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BIOCATALYSIS

Towards the discovery of alcohol dehydrogenases: NAD(P)H fluorescence-based screening and characterization of the newly isolated *Rhodococcus erythropolis* WZ010 in the preparation of chiral aryl secondary alcohols

Chi Yang · Xiangxian Ying · Meilan Yu · Yinjun Zhang · Bin Xiong · Qingqing Song · Zhao Wang

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Abstract A simple and reliable procedure was developed to screen biocatalysts with high alcohol dehydrogenase activity, efficient internal coenzyme regeneration, and high stereoselectivity. The strategy of activity screening in a microtitre plate format was based on the detection of fluorescence of NAD(P)H originating from the oxidation of alcohols. The primary and secondary screenings from soil samples yielded a versatile bacterial biocatalyst *Rhodococcus erythropolis* WZ010 demonstrating potential for the preparation of chiral aryl secondary alcohols. In terms of activity and stereoselectivity, the optimized reaction conditions in the stereoselective oxidation were 30 °C, pH 10.5, and 250 rpm, whereas bioreduction using glucose as co-substrate was the most favorable at 35 °C and pH 7.5 in the static reaction mixture. Under the optimized conditions, fresh cells of the strain stereoselectively oxidized the (*S*)-enantiomer of racemic 1-phenylethanol (120 mM) to acetophenone and afforded the unoxidized (*R*)-1-phenylethanol in 49.4 % yield and >99.9 % enantiomeric excess (*e.e.*). In the reduction of 10 mM acetophenone, the addition of 100 mM glucose significantly increased the conversion rate from 3.1 to 97.4 %. In the presence of 800 mM glucose, acetophenone and other aromatic ketones (80 mM) were enantioselectively reduced to corresponding (*S*)-alcohols with excellent *e.e.* values. Both stereoselective oxidation and asymmetric reduction required no external cofactor regeneration system.

Keywords NAD(P)H fluorescence · *Rhodococcus erythropolis* WZ010 · Stereoselective oxidation · Asymmetric reduction

Chi Yang and Xiangxian Ying contributed equally to this study.

C. Yang · X. Ying · Y. Zhang · B. Xiong · Q. Song · Z. Wang (✉)
College of Biological and Environmental Engineering, Zhejiang University of Technology, 18 Chaowang Road, Hangzhou 310014, Zhejiang, China
e-mail: hzwangzhao@163.com

M. Yu
College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou 310018, China

Introduction

Chiral aromatic alcohols are widely used as building blocks in the synthesis of organic chemicals and chiral medicines [16, 17, 30, 37]. Both (*R*)- and (*S*)-enantiomers are also used as chiral reagents for the determination of enantiomeric purity and are often more expensive than their racemic mixtures [7]. To replace well-established chemical methods, oxidoreductase-based biological approaches were intensively investigated for their high efficiency, mild reaction conditions, and outstanding stereospecificity [6, 11, 15, 29, 32]. Among them, asymmetric reduction catalyzed by alcohol dehydrogenases is gaining popularity as an excellent strategy for the preparation of chiral aryl secondary alcohols. Both whole-cell biocatalysts and isolated enzymes are routinely applied in asymmetric bioreduction. In comparison to isolated enzymes, whole-cell biocatalysts are more stable, obtained more easily, and offer the capability of internal coenzyme regeneration, enabling their industrial application to be less expensive [8]. In the whole-cell-catalyzed bioreduction, aromatic ketones are usually toxic to microbial cells and the substrate/product inhibition is still a great challenge in practical applications [12]. Although various microorganisms have been widely studied, the discovery of more competitive

biocatalysts, particularly those with excellent substrate/product tolerance, is still warranted.

The high-throughput screening strategies of oxidoreductases including alcohol dehydrogenases are commonly based on the use of NAD(P)H as coenzyme, which satisfies the demands of wider applicability, easier use, and higher efficiency. Redox reactions catalyzed by alcohol dehydrogenases can be monitored colorimetrically by the addition of a suitable dye, e.g., *p*-rosaniline, tetrazolium salts, or *p*-nitrosodimethylaniline; in this system, a hydrogen atom from the reduced form of the coenzyme is transferred to the dye leading to a detectable color change [5, 18, 22]. Unlike colorimetric methods, screening procedures based on fluorescence detection of the coenzyme or its derivative are simpler, highly sensitive, and require no coupled dye. After sequential treatment of strong acid and alkali, the product from $NAD(P)^+$ but not NAD(P)H shows absorbance at 360 nm and fluorescence emission at 455 nm upon excitation at 360 nm. Relying on the difference of fluorescence or absorbance between oxidized and reduced coenzymes, a high-throughput assay in a microtitre plate format was successfully applied for screening libraries of substrates against a cytochrome P450 monooxygenase as well as its mutants [28]. Furthermore, Reisinger et al. [19] developed a NADH fluorescence-based assay at the colony level to directly visualize the activity of dehydrogenases, providing a potential screening solution for a variety of enzymatic reactions.

The development of a rapid and reliable screening method for alcohol dehydrogenases remains challenging [27]. This work aims to develop a novel NAD(P)H fluorescence-based screening method in which fluorescent signals could directly reflect the activities of alcohol dehydrogenases. On that basis, the newly isolated biocatalysts would be investigated for their potential to afford both (*R*)- and (*S*)-enantiomers of chiral aryl secondary alcohols via stereoselective oxidation and asymmetric reduction. Here we present a reliable screening procedure that could rapidly detect alcohol dehydrogenases or related microorganisms originating from diverse environments. Furthermore, a versatile biocatalyst from the genus *Rhodococcus* was identified and characterized, which exhibited excellent stereoselectivity, desired yield, high substrate tolerance, and no need for external cofactor regeneration (Fig. 1).

Materials and methods

Materials

Soil samples used to isolate bacteria with alcohol dehydrogenase activities were collected from Hubei, Shandong,

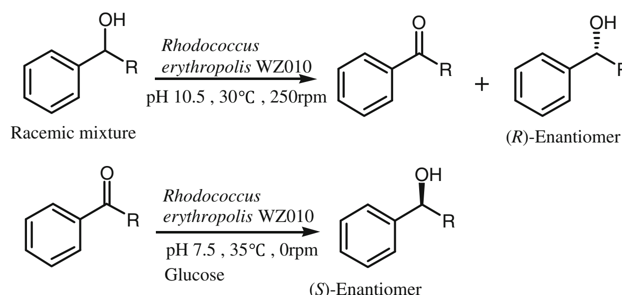


Fig. 1 Stereoselective oxidation and asymmetric reduction by *R. erythropolis* WZ010. R = CH_3, CH_2CH_3, CH_2OH, $COOCH_3$, or $COOCH_2CH_3$

and Zhejiang provinces of China. Coenzymes NAD^+, NADH, $NADP^+$, and NADPH were purchased from Sangon Biotech Co. Ltd (Shanghai, China). Alcohols and ketones were purchased from Shanghai Jingchun Reagent Co. Ltd (Shanghai, China). All other chemicals were of analytical grade and commercially available.

Media

The liquid culture medium consisted of the following (per liter): peptone 10 g, yeast extracts 5 g, NaCl 5 g. The initial pH of the medium was adjusted to 7.0–7.2. The nutrient agar medium for the study of colony characteristics of strain WZ010 was the liquid culture medium supplemented with 15 g/l agar. The enrichment agar medium was the nutrient agar, the surface of which was spread with 150 μl 1-phenylethanol per agar plate.

Microorganism and culture conditions

The strain *R. erythropolis* WZ010 was isolated from soil samples collected in Hubei Province, China and deposited in the China Center for Type Culture Collection (CCTCC 2011336, Wuhan, China). The bacterium was routinely cultivated using the liquid culture medium in a 250-ml Erlenmeyer flask at 30 °C on a rotary shaker at 200 revolutions per minute (rpm) for 48 h. The cells were harvested by centrifugation at 4 °C and 9,000×*g* for 10 min and washed with phosphate buffer (50 mM, pH 7.0). After centrifugation, the fresh cells were collected and directly applied for stereoselective oxidation and asymmetric reduction.

Fluorescence spectroscopy of NADH and NADPH

Fluorescence spectroscopy of NAD(P)H was determined using 0.5 mM coenzyme aqueous solution in a microplate spectrophotometer (Molecular Devices Corporation, USA). The emission spectrum was determined to measure

fluorescent light at different wavelengths (400–500 nm) by holding the excitation light at 360 nm. To determine the excitation spectrum, the emission light was held at 460 nm and the excitation light was scanned through different wavelengths (300–400 nm). The effect of pH on fluorescence was determined over the range of 7.0–10.5. The buffers (50 mM) used were Tris–HCl (pH 7.0–8.5) and glycine-NaOH (8.5–10.5). The effect of the temperature on fluorescence was examined at temperatures from 4 to 70 °C. The effect of cosolvents on fluorescence was investigated by adding each cosolvent into 0.5 mM coenzyme aqueous solution.

Screening procedure

Soil samples (0.5 g) were suspended in 1 ml of sterile water in a 2-ml Eppendorf tube. The suspended solution was further diluted with sterile water at a dilution factor of 1,000 and then 100 μl diluted sample was plated onto the enrichment agar plate. The enrichment agar plates were incubated at 30 °C for 48 h to obtain single colonies. The isolated pure colonies were transferred onto agar slants at 30 °C for 24 h. The fresh culture (2 loopfuls) from the agar slant was resuspended with 60 μl sterile water. A 96-well FLUOTRAC 200 black plate (Greiner, Germany) was filled per well with 300 μl reaction solution containing 50 mM glycine-NaOH buffer (pH 10.0), 40 mM 1-phenylethanol, 1 mM NAD^+, and 25 μl culture suspension. The corresponding control contained no 1-phenylethanol in the reaction mixture. The microplate was covered and incubated at 30 °C for 24 h. Fluorescence spectroscopy was determined at an excitation wavelength of 360 nm and emission wavelength of 460 nm. Cultures resulting in fluorescence intensity increase (>400) were considered to be positive candidates.

To validate activity and determine the stereoselectivity of selected strains, the reactions were performed at 30 °C for 48 h in 50 mM glycine-NaOH buffer (pH 10.0) containing 40 mM racemic 1-phenylethanol and 0.3 g wet cells/ml. After 48 h, 1-phenylethanol and acetophenone were determined by gas chromatography–mass spectrometry (GC–MS) and chiral gas chromatography (GC) methods as described below.

Molecular identification and Biolog phenotype microarray

To identify the isolated strain WZ010, 16S rDNA gene fragments were amplified by PCR with the following set of primers: 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR conditions were as follows: 5 min of denaturing at 98 °C followed by 35 cycles consisting of 35 s at 95 °C, 35 s at 55 °C, and 90 s at 72 °C and finally by 8 min of extension at 72 °C. The partial *rpo*B gene was amplified using a set of primers: rpoBF (5′-AGCTCATCCAGAACCAGATCC-3′) and rpoBR (5′-CTTCGGCGTGACCTTTCC-3′). The corresponding PCR conditions were as follows: 3 min of denaturing at 94 °C followed by 35 cycles consisting of 30 s at 94 °C, 30 s at 58 °C, and 90 s at 72 °C and finally by 10 min of extension at 72 °C. After the PCR products were purified using a DNA gel extraction kit (Sangon, Shanghai, China) according to the manufacturer's instructions, DNA was directly applied for sequencing on both strands. The obtained 16S rDNA gene sequence was BLASTed against the GenBank database and Ribosomal Database Project (RDP II) [4]. Multiple alignments of the determined 16S rDNA sequences and reference sequences obtained from the GenBank database were carried out using the Clustal W package (version 1.83) [26]. A phylogenetic tree was constructed with the software of MEGA5 based on the homologous 16S rDNA sequences [25].

To perform the testing of Biolog phenotype microarray, the strain WZ010 was grown on BUG agar with 5 % sheep blood at 30 °C for 48 h and then suspended in an inoculating fluid (IF-A) at the recommended cell density (95 % T). The cell suspension was inoculated into the GEN III MicroPlate (100 μl per well) and the MicroPlate was incubated at 30 °C for 48 h to allow the phenotypic fingerprint to form. After incubation, the phenotypic fingerprint of purple wells was compared to Biolog's extensive species library (database version: Biolog GEN III DB.15G).

Stereoselective oxidation of racemic alcohols

The standard reaction mixture (3 ml) contained 50 mM glycine-NaOH buffer (pH 10.5), 40 mM racemic 1-phenylethanol, and 0.3 g wet cells/ml. The wet weight/dry weight ratio of the cells was approximately 17.5. The reaction was carried out in an orbital shaker (200 rpm) at 30 °C for 48 h unless specified otherwise. Centrifugation and extraction were used to stop the reactions. One volume of ethyl acetate was mixed with the reaction solutions, and the mixtures were centrifuged at $10{,}000 \times g$ for 10 min. The organic layer was dried over anhydrous sodium sulfate overnight and then analyzed by GC as described below.

The effect of pH on stereoselective oxidation was determined over the range of 8.0–10.5. The buffers (50 mM) used were Tris–HCl (pH 8.0–9.0) and glycine-NaOH (9.0–10.5). The effect of the temperature on stereoselective oxidation was examined at temperatures from 4 to 50 °C. To determine the time course of stereoselectively oxidizing racemic 1-phenylethanol, the initial volume of reaction mixture was 15 ml and samples were taken for GC analyses periodically. Substrate specificity was determined using aromatic and aliphatic alcohols under standard reaction conditions except that the reaction time was 2 h.

Asymmetric reduction of aromatic ketones

The standard reaction mixture (3 ml) contained 50 mM Tris–HCl buffer (pH 7.5), 10 mM phenylacetone, 100 mM glucose as co-substrate, and 0.15 g wet cells/ml. The reaction was carried out at 30 °C for 48 h unless specified otherwise. Similar to stereoselective oxidation, the reactants in the asymmetric reduction were extracted, dried over anhydrous sodium sulfate, and then submitted to GC analyses. The effect of pH on asymmetric reduction was determined over the range of 6.0–8.5. The buffers (50 mM) used were Na_2HPO_4–NaH_2PO_4 (pH 6.0–7.5) and Tris–HCl (7.5–8.5). The effect of the temperature on asymmetric reduction was examined at temperatures from 4 to 50 °C. The effect of co-substrates on asymmetric reduction was determined by adding each co-substrate at a tenfold substrate concentration into the standard reaction mixture.

GC–MS and chiral GC analyses

Acetophenone evolved from the oxidation of 1-phenylethanol was validated in an Agilent 6890 N/5975C GS-MS system. The GC–MS analyses were performed under the following conditions: column, Agilent Hp-5, 30 m × 0.25 mm × 0.25 μm; carrier gas, nitrogen at a flow of 20 ml/min; split ratio, 1:50; solvent delay, 2 min; oven temperature program, 100–150 °C at a ramp rate of 5 °C/min; MS temperature, 230 °C (source) and 150 °C (quad); transfer line temperature, 250 °C. The reaction mixture after extraction (0.2 μl) was directly applied onto the injector (250 °C) for GC–MS analyses. The retention times of 1-phenylethanol and acetophenone were 4.031 and 4.174 min, respectively.

The substrates and products from reaction mixtures were quantitated in a Shimadzu GC-2014 gas chromatograph equipped with a flame ionization detector (FID, 250 °C). The GC analyses were performed under the following conditions: column, CP-Chirasil DEX CB, 30 m × 0.25 mm × 0.25 μm; carrier gas, nitrogen at a flow of 33 ml/min; split ratio, 1:49; oven temperature, 140 °C; air, 330 ml/min; hydrogen, 33 ml/min. The reaction mixture after extraction (1 μl) was directly applied onto the injector (250 °C) for GC analyses. The peak areas were quantitated using specific external standards, and the absolute configuration was identified by comparing the GC retention times with those of standard samples.

Nucleotide sequence accession number

The partial *rpo*B gene and 16S rDNA sequences of *R. erythropolis* WZ010 have been deposited in the GenBank database under the accession numbers of JQ964229 and JQ305102, respectively.

Results

Fluorescence spectroscopy of NAD(P)H

Reduced coenzyme NAD(P)H but not oxidized coenzyme $NAD(P)^+$ emitted fluorescence when excited by UV light of 340–360 nm [19]. To develop an activity screening method for alcohol dehydrogenases, the effects of various factors on the viability and stability of NAD(P)H fluorescence were investigated. Fluorescence emission spectroscopy of both NADH and NADPH showed a maximum at 460 nm upon excitation at 360 nm (Fig. 2a). When holding the emission wavelength at 460 nm, fluorescence excitation spectroscopy of NADH and NADPH exhibited the hightest values upon excitation of 350 and 360 nm, respectively (Fig. 2b). The effect of temperature on fluorescence was examined in the range of 4–70 °C using 0.5 mM NAD(P)H at pH 10.0, which indicated that the fluorescence intensities were stable for NADH but decreased for NADPH under higher temperatures (Fig. 3a). The redox reactions catalyzed by alcohol dehydrogenase commonly spanned from neutral to alkaline pHs and thus the effect of pH on fluorescence was tested in the range of 7.0–10.5. Fluorescence intensities at the tested pHs were 4,000–5,500 but appeared less stable at neutral pH than in alkaline environments (Fig. 3b). The substrates used by alcohol dehydrogenases, particularly aromatic alcohols and ketones, had lower solubility and therefore cosolvent might be required in the screening system. The addition of cosolvents such as 10–30 % (v/v) methanol, dimethyl sulfoxide (DMSO), and acetonitrile did not have a significant effect on fluorescence intensity (Fig. 4). Under the optimized conditions, the increase of NAD(P)H concentrations (0–1 mM) had a positive correlation with that of fluorescence intensity (0–5,500); however, 0.5 mM NAD(P)H was sufficient to be readily detected due to its fluorescence intensity (>4,000).

Screening of microorganisms oxidizing 1-phenylethanol

The screening strategy consecutively combined the enrichment with a targeted substrate 1-phenylethanol, the primary activity screening based on fluorescence of NADH originating from alcohol oxidation, and the secondary screening aiming to detect biocatalysts with efficient internal coenzyme regeneration and high stereoselectivity (Fig. 5). The reaction mixture in the primary activity screening was set at pH 10.0 because alcohol dehydrogenases commonly catalyze alcohol oxidation more efficiently at alkaline pH values [33, 34]. The initial concentration of 1-phenylethanol as substrate was 40 mM. A total of 214 colonies from enrichment agar plates were

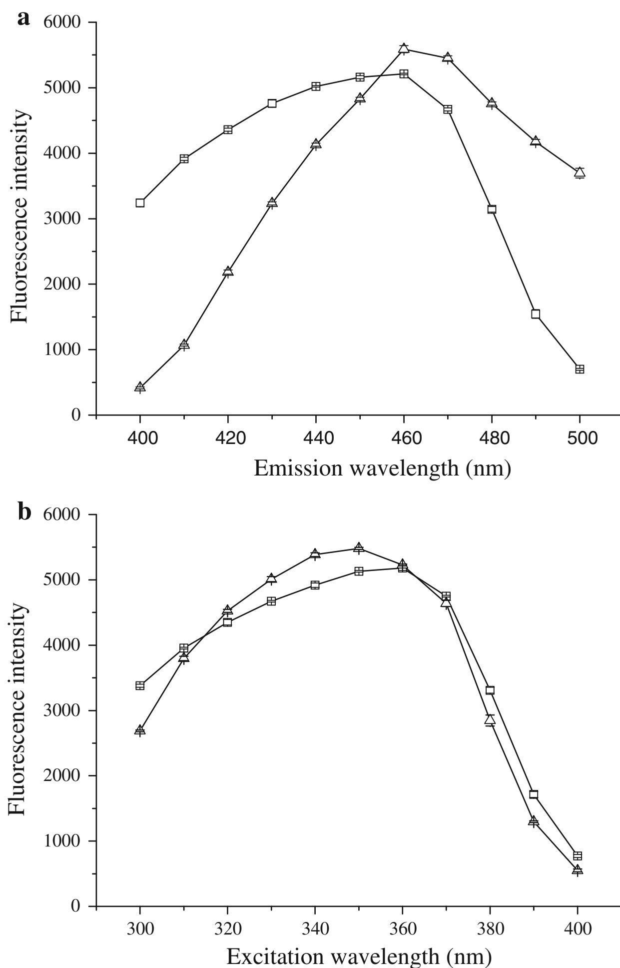


Fig. 2 Emission (**a**) and excitation (**b**) spectroscopy of NAD(P)H. *Triangles* NADH, *squares* NADPH

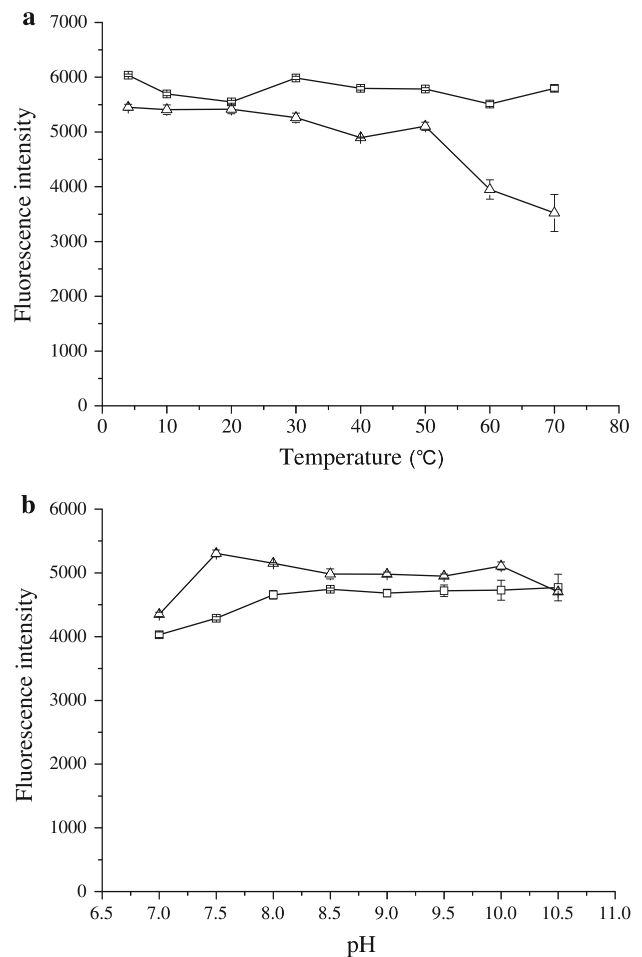


Fig. 3 Effect of temperature (**a**) and pH (**b**) on NAD(P)H fluorescence. *Triangles* NADH, *squares* NADPH

tested, 44 of which demonstrated an increase of NAD(P)H fluorescence intensity. The increase of fluorescence intensity of the hits was in the range of 400–4,000, whereas that of the non-hits was no greater than 400. To test the reproducibility of the activity screening, the newly isolated strain WZ010 was chosen as a biocatalyst and the assay was repeated five times under the same conditions. The increases of NADH fluorescence in five replications were 3,365 ± 46.2, 3,503.1 ± 113.4, 3,453 ± 105.5, 3,583.5 ± 348.8, and 3,284.8 ± 196.4, indicating that the assay was reproducible. As coenzymes are expensive and whole-cell catalysis usually takes advantage of its internal coenzyme regeneration, the reaction mixture of the secondary screening was devoid of exogenous oxidized coenzyme NAD^+ in order to further discover biocatalysts with an efficient internal coenzyme regeneration system. Chiral GC analyses indicated a total of 39 strains capable of efficiently oxidizing 10 mM 1-phenylethanol after 16 h, of which 17 showed higher stereoselectivity in the oxidation of

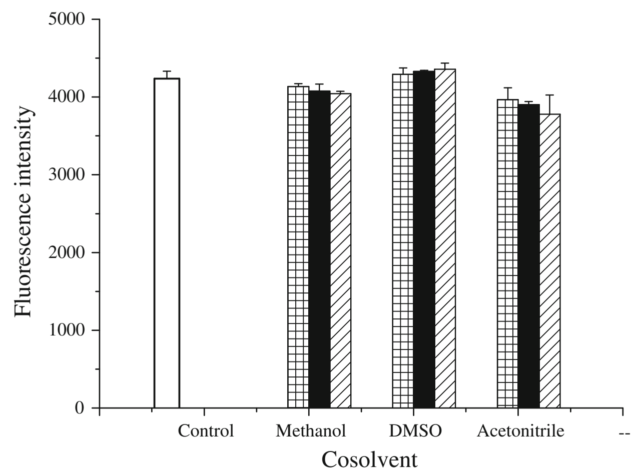


Fig. 4 Effect of solvents on NAD(P)H fluorescence. *Bars with grids* 10 % (v/v), *black bars* 20 % (v/v), *bars with diagonal lines* 30 % (v/v)

1-phenylethanol (*e.e.* > 80 %). When 1-phenylethanol was oxidized, all the gas chromatographs indicated a single product peak, which was validated to be acetophenone

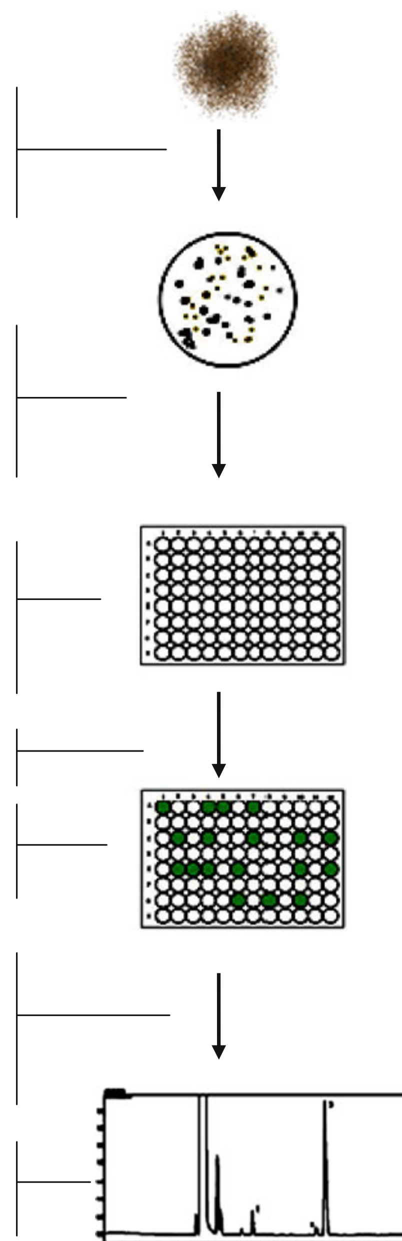


Fig. 5 Enrichment step and primary and secondary screenings for microorganisms oxidizing 1-phenylethanol

using a GC–MS method. Among these efficient bio-oxidizers of 1-phenylethanol, the strain WZ010 was chosen as the best strain for further study in terms of its higher activity and stereoselectivity. In addition, the screening procedure was adapted to investigate the substrate spectrum of strain WZ010 against various alcohols. The NADH fluorescence activity assay also indicated significantly increased fluorescence intensity for the tested substrates except methyl mandelate, which was affected by its auto-lysis at pH 10.5 (Fig. 6). To further improve the substrate spectrum of strain WZ010, the oxidation of various alcohols in the absence of exogenous NAD^+ was carried out at 30 °C and pH 10.5 for 10 h. Chiral GC analyses of products and substrates indicated that the conversions of the tested alcohols were significant and the strain WZ010 predominantly oxidized the (*S*)-enantiomer of aromatic and aliphatic alcohols to corresponding ketones.

Identification of strain WZ010

The morphological and physiological characteristics of strain WZ010 were thoroughly investigated. The rod-shaped cells were aerobic, gram-positive, non-sporulating, and surrounded with capsule-like materials under microscopic examination. Colonies on nutrient agar appeared round, smooth, mucoid, and light pink colored. It was noted that the cells in the liquid culture turned pink more significantly after 24 h incubation than the colonies on the

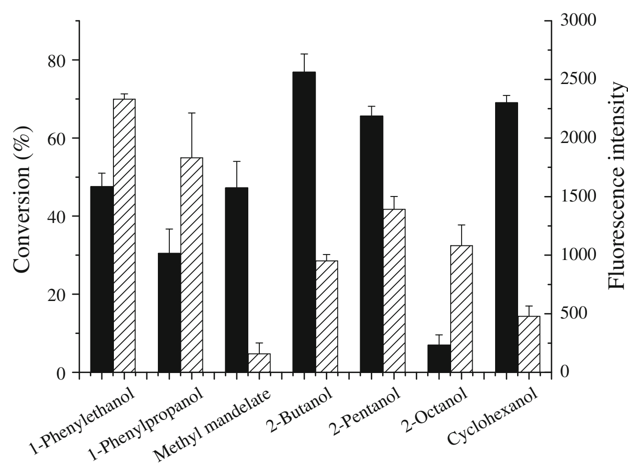


Fig. 6 Comparison of substrate spectrum of *R. erythropolis* WZ010 based on fluorescence assay and GC analysis. Fluorescence assays were carried out at 30 °C for 16 h using reaction mixtures containing 50 mM glycine-NaOH buffer (pH 10.0), 40 mM alcohol, 1 mM NAD^+, and 25 µl culture suspension described in "Screening procedure". Conversions in alcohol oxidation at 30 °C after 16 h were determined by GC using reaction mixtures containing 50 mM glycine-NaOH buffer (pH 10.0), 40 mM racemic alcohol, and 0.03 g wet cells/ml. *Black bars* conversions, *bars with diagonal lines* fluorescence intensities

nutrient agar. The 16S rDNA sequence with a length of 1,394 bp was obtained and the constructed phylogenetic tree indicated that the strain WZ010 was closely related with *Rhodococcus* species, i.e., *R. erythropolis* strain ZJB-09149 (1389/1391 identity; HQ179128), *Rhodococcus boritolerans* strain FW815 (1389/1391 identity; GU181 290), *Rhodococcus globerulus* strain DSQ17 (1389/1392 identity; HM217119), *Rhodococcus baikonurensis* strain KCTC 19793 (1386/1391 identity; AB288063), and *Rhodococcus erythreus* strain REG20 (1313/1313 identity; EU647695) (Fig. 7). However, the match of the 16S rDNA sequence against the Ribosomal Database Project (RDP II) indicated that the top five hits with full genus and species names all belonged to *R. erythropolis* such as *R. erythropolis* EK5 (AJ237967), *R. erythropolis* HXN2000 (AJ457058), *R. erythropolis* (AF512838), and *R. erythropolis* (AF512 839). Since the 16S rDNA sequence might not be reliable enough to clearly differentiate the closely related species, another housekeeping gene such as the *rpo*B gene was required for accurate identification [2]. The partial *rpoB* sequence (1,260 bp) was determined and the BLASTP search of its corresponding amino acid sequence showed that it had 99.8 % identity to that of *R. erythropolis* SK121 (EEN88338) or *R. erythropolis* PR4 (BAH32441). To further identify the strain, the carbon source utilization was examined by a standardized micromethod with the Biolog microstation (Biolog Inc., CA, USA). The reaction profile containing 94 biochemical tests indicated that strain WZ010 had 98.4 % probability (0.802 similarity and 2.401 distribution) of being *R. erythropolis*. Therefore, the strain WZ010 was designated as *R. erythropolis* WZ010.

Effect of co-substrate addition on asymmetric reduction

When 40 mM racemic 1-phenylethanol was used as substrate, *R. erythropolis* WZ010 could completely oxidize the (*S*)-enantiomer to acetophenone and almost retained (*R*)-1-phenylethanol unoxidized after 10 h (Fig. 8). The reaction did not require the addition of exogenous coenzyme NAD^+ or $NADP^+$, suggesting that internal coenzyme regeneration was sufficient for the oxidation. In the reduction reaction, however, the reaction conversion was only 3.1 % when 10 mM acetophenone was reacted for 48 h, indicating that co-substrate was essential to improve internal coenzyme regeneration. The effect of various co-substrates on conversion was examined by adding the co-substrate at a tenfold substrate concentration into the reaction mixture (Fig. 9). Sodium glutamate and fructose did not improve conversion. Addition of co-substrates such as sodium lactate, ethanol, sodium acetate, and isopropanol afforded the conversion rates of 7.7–37.1 %, whereas glucose and glycerol dramatically increased the conversions to 97.4 and 64.5 %, respectively. The asymmetric reduction without co-substrate addition yielded an *e.e.* value of 44.5 % against (*R*)-1-phenylethanol. It is of interest to note that the stereoselectivity of reactions supplemented with various so-substrates varied and the type of stereoselectivity in most cases was not consistent with that of the control (Fig. 9). Except fructose and glutamate, all other tested so-substrates reversed the stereoselectivity to (*S*)-type. In contrast to glucose, other co-substrates lowered the *e.e.* values to 47.9–93.9 %, implying that other endogenous enzymes might be present and compete with the desired alcohol dehydrogenase(s) [10].

Effect of shaking speed on stereoselective oxidation and asymmetric reduction

The effect of shaking speed (0–300 rpm) on the stereoselective biocatalysis was investigated to select a suitable level (Fig. 10). In the stereoselective oxidation of racemic 1-phenylethanol, it was found that the conversion increased clearly with the increasing shaking speed from 0 to 250 rpm, above which no obvious variation of the conversion was observed. The *e.e.* values also increased as the stirring speed increased from 0 to 250 rpm. In the asymmetric reduction of acetophenone, it appeared that the higher the stirring speed, the lower the conversion, whereas the highest conversion was obtained when the reaction mixture was kept static. The change of stirring speed in the range of 0–300 rpm did not lower the *e.e.* values.

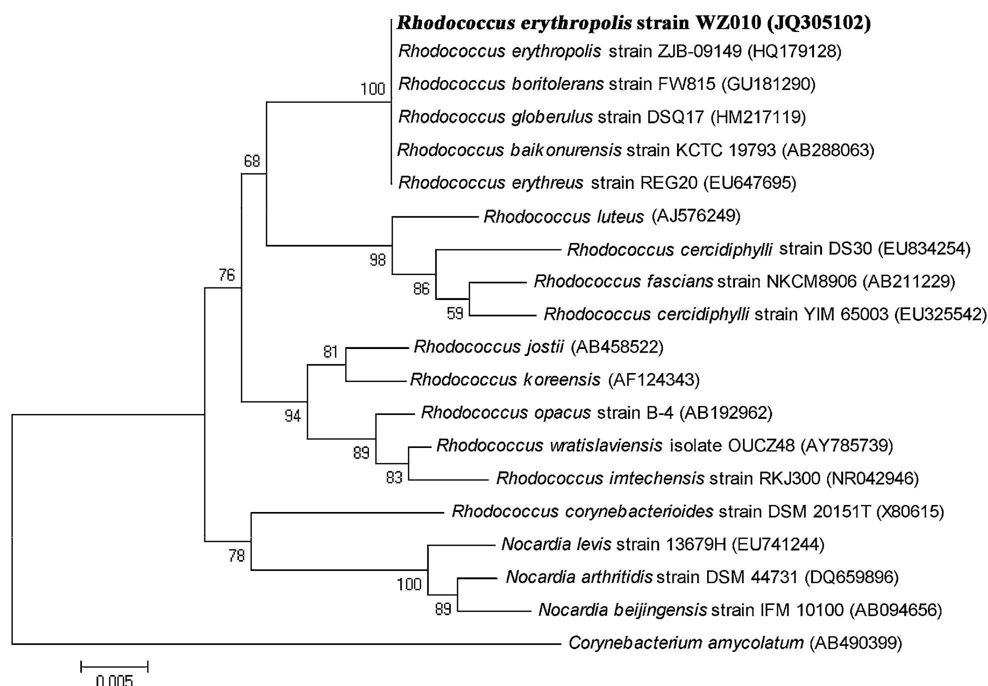


Fig. 7 Phylogenetic relationship between *R. erythropolis* WZ010 and related strains based on the 16S rDNA sequence. The sequences were aligned using Clustal W and subsequently a phylogenetic tree was constructed against 1,313-bp fragments shared by all the sequences using the neighbor-joining method. The number of bootstrap replications was set as 1,000. The *scale bar* represents 0.005 substitutions per sequence position. *Numbers* in *parentheses* are accession numbers of published sequences. *Corynebacterium amycolatum* was used as the outgroup

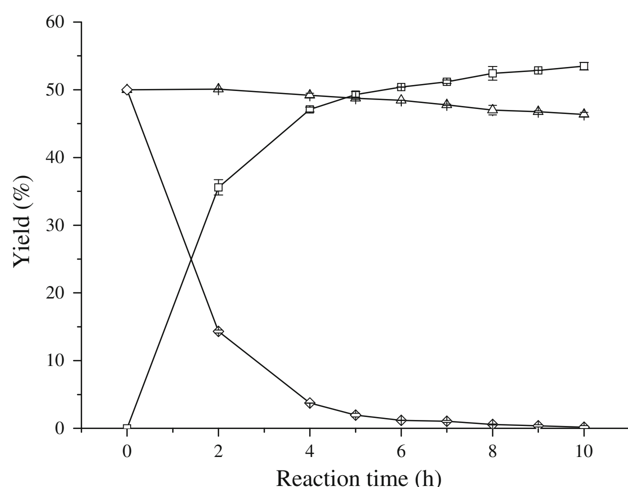


Fig. 8 Time course of stereoselective oxidation of racemic 1-phenylethanol by *R. erythropolis* WZ010. *Triangles* (*R*)-1-phenylethanol, *diamonds* (*S*)-1-phenylethanol, *squares* acetophenone

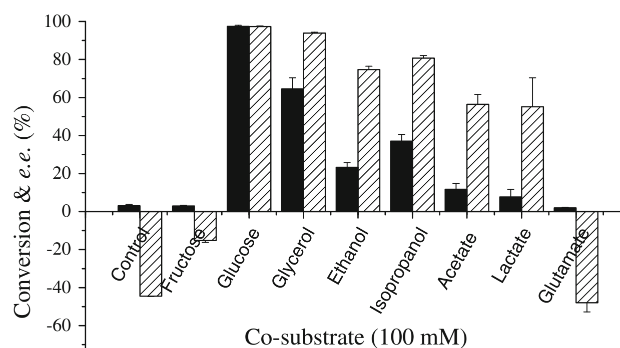


Fig. 9 Effect of various co-substrates on the asymmetric reduction of acetophenone by *R. erythropolis* WZ010. The reaction mixture (3 ml) contained 50 mM Tris–HCl buffer (pH 7.5), 10 mM acetophenone, 100 mM co-substrate, and 0.45 g wet cells. Bioreduction was carried out at 30 °C and 200 rpm for 48 h. Positive and negative *e.e.* values represent (*S*)- and (*R*)-enantioselectivities, respectively. *Black bars* conversion, *bars with diagonal lines e.e.* value

Effect of pH and temperature on stereoselective oxidation and asymmetric reduction

Temperature and pH are important parameters in industrial bioreaction. In the oxidation of 1-phenylethanol, *R. erythropolis* WZ010 was active in the range of 4–50 °C with an optimum value at 30 °C (Fig. 11a). The optimal temperature for bioreduction was 35 °C, with >99.9 % *e.e.* and 50.1 % of conversion, respectively. The conversion increased as the pH was elevated from 8.0 to 10.5 in the oxidation of racemic 1-phenylethanol, whereas *R. erythropolis* WZ010 displayed the highest activity at pH 7.5 in the reduction of acetophenone (Fig. 11b). The change of the corresponding *e.e.* values was in parallel to the change of conversions in the oxidation, whereas those of bioreduction were constant at high levels.

Stereoselective oxidation of different racemic aryl alcohols

When 40 mM racemic 1-phenylethanol was used as substrate at 30 °C and pH 10.5, the reaction retained the (*R*)-1-phenylethanol yield of 46.3 % after 10 h, whereas

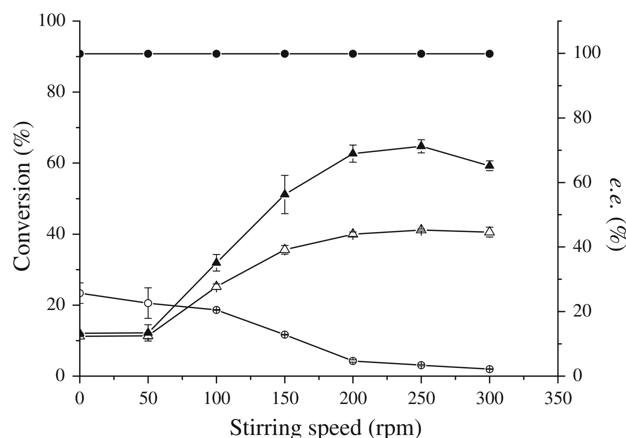
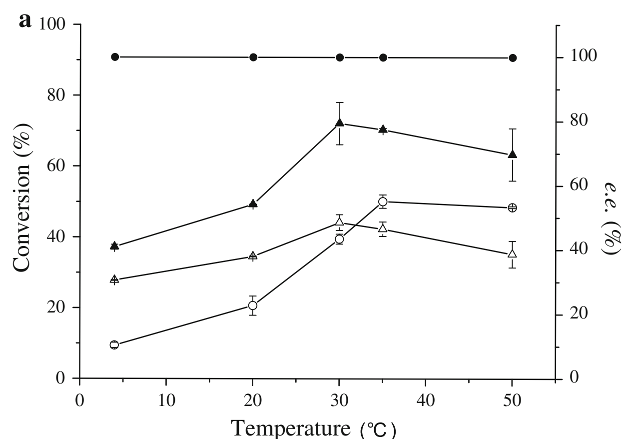


Fig. 10 Effect of stirring speed on stereoselective oxidation and asymmetric reduction. The reaction mixture (2 ml) of asymmetric reduction (*circles*) contained 50 mM Tris–HCl buffer (pH 7.5), 20 mM acetophenone, 200 mM glucose, and 0.09 g wet cells. The 2-ml reaction mixture of stereoselective oxidation (*triangles*) contained 50 mM glycine-NaOH buffer (pH 10.5), 20 mM 1-phenylethanol, and 0.09 g wet cells. Reactions were carried out at 30 °C for 4 h. *Filled symbols* conversion, *open symbols e.e.* value

(*S*)-1-phenylethanol was completely oxidized to acetophenone. When the concentration of 1-phenylethanol was increased to 120 mM, the reaction gave an (*R*)-1-phenylethanol yield of 49.4 % and still >99 % *e.e.* after 48 h (Table 1). When the concentration of 1-phenylethanol was further raised up to 140 mM, the *e.e.* value of the reaction decreased to 85 %, which might be improved by extending the reaction time or increasing the concentration of resting cells. To extend the application of *R. erythropolis* WZ010, racemic 1-phenylpropanol, methyl mandelate, and 1-phenyl-1, 2-ethanediol were subjected to stereoselective oxidation under the same conditions. *R. erythropolis* WZ010 oxidized the (*S*)-enantiomer of racemic alcohols in >99 % *e.e.* but the yields of (*R*)-1-phenylpropanol and methyl (*R*)-mandelate were relatively low. When 40 mM 1-phenyl-1, 2-ethanediol was used as substrate, neither oxidation nor degradation was observed.

Asymmetric reduction of different aryl ketones

Driven by 200 mM glucose in the reaction mixture, 20 mM acetophenone was predominantly reduced to (*S*)-1-phenylethanol with 93.9 % yield and 97.9 % *e.e.* (Table 2). When the concentration was increased up to 80 and 150 mM, the optical purity of (*S*)-1-phenylethanol was still satisfactory but the conversion was lowered to 64.5 and 33.5 %, respectively, indicating the occurrence of substrate/product inhibition. Similar cases were also observed when four other aromatic ketones (80 and 150 mM) were subjected to asymmetric reduction in the presence of 800 mM glucose as co-substrate. The biocatalyst reduced 80 mM 2-hydroxyacetophenone very effectively



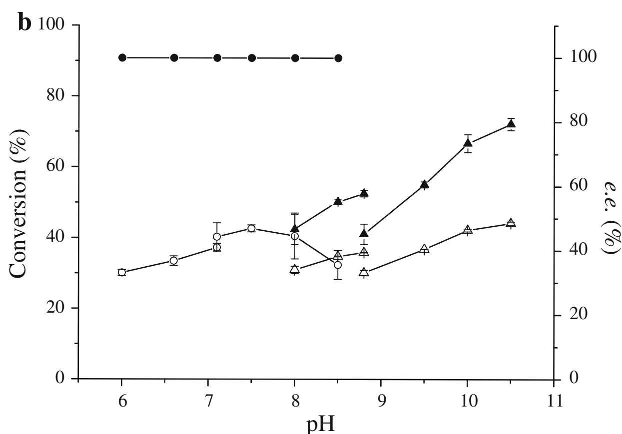


Fig. 11 **a** Effect of temperature on stereoselective oxidation and asymmetric reduction. The reaction mixture of asymmetric reduction (2 ml, *circles*) contained 50 mM Tris–HCl buffer (pH 7.5), 20 mM acetophenone, 100 mM glucose, and 0.18 g wet cells, whereas that of stereoselective oxidation (2 ml, *triangles*) contained 50 mM glycine-NaOH buffer (pH 10.5), 20 mM 1-phenylethanol, and 0.18 g wet cells. Oxidation and reduction were carried out for 4 h at 200 and 0 rpm, respectively. **b** Effect of pH on stereoselective oxidation and asymmetric reduction. The reaction mixture of asymmetric reduction (2 ml, *circles*) contained 50 mM buffer, 20 mM acetophenone, 100 mM glucose, and 0.18 g wet cells, whereas that of stereoselective oxidation (2 ml, *triangles*) contained 50 mM buffer, 20 mM 1-phenylethanol, and 0.18 g wet cells. Oxidation and reductions were carried out for 4 h at 200 and 0 rpm, respectively. *Filled symbols* conversions, *open symbols e.e.* values

to 1-phenyl-1, 2-ethanediol, affording a yield of 67.6 % and *e.e.* of 99.6 %. Although *R. erythropolis* WZ010 was not as efficient with ethyl benzoylformate (80 mM) and 4-chloroacetophenone (80 mM), it also reduced them in high *e.e.* values of >99.9 and 98.2 %, respectively. The reduction of 150 mM propiophenone did not achieve high stereoselectivity and the optical purity of product was only 72.5 % *e.e.*

Discussion

The presented activity screening based on NAD(P)H fluorescence has wide-ranging applications for the

discovery of alcohol dehydrogenases. The assay is target reaction-oriented and could be used to screen a library of microorganisms using a specific alcohol as substrate. When a pair of (*R*)- and (*S*)-alcohols were used to replace corresponding inexpensive racemic alcohol, it would be possible to directly screen biocatalysts with stereoselectvity by comparing the difference of NAD(P)H fluorescence evolved from the oxidation of (*R*)- and (*S*)-enantiomers. It could also be used to rapidly investigate the activities of a specific $NAD(P)^+$-dependent enzyme against a library of alcohols. The method could be adapted to broad pH and temperature ranges, thus making it applicable to discover extremophiles, particularly psychrophiles and thermophiles. In addition, the reverse process for detecting a decrease of NAD(P)H fluorescence intensity was also feasible. Combined with enrichment, chiral GC and GC–MS analyses, the screening procedure aims to find the bacterial biocatalysts that can tolerate a high concentration of substrate and efficiently carry out internal coenzyme regeneration, in addition to high activity and excellent stereoselectivity. The NAD(P)H fluorescence-based activity screening has advantages over known high-throughput screening methods. As opposed to the phenazine methosulfate/nitroblue tetrazolium method, the assay required no coupled dye and its simplicity greatly reduced the interference factors such as oxygen. In contrast to a P450 monooxygenase screening method [28], the assay was performed at more alkaline pHs such as pH 10 and thus lowered false positive results generated from other $NAD(P)^+$-dependent enzymes such as P450 monooxygenase. Furthermore, the NAD(P)H fluorescence evolved from the assay was directly visualized without the need for strong acid and alkali treatments. In contrast to a colony assay based on NADH fluorescence [19], the emission wavelength for NAD(P)H fluorescence was 460 nm, whereas the excitation wavelengths for NADH and NADPH fluorescence were 350 and 360 nm, respectively. More importantly, the use of a microplate instead of a solid support such as filter paper made the assay more reproducible and precise because the experimental conditions such as pH, temperature, and the amount of cells used could be quantitatively controlled.

Table 1 Stereoselective oxidation of racemic aromatic alcohols by *R. erythropolis* WZ010

Substrate	Concentration (mM)	Time (h)	Yield (%)	$e.e._S$ (%)[a]
1-Phenylethanol	40	10	46.3 ± 0.4	>99.9
	80	48	46.1 ± 1.3	>99.9
	100	48	46.9 ± 1.2	>99.9
	120	48	49.4 ± 0.6	>99.9
	140	48	49.7 ± 0.8	85.1 ± 0.6
1-Phenylpropanol	40	5	21.9 ± 0.7	>99.9
	80	48	32.0 ± 2.3	61.9 ± 0.9
Methyl mandelate[b]	60	48	33 ± 2.6	>99.9
1-Phenyl-1,2-ethanediol	40	48	ND	

Biooxidation was carried out at 30 °C, pH 10.5, and 250 rpm in the reaction mixture (3 ml) with 0.9 g wet cells

ND not detectable

[a] The enantiomeric excess of the substrate was (*R*)-enantioselective

[b] The pH was reduced to 9.5 because of severe autohydrolysis of methyl mandelate at pH 10.5

Table 2 Asymmetric reduction of aromatic ketones by *R. erythropolis* WZ010

Substrate	Concentration (mM)	Time (h)	Yield (%)	$e.e._P$ (%)[a]
Acetophenone	20	24	93.9 ± 2.0	97.9 ± 0.2
	80	48	64.5 ± 2.3	97.8 ± 0.01
	150[b]	72	33.5 ± 1.7	95.7 ± 0.04
Propiophenone	20	24	90.3 ± 2.8	87.6 ± 0.9
	80	48	40.4 ± 1.6	89.9 ± 0.5
	150[b]	72	13.6 ± 1.6	72.5 ± 3.9
Ethyl benzoylformate	20	24	63.9 ± 10.3	>99.9
	80	48	10.4 ± 0.05	>99.9
	150[b]	72	3.0 ± 0.02	>99.9
2-Hydroxyacetophenone	20	24	95.0 ± 1.0	>99.9
	80	48	67.6 ± 4.3	99.6 ± 0.5
	150[b]	72	13.6 ± 0.7	98.9 ± 0.1
4-Chloroacetophenone	20	24	49.3 ± 5.4	>99.9
	80	48	19.1 ± 5.0	98.2 ± 0.1
	150[b]	72	9.1 ± 0.1	99.1 ± 0.8

Bioreduction was carried out at 35 °C and pH 7.5 in the static reaction mixture (3 ml) with 0.45 g wet cells. The concentration of glucose as a co-substrate was 10 times higher than the corresponding substrate concentration

[a] The enantiomeric excess of the product was (*S*)-enantioselective

[b] The concentration of glucose as co-substrate was 800 mM

Up to now, the majority of biocatalysts reported for the preparation of chiral aromatic alcohols are yeasts and fungi because the cells are inexpensive and readily available [31]. The newly isolated strain WZ010 belongs to the genus *Rhodococcus* and is a new member of bacterial biocatalysts in this context. *Rhodococcus* species are important in bioremediation owing to their ability to metabolize aromatic pollutants [3, 23]. In enantioselective oxidoreduction for the synthesis of chiral aromatic alcohols, most research has concentrated on asymmetric reduction of ketones, whereas relatively little attention has been paid to stereoselective oxidation of racemic alcohols. The strain *R. erythropolis* JX-021 was recently used for the enantioselective reduction of 1-phenyl-2-propanone to prepare (*S*)-1-phenyl-2-propanol, a useful intermediate for the preparation of the amphetamines and amphetaminil [12]. To the best of our knowledge, the current report is the first in which a native *Rhodococcus* species was investigated as a whole-cell biocatalyst to afford various (*R*)-aromatic alcohols via stereoselective oxidation.

Rhodococcus erythropolis WZ010 possesses several outstanding features making it a versatile biocatalyst. The strain exhibits high activity and excellent stereoselectvity in both oxidation and reduction. The strain stereoselectively oxidized racemic 1-phenylethanol at a relatively high concentration to give (*R*)-1-phenylethanol with a yield of 49.7 %, which is very close to the 50 % theoretical value in kinetic resolution. Although stereoselective oxidation ideally gives only half of the substrate racemate as a desired product, the oxidation required no energy source other than atmospheric oxygen [9, 11]. Compared to asymmetric reduction, simplicity of stereoselective oxidation would be advantageous in cases where the cost of co-substrate for bioreduction cannot be ignored. In cases where the product ketone and the remaining alcohol can be easily separated, the sequential reactions of oxidation and reduction using the same *R. erythropolis* WZ010 would be a favorable way to obtain both (*R*)- and (*S*)-enantiomers from racemic alcohols (Fig. 1). *R. erythropolis* WZ010 has a broad substrate spectrum including 1-phenylethanol and its derivatives, some of which are valuable intermediates for synthesis of biologically or pharmacologically active compounds. (*S*)-1-Phenyl-1,2-ethanediol is a versatile chiral building block for the synthesis of pharmaceuticals, agrochemicals, and liquid crystals [17], whereas (*R*)-1-phenylethanol is used as a fragrance in the cosmetics industry, an ophthalmic preservative, and an inhibitor of intestinal cholesterol absorption [24]. In addition, the adaptability of *R. erythropolis* WZ010 to broad ranges of pH, temperature, and substrate concentration make it more applicable in large-scale processes. Similar to oxidation, bioreduction catalyzed by resting cells of *R. erythropolis* WZ010 required no external coenzyme regeneration but only glucose as a co-substrate. The internal coenzyme regeneration for bioreduction was significantly improved by the addition of glucose or glycerol, which has been highly preferred in terms of its availability and low cost [34]. All these features demonstrate that it is feasible to obtain the (*R*)-enantiomer by stereoselective oxidation of racemic aromatic alcohols and/or the (*S*)-enantiomer by asymmetric reduction of aromatic ketones using the same *R. erythropolis* WZ010 as biocatalyst.

Coenzyme recycling and its control are one of the most crucial issues encountered in redox reactions catalyzed by alcohol dehydrogenases. In the stereoselective oxidation of 1-phenylethanol, the strain predominantly oxidized the (*S*)-enantiomer to acetophenone. Since alcohol dehydrogenase-catalyzed redox reactions are usually reversible, it seemed logical to speculate that the product in the asymmetric reduction of acetophenone would be (*S*)-1-phenylethanol. However, the product without the addition of any co-substrate was (*R*)-enantioselective, indicating that (*R*)-enantioselective alcohol dehydrogenase was present in the cells. The speculation was supported by cloning and characterization of a chiral alcohol dehydrogenase from *R. erythropolis* ATCC 4277 reducing ethyl 4-chloroacetoacetate to (*R*)-4-chloro-3-hydroxybutyrate [36]. When various co-substrates were tested in the bioreduction, the *e.e.* values varied and the type of stereoselectivity was even reversed from (*R*)- to (*S*)-enantioselective, implying the presence of endogenous enzymes exhibiting competing or complementary activity to the desired reaction. The genome information of *R. erythropolis* strain PR4 demonstrated that it harbored 13 putative genes encoding zinc-containing alcohol dehydrogenases, of which a couple of (*S*)-enantioselective alcohol dehydrogenases had been cloned, overexpressed, and characterized [1, 13, 14, 21]. Owing to the diverse stereospecificies of these enzymes, overexpressing desired enzymes or knocking out non-desired enzymes would further improve the optical purity of the product in bioreduction [20].

Rhodococcus erythropolis WZ010 has distinct catalytic properties from other bacterial biocatalysts. Redox reactions catalyzed by alcohol dehydrogenases are usually reversible and exhibit the same optimal temperatures for both oxidation and reduction [33–35]. However, the optimal temperatures of oxidation and bioreduction for *R. erythropolis* WZ010 were 30 and 35 °C, respectively, implying that more than one alcohol dehydrogenase might be involved in those reactions. In contrast to *R. erythropolis* JX-021's performance in the bioreduction of 1-phenyl-2-propanone [12], the conversion of bioreduction catalyzed by *R. erythropolis* WZ010 appeared optimal when the reaction mixtures were kept static. Bioreduction was generally carried out under shaking conditions to improve the diffusion and the partitioning of the substrate and product

in the reaction system. The case of *R. erythropolis* WZ010 indicated that levels of dissolved oxygen played a more crucial role in small-scale reaction systems and lower dissolved oxygen facilitated its internal coenzyme regeneration for bioreduction. On the other hand, higher stirring speed significantly increased the conversion of biooxidation and proceeded without any energy source other than atmospheric oxygen.

Rhodococcus erythropolis stereoselectively oxidized 120 mM 1-phenylethanol affording 49.4 % (*R*)-1-phenylethanol yield and >99.9 % *e.e.* value after 48 h, whereas the bioreduction of 80 mM acetophenone after the same reaction time only gave 64.5 % (*S*)-1-phenylethanol yield. These findings suggested that aromatic ketones might be more toxic to the cells than the corresponding alcohols. Higher concentrations up to 150 mM did not significantly affect the stereoselectivity but lowered the conversions of bioreduction; this behavior was similar to that of *Bacillus* sp. ECU0013 with high substrate tolerance [31]. Although conversion at high substrate concentrations and consequently high product concentrations are advantageous in terms of economy, the bioreduction of aromatic ketones often encounters substrate/product inhibition. It was proposed that the substrate/product inhibition of *R. erythropolis* JX-021 was due to the leakage of coenzymes from the whole cells at high substrate concentrations. To protect the cells from permeabilization, the dual co-substrates (800 mM glucose and 1 M glycerol) were investigated in the bioreduction of 150 mM ketones but no obvious improvement was observed, indicating that high concentration of substrate might also inhibit the activities of alcohol dehydrogenases or the enzymes involved in internal coenzyme recycling. Other methods therefore appear necessary to resolve the substrate/product inhibition problem, e.g., removal of the product by absorption with resins, the use of organic solvent as the second phase, and the addition of suitable amounts of reduced coenzymes [12].

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