Appl Biochem Biotechnol (2010) 162:2075–2086
DOI 10.1007/s12010-010-8983-3

Improved Synthesis of (*S*)-1-Phenyl-2-Propanol in High Concentration with Coupled Whole Cells of *Rhodococcus erythropolis* and *Bacillus subtilis* on Preparative Scale

Jian-Zhong Jin · He Li · Jie Zhang

Received: 14 January 2010 / Accepted: 26 April 2010 /
Published online: 19 May 2010

Abstract Bioreduction of 1-phenyl-2-propanone to prepare (*S*)-1-phenyl-2-propanol, a useful pharmaceutical intermediate, was performed with growing cells of *Rhodococcus erythropolis* JX-021, giving 14 mM (1.9 g/L) product in 99% e.e. at 5 h in the catalysis of 15 mM substrate. The reduction stopped afterwards due to strong inhibition of substrate and formed product, a problem that is often encountered in biotransformation. While the substrate inhibition was solved by stepwise feeding, product inhibition was tackled by different methods: repeated removal of the product by centrifugation, by absorption with Amberlite XAD-7 resin, and by the use of dodecanol as the second phase gave the final product in 58, 68, and 61 mM in the catalysis of 80 mM substrate, respectively. The inhibition was caused by the partial permeabilization of cell membrane of *R. erythropolis* JX-021, and addition of NADPH or glucose 6-phosphate to such cell culture retained the reduction activity. Therefore, higher productivity in the reduction of **1** with resting cells of *R. erythropolis* JX-021 was achieved through cofactor regeneration and recycling by the addition of glucose and permeabilized cells of *Bacillus subtilis* BGSC 1A1 containing a glucose dehydrogenase, giving the product in 62 mM without addition of cofactor and 78 mM with the addition of 0.01 mM $NADP^+$ in the catalysis of 120 mM substrate. The product e.e. retained 99% during the process which showed industrial possibility.

Keywords Reductase · (*S*)-1-phenyl-2-propanol · Amphetaminil · Coupled microorganisms · NADPH regeneration · Preparative scale

J.-Z. Jin (✉) · H. Li
College of Biological and Environmental Engineering, Zhejiang Shuren University, Hangzhou 310015, China
e-mail: hzjjz@sohu.com

J. Zhang
Jiaxing Research Center for Applied Chemistry, Chinese Academy of Sciences, Jiaxing 314006, China

Introduction

Enantioselective oxidoreduction is the key reaction in asymmetric synthesis of enantiopure compounds that are useful synthetic intermediates for pharmaceuticals, flavors, and agrochemicals. Biocatalysis provides a green and highly selective alternative to the chemical catalysis for these reactions. One important class of these reactions is the enantioselective reduction of keto group giving rise to enantiopure alcohol [1, 2]. Baker's yeast is well known for such reduction with many synthetic applications [3]. Many other enzymes have been reported for the bioreduction [4, 5], and some of them have also been cloned and expressed in recombinant strains for achieving high productivity [6] or high selectivity [7]. Nevertheless, for a desired reduction, it is often necessary to screen for the appropriate enzymes with high selectivity and high activity. High-throughput screening/detection systems are needed to provide a useful method for the quick identification of microorganism containing the suitable enzyme [8].

Microorganisms are often used as catalysts for practical bioreductions due to their higher stability, easy availability, and the possibility of cofactor recycling with growing cells through metabolism. On the other hand, many other cofactor-recycling methods have also been developed [4, 9, 10], based on the "coupled substrate" [5] and "coupled enzyme" approach [11]. Recently, an enzyme-coupled system containing ketoreductase and mannitol dehydrogenase was successfully applied for the reduction of ketones with NADPH TTN of more than 4,000 times [12]. Furthermore, the use of recombinant strains expressing two necessary enzymes seems to be practical [13, 14]. Meanwhile, Li developed a new method for cofactor recycling based on the use of one or two permeabilized microorganisms [15, 16], providing a new possibility for efficient bioreduction with cofactor recycling.

Another challenge in practical bioreduction is to solve substrate or product inhibition. This is, however, an often-encountered problem in a general biotransformation. To keep the substrate/product concentration low enough in the system to avoid the inhibition, a variety of feeding strategies [17] or online product recovery by ion-exchange resins [18] or hydrophobic resins [19] are developed. The use of organic solvent as second liquid phase for biotransformation is another useful choice [20–22].

Recently, we did the high-throughput screening to discover the strain *Rhodococcus erythropolis* JX-021 for the enantioselective reduction of 1-phenyl-2-propanone **1** to prepare (*S*)-1-phenyl-2-propanol **2**, a useful intermediate for the preparation of the amphetamines and amphetaminil (sympathomimetics; Fig. 1) [23]. Bioreduction of **1** has been intensively investigated, and the best results prior to our study were achieved with *Zygosaccharomyces rouxii* ATCC 14462 as catalyst: (*S*)-**2** was obtained in 99% e.e. and 90% yield in the catalysis of 10 mM substrate [24]. In 1996, Julich GmbH in Germany carried out the industrial production of (*S*)-**2** in catalysis of 15 mM substrate with 72% conversion and 99% e.e. [25]. We recently screened 112 bacterial strains isolated from soil for the discovery of new reductases with better enantioselectivity for the reduction of **1**. Three strains were found to produce (*S*)-**2** in >95% e.e. Among them, *R. erythropolis* JX-021 showed the highest enantioselectivity, giving (*S*)-**2** in 99% e.e. Here, we report the development of efficient bioreduction of **1** with *R. erythropolis* JX-021, the investigation of substrate/product inhibition, the approaches for avoiding the inhibition, and the new method of coupling of in situ permeabilized cells of *R. erythropolis* JX-021 and *Bacillus subtilis* BGSC 1A1 for practical reduction and cofactor recycling.

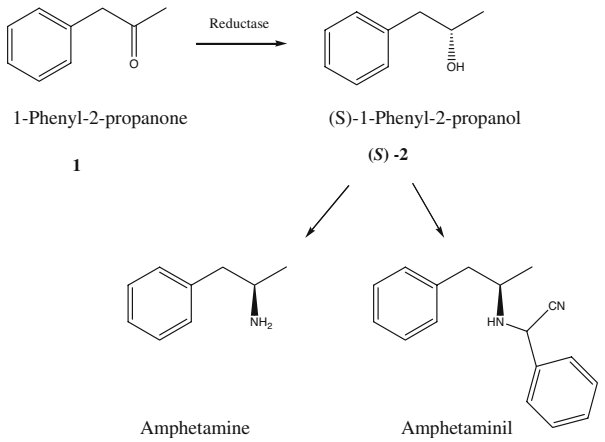

Fig. 1 Bioreduction of 1-phenyl-2-propanone for the synthesis of (*S*)-1-phenyl-2-propanol, the intermediate of amphetamines and amphetaminil

Materials and Methods

Chemicals

1-Phenyl-2-propanone **1** was purchased from Acros. (*RS*)-1-Phenyl-2-propanol (>99%) was synthesized by reduction of 1-phenyl-2-propanone **1** with $NaBH_4$ [23]. 1-Dodecanol (98%), Amberlite® XAD-7, toluene (99%), chloroform (99%), and $NaBH_4$ were bought from Acros (Shanghai, P. R. China); NADPH, $NADP^+$, and glucose-6-phosphate (99%) were purchased from Sigma (Shanghai, P. R. China)

Analytical Condition

Bioconversion of 1-phenyl-2-propanone **1** to (*S*)-1-phenyl-2-propanol **2** was analyzed by gas chromatography (GC; Fisons Instruments) on a fused silica capillary column OPTIMA-5 (25 m×0.32 mm) with hydrogen as the carrier gas. Temperature program was as follows: 100 °C for 0 min, increase to 280 °C at a rate of 20 °C/min, kept at 280 °C for 2 min. Retention time was 3.21 min for 1-phenyl-2-propanone **1** and 6.22 min for 1-phenyl-2-propanol **2**.

The e.e. of 1-phenyl-2-propanol **2** was analyzed by GC on a chiral column CP-Chirasil-Dex CB (25 m×0.25 mm). Temperature program was as follows: 100 °C to 120 °C at 10 °C/min, increase the temperature to 180 °C at 40 °C/min; retention time was 4.9 min for (*S*)-**2** and 6.1 min for (*R*)-**2**.

Bacterial Strains

R. erythropolis JX-021 is a benzene acetone degrader isolated from soils by Dr. He Li at Jiaxing city, Zhejiang Province; *B. subtilis* BGSC 1A1 was obtained from the Institute of Biotechnology, ETHZ, Switzerland.

Growth of *R. erythropolis* JX-021 on Fed Batch in a Bioreactor

R. erythropolis JX-021was grown on an LB agar plate at room temperature for 4 days and inoculated into 120 mL medium containing (g/L) KH_2PO_4 2.44, $Na_2HPO_4{\cdot}12H_2O$ 12.03,

$MgCl_2 \cdot 6H_2O$ 0.412, $CaCl_2 \cdot 2H_2O$ 0.001, $FeCl_3 \cdot 6H_2O$ 0.001, $MnCl_2$ $4H_2O$ 0.004, glycerol 1.12, NH_4Cl 2.11, 2% glucose, and 5 mM benzene acetone. The cells were grown at 25 °C and 200 rpm for 36 h, reaching an OD_{450} of 7.5 in the late exponential phase. The culture was transferred into a 3-L bioreactor containing 2-L of the same medium as the shaking flask grown at 1,000 rpm, 25 °C, and pH of 7.0 with air flow of 1.0 L/min. At 24 h, OD_{450} reached 9.9 and glucose concentration decreased to 1.0 g/L. Glucose (50%) was pumped at 15.0 g/h into the reactor to keep the glucose concentration around 2.0 to 3.0 g/L. At 28 h, glucose feeding was increased to 25.0 g/h, stirring rate was changed to 1,500 rpm, and airflow rate was adjusted to 1.5 L/min. At 35 h, glucose (50%) was pumped at 30 g/h, and the reactor was stirred at 2,000 rpm with an airflow rate of 2.0 L/min. A 210-mL concentrated growth medium (ten times) was added. At 42 h, OD_{450} reached 52, corresponding to a cell density of 15.1 g cdw/L. The cells were harvested by centrifugation at 4 °C, 4,000 rpm for 30 min, and the cell pellets were washed by phosphate buffer (pH= 7.0) and stored in a freezer at −80 °C.

The activities of cells at 8, 12, 24, and 36 h were determined by the following assay: 5 mL culture was taken out and centrifuged, and the cells were resuspended in 5 mL Tris buffer (pH=7.0) containing 2% glucose (100 mg, 0.56 mmol, 111 mM); 1-phenyl-2-propanone (3.4 mg, 0.025 mmol, 5.0 mM) was added, and the resulting mixture was shaken at 25 °C and 300 rpm for 30 min; after centrifugation, 2.0 mL supernatant was extracted with 2.0 mL chloroform, and the organic phase was dried, filtrated, and analyzed by GC.

Growth of *B. subtilis* BGSC 1A1

B. subtilis BGSC 1A1 were grown in 100 mL 1/2 Evans medium [14] containing 50 mM glucose (0.9 g, 5 mmol, 50 mM) and 0.05% yeast extract (50 μL) in 500-mL shaking flask at 37 °C and 250 rpm. The cells were harvested at the late exponential phase at 11 h with an OD_{450} of 6.2.

Reduction of 1-Phenyl-2-Propanone 1 with Growing Cells of *R. erythropolis* JX-021 on 1-L Scale

The cells of *R. erythropolis* JX-021 was grown to a cell density of 15.3 g cdw/L according to the procedure described above. 1-Phenyl-2-propanone **1** (1.34 g, 10 mmol, 10 mM) was added to the culture, and the cells grew slowly. It stopped growing after 2 h. Substrate **1** (1.34 g, 10 mmol, 10 mM) was added every 1 h with a cumulative concentration of 50 mM (6.7 g, 50 mmol, 50 mM) at 5 h. Ten grams of glucose (1%) was added each time, and 60 g of glucose (0.33 mol, 0.33 M) was added after 5 h, and the biotransformation was stopped at 20 h. In analyzing conversion by GC, 300 μL solutions were taken at different time points (0.5, 1, 2, 3, 5, 16, 18, 20 h). The analytic samples were prepared by centrifugation, mixing of 100 μL supernatant with 400 μL Tris buffer (pH=7.0), extracting with 500 μL chloroform, and drying the organic solution with Na_2SO_4.

Reduction of 1-Phenyl-2-Propanone 1 with Resting Cells of *R. erythropolis* JX-021 at Different pH

The frozen cells of *R. erythropolis* JX-021 were thawed and suspended to a density of 6.7 g cdw/L in 10 mL 100 mM Tris buffer (pH=7.0) containing 2% glucose (200 mg,

1.11 mmol, 111 mM) at pH of 6, 7, and 8, respectively. Substrate **1** (6.7 mg, 5 mmol, 5 mM) was added, and the mixture was shaken at 25 °C, 300 rpm for 30 min. Cells were removed by centrifugation, 1.0 mL supernatant was extracted with 1.0 mL chloroform, and the organic solvent was dried and analyzed by GC.

Investigation of Inhibition of Substrate and Product on the Whole Cell Bioreduction

To a 10 mL cell suspension of *R. erythropolis* JX-021 (6.7 g cdw/L) in 100 mM Tris buffer (pH=7.0) was added substrate **1** (0–134 mg, 0–1 mmol, 0–100 mM) or racemic product **2** (0–136 mg, 0–1 mmol, 0–100 mM). The mixture was shaken at 25 °C, 300 rpm for 30 min. After centrifugation, the cells were washed with Tris buffer (pH= 7.0) for three times and resuspended in 10 mL 100 mM Tris buffer (pH=7.0) containing 2% glucose (200 mg, 1.11 mmol, 111 mM). Substrate **1** (6.7 mg, 0.05 mmol) was added, and biotransformation was performed at 25 °C, 300 rpm for 5 h. At 0.5, 1, 2, 3, and 5 h, 0.5 mL aliquots were taken; samples were prepared as described and analyzed by GC.

Investigation of Cofactor Regeneration During Reduction of 1-Phenyl-2-Propanone **1** with Resting Cells of *R. erythropolis* JX-021

To 10 mL cell suspension (20 g cdw/L) in 100 mM Tris buffer (pH=7.0) containing 6% glucose (0.6 g, 3.33 mmol, 333 mM) was added substrate **1** (107.2 mg, 0.8 mmol, 80 mM). The mixture was shaken at 25 °C and 300 rpm, and 300 μL aliquot was taken for analysis at 0.5, 1, 3, 5, 7, 9, and 10 h. At 10 h, 3×2 mL mixture was taken and used for further transformation by the addition of (a) nothing, (b) 30 mM glucose-6-phosphate (10.2 mg, 0.03 mmol), and (c) 5 mM NADPH (4.5 mg, 5 μmol). Bioconversion was followed at 0.5, 1, 2, and 5 h.

Repeated Use of Resting Cells of *R. erythropolis* JX-021 for Bioreduction of 1-Phenyl-2-Propanone **1**

To 20 mL cell suspension (20 g cdw/L) in Tris buffer (pH=7.0) containing 2% glucose (400 mg, 2.22 mmol, 111 mM) was added 1-phenyl-2-propanone **1** (26.8 mg, 0.2 mmol, 10 mM), and the mixture was shaken at 25 °C and 300 rpm for 5 h. At 0.5, 1, 2, 3, and 5 h, 300 μL aliquots were taken out for analysis. The cells were harvested by centrifugation, washed twice with Tris buffer (pH=7.0), and reused for the biotransformation of **1** (10 mM). The procedure was repeated eight times with total 80 mM substrate **1**, yielding 58 mM product **2**.

Repeated Bioreduction Of 1-Phenyl-2-Propanone **1** with Resting Cells of *R. erythropolis* JX-021 by Repeated Removal of Product with Amberlite® XAD-7 Resin

To 20 mL cell suspension (20 g cdw/L) in Tris buffer (pH=7.0) containing 2% glucose (400 mg, 2.22 mmol, 111 mM) was added substrate **1** (26.8 mg, 0.2 mmol, 10 mM), and the mixture was shaken at 25 °C and 300 rpm for 5 h and analyzed by taking 300 μL aliquots at 0.5 h, 1 h, 2 h, 3 h, 5 h. At 5 h, 1 g resin Amberlite® XAD-7 in gauze was added to remove the products. The cell suspension was reused for the bioreduction of 10 mM substrate, and such procedure was repeated eight times to produce 68 mM product **2** from 80 mM substrate **1**.

Bioreduction of 1-Phenyl-2-Propanone **1** with Resting Cells of *R. erythropolis* JX-021 in a Two-Phase System Containing 1-Dodecanol

1-Phenyl-2-propanone 1 (134 mg, 1.0 mmol, 100 mM) in 10 mL 1-dodecanol was added to 10 mL cell suspension of *R. erythropolis* JX-021 (20 g cdw/L) in 100 mM Tris buffer (pH= 7.0) containing glucose (0.6 g, 3.33 mmol, 333 mM). The mixture was shaken at 25 °C and 300 rpm. At 15 h, more substrate **1** (31 mg, 0.23 mmol, 30 mM) was added. The biotransformation was performed for 58 h. Samples were taken at 0.5, 1, 3, 5, 7, 9, 10, 12, 15, 24, 29, 34, 39, 48, 53, and 58 h for the analysis. Analytic samples for aqueous phase were prepared as described before. Samples for dodecanol phase were prepared by drying over Na_2SO_4 and mixing with four times chloroform. Finally, 61 mM product **2** was obtained.

Bioreduction of 1-Phenyl-2-Propanone 1 with Resting Cells of *R. erythropolis* JX-021 Followed by the Addition of Permeabilized *B. subtilis* BGSC 1A1 [14] at 9 h with or Without the Supplying of $NADP^+$

1-Phenyl-2-propanone **1** (107.2 mg, 0.8 mmol, 80 mM) was added to 10 mL cell suspension of *R. erythropolis* JX-021 (40 g cdw/L) in 100 mM Tris buffer (pH=7.0) containing 6% glucose (600 mg, 3.33 mmol, 333 mM), and the mixture was shaken at 25 °C and 300 rpm for 10 h. At 0.5, 1, 3, 5, 7, 9, and 10 h ,250 μL aliquots were taken for analysis. At 10 h, 8.0 mL biotransformation mixture was mixed with 8.0 mL suspension of permeabilized cells of *B. subtilis* BGSC 1A1 [14] (80 g cdw/L) in the same buffer. The resulting mixture was divided into two portions of 8 mL: to one portion was added $NADP^+$ (0.063 mg, 16.0 μL solution of 4 mg/mL, 0.08 μmol, 0.01 mM); to another one, no $NADP^+$ was added. Another 40 mM substrate **1** (42.9 mg, 0.32 mmol, 40 mM) was also added in both cases, and the biotransformation continued until 59 h. Samples (300 μL) were taken at 10.5, 11, 12, 20, 22, 24, 29, 34, 44, 49, 54, and 59 h for the analysis. Glucose was added at 12 h (0.42 g, 2.33 mmol, 0.33 M), 20 h (0.2 g, 1.11 mmol, 0.16 M), 24 h (0.186 g, 1.06 mmol, 0.16 M), 29 h (0.177 g, 0.98 mmol, 0.16 M), 34 h (0.336 g, 1.87 mmol, 0.33 M), 44 h (0.159 g, 0.88 mmol, 0.16 M), 49 h (0.15 g, 0.83 mmol, 0.16 M), and 54 h (0.14 g, 0.77 mmol, 0.16 M). The product was obtained in 62 mM without addition of cofactor and in 78 mM with the addition of 0.01 mM $NADP^+$ in the catalysis of 120 mM substrate.

Results and Discussion

Growth of *R. erythropolis* JX-021 on Fed Batch in Bioreactor

For practical biotransformation with microbial cells, the microorganism should be easily and economically grown to a high cell density. *R. erythropolis* JX-021 was found to grow on glucose, giving good activity and enantioselectivity for bioreduction of **1**. Thus, in large-scale cultivation, glucose was used as carbon sources. Firstly, the cells were freshly grown on LB agar and then inoculated into 120 mL specific liquid medium containing 5 mM benzene acetone as the inducer. It was found in preculture that at 30 °C the cells grew faster but showed 5% lower product e.e. in the bioreduction. The preculture was prepared by shaking at 25 °C and 300 rpm for 36 h with a cell density of 2.4 g cdw/L (OD_{450}=8.3). The fermentation on 1-L scale in specific medium containing glucose (2%) was carried out on fed batch. It gives cell density of 24 g cdw/L (OD_{450}=82) at 37 h (Fig. 2).

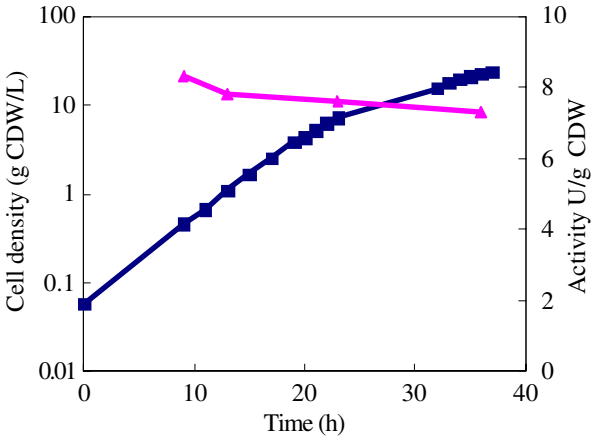

Fig. 2 Growth curve of *R. erythropolis* JX-021 with glucose as carbon source on 2-L scale in a fermenter: *squares*, cell growth curve; *triangles*, activity test

The catalytic activities of the cells produced at different stages were examined by bioreduction of 5 mM **1** in 10 mL 100 mM Tris buffer containing 2% glucose at pH=7.0, 25 °C and 300 rpm for 0.5 h. Specific activities of 7–8 U/g CDW were achieved for the cells produced between 10 and 36 h. Bioreduction at the pH 6.0 and 8.0 gave lower activity than at pH 7.0.

Obviously, biotransformation with the growing cells would give the maximum productivity. However, a strong substrate inhibition was observed for cells. In the 1-L scale fermentation, with the addition of 10 mM substrate **1** twice, the cells stopped growing. The reduction of **1** with these *non*growing cells gave more than 15 mM product **2** at 5 h with accumulative substrate concentration of 50 mM. This indicates the regeneration of the cofactor during this period, possibly through the metabolism of glucose by the enzymes inside the cells. After 5 h, the reduction became very slow. Obviously, the formed product also inhibited the reduction. Experiments with preincubation of cells with substrate or production demonstrated even higher inhibition effect of product than substrate.

The stopped conversion after 5 h in the bioreduction of 50 mM substrate with whole cells of *R. erythropolis* JX-021 may be caused by substrate/product inhibition. To investigate this effect, cell suspension (6.7 g cdw/L) of *R. erythropolis* JX-021 in Tris buffer (pH=7.0) was treated with substrate/product at 20 to 100 mM for 30 min. Cells were then washed twice with Tris buffer (pH=7.0) and examined for bioreduction of **1** (5 mM) under standard conditions. As listed in Table 1, 20 mM substrate did not influence the biotransformation of whole cells, but some inhibitory effect was already observed for 20 mM product. With the increase of concentration of substrate, fewer products were produced. Strong inhibition was observed at 60 mM substrate or 40 mM product. Clearly, the product was more toxic to the cells.

Substrate/product inhibition is a general problem in biotransformation. Similar inhibition was found in bioreduction of **1** with resting cells of *R. erythropolis* JX-021. Product inhibition was solved by different methods: repeated removal of the product by centrifugation, by absorption with Amberlite XAD-7 resin, and by the use of dodecanol as the second phase.

Table 1 Reduction of **1** with whole cells of *R. erythropolis* JX-021 that are pretreated with substrate or product.

Cells treated by substrate (mM)	Cells treated by racemic product (mM)	Activity of pretreated cells (U/g cdw)	Product formed with pretreated cells (mM) (5 h)
0		7.6	4.9
20		7.5	4.9
40		5.5	4.1
60		2.1	1.3
80		0.4	0.3
100		0	0
	0	7.6	4.9
	20	6.8	4.0
	40	2.8	1.7
	60	1.7	1.1
	80	0	0
	100	0	0

The reaction was performed on 10-mL scale, shaking at 25 °C, 300 rpm for 30 min. Afterwards, the cells were washed by Tris buffer (pH 7.0) twice and reused in the biotransformation of 5 mM substrate on 10-mL scale with 2% glucose for cofactor regeneration

Reduction of Product Inhibition by Repeated Use of the Cells

While the substrate was used in only 10 mM for bioreduction, the cells were repeatedly used. In each run, when the reaction is finished, the cell solution was centrifuged for separation from the product. The cells were washed and reused in the biotransformation of 10 mM substrate again. After eight times bioconversion, 58 mM product was formed from a total of 80 mM substrates (Fig. 3a).

Reduction of Product Inhibition by the Removal of Product **2** Through the Absorption With Resin Amberlite XAD-7

After testing different resins (Amberlite® XAD-2, XAD-4, XAD-7, XAD-16, XAD-1180, Diaion HP-20), the Amberlite® XAD-7 was found to be a good absorbent. A 0.5 g resin can absorb 42 mg racemic product, and the product can be extracted from the resin by chloroform, and the resin can be reused. For bioreduction of **1** (10 mM), after each run, 1 g Amberlite® XAD-7 was added to remove the product. After removal of the resin, new substrate (10 mM) was added to the cell suspension again for the reduction. Repeating this procedure eight times resulted in 68 mM product from 80 mM substrate (Fig. 3b).

Reduction of Substrate/Product Inhibition by the Use of Dodecanol as the Second Phase for Biotransformation

In two-liquid-phase system, substrate and product are dissolved in organic solvents, thus reducing their inhibitory effect on enzymatic reactions. After testing several solvents (hexane, ethyl acetate, methyl *tert*-butyl ether, dodecanol, and chloroform), dodecanol was found to be a good one for dissolving substrate **1** and product **2**. Extraction of 1 mL 200 mM substrate or racemic product with 1 mL dodecanol gave 64% substrate or 82%

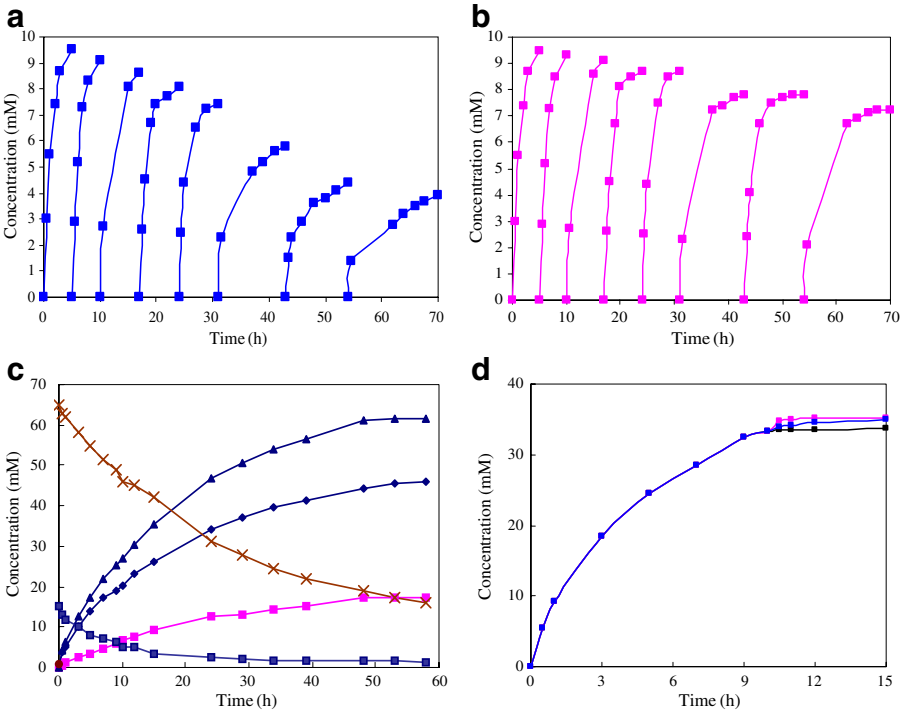

Fig. 3 **a** Bioreduction of substrate **1** with resting cells of *R. erythropolis* JX-021 (20 g CDW/L) on 10-mL scale. Repeated use of cells after removing the product by centrifugation: *blue squares*, product formation; repeated removal of the product by absorption with resin Amberlite XAD-7: *pink squares*, product formation; **b** bioreduction of 80 mM substrate **1** with resting cells of *R. erythropolis* JX-021 (20 g CDW/L) on 20-mL scale in two-liquid-phase system with 10 mL dodecanol as the organic solvent: *brown x marks*, substrate in organic phase; *blue squares*, substrate in aqueous phase; *blue triangles*, total product formed; *blue diamonds*, product in organic phase; *pink squares*, product in aqueous phase; **d** biotransformation of 80 mM substrate **1** with resting cells of *R. erythropolis* JX-021 (20 g CDW/L) on 10-mL scale. Product formation: *pink squares*, 5 mM NADPH was added at 10 h; *blue squares*, 30 mM G-6-P was added at 10 h; *black squares*, no addition

product in the organic phase. Meanwhile, the log *P* value of dodecanol is 5.0 which is nontoxic to the cells. As shown in Fig. 3c, biotransformation of 80 mM substrate **1** with such two-phase system gave 61 mM product, and reaction was performed for as long as 58 h.

Substrate/Product Inhibition Caused by the Damage of Cell Membrane

To further study the inhibition, bioreduction was performed with 80 mM substrate at a cell density of 20 g cdw/L. As shown in Fig. 3d, the product formation become very slow after 10 h. At this time points, two portions of cell suspension were taken out. To one of them was added 5 mM NADPH, while 30 mM G-6-P was added to the second portion. These gave 1.6 and 1.5 mM products, respectively, from 10 to 15 h. While in the control experiment with no addition, no more products formed from 10 to 15 h.

Repeated bioreduction of **1** at 10 mM with reharvested cells was shown to be possible and gave 58 mM product after eight times reaction. However, this does not provide a

practical vision for such transformation because of technical reuse of strains. Repeated removal of product by use of resin Amberlite® XAD-7 gave 68 mM product after eight runs. Here, again, it could not be the method of choice due to the cost of the resins. Another approach is the use of organic solvent as the second phase for bioreduction of **1** in two-liquid-phase system. The choice of suitable solvent is very important. Solvent with high log *P* value has less damage to the cells; however, it is also difficult to have high solubility. Here, we used 1-decanol as second phase, which can keep both substrate and product concentration lower than 15 mM during the bioreduction of 80 mM **1**. A 61 mM product was obtained after 58 h. Although further reduction seemed to be possible, the reaction rate would be slow.

The inhibition of substrate/product was caused by the damage of cell membrane, since the externally added NADPH could enter the cells and restore the bioreduction activity, shown in Fig. 3d. The glucose-6-phosphate dehydrogenase inside the cells permeabilized by the substrate/product was still active, and addition of glucose-6-phosphate enabled NADPH regeneration and contributed to further bioreduction.

Since the permeabilization of the cells of *R. erythropolis* JX-021 is difficult to be avoided during bioreduction with high concentration of substrate/product, it is more interesting to utilize this phenomenon for further development of practical process. The cells of *R. erythropolis* JX-021 were at first used for bioreduction of **1** for 10 h to form 38 mM product in the catalysis of 80 mM substrate and then coupled with permeabilized cells of *B. subtilis* BGSC 1A1 for further reduction and cofactor recycling (Fig. 4). Even without external addition of cofactor, another 24 mM product was produced due to the recycling of original $NADP^+$ in the system. The final product concentration reached 62 mM, which is a very good result in comparison with other methods described before. Moreover, the addition of 0.01 mM $NADP^+$ after the mixing of two microorganisms resulted in 40 mM more products. The final product concentration reached 78 mM with only the addition of 0.01 mM $NADP^+$; thus, the process is practical.

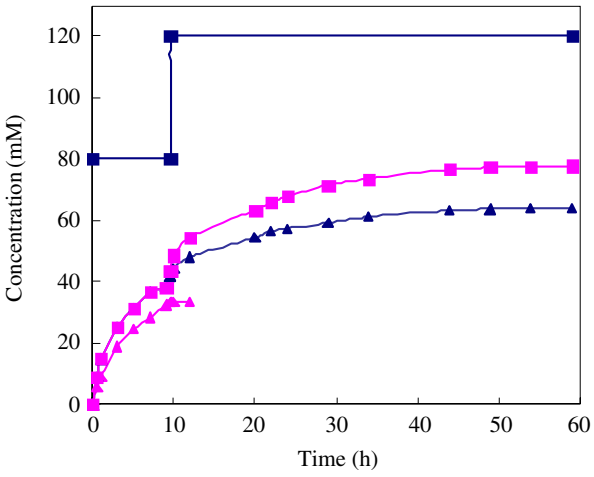

Fig. 4 Bioreduction of 120 mM substrate **1** with resting cells of *R. erythropolis* JX-021 (20 g CDW/L) on 10-mL scale: *blue squares*, cumulative substrate; *pink squares*, product formation; addition of permeabilized cells of *B. subtilis* and 0.01 mM $NADP^+$ at 9 h; *blue triangles*, product formation; addition of permeabilized cells of *B. subtilis* at 9 h; *pink triangles*, product formation in the reduction of 80 mM substrate **1** as control

Conclusions

Bioreduction of 1-phenyl-2-propanone to prepare (*S*)-1-phenyl-2-propanol, a useful pharmaceutical intermediate, was performed with newly isolated cells of *R. erythropolis* JX-021 in the catalysis of 80–120 mM substrate which is 5.3–10 times the concentration of industrial cases. Different methodologies were taken to tackle the substrate/product inhibition problem including repeated removal of the product by centrifugation, by absorption with resins, and by the use of organic solvent as the second phase. It firstly demonstrated that bioreduction with whole cells stopped in the catalysis of high concentration of substrate because of leakage of cofactors from the whole cells. Formerly, the enzymes inside the cells which lost the activity were thought to be responsible for that. Addition of NADPH or glucose 6-phosphate to such cell culture retained the reduction activity. Thus, it could be a general approach of using in situ substrate/product permeabilized microorganisms for efficient bioreduction and cofactor regeneration. A recombinant strain that expresses glucose dehydrogenase may be constructed for more efficient NADPH regeneration. Combination of this strain with reductase-containing microorganism/recombinant strain should enable practical application of bioreduction with inhibitory substrates/product.

Acknowledgement This work was supported by the Zhejiang Provincial Natural Science Foundation (grant no. y4080311)

References

1. Nakamura, K., & Matsuda, T. (2002). In K. Drauz & H. Waldmann (Eds.), *Enzyme catalysis in organic synthesis* (Vol. III, pp. 991–1047). Hoboken: Wiley-VCH. Chap 15.
2. Faber, K. (1997). *Biotransformations in organic chemistry, chap. 2.2* (pp. 160–200). Berlin: Springer.
3. Pereira, R. S. (1998). *Critical Reviews in Biotechnology, 18*, 25–64.
4. Kroutil, W., Mang, H., Edegger, K., & Faber, K. (2004). *Current Opinion in Chemical Biology, 8*, 120–126.
5. Stampfer, W., Kosjek, B., Moitzi, C., Kroutil, W., & Faber, K. (2002). *Angewandte Chemie International Edition, 41*, 1014–1017.
6. Yoshihiko, Y., Noriyuki, K., Junzo, H., Masaru, W., Michihiko, K., & And Shimizu, S. (2000). *Bioscience, Biotechnology, and Biochemistry, 64*, 1430–1436.
7. Rodriguez, S., Schroeder, K. T., Kayser, M. M., & Stewart, J. D. (2000). *The Journal of Organic Chemistry, 65*, 2586–2587.
8. Zhang, J., Duetz, W. A., Witholt, B., & Li, Z. (2004). *Chemical Communications*, 2120–2121.
9. Chenault, H. K., & Whitesides, G. M. (1987). *Applied Biochemistry and Biotechnology, 14*, 147–197.
10. Donk, W. A., & Zhao, H. M. (2003). *Current Opinion in Biotechnology, 14*, 421–426.
11. Wong, C. H., & Whitesides, G. M. (1981). *Journal of the American Chemical Society, 103*, 4890–4899.
12. Yang, W., Xu, J. H., Pan, J., Xu, Y., & Wang, Z. L. (2008). *Biochemical Engineering Journal, 42*, 1–5.
13. Kizaki, N., Yasohara, Y., Hasegawa, J., Wada, M., Kataoka, M., & Shimizu, S. (2001). *Applied Microbiology and Biotechnology, 55*, 590–595.
14. Kataoka, M., Yamamoto, K., Kawabata, H., Wada, M., Kita, K., Yanase, H., et al. (1999). *Applied Microbiology and Biotechnology, 51*, 486–490.
15. Zhang, J., & Witholt, B., Li, Z. (2006). *Chemical Communications*, 398–400.
16. Zhang, J., Witholt, B., & Li, Z. (2006). *Advanced Synthesis & Catalysis, 348*, 429–433.
17. Gbewonyo, K., Buckland, B. C., & Lilly, M. D. (1991). *Biotechnology and Bioengineering, 37*, 1101–1107.
18. Roddick, F. A., & Britz, M. L. (1997). *Journal of Chemical Technology and Biotechnology, 69*, 383–391.

19. Martin, H., Suske, W., Schmid, A., Engesser, K.-H., Kohler, H.-P., Witholt, B., et al. (1998). *Journal of Molecular Catalysis B, Enzymatic, 5*, 87–93.
20. Schmid, A., Sonnleitner, B., & Witholt, B. (1998). *Biotechnology and Bioengineering, 60*, 10–23.
21. Buhler, B., Bollhalder, I., Hauer, B., Witholt, B., & Schmid, A. (2003). *Biotechnology and Bioengineering, 81*, 683–694.
22. Filho, M. V., Stillger, T., Muller, M., Liese, A., & Wandrey, C. (2003). *Angewandte Chemie International Edition, 42*, 2993–2996.
23. Bracher, F., & Litz, T. (1994). *Archives de Pharmacie, 327*, 591–593.
24. Erdelyi, B., Szabo, A., Seres, G., Birincsik, L., Ivanics, J., Szatzker, G., et al. (2006). *Tetrahedron: Asymmetry, 17*, 268–274.
25. Kruse, W., Kragl, U., Wandrey, C. (1996) DE 4436149 A1.