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Full Length Research Paper

Rhodotorula mucilaginosa as a new biocatalyst for asymmetric reduction of acetophenone

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The biocatalysts for asymmetric reduction of aromatic ketones were successfully screened from soil samples polluted by substituted acetophenones. 12 strains could asymmetrically reduce acetophenone into phenethanol, while only strain YS62 possessed the best performance of reducing acetophenone into (*S*)-1-phenethanol. It was identified as *Rhodotorula mucilaginosa* based on phenotypic and genetics characteristics and it was used for further asymmetric reduction experiments of acetophenone as a new biocatalyst. *R. mucilaginosa* YS62 whole-cells could catalyze the asymmetric reduction of acetophenone (35 mM) into (*S*)-1-phenethanol (31.4 mM) with a conversion rate of 89.7% and enantiomeric excess (e.e.) of 99.9% under 60 g/L YS62 cell, pH 6.5 , 34°C for 30 h and 2% glucose as a co-substrate. These results have shown that *R. mucilaginosa* YS62 is a promising biocatalyst for the production of optically active phenylethanol derivatives.

Key words: Asymmetric reduction, acetophenone, *Rhodotorula mucilaginosa*.

INTRODUCTION

Chiral phenylethanol derivatives are very important intermediates to introduce chiral centers into pharmaceuticals, spices, agricultural chemicals and special materials (Ni and Xu, 2011; Kurbanoglu et al., 2011). In the past few years, a large number of chiral phenylethanol derivatives produced by biological and chemical methods have been reported in various documents (Ni and Xu, 2002; Kurbanoglu et al., 2007; Yang, 2011). Because of pharmaceutical industry's need for enantiopure compounds, it can be expected that their number will continuously increase in the next decades. Compared with conventional chemical processing, biocatalytic asymmetric reduction has recently become an important method to prepare enantiopure compounds because it has high enantioselectivity, environmentally benign processes and energy-effective operations (Ni and Xu, 2011). Both isolated enzymes and whole cells can be employed as biocatalysts in the asymmetric synthesis of chiral phenylethanol derivatives. In biocatalytic processes, the reaction of isolated enzymes needs the supply of external cofactors. As they are very expensive, large-scale applications of such reaction are rather scarce despite considerable research efforts to develop *in-vitro* cofactor regeneration systems for re-establishing the biocatalyst reduction potential (Valadez-Blanco and Livingston, 2009). On the contrary, whole cells can use their internal metabolism to regenerate such cofactors by utilizing inexpensive and readily available hydrogen-donor substrate materials (Nakamura et al., 2003). Moreover, the excellent enantioselectivity, high yield and volumetric productivity of whole-cell biotransformation are very attractive for the production of chiral synthons. Therefore, much attention in biocatalytic asymmetric reduction has turned to developing the whole cell biocatalysts with desired activity (Shaw et al., 2003).

It is well known that the screening of microorganisms

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with the desired enantiometric excess and conversion activity is an efficient way to obtain good whole-cell biocatalysts for the asymmetric reduction of substrates. In recent years, various microbial cultures belonging to the genera *Saccharomyces*, *Aspergillus*, *Alternaria*, *Rhodoturula, Paracoccus*, *Comamonas* and *Candida* have been reported for the enantiomeric reduction of acetophenone and its derivatives (Griffin et al., 1998; Mandal et al., 2004; Comasseto et al., 2004; Patel et al., 2004; Kurbanoglu et al., 2007a, b; Valadez-Blanco and Livingston, 2009). Among them, the tremendous potential of yeast cells of *S. cerevisiae* has been well recognized. However, in order to meet the new demand of whole cell biocatalysts in pharmaceuticals, agrochemicals and natural products, it is still necessary to discover new strains which are able to reduce various substrates with excellent enantiomeric purity and high yield.

In our previous work, we have found the chiral phenylethanols present in soil polluted by substituted acetophenones around some chiral drug factories. However, we still have no knowledge about whether they come from wastes discharged by the factories or from biological transformation products mediated by some microorganism living in soil. In this study, a microbial strain with excellent asymmetric reduction activity and high conversion yield of acetophenone have isolated from soil polluted by acetophenone in a chiral drug factory located in the eastern suburb of Chengdu, China. Morphological and physiological characteristics, 18S rRNA and 26S rRNA D1/D2 variable domain phylogenies and the asymmetric reduction of acetophenone were also analyzed. To our knowledge, this is the first report on the asymmetric reduction characteristics of acetophenone mediated by *R. mucilaginosa* with high conversion rate and enantioselectivity.

MATERIALS AND METHODS

Soil samples and isolation of microorganism with asymmetric reduction activity

Soil samples were collected from a factory where chiral drugs were produced in the eastern suburb of Chengdu, China. The isolation of microorganisms was performed as described by Kurbanoglu et al. (2008) with some modifications. YPD agar plates inoculated with soil suspension were incubated at 30°C for 48 h. The developing colonies were isolated and were then screened for the asymmetric reduction of acetophenone to (*S*)-1-phenylethanol. The most potent strains was selected and stored at -20°C in 5 ml vials containing 50% (v/v) glycerol until used.

Phenotypic analysis of the yeast isolate

Morphological and physiological characteristics of the isolated yeast were examined according to Yeast (Barnett et al., 1990). Cellular fatty acid composition was analyzed by gas chromatography according to Xiang et al. (2008). The methyl esters of fatty acid were obtained from 100 mg of freeze-dried yeast cells harvested from shaking culture at 30°C for 48 h at pH 7.0 in YPD liquid medium, then extracted twice with the mixture of $CHCl_3$/MeOH/H_2O (65:25:4, v/v). Methyl esters of different fatty acids obtained were then separated and analyzed by an automated gas chromatograph mass spectrometer (GCMS-QP2010, Shimadzu, Japan) on a fused silica capillary column (25 m × 0.25 mm ID) coated with 5% methyl phenyl silicone.

Molecular analysis of the yeast isolate

Genomic DNA was extracted and purified from cells in the mid-logarithmic growth phase according to Methods described by Adams et al. (1997). The polymerase chain reaction (PCR) amplification of 18S rRNA gene of the isolate was performed using the universal primers NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') and NS8 (5'-TCC GCA GGT TCA CCT ACG GA-3') (Anderson et al., 2003). The amplification conditions were as followed: pre-incubation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension at 72°C for 10 min. The primers NL1 (5'-GCA TCA ATA AGC GGA GGA AAA G-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') were employed to amplify the 26S rRNA D1/D2 variable domain of the isolate (White et al., 1990). The thermal cycling conditions were 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and 1 cycle of 10 min extension at 72°C. The amplified 18S rRNA and 26S rRNA D1/D2 fragments were respectively cloned into a plasmid pMD18-T T-vector (Takara, Kyoto, Japan) using DNA Ligation Kit version 2 (Takara, Kyoto, Japan) according to the manufacturer's protocol. The recombined plasmids were then transformed into the chemically competent *E. coli* DH 5α cells and transformants were identified on LB agar plates containing 100 µg/ml ampicillin, 40 µg/ml X-gal and 24 µg/ml IPTG. The white clones were used to sequence the 18S rRNA and 26S rRNA D1/D2 variable domain.

The data of 18S rRNA and 26S rRNA D1/D2 sequences have been respectively deposited in GenBank Database by using the BLAST program and then the similarity percent was obtained. Multiple alignments were run via the Clustal X1.83 program (Thompsom et al., 1997). The 18S rRNA and 26S rRNA D1/D2 phylogenetic trees were respectively constructed with the MEGA program version 5.0 by using the neighbor-joining method with the Kimura two-parameter model (Tamura et al., 2011).

Evaluation of asymmetric reduction of the yeast isolate

3 g wet living cells were harvested from Erlenmeyer flask by centrifugation and re-suspended into 250 ml shaking flask containing 50 ml of potassium phosphate buffer (pH 6.5). Subsequently, acetophenone and glucose were directly added into the mixture until final concentration of 70 mmol/L and 2% (w/v), respectively. The reaction mixture was incubated at 30°C and 170 rpm for 30 h to investigate the effect of co-substrate category, initial pH, reaction temperature, cell concentration and substrate concentration on asymmetric reduction reaction. Supernatant of reaction mixture was separated by centrifugation and extracted twice with ethyl acetate. The obtained ethyl acetate layer was dried with anhydrous $MgSO_4$ and then used for GC analysis. The e.e. value and yield of the product were evaluated as follows: yield = C_P/C_0×100% where C_P and C_0 are the final molar concentration of the product and the initial molar concentration of the substrate, respectively, e.e. = (C_S-C_R) / (C_S-C_R) ×100% where C_R and C_S are the molar concentrations of (*R*) - 1- phenethanol and (*S*) -1- phenethanol, respectively (Wang et al., 2011).

Nucleotide sequence accession numbers

The 18S rRNA sequences GenBank ID: EU380239; the 26S rRNA D1/D2 sequences GenBank ID: EU380240.

Table 1. The catalytic characteristics of different strains for asymmetric reduction of acetophenone.

Strain	Conversion (%)	e.e. value (%)	Conformation
YM62	8.4	94.6	*S*
YM63	21.3	98.5	*S*
YM64	19.0	98.7	*S*
YM67	21.4	98.7	*S*
YM68	16.9	98.7	*S*
YM82	21.5	98.6	*S*
YS51	22.0	98.9	*S*
YS62	26.8	98.8	*S*
YN41	11.5	98.2	*S*
YQ21	6.6	99.6	*S*
YL13	3.2	99.6	*S*
Y113	2.5	94.5	*R*

e.e.: enantionmeric excess; *S*: (*S*)-1- phenethanol; *R*: (*R*)-1- phenethanol

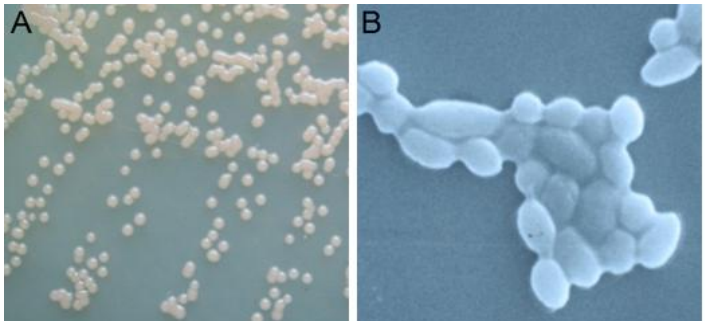

Figure 1. Colonies of *R. mucilaginosa* strainYS62 on the YPD medium (A) and cell morphology by scanning electron microscopy (B, × 2000).

RESULTS AND DISCUSSION

Isolation of strains

Out of 145 strains isolated from soil samples in the 70 mmol/L acetophenone medium, 12 strains could asymmetrically reduce acetophenone into phenethanol. Among them, 11 strains could asymmetrically reduce acetophenone into (*S*)-1-phenethanol and 1 strain into (*R*)-1-phenylethanol (Table 1). However, only the strain YS62 possessed the best performance of asymmetric reduction. Its substrate transformation efficiency and enantiomeric excess value of (*S*)-1-phenethanol reached 26.8 and 98.8%, respectively. Therefore, YS62 was selected to carry out further investigations.

Phenotypic characteristics

On the YPD medium plate, the YS62 colonies are round configurations raised elevations, regular margin, smooth surface, ropy texture and opacitas pink, but no pigment diffusing into the surrounding medium, no filamentous and pseudohyphae growth. Its cells were ellipse or long ellipse, pullulating but only one side by scanning electron microscopic examination. The colonial morphology of strain YS62 on the YPD medium and cell morphology under scanning electron microscopy were shown in Figure 1. The glucose, sucrose, trehalose, D-xylose, raffinose and ethylamine but no potassium nitrate, lactose, melibiose, inositol, soluble starch and cadaverine were assimilated by YS62 isolate, while the galactose, melezitose, maltose, glycerol, cellobiose, L-arabinose and citric acid as substrates were variable. YS62 can grow on 0.1% cycloheximide medium at 25 and 30°C, but not at 37°C. To obtain further information about the taxonomic affiliation of the isolate, cellular fatty acid compositions of YS62 were characterized (data not shown). Content of major cellular fatty acids of YS62 isolate was similar to those of *R. mucilaginosa* reported by Shi and Zhou (1993). Compared with *R. mucilaginosa*, the morphological, physiological and cellular fatty acid characteristics of YS62 strain were highly similar to those of *R. mucilaginosa*. Therefore, the YS62 was classified as *R. mucilaginosa*.

Phylogenetic analysis of 18S rRNA and 26S rRNA D1/D2 domain

Tao et al. (2007) thought the essence of all phenotypic characteristics in all organisms is outward manifestation of their gene molecular level. However, in some cases, there is a pattern of disparity between isolate and type strains by comparison of morphological traits and physiological features (Querol et al., 2003). This may lead to an incorrect classification of species or a false identification of strains. In recent years, comparison of rRNA has been used extensively to assess both close and distant relationships among many types of organisms including

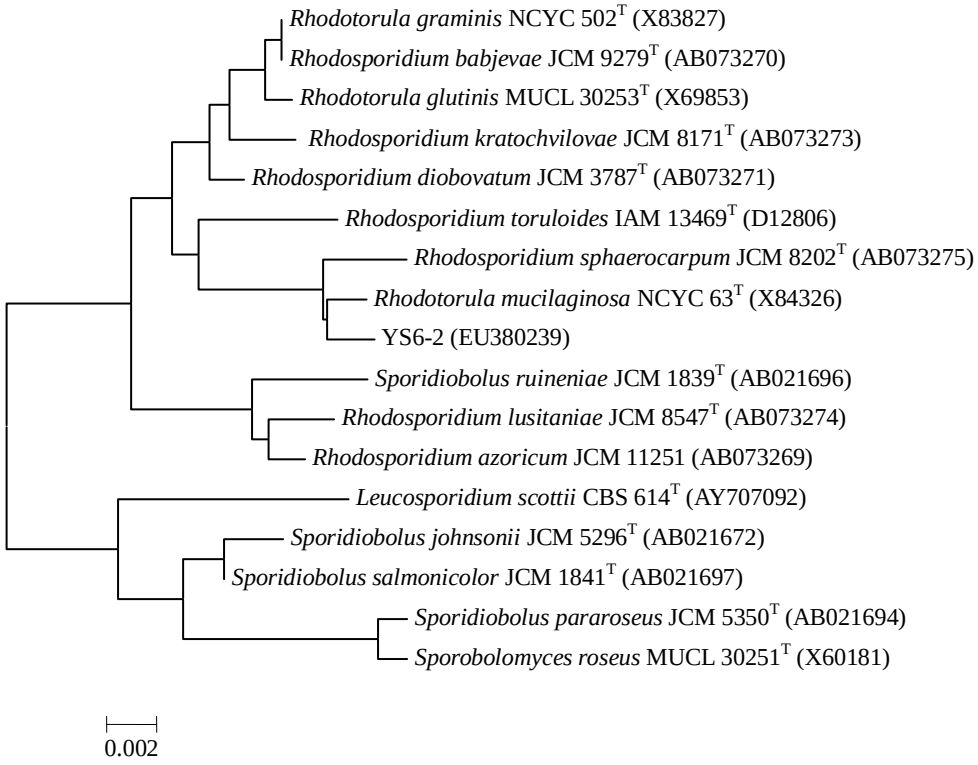

Figure 2. Neighbour-Joining tree showing the phylogenetic position of strain YS62 and representatives of some other related taxa based on 18S rRNA sequences. Bar, 0.002 substitutions per nucleotide position.

yeast species. Some of these methods are based on sequence analysis, primarily of the 26S rRNA D1/D2 variable domain (Kurtzman and Robnett, 1998) and of the 18S subunit (James et al., 1997). In the current study, the 18S rRNA and the 26S rRNA D1/D2 variable domain of YS62 isolate respectively shared 99.44% similarity to that of *R. mucilaginosa* NCYC63^T and 100% similarity to that of *R. mucilaginosa* CBS 8383. The high similarity values as well as above morphological, physiological and cellular fatty acid results suggested that the YS62 should be assigned to *R. mucilaginosa*.

To disclose the taxonomic position and relationships, the phylogenetic trees based on the 18S rRNA and 26S rRNA D1/D2 domain of YS62 and the relational strains in GenBank by using neighbor-joining method were respectively constructed (Figure 2,3). Two evolutionary distance dendrograms further revealed that the isolate YS62 was affiliated to the pedigree of *R.mucilaginosa*, a lineage of domain yeast.

Evaluation analysis of asymmetric reduction

Biocatalysts employed in organic reactions have opened up a new field in the industrial applications of chemical compounds. However, the number of effective biocatalysts remains limited. Is *R.mucilaginosa* YS62 a promising biocatalyst for asymmetric reduction reaction or not? In this study, parameters such as co-substrate, pH, temperature, cell concentration and acetophenone concentration were investigated in the shake flasks. When 2% (w/v) glucose, sucrose, methanol, ethanol, 2-propanol, glycerol and 1-butanol as co-substrate were respectively added into the reduce system, the acetophenone conversion and corresponded product's e.e. value stimulated by glucose have increased up to higher levels, 50.2 and 99.2% (Table 2). Yan et al. (2011) thought that it is possible that multiple cofactor recycling systems run in parallel in cell by adding several hydrogen donors, and hence greatly enhance the cofactor regeneration. Thus, comparing with no co-substrate, conversion rate of acetophenone and e.e. value of product were greatly improved. While, the conversion rate of acetophenone would decrease when the glucose content was more or lower than 2% (w/v), specifically above 10%. Interestingly, influence of glucose concentration on stereo selectivity was not obvious, e.e.≥ 99.1% (data not shown).

To determine the optimum pH of the YS62 catalytic reduction, the reaction was carried out from pH 5.0 to 9.0 media containing 2% (w/v) glucose (Table 3). It should be noted that conversion of acetophenone and the e.e. value of product were affected by pH value less than 7.0. The percentage conversion and e.e. value sharply increased when the buffer pH value from 5.0 to 6.5. At pH value

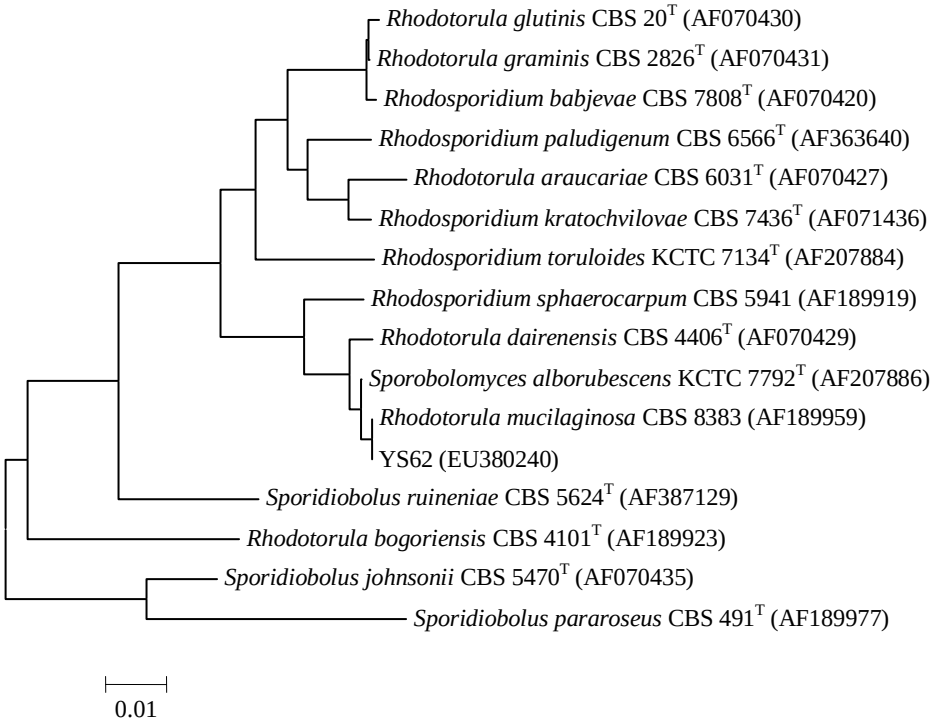

Figure 3. Neighbour-Joining tree showing the phylogenetic position of strain YS62 and representatives of some other related taxa based on 26S rRNA D1/D2 domain. Bar, 0.01 substitutions per nucleotide position.

Table 2. The effect of co-substrate category on asymmetric reduction ability of *R. mucilaginosa* YS62 at 70 mmol/L acetophenone, 2% (w/v) co-substrate and 30 h reaction time at agitation of 170 rpm.

Substrate	Conversion (%)	e.e. value (%)
Glucose	50.2	99.2
Sucrose	46.2	99.1
Methanol	45.0	99.0
Ethanol	32.4	99.9
2-propanol	43.1	98.9
Glycerol	43.6	99.1
1-butanol	14.8	97.7

between 6.5 and 9.0, the conversion rapidly decreased again, but the e.e. value of the reaction had no notable decreasing (Table 3). Thus, a reaction pH 6.5 of buffer was thought as optimum catalytic pH.

The reaction temperature was also investigated and the results are shown in Table 3. It was observed that the conversion increased gradually when raising the temperature from 18 to 34°C. However, further increasing the temperature could lead to a sharp decrease in the conversion, which could be attributed to the partial inactivation of the cells at higher temperatures (above 37°C). On the contrary, 22 to 45°C did not have an effect on the enantiomeric purity of the product. The highest conversion of the substrate and the e.e. value of products were 52.3 and 99.7%, respectively. Therefore, 34°C was chosen as the optimum temperature for subsequent investigation. The effect of cell concentration on the reduction reaction of acetophenones was illustrated in Figure 4. The conversion rate of acetophenones increased with the cell concentration from 60 to 180 g/L. When the cell concentration increased to 180 g/L, the conversion rate of acetophenones was close to 100%, where it reached its maximum. This phenomenon was due to increase concentration of cells, which augments the amount of catalyst, thus it raised the conversion rate of acetophenones. When the cell concentration reached above 180 g/L, the influence of cell concentration on the conversion rate of acetophenones was not evident.

Table 3. The effect of different pH values and temperatures on asymmetric reduction ability of *R. mucilaginosa* YS62 at 70 mmol/L acetophenone, 2% (w/v) glucose and 30 h reaction time at agitation of 170 rpm.

pH	Conversion (%)	e.e. value (%)	Temperature °C	Conversion (%)	e.e. value (%)
5.0	5.9	96.8	18	37.2	96.7
6.0	29.8	99.1	22	41.4	99.4
6.5	47.8	99.2	26	45.6	99.5
7.0	45.2	99.2	30	50.1	99.5
7.5	41.0	99.1	34	52.3	99.7
8.0	30.1	98.9	37	48.9	99.6
9.0	9.5	98.7	45	32.8	99.9

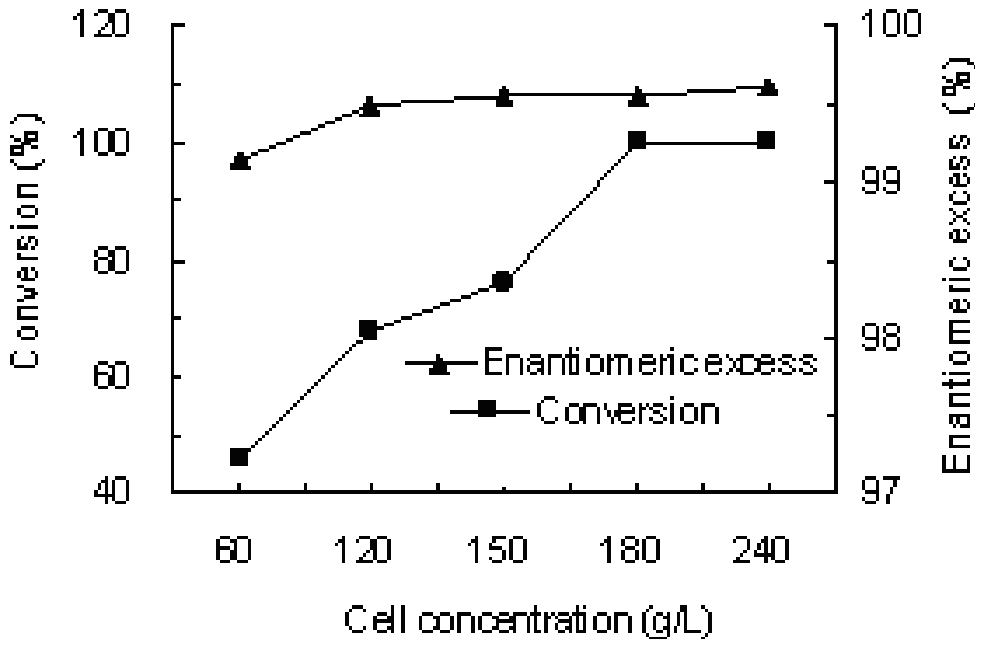

Figure 4. The effect of cell concentration on asymmetric reduction ability of *R. mucilaginosa* YS62 at 70 mmol/L acetophenone, pH 6.5 and 34°C at agitation of 170 rpm for 30 h and 2% glucose as a co-substrate.

Furthermore, the effect of cell concentration on stereo selectivity of reduction reaction is not obvious, e.e. value remaining around 99.5% (Figure 4).

In enzyme-catalyzed reaction systems, enzyme and substrate are the most basic influencing factors. The effect of different substrate concentration on the reduction reaction was shown in Figure 5. In YS62 isolate catalytic reaction, the conversion rate of acetophenones and the stereo selectivity decreased with the increase of substrate concentration (Figure 5). Under the initial acetophenones concentration from 18 to 35 mmol/L, the conversion rate and the e.e. values were higher. However, the accumulation of phenethanol was maintained at 35 to 38.6 mmol/L although conversion rate and e.e. values were also decreased when the substrate concentration was increased to 35 to 70 mmol/L. When acetophenones was above 70 mmol/L, the conversion rate, e.e. value and accumulation of phenethanol dropped to some degree. It is thought that the amount of enzyme is fixed in YS62 cell.

At low substrate concentrations, accumulation of product is increased with increasing substrate concentration. When substrate concentration makes the reduction ability of enzyme reaching to saturation, the accumulation of product maintains a certain level although substrate concentration increases. At higher substrate concentration, the conversion and stereo selectivity would be inhibited because of the toxicity effect of aromatic compounds and reactive carbonyl groups for cell (Pfruender et al., 2006). Thus, when considering the conversion rate of the acetophenones, stereo selectivity and accumulation of phenethanol, it could be stated that the optimal parameters of asymmetric reduction reaction of acetophenones was 35 mmol/L under 60 g/L cell concentration. Under these con-

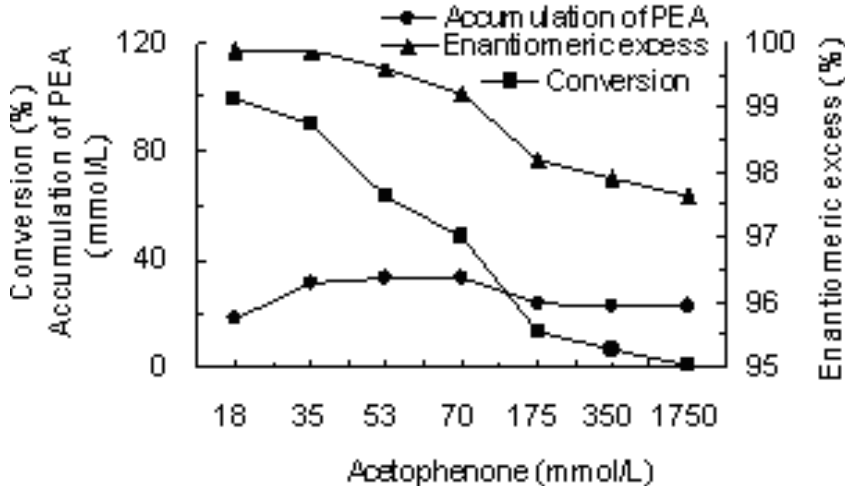

Figure 5. The effect of acetophenone concentration on asymmetric reduction ability of *R. mucilaginosa* YS62 at 60 g/L cell, pH 6.5 and 34°C at agitation 170 rpm for 30 h and 2% glucose as a co-substrate.

ditions, the conversion rate, e.e. value and accumulation of phenethanol respectively reached 89.7, 99.9% and 31.4 mmol/L.

Conclusions

A new yeast strains with high activity and excellent enantioselectivity for the reduction of acetophenones has successfully been obtained from acetophenone-polluted soil. It was identified as *R. mucilaginosa* through phenoltypic and genetic characteristics. Though many enantioselective biotransformations of acetophenones mediated by microbes have been described in literatures, *R. mucilaginosa* YS62 has been reported for the first time as a novel biocatalyst for the asymmetric synthesis of (*S*)-phenylethanol derivatives with excellent e.e. value and conversion rate. The *R. mucilaginosa* YS62 whole-cells catalyzed the asymmetric reduction of acetophenone (35 mM) into (*S*)-1-phenethanol (31.4 mM) with a conversion rate of 89.7% and enantiomeric excess of 99.9% at 60 g/L YS62 cell, pH 6.5 and 34°C for 30 h and 2% glucose as a co-substrate. The results suggest that *R. Mucilaginosa* YS62 could be a promising biocatalyst, which could be of great importance to chemical reactions used for preparing optically active phenylethanol derivatives.

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