeSO_4 \cdot 7H_2O$ and 0.1 mg of $ZnSO_4$ in 1 l of distilled water. Nutrient-rich medium (NRM, pH 7.0) contains 10.0 g of glucose, 5.0 g of
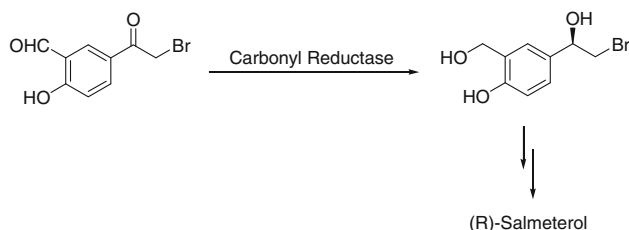
peptone, 5.0 g of yeast extract, 1.0 g of $KH_2PO_4 \cdot 12H_2O$, 1.0 g of $K_2HPO_4 \cdot 3H_2O$, 1.0 g of NaCl and 0.5 g of $MgSO_4 \cdot 7H_2O$ in 1 l of distilled water. For cell growth on plates, MSM or NRM with 1.5 % (w/v) agar was used. *para*-Acetylphenol and other chemicals were purchased from commercial source (Alfa Aesar). Restriction enzymes, TaqMix DNA polymerase, and their corresponding buffers were purchased from TaKaRa.

Isolation of microorganisms

Soil samples collected from orchards, farmland, and unearthed ecological areas in several regions of China were used to screen for strains with carbonyl reductase activity. Each soil sample (5 g) was suspended in 50 ml of sterilized water in a 250-ml Erlenmeyer flask with a number of glass beads. The flask was shaken at 30 °C and 200 rpm for 10 min and rested for 10 min, and then 1 ml of the supernatant was transferred into 50 ml of sterilized MSN in a 250-ml flask. Different concentrations of substrate salicylaldehyde (0.5–10 mmol l^{-1}) and *para*-acetylphenol (1–10 mmol l^{-1}) was separately mixed with the MSM for enrichment culture without any other carbon and energy sources at 30 °C and 200 rpm. After 2–5 days, 1 ml of the culture liquid was added into a fresh medium with the same composition for another round of enrichment culture until the start of the microbial growth (about 2–5 days), 0.2 ml of the culture were sprayed onto the MSM agar plates with substrate (5 mmol l^{-1} of *para*-acetylphenol). Moreover, salicylaldehyde was supplied in vapor state by placing the plates and a 25-ml beaker filled with salicylaldehyde in a sealed plastic bag [32]. The plates were placed in an incubator at 30 °C and monocolonies were individually inoculated into NRM agar slopes. The isolated strains were inoculated in 20 ml of sterilized NRM in a 100-ml flask which was constantly shaken for 24–48 h, and then the appropriate amount of substrates (5 mmol l^{-1}) was added for transformation reaction. The reaction mixtures were extracted with ethyl acetate (1:1 v/v) and analyzed by thin-layer chromatography (TLC). The samples with an obvious product spot were further analyzed by HPLC for determination of conversion and enantiomeric excess (e.e.). The strain with high conversion and giving single product was selected and labeled as 1-0130.

16S rDNA sequence analysis and physiological characteristics

Genomic DNA of strain 1-0130 was extracted using the Tiangen bacteria genomic DNA isolation kit (Tiangen Biotech CO., Ltd. Beijing). The 16S rDNA was amplified by PCR (94 °C for 5 min, one cycle; 94 °C for 30 s/52 °C for 30 s/72 °C for 90 s, 30 cycles; 72 °C for 10 min, one

Scheme 1 A concise approach to the synthesis of (R)-salmeterol

cycle), universal primers (F: 5′-GAGTTTGATCCTGGCT CAG-3′, R: 5′-GTACCTTGTTACGACTT-3′; 0.5 µmol l^{-1} each). For the identification of the microorganism, the 16S rDNA gene was isolated and sequenced, and the obtained sequence was submitted to GenBank on NCBI for BLAST search. The 16S rDNA sequences were aligned and the phylogenetic tree was constructed by using MEGA version 4.0 [41].

The physiological characterization was carried out using physiological and biochemical kit, and Bergey's manual was used as the reference to determine the characteristics of strain 1-0130. The strain was inoculated on NRM agar (30 °C culture) and its phenotypic features were observed for the next 5 days.

Cultivation conditions and preparation of resting cells

The strain was inoculated in 20 ml of NRM for 20 h and 10 ml of the culture liquid (inoculum size 1:50) were transferred into 500 ml of NRM, which was constantly shaken at 30 °C and 200 rpm for 24 h. The cells were collected by centrifugation (6,750 × g, 10 min) and washed twice with 0.1 mol l^{-1} potassium phosphate buffer (pH 7.0). The cells were re-suspended in 0.1 mol l^{-1} potassium phosphate buffer (pH 7.0) and stored at 4 °C.

Characterization of the hydroxylating product 1-(3,4-dihydroxyphenyl)ethanone

para-Acetylphenol (68 mg, 5 mmol l^{-1}) and glucose (0.3 g, 3 g l^{-1}) were added into a suspension of resting cells (4 g, wet weight) in 100 ml of potassium phosphate buffer (0.1 mmol l^{-1}, pH 8.0). The hydroxylation reaction was carried out in the air at 30 °C by shaking the flask at 220 rpm. After incubation for 5 h, TLC analysis indicated that the substrate was consumed, and the mixture was centrifuged at 6,750 × g for 10 min. The supernatant was saturated with NaCl and then extracted three times with ethyl acetate or methyl *tert*-butyl ether. The cells were soaked in ethyl acetate. The combined organic solution was dried over anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by silica gel chromatography with eluent (petroleum ether/ethyl acetate, 4:1, v/v), giving 1-(3,4-dihydroxyphenyl)ethanone (52.4 mg, 69 % yield) [21]. ^{1}H NMR (CDCl$_3$) δ: 2.55 (s, 3H, CH$_3$), 6.07 (br, 1H), 6.15(br, 1H), 6.92 (d, 1H,), 7.50 (d, 1H), 7.60 (d, 1H).

Characterization of the reduction product *S*-(−)-1-(*para*-hydroxyphenyl)ethanol

para-Acetylphenol (68 mg, 5 mmol l^{-1}) and glucose(0.3 g, 3 g l^{-1}) were added to a suspension of resting

cells (6 g, wet weight) in 100 ml of potassium phosphate buffer (0.1 mol l^{-1}, pH 7.0). The reduction reaction was carried out under anaerobic conditions (nitrogen) at 30 °C and pH was controlled at 7.0. After incubation for 24 h, the mixture was centrifuged at 6,750 × g for 10 min. The supernatant was saturated with NaCl and then extracted three times with ethyl acetate or methyl *tert*-butyl ether. The cells were soaked in ethyl acetate. The combined organic solution was dried over anhydrous sodium sulfate and concentrated under vacuum. The crude product was purified by silica gel chromatography with eluent (petroleum ether/ethyl acetate, 4:1, v/v) to afford *S*-(−)-1-(*para*-hydroxyphenyl)ethanol (40 mg, 58 % yield, 95 % ee) [26, 27]. ^{1}H NMR (CDCl$_3$) δ: 1.37 (d, 3H, CH$_3$), 4.69 (q, 1H), 6.70 (m, 2H), 7.15 (m, 2H). $[\alpha]_D^{25} = -40°$ (c = 0.875, MeOH), lit. $[\alpha]_D = -47.5°$ (c = 5.0, MeOH) for *S*-enantiomer [27].

Determination of the growth curve and enzyme activity curves for hydroxylase and carbonyl reductase

The strain was inoculated in 20 ml of NRM for 20 h and 4 ml of the culture liquid (inoculum size 1:50) was transferred into a flask containing 200 ml of NRM, which was constantly shaken at 30 °C and 200 rpm, the OD value of the cell culture was measured at an interval of 4 h.

Similarly, the strain cells were cultured to a different growth period and the resting cells were prepared as described above. The hydroxylation (substrate concentration: 5 mmol l^{-1}, 40 g l^{-1} wet weight cells) was carried out at 30 °C and 220 rpm for 5 h. The reduction (substrate concentration: 5 mmol l^{-1}, 60 g l^{-1} wet weight cells) was carried out at 30 °C for 24 h under nitrogen. The conversion was determined by HPLC analysis.

pH and temperature dependence of strain 1-0130 for hydroxylation and reduction by resting cells

To evaluate the pH dependence of strain 1-0130 for biotransformation of *para*-acetylphenol, the reaction was carried out at various pHs using 0.1 mol l^{-1} buffers (citric acid/Na$_2$HPO$_4$ for pH 4–6, Na$_2$HPO$_4$/NaH$_2$PO$_4$ for pH 6–8, Tris–HCl for pH 8–9, Glycine/NaOH for pH 9-10). For hydroxylation, the resting cell (40 g l^{-1}, wet cells weight), glucose (3 g l^{-1}) and *para*-acetylphenol (5 mmol l^{-1}) were added into the buffer. For reduction, the resting cell (60 g l^{-1}, wet cells weight), glucose (2 g l^{-1}) and *para*-acetylphenol (5 mmol l^{-1}) were added into the buffer. The reaction and analysis were carried out as described above.

To determine the optimum temperature for biotransformation, the reaction was carried out at different temperatures from 25 to 60 °C. The reaction and analysis were carried out as described above.

Effects of glucose and metal ions on hydroxylation and reduction

For hydroxylation, the resting cell (40 g l^{-1}, wet cells weight) and *para*-acetylphenol (5 mmol l^{-1}) were mixed with different concentration of glucose (0–100 g l^{-1}) or different metal ions such as Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2} (1 mM) in the potassium phosphate buffer (0.1 mol l^{-1}, pH 7.0). For reduction, the resting cell (60 g l^{-1}, wet cells weight) and *para*-acetylphenol (5 mmol l^{-1}) were mixed with different concentration of glucose (0-10 g l^{-1}) or different metal ions such as Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2} (1 mM) in the potassium phosphate buffer (0.1 mmol l^{-1}, pH 7.0). The reaction and analysis were carried out as described above.

Substrate profile for the hydroxylation and reduction

To determine the hydroxylating and reducing ability of strain 1-0130, some *para*-substituted phenol derivatives and various substituted acetophenone derivatives were used as substrates to carry out the hydroxylation and reduction reaction by resting cells as described above.

Analyses

Thin-layer chromatography analysis (TLC) was carried out by the ascending method using silica gel 60 F254 plates. The solvent system was petroleum ether/ethyl acetate (2:1 v/v) for 1-(3,4-dihydroxyphenyl)ethanone and (*S*)-1-(*para*-hydroxyphenyl)ethanol. 1-(3,4-dihydroxyphenyl)ethanone was visualized under UV light (254 nm) and FeCl$_3$ color developing reagent. (*S*)-1-(*para*-hydroxyphenyl)ethanol was visualized by phosphato-molybdic acid color developing reagent.

The HPLC analysis was performed on an Agilent 1200 high-performance liquid chromatography system with Agilent Eclipse XDB-C18 column (4.6 × 150 mm) or Chiralcel OD-H column (4.6 × 250 mm) (for chiral analysis). A mixture of water and methanol (40/60, v/v) was used as eluent for Eclipse XDB-C18 column (4.6 × 150 mm) and a mixture of isopropanol and *n*-hexane (10/90, v/v) was used as eluent for OD-H column (4.6 × 250 mm). The corresponding alcohols were either purchased from commercial sources or prepared by the reduction of the corresponding ketones using sodium borohydride in methanol.

The ^{1}H-NMR spectra were obtained in deuterated chloroform (CDCl$_3$) using a Bruker Avance 600 spectrometer (600 MHz). The chemical shifts were reported in ppm with tetramethylsilane as the internal reference. The absolute configuration of *S* -(−)-1-(*para*-hydroxyphenyl)ethanol was determined by comparison of the sign of optical rotation with the literature data [27].

Results

Strain identification, and phenotypic and biochemical properties

In the screening study, an isolated strain (1-0130) was found to convert *para*-acetylphenol to a product with similar R$_f$ as the reduction product 1-(*para*-hydroxyphenyl)ethanol, but further HPLC analysis showed that it was not 1-(*para*-hydroxyphenyl)ethanol. The product was then isolated and characterized as 1-(3,4-dihydroxyphenyl)ethanone by ^{1}H NMR analysis, indicating that strain 1-0130 had hydroxylase activity toward *para*-acetylphenol. At this point, we were wondering what would happen when the biotransformation was performed anaerobically (under nitrogen). Surprisingly, another product was obtained under anaerobic conditions and characterized as the reduction product, 1-(*para*-hydroxyphenyl)ethanol. As such, strain 1-0130 exhibited *para*-acetylphenol hydroxylase or carbonyl reductase activity under aerobic or anaerobic conditions, respectively.
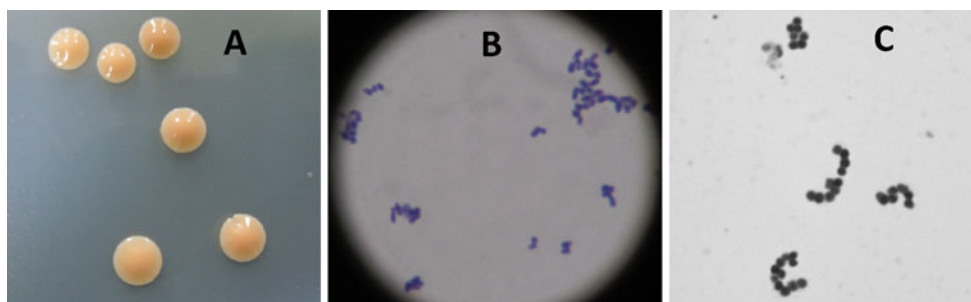
Cells of strain 1-0130 grown on nutrient-rich medium (NRM) culture were short rhabditiform and each cell was divided into a few coccoid-shaped isolates at the later stage (Fig. 1). Gram staining was positive. Colonies were circular, moist, and the color was transformed from soft pink to orange-red after 2–5 days at 30 °C. Strain 1-0130 could grow on sugars such as D-glucose, D-galactose, D-maltose, D-fructose, D-xylose, D-sorbitol, L-mannose, lactose, and sucrose, but not on L-sorbose. Sugar fermentation tests were negative in all the cases.

The phylogenetic position of strain 1-0130 was determined using its 16 s rRNA gene sequence. Accordingly, this strain belongs to the genus *Rhodococcus* with *R. pyridinivorans* as its closest neighbor (Fig. 2). Strain 1-0130 was deposited into the China General Microbiological Culture Collection as *Rhodococcus* sp. and assigned Accession # CGMCC 1.12281.

Growth and enzyme activity curves, effects of glucose, metal ions, pH, and temperature

The growth curve of *Rhodococcus* sp. 1-0130 is shown in Fig. 3. The enzyme activity at different phase of microorganism growth was measured based on the conversion rate as described in the "Materials and methods" section. For both reduction and hydroxylation, the enzyme activity of the resting cells increased at the early stage of the cell growth and reached the maximum activity at logarithmic growth phase. After that, the enzyme activity decreased and then stabilized at a certain level (Fig. 4).

In the hydroxylation reaction of *para*-acetylphenol, glucose addition greatly affected the enzyme activity

Fig. 1 Morphological characteristics of strain 1-0130 grown on nutrient-rich medium at 30 °C. **a** The colonial morphology of strain 1-0130. **b** The thalli was short rhabditiform at the early stage. **c** Each cell was divided into many coccoid-shaped isolates at later growth stage

Fig. 2 Phylogenetic tree of strain 1-0130 and its related micro-organisms. The 16S rDNA gene sequence of strain 1-0130 was aligned with those strains in *Rhodococcus* species collected at NCBI as well as *R. pyridinivorans* using molecular evolutionary genetics analysis (MEGA) software version 4.0 [41]. A phylogenetic tree was constructed using the neighbor-joining method. The *numbers on the tree* indicate the percentages of interior branch test sampling, derived from 1,000 replications
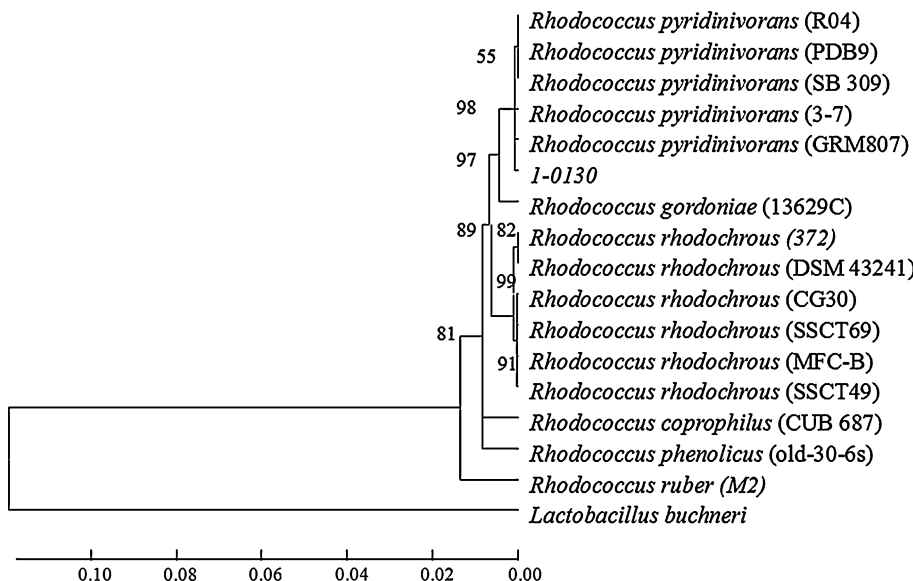
(Fig. 5). With 1 g l^{-1} glucose in the reaction system, the conversion increased more than threefold. The enzyme activity increased as more glucose was added into the reaction system, and the conversion was complete in 5 h with 3 g l^{-1} of glucose. The glucose concentration had no effect on hydroxylation reaction beyond 3 g l^{-1}. For the reduction of *para*-acetylphenol, the glucose enhanced the reduction rate with less than 2 g l^{-1} of glucose. The addition of 3 g l^{-1} of glucose into the reaction decreased enzyme activity (Fig. 5). Therefore, the optimal initial glucose concentration was 2 g l^{-1} for the reduction of *para*-acetylphenol with *Rhodococcus* sp. 1-0130.
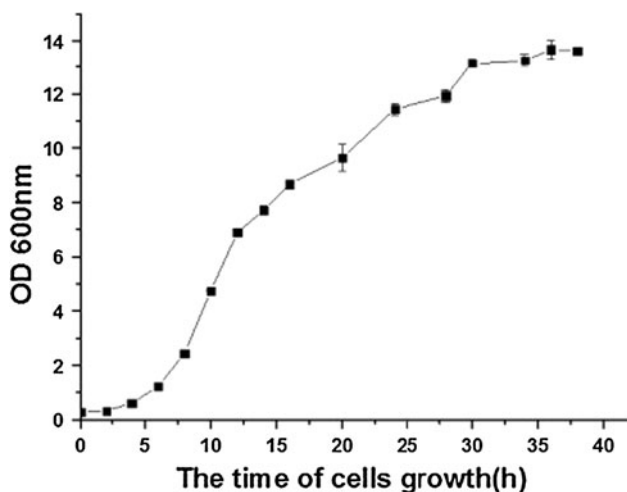
The effects of initial pH and temperature on the hydroxylation and reduction of *para*-Acetylphenol by *Rhodococcus* sp. 1-0130 were studied, and the results are presented in Figs. 6, 7. As shown in Fig. 6, for hydroxylation, the reaction reached maximum conversion at the pH range of 7–8, but no activity was observed with pH below 5 or above 9. The optimal reaction temperature for hydroxylation of *para*-acetylphenol by *Rhodococcus* sp. 1-0130 was 35 °C (Fig. 7).

An unusual pH dependence was observed for the reduction of *para*-acetylphenol by *Rhodococcus* sp. 1-0130. As shown in Fig. 6, the reduction of *para*-acetylphenol had an optimal reaction pH at 7 with a broad pH range, but a surprisingly high activity was observed for pH being 4 and 5. A possible reason for this unusual behavior of *Rhodococcus* sp. 1-0130 for the reduction of *para*-acetylphenol is that the strain may promote the metabolism of *para*-acetylphenol via other pathways, because lower yield was obtained for the reduction product under acidic conditions (data not shown). *Rhodococcus* sp. 1-0130 exhibited a broad optimal temperature range (35–45 °C) for the reduction of *para*-acetylphenol (Fig. 7).
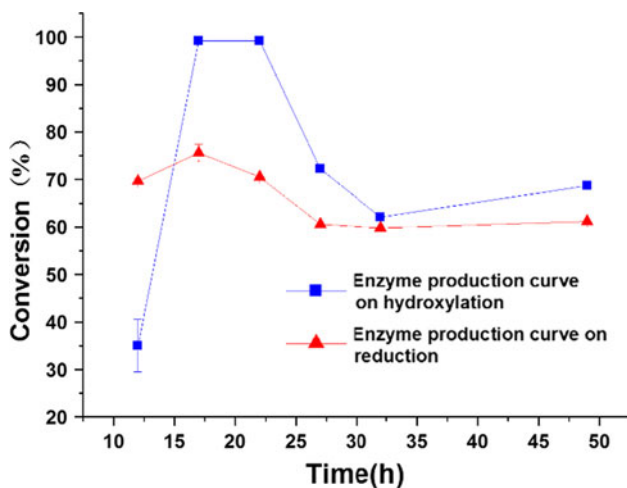
As shown in Fig. 8, metal ions such as Cu^{2+}, Ni^{2+} and Co^{2+} strongly inhibited the hydroxylase activity, while Cu^{2+}, and Ni^{2+} reduced the activity for the reduction of *para*-acetylphenol. The chelating reagents such as EDTA did not inhibit the hydroxylase and carbonyl reductase activities obviously at a concentration of 1 mM. This indicates that metal ions are not essential for the manifestation of activities (Fig. 8).

Hydroxylation of various substituted phenols

To investigate the substrate profile for the hydroxylase activity of *Rhodococcus* sp. 1-0130, various substituted phenols were studied under aerobic conditions, and the results are summarized in Table 1. *Rhodococcus* sp. 1-0130 hydroxylated 4-cyanophenol to give *para*-cyanocatechol, but showed little or no activity toward 4-nitrophenol, 4-chlorophenol, and 4-methylphenol. This indicated that the substituent on phenol exert great effect on the hydroxylase activity of *Rhodococcus* sp. 1-0130. Under the

same conditions, 4′-methoxyacetophenone was oxidized to 4′-methoxybenzoic acid, suggesting that *Rhodococcus* sp. 1-0130 also exhibited Baeyer–Villiger monooxygenase activity [14].

Bioreduction of substituted acetophenone derivatives

To further evaluate the carbonyl reductase activity of *Rhodococcus* sp. 1-0130, other substituted acetophenone


Fig. 3 Growth curve of strain 1-0130. The strain was cultivated in nutrient-rich medium at the 30 °C and 200 rpm. The values were the average of three replicates
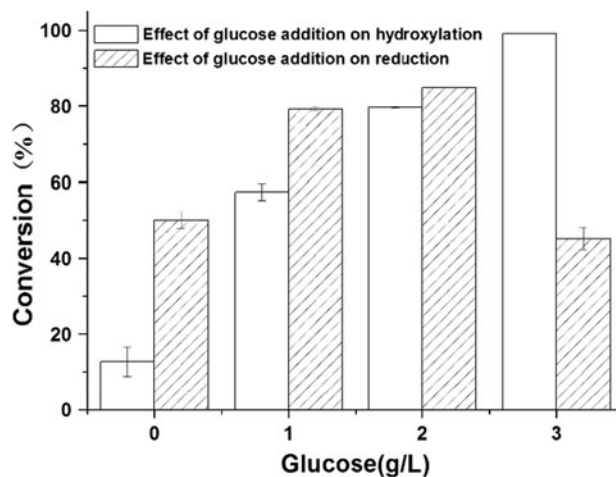
Fig. 5 Effect of glucose addition on hydroxylation and reduction of *para*-acetylphenol: (*open square*) the reaction of hydroxylation(40 g l^{-1}, wet cells weight, 30 °C and 220 rpm for 5 h, pH 8.0; substrate concentration 5 mmol l^{-1}); (*right to left arrow*) the reaction of reduction (60 g l^{-1}, wet cells weight, 30 °C in nitrogen for 24 h, pH 7.0; substrate concentration 5 mmol l^{-1}). The values were the average of three replicates

Fig. 4 Enzyme production curve of *Rhodococcus* sp. 1-0130: (*filled square*) the curve of hydroxylation (40 g l^{-1}, wet cells weight, 30 °C and 220 rpm for 5 h, pH 8.0; substrate concentration 5 mmol l^{-1}); (*filled triangle*) the curve of reduction(60 g l^{-1}, wet cells weight, 30 °C in nitrogen for 24 h, pH 7.0; substrate concentration 5 mmol l^{-1}). The values were the average of three replicates
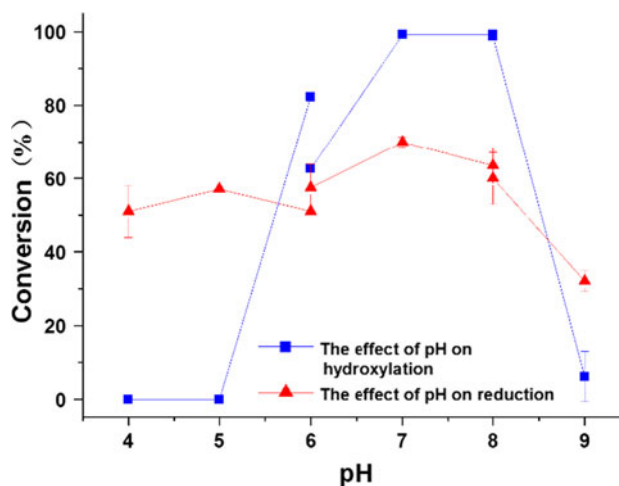
Fig. 6 Effect of pH on hydroxylation and reduction of *para*-acetylphenol by *Rhodococcus* sp. 1-0130; various pHs using 0.1 mol l^{-1} buffers (citric acid/Na$_2$HPO$_4$ for pH 4–6, Na$_2$HPO$_4$/NaH$_2$PO$_4$ for pH 6–8, Tris–HCl for pH 8–9, Glycine/NaOH for pH 9–10): (*filled square*) pH dependence of hydroxylation; (*filled triangle*) pH dependence of reduction. The values were the average of three replicates
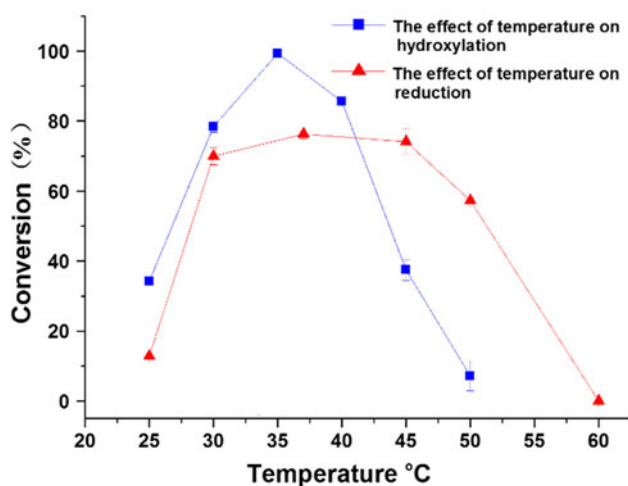
Fig. 7 Effect of temperature on hydroxylation and reduction of *para*-acetylphenol by *Rhodococcus* sp. 1-0130; different temperatures from 25 to 60 °C: (*filled square*) temperature dependence of hydroxylation; (*filled triangle*) temperature dependence of reduction. The values were the average of three replicates
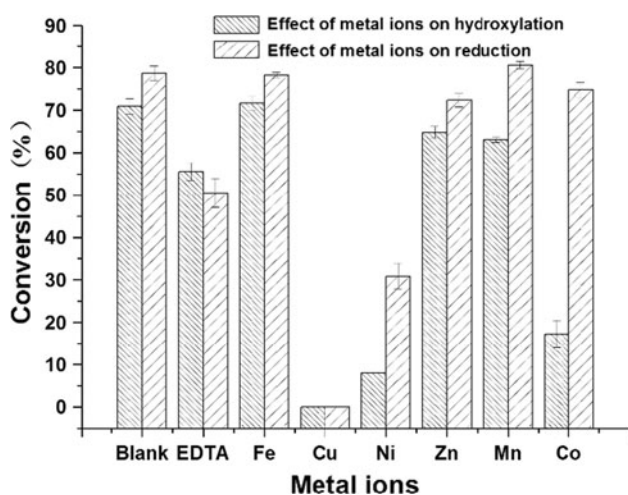
Fig. 8 Effect of metal ions on hydroxylation and reduction; different metals such as 1 mM of Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^2 (the adding chelating reagents such as EDTA (1 mM) and no adding metal ions were as controlled experiment) (*left to right arrow*) effect on hydroxylation; (*right to left arrow*) effect on reduction. The values were the average of three replicates

stereoselectivity of their reduction. For example, while α-aminoacetophenone and α-hydroxyacetophenone were reduced to (*R*)-2-amino-1-phenylethanol and (*R*)-2-hydroxy-1-phenylethanol with e.e. being 98 % and >99 %, respectively, (*R*)-2-chloro-1-phenylethanol and (*S*)-2-cyano-1-phenylethanol were obtained in e.e. of only 61 % and 68 % for the reduction of α-chloroacetophenone and α-cyanoacetophenone by *Rhodococcus* sp. 1-0130. For the diketone substrate, 1-phenylbutane-1,3-dione, only the 3-keto group was reduced to give (*S*)-1-phenyl-3-hydroxybutanone with 98 % e.e.

Discussion

It has been reported that the initial steps of *para*-acetylphenol mineralization comprise a Baeyer–Villiger oxidation, in which *para*-acetylphenol is converted to 4-hydroxybenzoate [14, 29] or 4-hydroxyphenyl acetate [13, 19, 20, 36, 42]. 4-Hydroxybenzoate is then hydrolyzed to form 3,4-dihydroxybenzoate, which enters the β-ketoadipate pathway via the ring cleavage [17]. 4-Hydroxyphenyl acetate is subsequently hydrolyzed to hydroquinone (1,4-dihydroxybenzene) as reported for *Pseudomonas fluorescens* ACB [13, 29], *Pseudomonas putida* JD1 [5], and *Aspergillus fumigatus* ATCC 28282 [18]. Although many microorganisms utilize aromatic hydroxylases to initiate the degradation of phenolic compounds [6, 12], microbial hydroxylation of *para*-acetylphenol to *para*-acetylcatechol has only reported for *Pseudomonas putida* [33]. In this study, a genus *Rhodococcus* with *para*-acetylphenol hydroxylase and carbonyl reductase activity was identified by using *para*-acetylphenol as the carbon source in enrichment and screening. The isolated *Rhodococcus* sp. 1-0130 could catalyze hydroxylation of *para*-acetylphenol to form *para*-acetylcatechol under aerobic conditions, suggesting that catabolism of *para*-acetylphenol may also occur via initial hydroxylation of benzene ring in genus *Rhodococcus*.

Para-phenol hydroxylase activity has been reported in *Rhodococcus* sp. for *para*-nitrophenol and *para*-halophenols [9, 39, 40, 47]. Recently, a phenol hydroxylase from *Rhodococcus*, which catalyzed the conversion of phenol to catechol, was cloned and characterized for the first time, but no information about its activity toward phenol analogues is revealed [38]. In this study, *Rhodococcus* sp. 1-0130 was found to possess *para*-acetylphenol hydroxylase activity. It is interesting to note that methylation of the hydroxy group of *para*-acetylphenol played an critical role for the activity and could switch the oxidation from phenyl hydroxylation to Baeyer–Villiger oxidation of ketone group. This suggests the diversity of oxygenases in this newly isolated strain.

substrates were subjected to the bioreduction under the same conditions as those for *para*-acetylphenol reduction (Table 2). The results showed that *Rhodococcus* sp. 1-0130 reduced all the tested acetophenone derivatives by following "Prelog's rule" [34]. Acetophenone and its derivatives with substituent on the benzene ring were reduced to afford the corresponding (*S*)-configured alcohols with high e.e., although the substituent affected the activity and enantioselectivity to some extent. The substituent at the α-position of acetophenone exerts larger effect on the

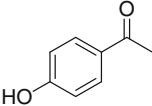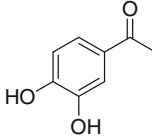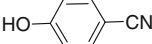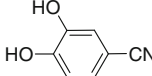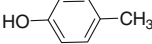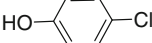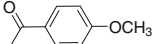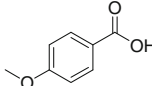**Table 1** Hydroxylation of various substituted phenols

Substrate	Product	Conversion (%)[a]
4-acetylphenol		>99
4-cyanophenol		25
4-nitrophenol	[b]	<5
4-methylphenol	–	[c]
4-chlorophenol	–	[c]
4′-Methoxyacetophenone		>99
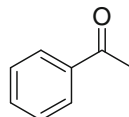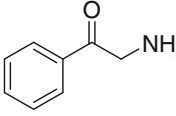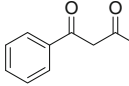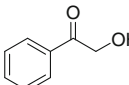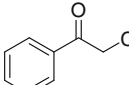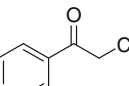
[a] The conversions were determined by HPLC analysis

[b] Since the conversion was too low, the product was not identified

[c] No conversion was observed

Table 2 Bioreduction of various substituted acetophenones by *Rhodococcus* sp. 1-0130

Acetophenone	Conversion (%)[a]	e.e. (%)[b]
Acetophenone	>99	97(S)
α-Aminoacetophenone	81	98(R)
1-Phenylbutane-1,3-dione	>99	98(S)[c]
α-Hydroxyacetophenone	92	>99(R)
α-Chloroacetophenone	>99	61(R)
α-Cyanoacetophenone	>99	68(S)
4′-hydroxyacetophenone	67	95(S)
4′-Methoxyacetophenone	51	97(S)
4′-Chloroacetophenone	>99	87(S)
3′-hydroxyacetophenone	97	>99(S)[d]

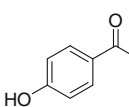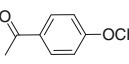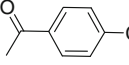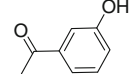[a] The conversions were determined by HPLC analysis

[b] The enantiomeric excess values were measured by chiral HPLC analysis

[c] The reduction product was (S)-1-phenyl-3-hydroxybutanone

[d] $[\alpha]_D^{20.} = -36°$ (c 0.2, MeOH), lit. $[\alpha]_D^{20} = -24.5$ (c 0.2, MeOH)

Biocatalytic carbonyl-reducing reaction plays an important role in the synthesis of chiral alcohols which are widely employed as useful building blocks for the production of pharmaceuticals and agricultural chemicals [30]. However, biocatalytic reduction of acetophenones with an unprotected hydroxy group on the benzene ring to give hydroxy-substituted 1-phenylethanols has been rarely reported [23, 31, 45]. In this study, 3′-hydroxyacetophenone and 4′-hydroxyacetophenone was reduced to the corresponding alcohols with 99 and 95 % e.e., respectively. In addition, *Rhodococcus* sp. 1-0130 catalyzed the reduction of a diversity of acetophenone derivatives under anaerobic conditions to afford the corresponding alcohols with high enantioselectivity. Especially, (R)-2-amino-1-phenylethanol and (R)-2-hydroxy-1-phenylethanol, which are valuable chiral compounds used in pharmaceuticals and materials, were obtained with excellent optical purity. Although carbonyl reductases have been previously characterized in *Rhodococcus* strains such as *Rhodococcus ruber* and *Rhodococcus erythropolis* [22, 43, 48], the present study showed that the *Rhodococcus* sp. 1-0130 carried a highly enantioselective carbonyl reductase, which was unique in terms of substrate profile and enantioselectivity, and would be very useful as a biocatalyst in the synthesis of optically active alcohols.

In conclusion, the first *Rhodococcus* strain with *para*-acetylphenol hydroxylase activity was identified. This strain also exhibited highly enantioselective carbonyl reductase activity with potential applications. In particular, *Rhodococcus* sp. 1-0130 catalyzed the reduction of α-aminoacetophenone, 3′-hydroxyacetophenone and 4′-hydroxyacetophenone to afford the corresponding alcohols with high enantiomeric purity. Studies on the biocatalytic reduction of these ketones with unprotected amino or hydroxy group are very limited [23, 31, 45, 46, 49]. *Rhodococcus* sp. 1-0130 represents a novel biocatalyst that showed great potential for the asymmetric reduction of these less-explored but important ketones. The *para*-acetylphenol hydroxylase and carbonyl reductase activity were switched by carrying the reaction in the presence or absence of air.

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