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Biocatalytic synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate with *Candida krusei* SW2026: A practical process for high enantiopurity and product titer

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ABSTRACT

Ethyl (*R*)-2-hydroxy-4-phenylbutyrate ((*R*)-HPBE), a key intermediate in the production of angiotensin-converting enzyme (ACE) inhibitors, was prepared by the microbial reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE). Among 63 microorganisms tested, *Candida krusei* SW2026, for the first time, was proven to be a highly effective biocatalyst in this reduction process, leading to the (*R*)-enantiomer in 99.7% ee and 95.1% yield at 2.5 g/L of OPBE (under optimal conditions of 30 °C, pH 6.6, and in the presence of 5% glucose as co-substrate). In order to achieve higher product concentration with desired enantiopurity and yield for application in large-scale production, strategies such as substrate fed-batch and aqueous/organic biphasic system were successfully conducted in the biotransformation reaction. At 20 g/L of OPBE, the enantiomeric excess (ee), yield, and product concentration were enhanced to 97.4%, 82.0%, and 16.6 g/L, respectively, in water/dibutyl phthalate biphasic system, compared with 87.5%, 45.8%, and 9.2 g/L in aqueous medium. This study provides an attractive process of (*R*)-HPBE production for potential green chemistry applications.

1. Introduction

Biocatalytic asymmetric reduction of prochiral ketoesters to prepare optically pure pharmaceuticals and other compounds with wide applications is one of the most investigated and exploited methods [1–3]. Biosynthesis has remarkable advantages due to its high enantioselectivity, mild reaction conditions, and environmental compatibility [4].

Ethyl (*R*)-2-hydroxy-4-phenylbutyrate ((*R*)-HPBE) is a versatile building block for the synthesis of a variety of angiotensin-converting enzyme (ACE) inhibitors for the treatment of hypertension, such as cilazapril, enalapril, benazepril, ramipril and quinapril [5–7]. A series of routes for the synthesis of (*R*)-HPBE have been explored, including kinetic resolution of the corresponding racemate [8–11], asymmetric reduction of the prochiral precursor 2-oxo-4-phenylbutanoic acid and its derivates [7,12–20], enzymatic esterification of 2-hydroxy-4-phenylbutanoic acid [21], and chemical multi-step synthesis [22,23]. Among these strategies, although several methods gave (*R*)-HPBE in favorable enantiomeric excess (ee), they inherited drawbacks such as involvement of multiple steps and consumption of large quantity of chemical reagents in chemical processes, and in case of resolution, the maximal yield is only 50% theoretically. In the past decade, Oda et al. reported the microbial reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE) in an interface bioreactor, resulting (*R*)-HPBE in 58% yield and 90% ee [7]. Kaluzna et al. attempted the reduction by using genetically engineered *Escherichia coli* overexpressing alcohol dehydrogenase from baker's yeast, leading to the (*R*)-enantiomer in 46% yield and 87% ee [14]. Lacerda et al. described the asymmetric reduction of OPBE with *Pichia angusta*, which afforded (*R*)-HPBE in moderate ee (81%). Although 100% conversion was reached, the applicable substrate concentration in the reaction was extremely low (0.14%, approximately 1.5 g/L) [18].

Besides, Chen et al. were successful in preparing (*R*)-HPBE with favorable ee and yield (99% and 92%, respectively) through *Candida boidinii*-mediated enantioselective reduction at 20 mM (approximately 4.1 g/L) of OPBE [20]. Unfortunately, the relatively low concentrations of substrate (around 4.1 g/L) and product (around 3.8 g/L) would restrict its application in large-scale production. And according to our previous work, unsatisfactory performance of *C. boidinii* as the biocatalyst was observed at relatively higher substrate concentration [24].

In this work, we reported, for the first time, the biocatalytically enantioselective synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate (**2**) by yeast strain *Candida krusei* SW2026 with desirable ee (99.7%) and yield (95.1%) in aqueous medium at 2.5 g/L of OPBE (**1**) (the bioreduction route is shown in Scheme 1). Due to the toxic and inhibitory effect of non-natural substrates on microorganisms,

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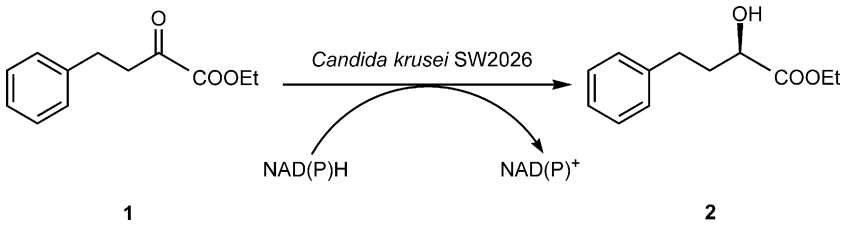

Scheme 1. *Candida krusei* SW2026-mediated asymmetric reduction of OPBE (**1**) to (*R*)-HPBE (**2**).

poor performance was commonly exhibited by biocatalysts at high substrate concentration, which was an obstacle for large-scale application. To achieve higher product concentration with excellent stereoselectivity, batch feeding of the substrate was effectively adopted to diminish the toxicity of high substrate concentration. Additionally, aqueous/organic biphasic system has been demonstrated to be an advantageous alternative to conventional monophasic system in biocatalysis [25–29]. The organic solvent strategy, although did not function well in previous study [20], was successfully applied in this study to solve the substrate-tolerance obstacle and enhance the process productivity, which greatly increased the feasibility of this work to become an industrial approach. Further investigation towards the industrialization of this reaction process is currently undergoing. This approach obviously provides a new access to (*R*)-HPBE with high enantioselectivity, mild reaction conditions, and most importantly, the potential for large-scale production.

2. Materials and methods

2.1. Chemicals

Ethyl (*R*)-2-hydroxy-4-phenylbutyrate was purchased from Sigma–Aldrich Chemical Co. Ethyl 2-oxo-4-phenylbutyrate was supplied by Porton Co., China. All other reagents and solvents were from local commercial suppliers, and were of analytical grade or biochemical reagents.

2.2. Microorganisms and cultivation conditions

Microorganisms for screening were preserved in our laboratory. Yeasts were grown in the following medium: 4.5% glucose, 3% peptone, and 1.5% beef extract, pH 6.0; mold was grown in the following medium: 3% maltose, 2% yeast extract, 0.5% peptone, 0.5% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.07% $MgSO_4{\cdot}7H_2O$, pH 6.0. Strains were incubated aerobically at 180 rpm and 28 °C in 250-mL Erlenmeyer flasks with the appropriate sterilized cultivation medium (30 mL). After 48 h of growth, cells were harvested by centrifugation and washed twice with saline (0.85%).

2.3. Asymmetric bioreduction of OPBE in aqueous medium

The reaction mixture, potassium phosphate buffer (0.1 mol/L, pH 6.6, 10 mL) together with wet cells (1 g), glucose (5%, w/v) and a certain amount of **1**, was added into a 50-mL Erlenmeyer flask capped with a septum. For fed-batch reaction, **1** was fed in two, three, and four batches, and each with half, one-third, and one-fourth of the overall quantity every 2 h, respectively. The flask was shaken in a water-bath shaker at 180 rpm and 30 °C for 14 h. Then the mixture was centrifuged to remove the cells. The supernatant was extracted three times with ethyl acetate and dried over anhydrous Na_2SO_4 for GC analysis.

2.4. Asymmetric bioreduction of OPBE in water/organic biphasic system

The reaction mixture, comprising wet cells (1 g), organic solvent (5 mL), potassium phosphate buffer (0.1 mol/L, pH 6.6, 5 mL) with 5% (w/v) glucose and a certain amount of **1**, was added into a 50-mL Erlenmeyer flask capped with a septum. The incubation proceeded by shaking at 180 rpm and 30 °C. After incubation for 14 h, the reaction mixture was centrifuged. The organic layer was analyzed directly by GC.

2.5. Cell activity assay

The enzyme activity of the cell was determined at 30 °C under the above mentioned reaction conditions by measuring the initial velocity of the product increase in the first hour. One unit of cell activity (U) is defined as the amount of dry cells required for catalyzing the generation of 1 μmol (*R*)-HPBE per minute. The activity is described in terms of U/g DCW (dry cell weight).

2.6. Analysis

The enantiomeric excess values were determined by GC analysis on a Chirasil-Dex CB column (CP7502, Varian, USA) and concentrations of OPBE and HPBE were measured using a CP WAX 52CB capillary column (CP8713, Varian, USA).

The enantiomeric excess and the yield of (*R*)-HPBE are calculated as follows:

$$\text{ee} = \frac{C_R - C_S}{C_R + C_S} \times 100\%$$

C_R and C_S are the molar concentrations of (*R*)-HPBE and (*S*)-HPBE, respectively:

$$\text{yield} = \frac{C_P}{C_S} \times 100\%$$

C_P and C_S are the final molar concentration of the product and the original molar concentration of the substrate, respectively.

2.7. Purification and identification of the product

The biosynthesis of **2** was carried out on a 600-mg scale for identification purpose as follows. The reaction mixture, potassium phosphate buffer (0.1 mol/L, pH 6.6) together with wet cells (10 g), glucose (5%, w/v), and 600 mg of **1** in a final volume of 100 mL, was added into a 500-mL Erlenmeyer flask capped with a septum. The flask was shaken in a water-bath shaker at 180 rpm and 30 °C for 14 h. After centrifugation, the supernatant was extracted three times with ethyl acetate and dried over anhydrous Na_2SO_4, and then the dried product was concentrated under vacuum. Then, the column chromatography was performed using silica gel (200–300 mesh) with ethyl acetate/petroleum ether (1:6) to purify the crude product. The enantiopurity and the yield of the product were determined by GC as described above. After the purification, 436 mg of **2** was obtained, with approximately 99% of ee and 99% of the purity. The overall yields of the bioreduction and the purification processes were 91% and 73%, respectively. Finally, the purified product was characterized by ^{1}H and ^{13}C NMR spectra, recorded on a Bruker AV-300 spectrometer using $CDCl_3$ as a solvent. The ^{1}H NMR and ^{13}C NMR spectra of (*R*)-HPBE was as follows: ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.28 (3H, t, J = 7.2 Hz, OCH_2CH_3), 1.93–2.01 (1H, m, $ArCH_2CHH$), 2.10–2.13 (1H, m, $ArCH_2CHH$), 2.74–2.79 (2H, m, $ArCH_2$), 2.92 (1H, s, O*H*), 4.15–4.23 (3H, m, OCH_2CH_3, *CH*OH), 7.18–7.30 (5H, m, Ar-*H*); ^{13}C NMR ($CDCl_3$, 75.5 MHz): δ 14, 31, 36, 62, 70, 126, 128, 129, 141, 175.

3. Results and discussion

3.1. Screening of microorganisms

The microorganisms capable of reducing **1** to the (*R*)-enantiomer **2** were screened, using absolute configuration, stereoselectivity, and yield as benchmarks. One mold strain (*Aureobasidium pullulans*) and 62 yeast strains (*Candida albicans*, *C. boidinii*, *C. krusei*, *Candida rugosa*, *Candida tropicalis*, *Candida utilis*, *Hansenula anomala*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, *Yarrowia lipolytica*, etc.) were tested. As shown in Fig. 1, eight strains were found potentially useful for the preparation of (*R*)-HPBE, with desired enantioselectivity ranging from 95% to 99% and moderate to high yields (circled). We further screened for the most suitable microbe by observing their performance at a relatively high substrate concentration (10 g/L, data not shown). The results showed that *C. krusei* SW2026 exhibited the greatest synthetic potential and substrate-tolerant capability, and was therefore chosen for our further study.

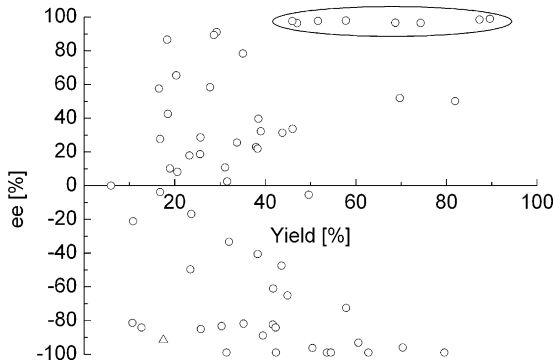

Fig. 1. Screening of microorganisms for the production of **2**. (△) Mold strain; (○) yeast strains. Minus values of ee stand for (*S*) absolute configuration. Reaction conditions: 1 g wet cells, 5% (w/v) glucose, 5 g/L of **1** in 10 mL potassium phosphate buffer (0.1 mol/L, pH 6.6), 30 °C, 14 h.

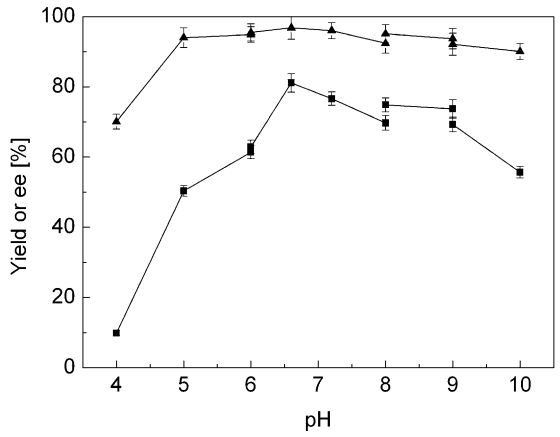

Fig. 3. Effect of pH on the asymmetric reduction of **1**. The product ee (▲) and yield (■) were assayed under various pH conditions. Reaction conditions: 1 g wet cells, 5% (w/v) glucose, 10 g/L of **1** in 10 mL buffer, 30 °C, 14 h.

3.2. Optimization of reaction conditions in aqueous medium

Major parameters affecting the biocatalytic reduction process mediated by *C. krusei* SW2026 were investigated, including reaction temperature, initial pH value of buffer, substrate concentration, as well as the co-substrate for cofactor regeneration.

3.2.1. Effect of reaction temperature on the asymmetric reduction of OPBE

Temperature can affect not only the activity, stereoselectivity, and stability of microbial enzymes, but also the equilibrium of a reaction [30]. The ee values and yields of the product at different temperatures are shown in Fig. 2. When temperature was increased from 20 to 30 °C, both ee and yield increased significantly. Maximum levels of 96.7% ee and 84.3% yield were reached at 30 °C in the asymmetric reduction of **1**–**2**. Further increase of temperature to 45 °C, however, caused a 23% drop in yield and a slighter decrease in ee value, indicating a lower catalytic efficiency at a higher temperature due to the deactivation of the enzymes.

3.2.2. Effect of pH on the asymmetric reduction of OPBE

The enantioselectivity and activity of enzymes can be strongly influenced by buffer pH [31]. From the results shown in Fig. 3, the optimum buffer pH was determined at 30 °C using four different buffers of various pH values: acetate buffer (pH 4–6), potassium phosphate buffer (pH 6–8), Tris–HCl buffer (pH 8–9), and carbonate buffer (pH 9–10). Best performances were observed under neutral pH conditions (pH 6.6–7.2), particularly in pH 6.6 potassium phosphate buffer. Lower or higher pH caused a drop of the yield especially under acidic environment (pH < 5). A sharp decrease in yield from 81.1% to 9.9% was evident when pH was decreased from 6.6 to 4. The enantiopurity remained stable over a pH range from 5 to 10, and decreased dramatically to 70.1% at pH 4. The results indicated that the enzyme system responsible for the reduction reaction was sensitive to strong acidic environment, while stayed active over a wide pH range (5–10). As a result, pH 6.6 was chosen as the favorable pH.

3.2.3. Effect of substrate concentration on the asymmetric reduction of OPBE

Microbial cells, when being employed in the organic synthesis as biocatalysts, can always be seriously repressed by the synthetic substrates, especially in the presence of high concentration of substrates [32]. This phenomenon was also reported for the bioreduction of **1**–**2** [7]. Substrate tolerance of *C. krusei* SW2026 cells was examined in this study (Fig. 4). With the increase of substrate concentration, the ee value and yield continuously decreased, owing to the known inhibitory effect on the cells. The ee value and yield reached the highest levels (99.7% and 95.1%,

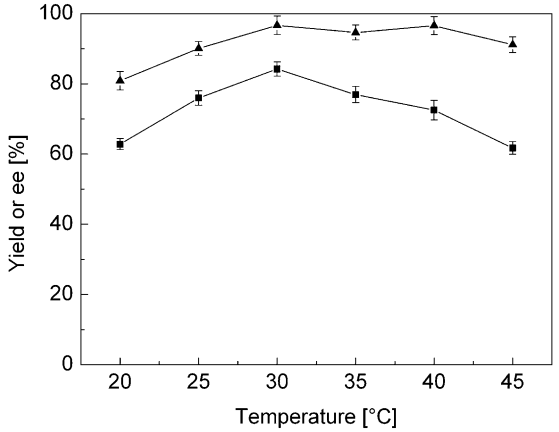

Fig. 2. Effect of temperature on the asymmetric reduction of **1**. The product ee (▲) and yield (■) were assayed at different temperatures. Reaction conditions: 1 g wet cells, 5% (w/v) glucose, 10 g/L of **1** in 10 mL potassium phosphate buffer (0.1 mol/L, pH 6.6), 14 h.

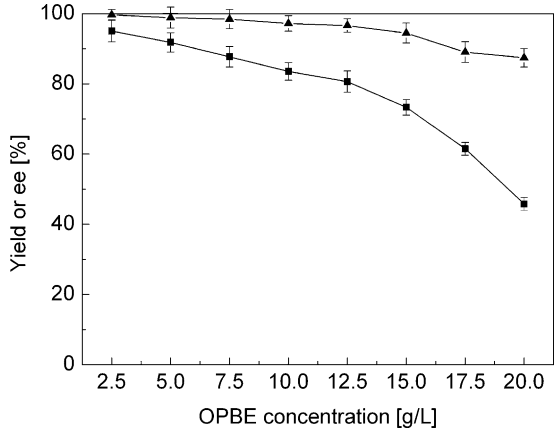

Fig. 4. Effect of substrate concentration on the asymmetric reduction of **1**. The product ee (▲) and yield (■) were assayed at different substrate concentrations. Reaction conditions: 1 g wet cells, 5% (w/v) glucose, 10 mL potassium phosphate buffer (0.1 mol/L, pH 6.6), 30 °C, 14 h.

Table 1
Effect of co-substrate on the asymmetric reduction of **1**.

Co-substrate	ee [%]	Yield [%]
Control	96.3	53.3
Lactose	95.5	72.6
Ethanol	97.2	65.3
Xylose	95.4	67.0
Inulin	96.0	64.2
Fructose	96.4	65.9
Glucose	97.4	80.8
Sucrose	91.5	37.9
Methanol	83.5	32.9
Glycerol	92.9	45.7
Isopropanol	89.9	52.8
Galactose	91.7	56.3

Reaction conditions: 1 g wet cells, 5% (w/v) co-substrate, 10 g/L of **1** in 10 mL potassium phosphate buffer (0.1 mol/L, pH 6.6), 30 °C, 14 h.

respectively) at the lowest substrate concentration (2.5 g/L) tested, and as expected, both of them dropped dramatically to their minimums (87.5% and 45.8%, respectively) at 20 g/L of **1**. Obviously, in aqueous medium, the repression effect of **1** increases with its concentration, and thus high productivity could not be readily attained.

3.2.4. Effect of the co-substrate on the asymmetric reduction of OPBE

The co-substrate, functioning as the electron donor, plays an important role in the cofactor recycling mechanism, and consequently is essential to the continuous and smooth proceeding of the biocatalysis reduction, since cells contain only catalytic amount of cofactors [33–35]. The cofactor regeneration ($NAD(P)^+$/NAD(P)H) catalyzed by glucose dehydrogenase or alcohol dehydrogenase existed in microbe cells, is realized by the oxidation of co-substrates, such as glucose and ethanol [33]. Herein, various sugars and alcohols were studied and the results were listed in Table 1. Compared with the control, in which no co-substrate was added, the highest product yield was reached when glucose was used as co-substrate. Meanwhile, the ee value did not change much with the addition of glucose, demonstrating that glucose provided a sustained supply of electron for the cofactor regeneration in the reduction reaction, while did not influence the (*R*)-preference of the enzyme and hence the product ee. The reason some co-substrates (such as methanol and isopropanol) led to much lower ee values was however not completely understood.

No obvious improvement was observed in our further attempts to find out the influence of some surfactant (such as Tween 40, Tween 60, Tween 80, CTAB, DMSO, and Triton X-100) and the inducers (**1** and **2**) of the reaction.

3.3. Strategies for higher product titer

In order to raise the substrate concentration and subsequently accumulate higher product titer, it is necessary to develop strategies to alleviate the inhibition of substrate on the biocatalyst. Herein, methods such as substrate fed-batch as well as aqueous/organic two-phase system were adopted.

3.3.1. Substrate fed-batch strategy

As previously mentioned, significant repression of enzyme activity in the presence of high substrate concentration was observed. We, therefore, carried out the reaction with substrate feeding to mitigate its toxicity and deactivation effect on the enzyme. As shown in Fig. 5, at a relatively higher substrate concentration of 20 g/L, the fed-batch strategies (mode A–C) advanced the reaction process, in which the highest product concentration was obtained in mode B (12.8 g/L, compared with 9.2 g/L of the control). Little improvement in enantiopurity was

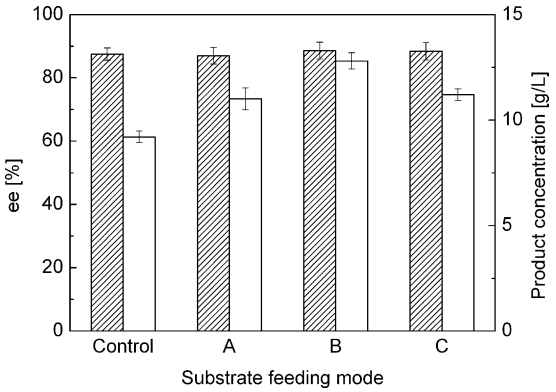

Fig. 5. Effect of substrate feeding strategy on the asymmetric reduction of **1**. The product ee (▨) and concentration (□) were assayed under different substrate feeding modes. Control: substrate was not fed in batches; A: substrate was fed in two batches, and each with the half of the overall quantity; B: substrate was fed in three batches, and each with one-third of the overall quantity; C: substrate was fed in four batches, and each with one-fourth of the overall quantity. Reaction conditions: 1 g wet cells, 5% (w/v) glucose, 20 g/L of **1** in 10 mL potassium phosphate buffer (0.1 mol/L, pH 6.6), 30 °C, 14 h.

observed as expected. Clearly, this strategy effectively contributed to the desired improvement in the product titer at a higher substrate concentration, and more importantly, could be applied conveniently.

3.3.2. Aqueous/organic biphasic system strategy

Although the substrate fed-batch strategy showed certain advantages at 20 g/L of **1**, dramatically decreased ee value (71.6%) was observed at 30 g/L of **1** (data not shown). To further enhance the substrate concentration, we endeavored to conduct the reaction in aqueous/organic biphasic system, a commonly accepted solution to the substrate-tolerance obstacle [28,29]. In this study, the concentrations of substrate and product are calculated based on the volume of the organic phase. With the introduction of organic solvents, the solubility of the water-insoluble substrate could be enhanced on the one hand, and on the other hand, the hydrophilic microbial cells (in aqueous phase) could be isolated from the hydrophobic substrate and the newly generated product (in organic phase). In our investigation into the effects of organic solvent system, ten organic solvents with different log *P* values ranging from 0.28 to 7.7 were screened for the bioreduction, as listed in Table 2. It has been suggested that solvents with a higher log *P* are more hydrophobic and thus more advantageous for enzymatic reactions [36]. The results obeyed this rule approximately, but still with some exceptions, such as *n*-pentane and *n*-hexane. Outstanding yield was

Table 2
Screening of organic solvents in the biphasic system for the production of **2**.

Organic solvent	Log *P*[a]	ee [%]	Yield [%]	Product concentration [g/L]
Control	–	87.5	45.8	9.2
n-Propanol	0.28	38.7	0.4	0.1
Butyl acetate	1.7	92.3	31.7	6.4
n-Pentane	3.0	42.2	2.2	0.4
Cyclohexane	3.2	72.3	98.5	19.9
n-Hexane	3.5	79.3	10.6	2.1
n-Heptane	4.0	78.9	72.0	14.5
n-Octane	4.5	86.1	59.2	12.0
n-Nonane	5.1	84.1	47.2	9.5
Dibutyl phthalate	5.4	97.4	82.0	16.6
Oleic acid	7.7	76.2	54.6	11.0

Reaction conditions: 1 g wet cells, 5% (w/v) glucose, 20 g/L of **1** in 5 mL potassium phosphate buffer (0.1 mol/L, pH 6.6) and 5 mL organic solvent, 30 °C, 16 h.
[a] Ref. [36].

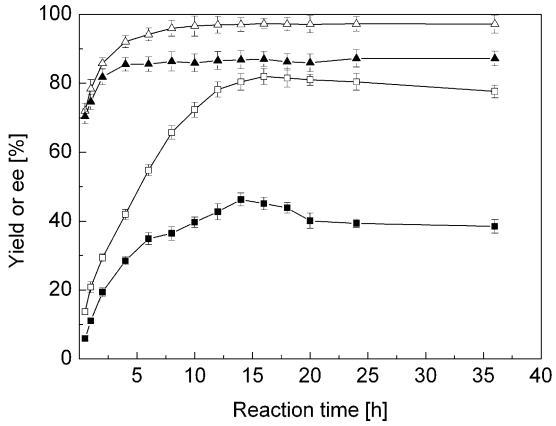

Fig. 6. Time courses of the asymmetric reduction of **1** in aqueous medium (solid) and aqueous/organic biphasic system (empty). (▲, △) ee; (■, □) yield. Reaction conditions in aqueous medium: 1 g wet cells, 5% (w/v) glucose, 20 g/L of **1** in 10 mL potassium phosphate buffer (0.1 mol/L, pH 6.6), 30 °C. Reaction conditions in aqueous/organic biphasic system: 1 g wet cells, 5% (w/v) glucose, 20 g/L of **1** in 5 mL potassium phosphate buffer (0.1 mol/L, pH 6.6) and 5 mL dibutyl phthalate, 30 °C.

obtained (98.5%) when cyclohexane was used, however, with a dissatisfactory ee (72.3%). Consequently, dibutyl phthalate (log P = 5.4) was determined to be the most suitable solvent as the non-aqueous phase, considering better enantioselectivity and yield, which were 11% and 79% higher than that of the control (organic solvent-free system). The yeast cells performed well in the water/dibutyl phthalate two-phase system at an even elevated substrate concentration (40 g/L), resulting a product ee of 95.0%, an yield of 45.7%, and a final product concentration up to 18.5 g/L (data not shown).

Moreover, time courses of biocatalysis in aqueous medium and aqueous/organic biphasic system were compared, as shown in Fig. 6. The reaction rate in the biphasic system was faster than that in the monophasic aqueous medium especially in the first 10 h, indicating serious substrate inhibitory effect in the aqueous medium at the beginning of the reaction. The introduction of dibutyl phthalate in the biphasic system not only mitigated the substrate toxicity effect on microbial cells, but also forced the reaction equilibrium towards the reduction of **1** by accumulating product in the organic phase. The specific activity of the cells in the biphasic system was 37.0 U/g DCW, which is around 2-fold of that in the aqueous system (19.6 U/g DCW). Consequently, a much higher yield was achieved in the biphasic system (82.0%, 16 h), which was almost twice of that in the aqueous medium (46.3%, 14 h). It has also been reported that the enzyme selectivity could be significantly influenced by the organic solvent [25,29]. In this study, the enantiopurity of the product was improved for almost 10% (from around 87% to 97%) in the presence of dibutyl phthalate. For its potential pharmaceutical application, higher product ee of up to 99% could be achieved by either purification procedure (such as recrystallization) or decrease of substrate concentration (to 15 g/L). Apparently, the aqueous/organic biphasic approach effectively minimized the adverse influence of substrate inhibition through the instant isolation of microorganism cells from toxic compounds, and therefore elevated the applicable substrate concentration in the asymmetric reduction.

Despite of the fine performance in the aqueous/organic biphasic system, the separation of (*R*)-HPBE was however quite difficult due to the high boiling point of dibutyl phthalate. The aqueous/ionic liquid biphasic system was therefore investigated to circumvent the product separation issue. Our preliminary results demonstrated that the reaction proceeded smoothly in aqueous/[Bmim]PF_6 biphasic system with excellent product ee and yield (data not shown), and importantly, the product could be isolated easily and efficiently by simple extraction with isopropyl ether.

4. Conclusion

The asymmetric reduction of OPBE to the corresponding (*R*)-HPBE catalyzed by a yeast strain *C. krusei* SW2026 was developed. In monophasic aqueous system, *C. krusei* SW2026 exhibited excellent catalytic capability, giving product in excellent ee (99.7%) and yield (95.1%), under optimum reaction conditions (30 °C, pH 6.6, 2.5 g/L of **1**). In order to mitigate the substrate inhibitory effect, a higher substrate concentration of 20 g/L was added in batches, and the accumulated product concentration reached 12.8 g/L, without obvious sacrifice in ee value. Additionally, the adoption of water/dibutyl phthalate biphasic system performed even better at 20 g/L of **1**, with 97.4% ee, 82.0% yield, and 16.6 g/L of product, and the product concentration could be further increased to an even higher level of 18.5 g/L at 40 g/L of **1**. As an important index indicating the efficiency of microorganisms, the substrate/biocatalyst ratio of this reaction (2 g/10 g wet cell, around 2 g/1.7 g dry cell) showed that *C. krusei* SW2026 was more efficient than many other strains such as *Daucus carota* (0.1 g/10 g callus) [13], *P. angusta* (0.14 g/3.8 g dry cell) [18], *C. boidinii* ClOC21 (1 g/20 g wet cell) [20], and *Bacillus pumilus* Phe-C3 (5.7 g/12 g dry cell) [37]. Apparently, the specific strain, *C. krusei* SW2026, coupled with above strategies, demonstrates great potential in the practical applications of (*R*)-HPBE synthesis. Optimization studies on the reaction process in the aqueous/organic biphasic system are currently under investigation. Further research on other reaction systems for higher substrate concentration and product titer, such as aqueous/ionic liquid biphasic system, should also be of great interest.

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