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Highly enantioselective reduction of 2-hydroxy-1-phenylethanone to enantiopure (*R*)-phenyl-1,2-ethanediol using *Saccharomyces cerevisiae* of remarkable reaction stability

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ABSTRACT

Saccharomyces cerevisiae JUC15 was successfully obtained by target reaction-oriented screening, which reduced 2-hydroxy-1-phenylethanone (HPE) to (*R*)-phenyl-1,2-ethanediol ((*R*)-PED) of excellent enantiomeric excess (e.e. >99.9%). There was no significant decrease in the yield and optical purity of (*R*)-PED when the free cells were reused for 40 repeated cycles at 2 g L^{-1} substrate concentration. The strain used cheap sucrose for cofactor regeneration and worked over a considerably wider range of pH (4–9). The optimum substrate concentration was 8 g L^{-1}, which was higher than any other biocatalysts reported so far. Interesting, when HPE concentration reached 20 g L^{-1} in reaction system, where 43.2% of the substrate was present in suspended solid form, the reaction still gave enantiopure (*R*)-PED in 71% yield. Last but not least, the product e.e. kept above 99.9% in all examined conditions. These results suggest the potential of this strain for the industrial production of optically active (*R*)-PED.

1. Introduction

Optically active secondary alcohols are versatile chiral building blocks for the synthesis of pharmaceuticals, agrochemicals, pheromones and liquid crystals (Gamenara and de Maria, 2009). Among them, enantiomerically pure (*R*)-1-phenyl-1,2-ethanediol ((*R*)-PED) can serve as a synthon for the preparation of (*R*)-norfluoxetine, (*R*)-fluoxetine and β-lactam antibiotics (Cao et al., 2006; Kumar et al., 2004), which can be used for the treatment of psychiatric disorders and metabolic problems (Kumar et al., 2004).

A series of bio-methods for preparation of (*R*)-PED have been explored, including stereospecific dihydroxylation of styrene catalyzed by naphthalene dioxygenase (Lee and Gibson, 1996), the lipase-mediated resolution of racemic PED (Bosetti et al., 1992), enantioselective microbial reduction of 2-hydroxy-1-phenylethanone (HPE) (Ridley and Stralow, 1975), and enantioconvergent hydrolysis of racemic styrene oxide (SO) using recombinant whole cells (Cao et al., 2006). However, even given a low product concentration, the optical purity of (*R*)-PED prepared by bio-methods so far has not met the stringent criteria of the pharmaceuticals industry, in which an enantiomeric excess (e.e.) of 98% is a minimum acceptable level (Pollard and Woodley, 2007). Furthermore, several strategies inherited weaknesses such as involvement of the carcinogenic SO and in case of kinetic resolution, the maximal yield can never exceed 50%. Up to now, only little description in the literature of synthesis of (*R*)-PED via bioreduction has been found (Manzocchi et al., 1988; Ridley and Stralow, 1975). As early as the 1970s, Ridley and Stralow (1975) firstly demonstrated that the baker's yeast-mediated reduction of HPE afforded (*R*)-PED in 45% yield. Later, Manzocchi et al. (1988) further enhanced the product yield with a substrate concentration of 0.68 g L^{-1} over 7 days, and the e.e. of (*R*)-PED was only 92%. However, problems such as low substrate concentration and long reaction period still remain to be solved.

It was very recently that Nie et al. (2008) were successful in preparing (*R*)-PED of 95.5% e.e. by using a recombinant *Escherichia coli* strain expressing (*R*)-specific carbonyl reductase from *Candida parapsilosis* CCTCC M203011. Unfortunately, low biomass yield and high biocatalyst consumption would restrict its application in large-scale production (Schoemaker et al., 2003). In addition, according to our former research (Nie et al., 2008), unsatisfactory performance of the recombinant biocatalyst was observed at relatively higher substrate concentration.

In this work, we aimed to screen for a microbial strain that could excellent stereoselectively reduce HPE at a high substrate concentration (the reduction route was shown in Scheme 1). Strategies for target-oriented screening were designed, and *Saccharomyces cerevisiae* JUC15 was discovered, which displayed excellent operational stability and high substrate tolerance. For further dem-

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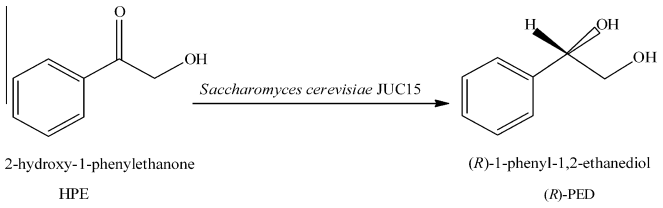

Scheme 1. *Saccharomyces cerevisiae* JUC15-mediated asymmetric reduction of 2-hydroxy-1-phenylethanone to (*R*)-phenyl-1,2-ethanediol.

onstration of the advantages of such system, crucial requirements for the successful preparation of (*R*)-PED were also investigated.

2. Methods

2.1. Microorganisms and chemicals

Microorganisms for screening were obtained from the collections of Spanish Type Culture Collection (CECT), American Type Culture Collection (ATCC), Institute for Fermentation of Osaka (IFO), the Brewing Society of Japan, China Center for Type Culture Collection (CCTCC), China Center of Industrial Culture Collection (CICC), Microbial Culture Collection Center of Guangdong Institute of Microbiology (GIM), and Chinese Academy of Science (AS). Microorganisms preserved in our laboratory (JUC) were also tested. (*R*)-PED, (*S*)-PED and HPE were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Hexane and 2-propanol used for high-performance liquid chromatography (HPLC) were of chromatographic grade (Honeywell Co. Ltd., USA). All other chemicals were from commercial sources and were of analytical grade.

2.2. Medium and cultivation

Yeasts were cultivated in the medium comprising 4% (w/v) glucose, 0.42% (w/v) yeast extract, and 10% (v/v) mineral solution (pH 7.0). Bacteria were cultivated in the medium comprising 0.5% (w/v) glucose, 0.2% (w/v) yeast extract, 1% (w/v) beef extract, 1% (w/v) peptone and 5.0% (v/v) mineral solution (pH 7.0). Molds were cultivated in medium containing 2% (w/v) glucose, 0.3% (w/v) yeast extract, 0.5% (w/v) meat extract, 0.5% (w/v) peptone, and 5% (v/v) mineral solution (pH 7.0). The mineral solution was consisted of 13% (w/v) $(NH_4)_2HPO_4$, 7% (w/v) KH_2PO_4, 0.8% (w/v) $MgSO_4?7H_2O$, and 0.1% (w/v) NaCl. All microorganisms were cultured in 250 mL flasks with 50 mL medium at 30 °C and 150 rpm. After 48 h of growth, the cells were harvested by centrifugation, washed twice with physiological saline and then used for further reaction.

2.3. Determination of the solubility of HPE and the dry weight of cells

The solubility of HPE in phosphate buffer (0.1 M, pH 6.5) was measured similar to the procedures described for 2,4-dinitrotoluene solubility (Prak and O'Sullivan, 2006), with slight modification. An excess amount of HPE was mixed with 5 mL of phosphate buffer in sealed vials and incubated at 30 °C in a shaking bath (150 rpm), which maintained the temperature within 0.5 °C. After 24 h, samples of saturated water were quickly withdrawn from the mixture using a syringe and filtered through 0.22 μm filters to remove particles. Finally, the filtrate was extracted with ethyl acetate and analyzed by HPLC.

The dry weight of cells was determined by drying a known amount of wet cells in an oven at 110 °C to constant weight.

2.4. Strategies for target-oriented screening

In order to obtain biocatalysts with high stereoselectivity, the first screening round was carried out as follow. An amount of cells equivalent to 0.04 g of dry weight were suspended in 2 mL of potassium phosphate buffer (0.1 M, pH 6.5) with HPE (1 g L^{-1}, w/v) and glucose (10 g L^{-1}, w/v). The reaction was conducted at 150 rpm, 30 °C for 48 h. The cells then were removed by centrifugation and the supernatant was extracted with ethyl acetate prior to HPLC analysis.

In order to further obtain strains with high substrate tolerance and favorable operational stability, the second screening round was performed on the strains from the first step selection. The cells of each microorganism were employed to catalyze the reduction of HPE with gradually increased concentrations (1, 1.5, and 2 g L^{-1}) in successive batch mode. Initially, whole-cell biocatalysts were used to reduce 1 g L^{-1} HPE under the above-described conditions for 48 h. The cells then were recovered by centrifugation and reused to catalyze the reduction of HPE at 1.5 g L^{-1} substrate concentration. The procedures were repeated following the same scheme but with 2 g L^{-1} HPE. After three successive batch cycles, the supernatant was analyzed by HPLC.

2.5. General procedure for the asymmetric reduction of HPE with S. cerevisiae cells

In a typical experiment, a weighed amount of HPE was mixed with 2 mL of phosphate buffer (0.1 M) containing co-substrate and 0.2 g of wet cells (equivalent to 0.04 g of dry cell). Then the reactions were carried out at an appropriate temperature, 150 rpm for 24–48 h under the limited oxygen conditions or aerobic conditions, which were maintained by closing flasks with rubber or cotton stoppers, respectively. Unless otherwise specified, all the reactions were run under the limited oxygen conditions. Details about reaction temperature, buffer pH, co-substrate and substrate concentration were specified for each case.

2.6. Operational stability of S. cerevisiae cells

To assess the operational stability of *S. cerevisiae* JUC15 cells, their reuse was investigated over 40 successive reaction cycles. Initially, 0.2 g of wet cells were suspended into 2 mL of phosphate buffer (0.1 M, pH 6.5) containing 10 g L^{-1} glucose and 2 g L^{-1} HPE. The reaction was then repeated over 40 reaction cycles (24 h per cycle) without changing the cells at 30 °C and 150 rpm. Between cycles, the cells were centrifuged, washed twice with saline and resuspended in fresh reaction mixture containing raw material and glucose.

2.7. Crude cell extract preparation

The yeast cells were resuspended in an appropriate volume of potassium phosphate buffer (20 mM, pH 6.5). If the cells had been used in the reaction, they were first recovered from the reaction medium, washed twice with saline solution. The cell suspension was then disrupted by sonication, at 200 W for 30 min at 0 °C, with an ultrasonic oscillator (Sonic Materials Co., USA) and after removing the cell debris by centrifugation, the supernatant was used as the cell-free extract.

2.8. Analytical methods

HPE reductase (HPER) activity measurements were performed as described previously (Nie et al., 2008), based on monitoring the oxidation rate of NADH at 340 nm. The assay mixture in 250 μL comprised potassium phosphate buffer (0.1 M, pH 6.5),

2 mM NADH, 10 mM HPE and an appropriate amount of crude cell extract. One unit (U) of enzyme activity refers to 1 μmol of NADH consumed per minute under the assay conditions. Protein content was determined by Bradford's method (1975) using bovine serum albumin as standard. Glucose was measured by the dinitrosalicylic acid (DNS) method (Jiang et al., 2002). Cell viability was estimated by methylene blue staining (Sinha et al., 2005). The reaction samples were withdrawn and a suitably diluted cell suspension (approximate 2×10^8 cells/mL) was mixed with an equal volume of methylene blue dye solution for 3 min and then mounted on a hemocytometer to count the percentage of live cells in the total cell number.

The e.e. of PED was determined by HPLC (HP 1100, Agilent, USA) equipped with a Chiralcel OB-H column (4.6 mm × 250 mm; Daicel Chemical Ind. Ltd., Japan) using *n*-hexane: 2-propanol (90:10, v/v) as eluent at a flow rate of 0.4 mL min^{-1} with detection at 215 nm. The column temperature was set at 38 °C. Under these conditions, (*R*)-PED, (*S*)-PED and HPE were eluted at 19.5, 23.5 and 27.5 min, respectively. The concentration of each compound was calculated from the peak area using a calibration curve determined for the pure compounds. All data were obtained from experiments that were repeated at least two times except the first screening round, which were only performed once, and averaged values are presented in this study.

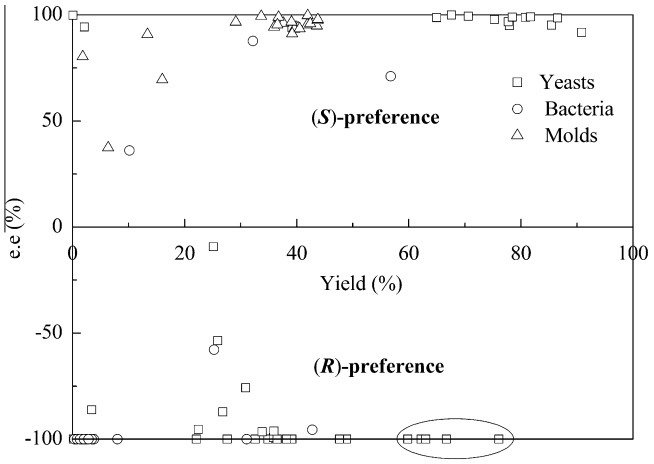

Fig. 1. The performance of microorganisms for the bioreduction of HPE to (*R*)-PED in the first screening step. Reaction conditions: cells equivalent to 0.04 g of dry weight, 2 mL phosphate buffer (0.1 M, pH 6.5), 1 g L^{-1} HPE, 10 g L^{-1} glucose, 150 rpm, 48 h.

3. Results and discussion

3.1. Screening of microorganisms

Previous studies have shown that many native microorganisms such as *Geotrichum* sp. 38, *Yamadazyma farinosa* IFO 10896 and *C. parapsilosis* CCTCC M203011 can stereoselectively catalyze the reduction of HPE to (*S*)-PED (Nie et al., 2004; Tsujigami et al., 2001; Wei et al., 1998). However, those capable of reducing HPE to (*R*)-PED were less common (Ridley and Stralow, 1975). In this study, more than 90 microorganisms, including yeasts, bacteria and molds, were evaluated for their potentiality to reduce HPE to (*R*)-PED. As shown in Fig. 1, all of the molds tested stereospecifically reduced HPE to (*S*)-PED, whereas bacteria exhibited an enantiocomplementarity behavior producing (*R*)-PED except for three isolates (*Brevibacterium protophormiae* JUC57, caproic acid bacteria JUC65 and *Bacillus* sp. JUC56). A total of 14 strains of *S. cerevisiae* followed "Prelog's rule" generating (*R*)-PED, and only one strain of *S. cerevisiae* JUC13 showed anti-Prelog's specificity giving (*S*)-PED. These results were coherent with "Prelog's rule", but it was the first demonstration that *S. cerevisiae* successfully catalyzed the reduction of HPE to enantiopure (*S*)-PED (98.1% e.e., 73.2% yield). To select the most suitable strain with high substrate tolerance and the long-term operational stability, biocatalysts displaying good performance in the first screening round (Fig. 1, circled) were further investigated in the second-stage screening. As shown in Table 1, among five strains compared, *S. cerevisiae* JUC15 exhibited the greatest catalytic activity and the best operational stability, and was therefore chosen for our further study. Screening revealed different microorganisms, even the species of *S. cerevisiae* from different sources, could have distinctions on the gene encoding the oxidoreductase (Kaluzna et al., 2004), which lead to different catalytic characteristics of biocatalysts in reaction. One interesting feature of the present reduction was that aerobic conditions had no influence on the stereoselectivity of reaction (Table 1, column 4) in contrast to the reduction of other compounds so far investigated, in which the presence of air reduced the optical purity of the desired products (Tsujigami et al., 2001). Compared with aerobic conditions, on the other hand, the limited oxygen conditions caused marked enhancement of the product yield. Although *S. cerevisiae* can use glucose as co-substrate under both aerobic

Table 1
The performance of microorganisms for the bioreduction of HPE to (*R*)-PED in the second screening step.

Strain	Oxygen[a]	Yield[b] (%)	e.e.[b] (%)	$Q_{glucose}$[c] (mg h^{-1} g^{-1}_{DCW})	$Q_{(R)\text{-PED}}$[d] (mg h^{-1} g^{-1}_{DCW})
S. cerevisiae	L	80.2	>99.9	49.1	4.0
JUC15	A	16.6	>99.9	40.8	1.2
S. ellipsoideus	L	72.3	>99.9	45.7	3.7
GIM2.105	A	22.1	>99.9	35.5	1.1
S. cerevisiae	L	71.1	>99.9	49.6	4.5
AS2.1392	A	14.6	>99.9	42.1	1.1
S. cerevisiae	L	42.0	>99.9	47.3	2.1
K7[e]	A	17.1	>99.9	38.4	1.2
S. cerevisiae	L	44.8	>99.9	46.0	1.8
701[f]	A	13.6	>99.9	38.9	0.8

Reaction conditions: cells equivalent to 0.04 g of dry weight, 2 mL phosphate buffer (0.1 M, pH 6.5), 10 g L^{-1} glucose, 30 °C, 150 rpm, each cycle 48 h. Substrate concentration in three successive batch was 1, 1.5, and 2 g L^{-1}, respectively.

[a] L and A mean the limited oxygen and aerobic conditions, respectively.

[b] The yields and e.e. of (*R*)-PED under the limited oxygen conditions were the results after three successive batch cycles. The yields and e.e. of (*R*)-PED under aerobic conditions were the results after the first batch.

[c,d] $Q_{glucose}$ and $Q_{(R)\text{-PED}}$ denote the specific glucose consumption rate and the specific (*R*)-PED formation rate, respectively. They were calculated for the first 2 h of reaction at 1 g L^{-1} HPE concentration.

[e,f] They were from the Brewing Society of Japan.

and anaerobic conditions for either NADH or NADPH-dependent bioreductions (Katz et al., 2003; Perles et al., 2008), the availability of oxygen not only affected the specific glucose consumption rate related to the metabolism of *S. cerevisiae*, but also the specific (*R*)-PED formation rate (Table 1). This might be from the fact that the regeneration rate of the reduced coenzyme through the dissimilatory metabolism of glucose matches up relatively well with the reduction rate of the carbonyl compound in some whole-cell reduction systems under the limited oxygen conditions (Katz et al., 2003). As the coenzyme involved in present reductions were not known, representative enzymatic assays were performed on the cell-free extracts of *S. cerevisiae* JUC15 harvested after growth for 48 h. A NADH-dependent reduction activity of 869 U g^{-1} total protein was observed and the corresponding NADH-dependent activity was 536 U g^{-1} total protein using HPE as substrate. Besides, Perles et al. (2008) found that enzymes involved in bioreduction used preferentially different coenzymes when the availability of oxygen in the reactor was different, this makes the analysis more complicated than if HPER had only utilized either NADH or NADPH as the coenzyme. A more systematic investigation of the relationship between the availability of oxygen and glucose yield (molar ratio of formed chiral alcohol to consumed glucose) is underway in our laboratory.

3.2. Reusability stability of S. cerevisiae JUC15 cells

Stability of biocatalyst during successive operation is an essential factor for practical application of biocatalyst, which affects frequency of catalyst replacement, reaction times and product yields (Burton et al., 2002). Therefore, the evaluation of the operational stability of *S. cerevisiae* cells was necessary prior to optimizing the reaction parameters. Surprisingly, there was no significant decrease in the yield and optical purity of (*R*)-PED even after being successively reused for 40 cycles (Fig. 2A). To date, there has been no report of whole-cell biocatalyst capable of being reused for more than 30 cycles without a significant lowering of the reaction yield in bioreduction reactions. The operational stability of biocatalyst attributed to a high cell viability (>80%) and a small loss of HPER activity (<30%) in the repeated batch cycles (Fig. 2B), which was mainly associated with suitable reaction conditions such as pH, temperature, the presence of glucose and a relatively lower substrate concentration (a detailed discussion in each part of optimization). Furthermore, compared with the immobilized cells, free cells boast the advantages of faster reaction rate, lower catalyst cost and eliminating additional processes for immobilization.

Encouraged by this result, we wanted to get a deeper insight into the reaction and further improve results with regard to substrate and product concentration. A systematic investigation was undertaken of the effects of several key factors, such as reaction temperature, buffer pH, co-substrate and substrate concentration on the reaction.

3.3. Effect of temperature and pH on the reduction of HPE

In general, the reaction temperature and pH influence the enantioselectivity and stability of biocatalysts (Polizzi et al., 2007). Therefore, the *S. cerevisiae*-mediated reduction was conducted at different temperatures and pH to investigate their influences on the reduction reaction. As shown in Table 2, the highest yield of 80.5% was obtained at 30 °C, and a further increase in the temperature up to 40 °C led to a 6.3% decrease in the yield of (*R*)-PED, mainly owning to a greater loss of HPER activity. Table 2 also showed the effect of pH on asymmetric reduction of HPE. Differing from most of the reported biocatalysts involved in redox reactions, *S. cerevisiae* JUC15 showed high and stable catalytic activity over a considerably wider range of pH (4–9), with an optimum at pH 6.5. This was similar to the reduction of 2′-chloroacetophenone by *S. cerevisiae* B5 (Ou et al., 2008). Slavik and Kotyk

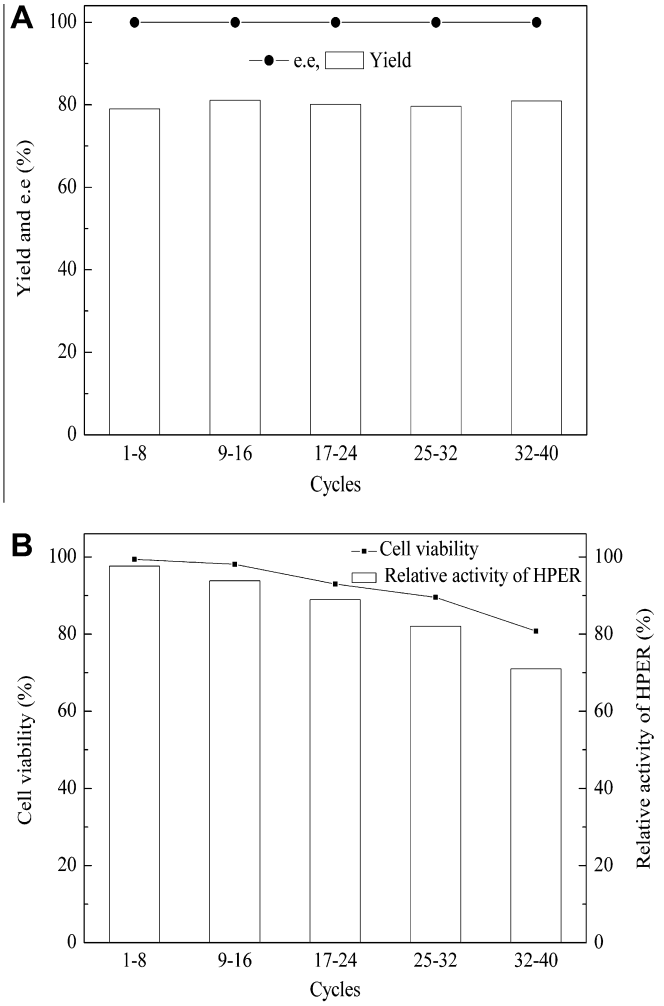

Fig. 2. Operationaland stability of *S. cerevisiae* JUC15 (A) and the changes of cell viability and intracellular HPER activity in the repeated batch cycles (B). Reaction conditions: 0.2 g wet cells, 2 mL phosphate buffer (0.1 M, pH 6.5), 2 g L^{-1} HPE, 10 g L^{-1} glucose, 150 rpm, 24 h per cycle. HPER activity in the fresh cells grown for 48 h was set as 100% relative activity. There was no significant difference between the cycles in the product e.e. and yield ($P > 0.05$).

Table 2
Effects of temperature and pH on reduction of HPE.

Variable condition		Yield (%)	e.e. (%)	Cell viability (%)	Relative activity of HPER (%)[a]
Temperature	20	76.9	>99.9	99.5	93.6
	25	79.5	>99.9	99.3	92.9
	30	80.5	>99.9	99.2	92.3
	35	80.1	>99.9	98.8	91.4
	40	75.4	>99.9	96.1	84.9
pH[b]	4.0	74.8	>99.9	95.2	88.7
	5.5	77.0	>99.9	97.4	89.8
	6.5	80.0	>99.9	98.5	93.3
	7.5	76.5	>99.9	96.0	70.2
	9.0	75.4	>99.9	94.8	66.8

Reaction conditions: 0.2 g wet cells, 2 mL buffer (0.1 M), 8 g L^{-1} HPE, 10 g L^{-1} glucose, 150 rpm. HPER activity, the optical purity and yield of (*R*)-PED were determined after whole-cell-catalyzed reaction for 48 h.

[a] HPER activity in the fresh cells grown for 48 h was set as 100% relative activity.

[b] pH 4.0–5.5 (HAc–NaAc), pH 6.5–7.5 (K_2HPO_4–KH_2PO_4), pH 9.0 (Na_2CO_3–$NaHCO_3$).

(1984) reported that when resting cells of *S. cerevisiae* were suspended in buffer of different pH values, the magnitude of pH shift inside the cells was far less than that of the external environment, which could contribute to the stability of the enzyme in the cells. In this work, after the reaction (48 h), the cell viability and HPER activity still retained more than 94% and 66% of their initial values, respectively, which was essential for whole cells to maintain reaction efficiency and obtain good yields (Chin-Joe et al., 2001). In addition, within the range examined, the reaction temperature and pH had no effect on the enantioselectivity of the reduction (Table 2). Those results indicated that *S. cerevisiae* JUC15-catalyzed reduction of HPE was weakly influence by pH variation (4–9), which avoided additional adjustment of pH value to maintain the catalytic activity of cells (Amidjojo et al., 2005).

3.4. Effect of co-substrate on asymmetric reduction of HPE

Yeast cells contain only a catalytic amount of NAD(P)H, whereas biocatalytic oxidoreductions at high concentration require efficient cofactor recycling during a sufficiently long time span (Hilker et al., 2008). In this case, many saccharides and alcohols, such as glucose, sucrose, D-xylose, and 2-propanol, can be used as sacrificial co-substrates to supply redox equivalents for biosynthetic processes (Carballeira et al., 2009; Goldberg et al., 2007). As shown in Table 3, compared with the systems without co-substrate, the addition of co-substrates to the reaction media remarkably enhanced the conversion efficiency (Table 2, entries 2–5) except for D-xylose (Table 2, entry 6), while co-substrates exhibited no significant promotion to HPER activity. Although co-substrate consumption might result in an increase in the dry weight of biomass through carbohydrate accumulation and/or lipid synthesis (Chin-Joe et al., 2001), we did not observed significant increase in the number of yeast cells (hemocytometer count) during the bioreduction, which was in agreement with the results reported by Chin-Joe et al. (2001). Those results suggested that co-substrates did not act as a protector of HEPR, and its positive effect on this whole-cell-catalyzed reaction was mainly achieved through the cellular metabolism of co-substrate to promote the regeneration of coenzymes. Quezada et al. (2009) postulated that 1 mol of glucose produced 2 mol of NADH by Embden–Meyerhof–Parnas (EMP) pathway and that 1 mol of 2-propanol produced 1 mol of NADH. Associating the fact that glucose and fructose (55.5 mM each) have nearly the same positive effect on this reduction (Table 2, entries 2–3), we can understand why saccharose is the best candidate. In addition, *S. cerevisiae* does not naturally use xylose (Jeffries, 2006), the addition of D-xylose had no significant ($P > 0.05$) yield advantage over the control group (Table 2, entry 6). The optimum concentration of sucrose was then determined to be 20 g L^{-1}.

Table 3
Effect of co-substrate on reduction of HPE.

Entries	Co-substrate	Yield (%)	e.e. (%)	Cell viability (%)	Relative activity of HPER (%)[a]
1	Control	13.3	>99.9	96.2	92.5
2	Glucose	80.2	>99.9	98.9	91.8
3	Fructose	78.4	>99.9	98.1	92.2
4	Sucrose	92.5	>99.9	99.3	91.3
5	2-Propanol	70.4	>99.9	96.8	90.4
6	D-xylose	12.9	>99.9	95.7	91.7

Reaction conditions: 0.2 g wet cells, 2 mL phosphate buffer (0.1 M, pH 6.5), 8 g L^{-1} HPE, 55.5 mM co-substrate, 30 °C, 150 rpm. HPER activity, the optical purity and yield of (*R*)-PED were determined after whole-cell-catalyzed reaction for 48 h.

[a] HPER activity in the fresh cells grown for 48 h was set as 100% relative activity. There was no significant difference between the co-substrate and control in the activity of HPER ($P > 0.05$).

3.5. Effect of substrate concentration on the asymmetric reduction of HPE

A high initial substrate concentration makes for the synthesis of the targeted product, and the substrate inhibition, however, may

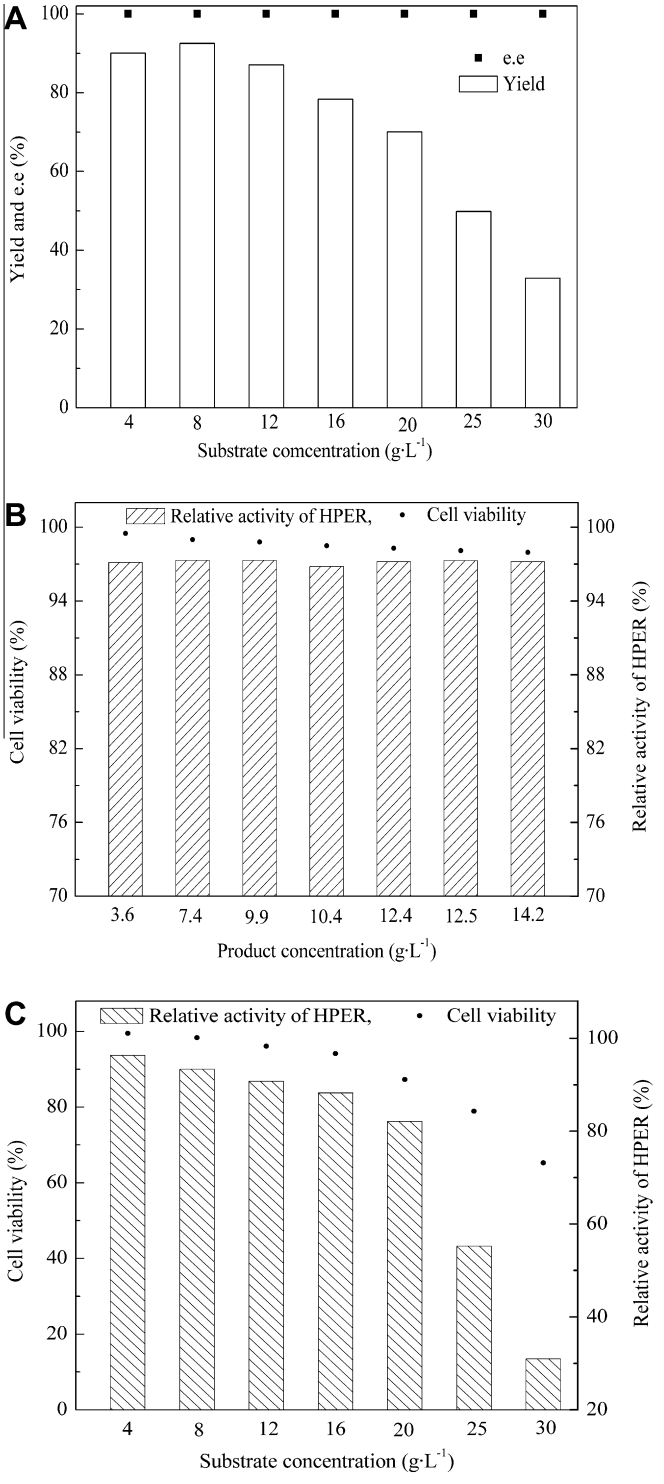

Fig. 3. Effect of substrate concentration on the reduction of HPE (A) and effect of product (B) or substrate (C) concentration on the activity of HPER and cell viability. Reaction conditions: 0.2 g wet cells, 2 mL phosphate buffer (0.1 M, pH 6.5), 20 g L^{-1} sucrose, 30 °C, 150 rpm, 48 h. HPER activity, cell viability, the optical purity and yield of (*R*)-PED were determined after whole-cell-catalyzed reaction for 48 h. HPER activity in the fresh cells grown for 48 h was set as 100% relative activity.

occur at excessively high concentration (Kim et al., 2007). Moreover, aromatic compounds are often toxic to most living cells (Schmid et al., 2001), which could reduce performance of a whole-cell biocatalyst (Oda et al., 1992). As shown in Fig. 3A, a substrate concentration of 8 g L^{-1} supported the highest product yield of 92.5%. However, a progressive decline in the product yield was observed beyond 8 g L^{-1} substrate concentration, probably due to one or several of the following reasons: toxic effect of product on whole cells, poor substrate solubility and inhibitory effect of product on enzyme. However, toxic effect of product on whole cells was consequently excluded on the basis of following result. After being incubated in different concentrations of (*R*)-PED solution for 48 h, in which the concentrations of the products used were the same as the final concentrations at the end of a reaction, the cells still had a viability of more than 97% and HPER also retained a high (>96%) residual activity (Fig. 3B). Furthermore, adequate HPE was always available in the aqueous phase during the bioreduction, the dissolution of the solid substrate was not the rate-limiting step for biotransformation. Based on above experimental results, we attempted to investigate the major reason for decline of product yield. When HPE concentration was below solubility limit (11.36 g L^{-1}), the cell viability and HPER activity still retained more than 95% and 90% of their initial values after the reaction (Fig. 3C). This suggested that product inhibition was the primary bottleneck to the reduction of HPE in this case. When the substrate concentration exceeded solubility, especially more than 20 g L^{-1}, both the cell viability and HPER activity decreased sharply with increasing substrate concentration (Fig. 3C). Associating the observed result that the levels of product concentration (Fig. 3B) and soluble substrate concentration (Fig. 3C) had little influence on the viability of the whole cells and HPER activity, therefore, the lowering of cell viability and HPER activity was related to the substrate particles. Lee and Kim (1998) demonstrated that excessive substrate particles in the reactor had a detrimental effect on activity of the biocatalyst. Therefore, the observed decrease in the yield of (*R*)-PED at higher substrate levels (above solubility limit) can be attributed to a combination of product inhibition and the inactivation of the biocatalyst, though the deactivation extent of the biocatalyst may vary with substrate concentration. Surprisingly, when HPE concentration reached 20 g L^{-1} in reaction system, where 43.2% of the substrate was present in suspended particles, the reaction still gave (*R*)-PED with >99.9% e.e. in 71% yield (Fig. 3A) owning to high cell viability and HPER activity (Fig. 3C). To the best of our knowledge, very few heterogeneous reactions containing solid substrates have been described in biocatalytic redox reaction so far (Kim et al., 2007).

The stereoselectivity of whole-cell-catalyzed reduction usually depends on substrate concentration because of the presence of multiple competing enzymes with conflicting stereopreferences and different affinities (Kaluzna et al., 2004). Modern molecular biology techniques provide an alternative approach to eliminating the catalytic activities of competing oxidoreductases (Carballeira et al., 2009). Unfortunately, when tailor-made *E. coli* cells were used to reduce HPE, substrate concentration still had a substantial influence on the stereoselectivity of reaction products (Nie et al., 2008). However, the product e.e. kept above 99.9% in all examined cases (such as different pH, temperature, co-substrate and substrate concentration), which might be accounted for the existence of one or several HPE reductases with high stereoselectivity inside the cells, which needs further confirmation.

3.6. Time courses of the asymmetric reduction of HPE

Under the optimal conditions (see Fig. 4), a typical time course of the *S. cerevisiae*-mediated reduction of HPE was examined at the substrate concentration of 8 g L^{-1}. Within the first 6 h, more than 40% of substrate was converted to the expected (*R*)-PED, after which the reaction rate decreased. At reduction time 24 h, the reaction afforded (*R*)-PED in 92.4% yield and no trace of the ketone was detected. The (*R*)-PED concentration of 7.4 g L^{-1} was significantly higher than any other biocatalysts reported so far. Nevertheless, too long incubation time resulted in a loss of (*R*)-PED, revealing that decomposition of (*R*)-PED occurred during the reaction. Since product inhibition and excessive solid substrate was adverse to the whole-cell-catalyzed reaction, an aqueous organic two-phase system may be useful to further enhance the productivity of (*R*)-PED and minimize the loss of product.

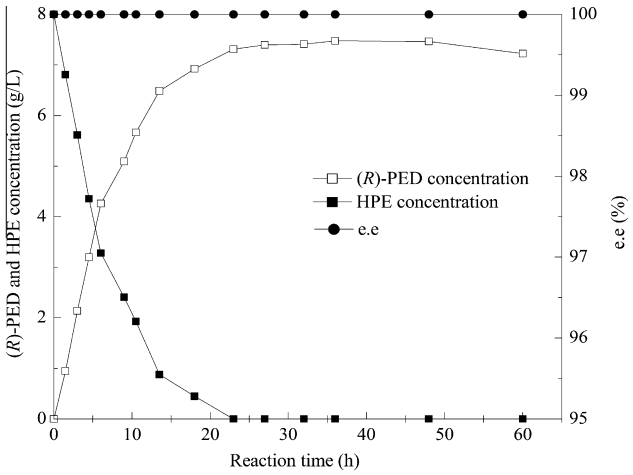

Fig. 4. A typical time course of the *S. cerevisiae*-mediated reduction of HPE. Reaction conditions: 0.2 g wet cells, 2 mL phosphate buffer (0.1 M, pH 6.5), 8 g L^{-1} HPE, 20 g L^{-1} sucrose, 30 °C, 150 rpm.

4. Conclusions

S. cerevisiae JUC15 was successfully obtained by target reaction-oriented screening to be the preferred strain for the reduction of HPE to (*R*)-PED with excellent e.e. The remarkable reusability of *S. cerevisiae* cells made it quite attractive for industrial application. Moreover, the strain used cheap sucrose for cofactor regeneration and worked over a considerably wider range of pH. Even in heterogeneous reaction systems containing the solid substrate, the reaction still afforded (*R*)-PED in high yield. All these features indicate the potential of this strain for industrial production of chiral (*R*)-PED meeting the stringent criteria of the pharmaceuticals industry.

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