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Production of a key chiral intermediate of Betahistine with a newly isolated *Kluyveromyces* sp. in an aqueous two-phase system

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ABSTRACT

(*S*)-(4-Chlorophenyl)-(pyridin-2-yl)methanol [(*S*)-CPMA] is an important chiral intermediate of anti-allergic drug Betahistine. Carbonyl reductase-producing microorganisms were isolated from soil samples for the stereoselective reduction of (4-chlorophenyl)-(pyridin-2-yl)methanone (CPMK) to (*S*)-CPMA. Among over 400 microorganisms isolated, one strain exhibiting the highest activity was selected and identified as *Kluyveromyces* sp. After optimization, the biotransformation reaction catalyzed by *Kluyveromyces* sp. CCTCC M2011385 whole-cell gave product (*S*)-CPMA in 81.5% *ee* and 87.8% yield at substrate concentration of 2 g/L in aqueous phase. Using an aqueous two-phase system (ATPs) consisted of PEG4000 (20%, w/w) and Na_2HPO_4 (14%, w/w), the product reached 86.7% *ee* and 92.1% yield at a higher substrate concentration of 6 g/L. The substrate tolerance and biocompatibility of microbial cells are greatly improved in ATPs by accumulating substrate/product in the upper PEG solution. This study, for the first time, reports the production of (*S*)-CPMA catalyzed by microbial cells.

1. Introduction

Biocatalytic synthesis is usually preferred to chemical synthesis due to number of advantages including high enantioselectivity, mild reaction conditions, environmental friendly, etc. [1–3]. The production of chiral alcohols by reducing prochiral ketones with microbial cells (such as *Saccharomyces cerevisiae*) has been applied in a in a number of biocatalytic processes [4]. Nakamura et al. reported the reduction of ethyl 4-chloro-3-oxobutanoate by *Candida magnolia* in 96.6% *ee* [5]. Nanduri et al. successfully synthesized enantiopure alcohols such as (*S*)-4-chloro-3-hydroxy-butanol using *Pichia methanolica* [6]. In our previous study, several chiral alcohols including (*R*)-(−)-2-bromo-1-phenylethanol (99.9% *ee*), (*R*)-2-hydroxy-4-phenylbutyrate (99.7% *ee*), and (*S*)-4-chloro-3-hydroxybutanoate (97% *ee*) were prepared by microbial reduction of their corresponding prochiral ketones [7–10]. Whole-cell biocatalysts are often more stable than free enzymes due to the presence of natural environment inside the cell. Additionally, oxidoreductase-catalyzed reactions require cofactor regeneration system which could be offered by whole-cell, and the addition of cheap co-substrates, glucose for example, is usually sufficient to drive the reaction [11].

(*S*)-(4-Chlorophenyl)-(pyridin-2-yl)methanol [(*S*)-CPMA] is an important chiral intermediate for the synthesis of anti-allergic drug Betahistine. A survey conducted by World Allergy Organization (WAO) in 30 countries reveals that the morbidity ratio of hypersensitiveness diseases was increasing in recent decades, 22% of people suffered from hypersensitiveness disease, and the prevalence of perenial allergic rhinitis and allergic asthma were 17% and 11%, respectively [12]. In fact, few studies on the bioreduction of diaromatic ketones have been reported. Roy et al. reported the asymmetric bioreduction of a bulky ketone 1-phenyl-1-(2-phenylthiazol-5-yl)-methanone to two enantiomeric alcohols (*S*-alcohol in 96% *ee* at 1.5 g/L substrate concentration; *R*-alcohol in 91% *ee* at less than 0.1 g/L substrate concentration) by two yeast strains [13]. Chartrain et al. reported the asymmetric bioreduction of a hindered ketone to (*S*)-bisaryl alcohol (>96% *ee*) by *Rhodotorula pilimanae* [14]. As a potential route to the chiral synthesis of (*S*)-CPMA, the asymmetric reduction of the highly hindered diaryl ketone precursor was investigated in this study. In ketone substrate (4-chlorophenyl)-(pyridin-2-yl)methanone (CPMK), the carbonyl group is surrounded by two bulky groups, pyridine and chlorophenyl. There have been only three reports involving the asymmetric synthesis of (*S*)-CPMA and its derivative so far. Chiral catalyst *trans*-$RuCl_2$[(*R*)-xylbinap][(*R*)-daipen] was utilized for the bioreduction of CPMK to (*S*)-CPMA in 60.6% *ee* [15]. Corey and Helal utilized catecholborane and BF_3/BBr_3 to reduce (4-phenyl)-(pyridin-2-yl)methanone to (*S*)-(4-phenyl)-(pyridin-2-yl) methanol, resulting product in 19–30% *ee* [16]. Truppo et al.

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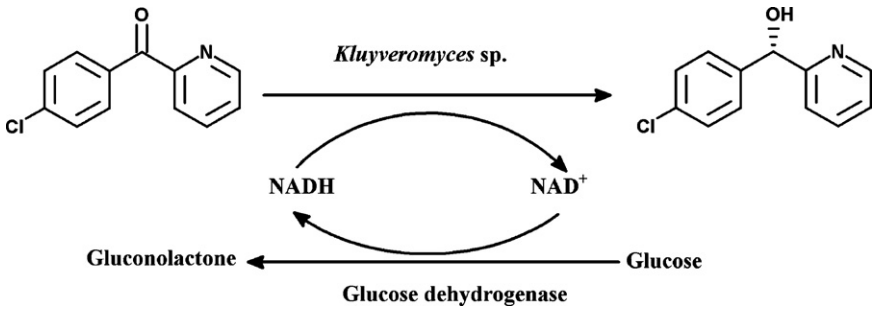

Scheme 1. Asymmetric reduction of CPMK to (*S*)-CPMA catalyzed by microbial cells.

reported the asymmetric synthesis of (*S*)-CPMA using commercial ketoreductases, and the product *ee* is only 60%, representing the only report on the bioreduction process for the production of (*S*)-CPMA [17]. Thus, the isolation of microorganisms with high carbonyl reductase activity toward CPMK is of necessity and could potentially provide a green approach for the industrial synthesis of (*S*)-CPMA.

Aqueous two-phase system (ATPs) is composed of two polymers (polyethylene glycol (PEG) and dextrans), or one polymer (PEG) and one inorganic salt (such as phosphates, citrates and sulfates) with appropriate concentration in aqueous solution. ATPs is a non-volatile, non-denaturing, and benign system for biomaterial separation, environmental remediation, and also as reaction media. Furthermore, ATPs shows excellent biocompatibility toward enzyme and microbial cells, and is regarded as a mild system for extractive fermentation as well as biocatalytic reaction [18]. In the fermentation and biocatalysis processes using ATPs as reaction system, the bioconversion could be conveniently coupled with product separation, such as enzymatic production of antibiotic cephalexin [19,20], extractive production of lactic acid [21], extractive fermentation of enzymes such as chitinase [22], endoglucanase [23], and fermentative solvent production [24].

In this study, an effective method was established to isolate microorganisms. *Kluyveromyces* sp. CCTCC M2011385 was selected from over 400 microbial strains for the enantioselective reduction of CPMK (Scheme 1). The whole-cell catalyzed reaction was carried out in an aqueous two-phase system (ATPs) for improved substrate tolerance and biocompatibility of *Kluyveromyces* sp. cells at higher substrate concentrations.

2. Methods

2.1. Chemicals

(4-Chlorophenyl)-(pyridin-2-yl)methanone (CPMK), all other reagents and solvents are of analytical grade or biochemical reagents, and were obtained from Sinopharm Chemical Reagent Co. Ltd. Racemic (*R*/*S*)-(4-chlorophenyl)-(pyridin-2-yl)methanol (CPMA) was prepared by reducing substrate CPMK with $NaBH_4$.

2.2. Microorganisms and cultivation conditions

The soil samples were collected from the campus and Changguangxi Park (Wuxi, China). Microorganisms were isolated from soil samples by initial screening in Enrichment medium and screening agar medium I and II supplemented with substrate CPMK. Then the isolated colonies were incubated in fermentation medium for 48 h at 30 °C and 200 rpm shaking, and the cells were harvested for further biotransformation.

Enrichment medium (g/L): glucose 50, yeast extract 20, KH_2PO_4 4, $MgSO_4{\cdot}7H_2O$ 1.5, adjust pH 6.0. One milliliter of 500 g/L CPMK solution (in tetrahydrofuran) was added into 1 L medium.

Screening agar medium I (SAMI) (g/L): $(NH_4)_2SO_4$ 0.2, KH_2PO_4 0.2, NaCl 0.1, $MgSO_4{\cdot}7H_2O$ 0.02, agar 20, adjust pH 6.0. To each plate (9 cm diameter), 1 mL of 1 g/L CPMK solution (in ethanol) was spread on the surface of agar medium.

Screening agar medium II (SAMII) (g/L): glucose 25, yeast extract 10, KH_2PO_4 4, $MgSO_4{\cdot}7H_2O$ 1.5, agar 20, adjust pH 6.0. To each plate (9 cm diameter) 1 mL of 0.5 g/L CPMK solution (in ethanol) was spread on the surface of agar medium.

Fermentation medium (g/L): glucose 50, yeast extract 20, KH_2PO_4 4, $MgSO_4{\cdot}7H_2O$ 1.5, adjust pH 6.0.

2.3. Screening of microorganisms

Ten grams of soil sample was mixed with 50 mL of saline (0.85%), and the supernatant was inoculated into enrichment medium supplemented with substrate CPMK. After incubation at 30 °C for 12 h, the supernatant was transferred onto SAMI plates. Tiny colonies could be observed after 3–5 days, and then they were transferred onto richer SAMII plates to obtain larger colonies (2–3 mm diameter). The isolated colonies were cultivated in fermentation medium at 30 °C and 200 rpm shaking for 48 h. The cells were harvested by centrifugation. The cell pellets were washed twice for further biotransformation. The reaction system comprising 5% glucose and 3 g/L substrate in 0.2 M phosphate buffer solution (PBS, pH6.5), was carried out at 30 °C and 200 rpm shaking for 24 h. The reaction was extracted with ethyl acetate and analyzed by thin layer chromatography (TLC) using hexane/isopropanol (9/1, v/v) as eluent. The samples showing diminished substrate spot on TLC plate indicate possible catalytic activity toward CPMK, and were further analyzed by HPLC to determine product enantiomeric excess (*ee*) and yield.

2.4. Asymmetric bioreduction of CPMK in aqueous system

The reaction mixture containing 1 g of wet cells, 2 g/L of CPMK, 3% of glucose, and 0.2 M PBS (pH 6.5) in a final volume of 10 mL, was conducted in a 50-mL Erlenmeyer flask with stopper at 200 rpm and 30 °C for 24 h. The reaction mixture was extracted with ethyl acetate, and the organic phase was evaporated and re-dissolved in ethanol for HPLC analysis.

2.5. Asymmetric bioreduction of CPMA in water/organic biphasic system

The reaction mixture consists of 1 g of wet cells, 2 g/L of CPMK, 3% of glucose, 0.2 M PBS (pH 6.5) and various organic solvent (50%, v/v) in a final volume 10 mL. The reaction was carried out at 30 °C and 200 rpm for 36 h. The reaction was analyzed by HPLC.

2.6. Asymmetric bioreduction of CPMA in ATPs

Aqueous two-phase system (ATPs) contains aqueous solution of PEG and inorganic salts. PEG of Mw from 2000 to 20,000 and various salts including Na_2SO_4, $(NH_4)_2SO_4$, Na_2HPO_4, NaH_2PO_4, and $MgSO_4$ were tested. Reaction mixture consists of 1 g of wet cells, 3% of glucose, and ATPs with various compositions in a final volume of 20 mL. The reaction was carried out at 30 °C and 200 rpm for 36 h, and was analyzed by HPLC.

2.7. Cell activity assay

The enzyme activity of *Kluyveromyces* sp. cell was determined at 30 °C under the above mentioned reaction conditions (Section 2.4) by measuring the initial velocity of the product formation in the first hour. One unit of the cell activity (U) was defined as the amount of wet cells required for catalyzing the reduction of 1 μmol of CPMK/minute.

2.8. HPLC analysis

The conversion and *ee* value was determined using an Agilent 1100 HPLC system (USA) equipped with a Chiralcel OB-H column (0.46mm × 250 mm, 5 μm, Diacel, Japan). The HPLC was performed at 254 nm using hexane:ethanol (95:5, v/v) as eluent at a flow rate of 1.0 mL/min.

The enantiomeric excess (*ee*) and the yield of (*S*)-CPMA are calculated as follows, C_S and C_R are the molar concentrations of (*S*)-CPMA and (*R*)-CPMA, C_{Pro} and C_{Sub} are

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

$$ee = \frac{C_s - C_R}{C_s + C_R} \times 100$$

$$\text{yield} = \frac{C_{\text{Pro}}}{C_{\text{Sub}}} \times 100$$

2.9. Preparative biosynthesis and purification of (S)-CPMA

The biosynthesis of (*S*)-CPMA was carried out on a 6-g scale as follows. The reaction mixture, ATPs containing PEG4000 (20%, w/w)/Na_2HPO_4 (14%, w/w) with wet cells (100 g), glucose (3%, w/v), and 6 g of CPMK in a final volume of 1 L, was added into a 3-L Erlenmeyer flask. The reaction was carried out at 180 rpm and 30 °C for 72 h. The product was extracted three times with ethyl acetate, dried over anhydrous Mg_2SO_4, and concentrated by rotatory evaporator. One gram of the crude product was further purified by silica gel (200–300 mesh) column chromatography using ethyl acetate/petroleum ether (1:7) as eluent.

3. Results and discussion

3.1. Screening of microorganisms

More than 400 microorganisms were obtained in preliminary screening using enrichment cultivation and agar plate isolation from soil samples collected from four different locations. In TLC analysis, 89 strains showed catalytic activity toward CPMK. Since the pyridine moiety is bulkier than chlorophenyl group in the prochiral ketone substrate, the reduction of CPMK catalyzed by microorganisms, following Prelog's rule, is expected to prevalently provide (*S*)-CPMA instead of (*R*)-CPMA. As shown in Fig. 1, our screening results are in accordance with Prelog specificity. In further screening by HPLC analysis, 76 strains gave product in *S*-configuration, among which strains exhibited *ee* values above 60% were re-screened. Only one strain z.2 (solid circle) showed stable enzymatic activity and enantioselectivity among generations, and the *ee* value and yield were 64.8% and 24.6%, respectively. For unknown reasons, two other strains failed to maintain high enantioselectivity in the next generation.

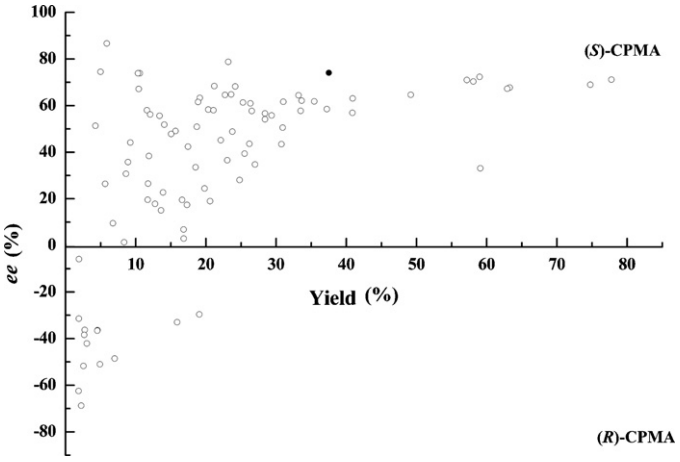

Fig. 1. Screening of microorganisms for the asymmetric reduction of CPMK. Reaction conditions: 1 g wet cells, 5% (w/v) glucose, and 3.5 g/L of CPMK in 10 mL 0.2 M, pH 6.5 PBS at 30 °C for 48 h.

3.2. Identification of the strain

Universal primers of yeast were used to amplify the 18S rRNA gene sequence of strain z.2: EF3: 5′-TCCTCTAAATGACCAAGTTTG-3′, EF4: 5′-GGAAGGGRTGTATTTATTAG-3′. The 1500-bp PCR product was sequenced, and a phylogenetic tree (Fig. 2) derived from 18S rRNA gene sequences was constructed by the neighbor joining method to illustrate the relative position of strain z.2 and related yeast genus. The similarities (%) in 18S rRNA sequences of strain z.2 with other related yeast genus are: *Kluyveromyces marxianus*, 99.3%; *Kluyveromyces yarrowi*, 99.3%; *Kluyveromyces lactis*, 99.3%; *Saccharomyces bayanus*, 96.0%; *S. cerevisiae*, 96.0%; *Torulaspora delbruekii*, 96.0%. Based on above results, strain z.2 is identified to be a *Kluyveromyces* sp.

3.3. Cell growth and carbonyl reductase production of Kluyveromyces sp.

To understand the correlation between the biomass and its carbonyl reductase activity, time course of cell growth and enzyme

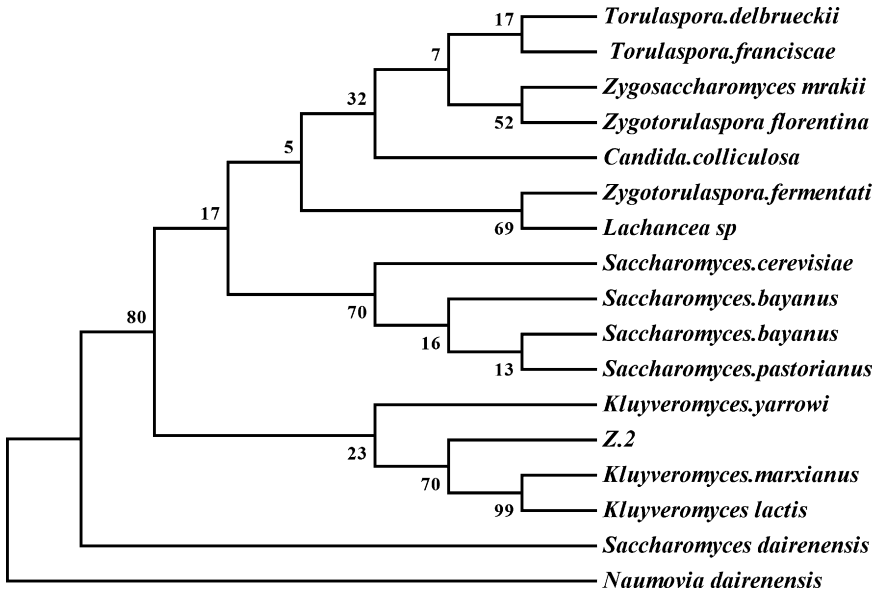

Fig. 2. Phylogenetic tree indicating the estimated relationships between the strain z.2 and related yeast genus.

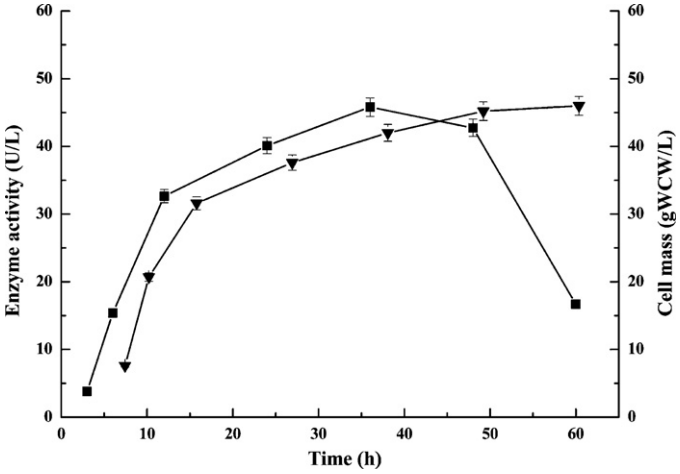

Fig. 3. Time course of cell growth and carbonyl reductase activity of *Kluyveromyces* sp. Fermentation conditions: the strain was grown in 100 mL fermentation medium under 2% inoculum, 30 °C and 200 rpm in 500-mL shake flask for 60 h.

production of *Kluyveromyces* sp. was investigated. As shown in Fig. 3, cells grew rapidly to 31.6 g WCW/L in the first 10 h, and then slow down in the remaining 50 h. The increase of carbonyl reductase activity was closely related to the cell growth, and a maximum activity of 45.8 U/L was attained at 36 h of the fermentation. A sharp decrease in reductase activity was evident after 36 h, possibly due to the ceased cell growth or even cell lysis caused by depletion of nutrient and inhibitory effect of metabolites in the end of fermentation. Therefore, cells were collected at 36th hour of fermentation for bioreduction study.

3.4. Asymmetric reduction in aqueous system

3.4.1. Optimization of reaction conditions in aqueous system

Buffer pH has significant effect on the enantioselectivity and activity of the biocatalyst. As shown in Fig. 4A, the optimum pH of bioreduction was determined in three different buffers including acetate buffer (pH 4.5–5.5), potassium phosphate buffer (pH 6–7.5), and Tris–HCl buffer (pH 8–8.5). The cells exhibited high activity under neutral conditions of pH 6.0–8.0. When pH was decreased from 6.0 to 5.5, the yield was dramatically reduced from 68.1% to 17.0%. The *ee* value was stabilized at 70–80% over the pH range tested. The highest activity was observed in pH 7.0 potassium phosphate buffer, 74.6% *ee* and 81.3% yield were achieved. Thus, pH7.0 was used as the suitable pH for the bioreduction in aqueous phase.

Temperature is one of the major parameters in the biocatalytic reaction, it could affect the reaction velocity, enzymatic activity and stability, even enantioselectivity of the enzyme. Fig. 4B shows product *ee* value and yield at different temperatures in the bioreduction. Both *ee* (78.2–78.3%) and yield (75.4–79.2%) were quite stable when temperature was increased from 25 °C to 30 °C, whereas they were dramatically dropped to −13.2% *ee* and 3.8% yield at 35 °C, and remained at low levels at higher temperature. It indicates that the carbonyl reductase responsible for the bioreduction of CPMK is extremely unstable at 35 °C and above. Therefore, the bioreduction was carried out at 30 °C in further study.

3.4.2. Effect of substrate concentration in aqueous system

The activity of whole-cell biocatalyst could be strongly inhibited by synthetic substrate in the organic synthesis applications, especially when substrate concentration is rather high. Similar situation existed in the microbial reduction of CPMK. As shown in Fig. 5A, the yield declined from 79.2% to 14.9% as substrate concentration was increased from 2 g/L to 6 g/L and above. At 2 g/L of CPMK, product of 76.0% *ee* and 79.2% yield was reached. Despite a slight increase in *ee* value at CPMK concentrations of 4–6 g/L, 2 g/L is regarded as the suitable substrate concentration in the aqueous reaction system to achieve desirable yield and enantioselectivity.

3.4.3. Effect of product inhibition in aqueous system

Product inhibitory effect in the microbial reduction of CPMK was also investigated in this study. Various concentrations of racemic CPMA (0–2 g/L) was added into the aqueous reaction system containing 2 g/L CPMK. As shown in Fig. 6, the yield was decreased rapidly from 77.9% to 54.1% when 0.4 g/L CPMA was added. Meanwhile, a continuous decrease in *ee* value was also observed with the increasing CPMA concentration. At 2 g/L of CPMA, only 15.3% yield and 36.1% *ee* was remained. This result validates that, not only higher substrate concentration, product accumulation could also negatively affect the biotransformation in both yield and enantioselectivity.

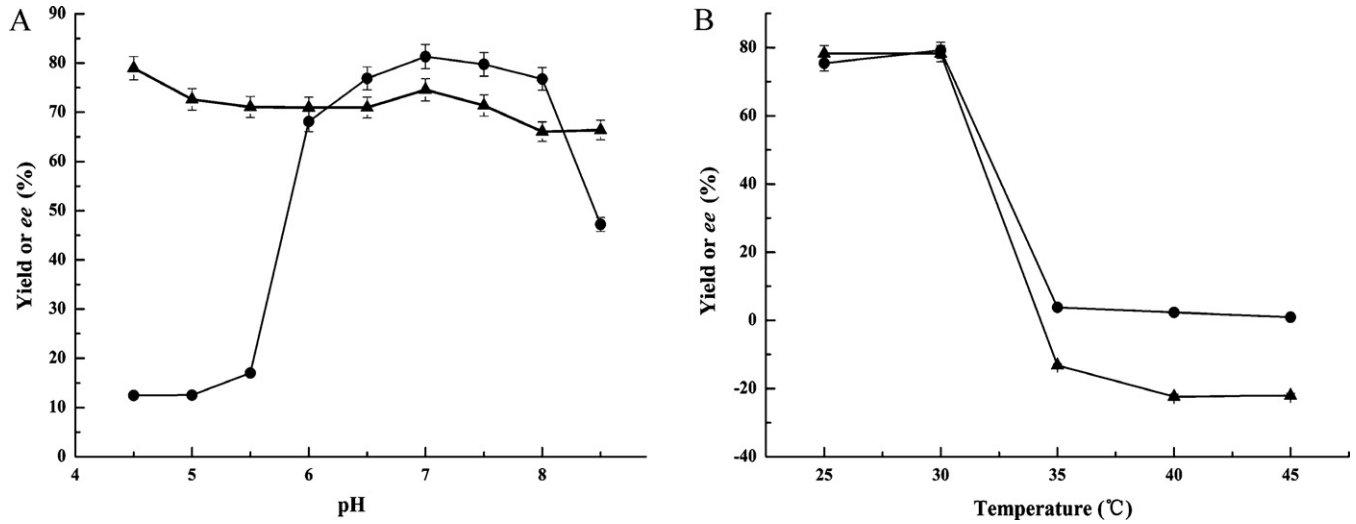

Fig. 4. Effect of pH (A) and temperature (B) on the asymmetric reduction of CPMK in aqueous system. Yield (●), *ee* (▲). Reaction conditions (A) 1 g wet cells, 3% (w/v) glucose, 2 g/L of CPMK in 10 mL buffer of various pHs at 30 °C for 48 h; (B) 1 g wet cells, 3% (w/v) glucose, 2 g/L of CPMK in 10 mL 0.2 M PBS (pH 7.0) at different temperatures for 48 h.

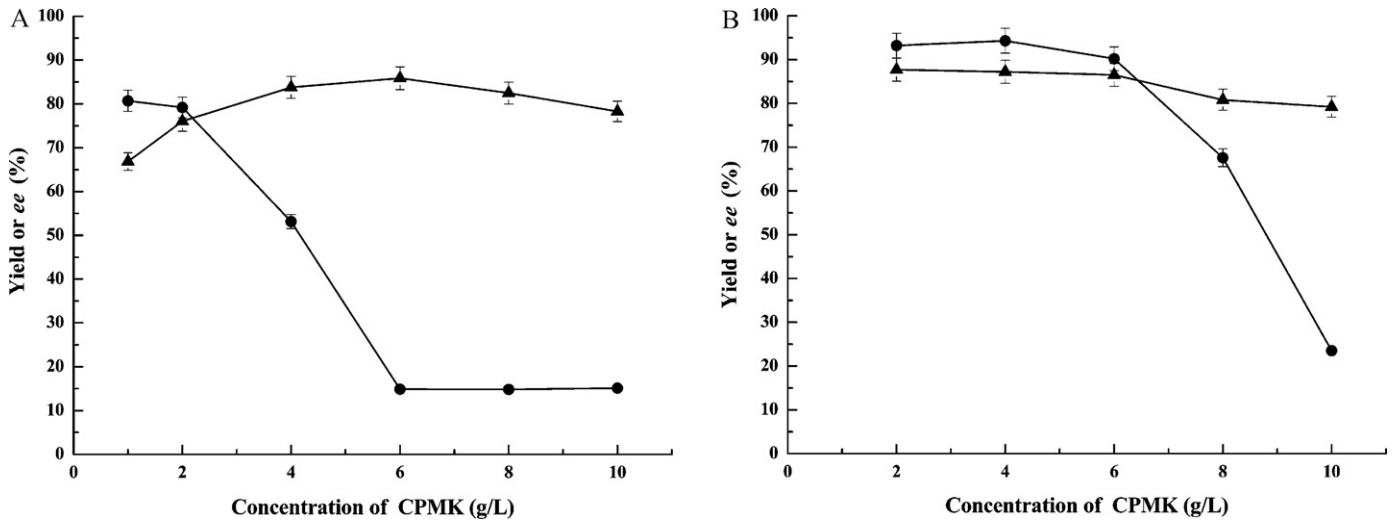

Fig. 5. Effect of substrate concentration on the asymmetric reduction of CPMK in aqueous system (A) and ATPs (B). Yield (●), *ee* (▲). Reaction conditions: 2 g wet cells, 3% (w/v) glucose, in 20 mL 0.2 M pH 7.0 PBS (A) or 20 mL (20%, w/w)/Na_2HPO_4 (14%, w/w) (B) at 30 °C for 48 h.

3.5. Asymmetric reduction in aqueous two-phase system

Aqueous/organic or aqueous biphasic systems allow easy product–catalyst separation, and have been proven to be beneficial not only in economic but also in environmental and green chemistry. In a biphasic system, the hydrophobic substrate could be extracted from the aqueous phase where biocatalyst resides, and also a higher substrate concentration could often be achieved. Here, various solvents and PEG/salt were attempted in the reaction system to alleviate the substrate/product inhibitory effect on the microbial cells. Our results indicate that all the alkane, alcohols, and ethers tested had negative influence on product *ee* and yield. It is noticed that the *ee* value (>70%) is quite stable in two ATPs tested (PEG4000/K_2HPO_4 and PEG6000/K_2HPO_4) (data not shown).

3.5.1. Composition of ATPs

In bioconversion reactions, ATPs provide mild condition that reduce substrate/product inhibition, and do not harm or denature the enzyme or the cells. Therefore, ATPs with different concentrations of PEG and K_2HPO_4/Na_2HPO_4 were investigated to further improve the asymmetric reduction. Partition coefficient *K* was calculated by dividing CPMA concentration in PEG phase (upper) by CPMA concentration in salt phase (bottom). The result indicates that the highest product *ee* and yield (87.3% *ee* and 93.6% yield) were achieved in the system containing PEG4000 (20%, w/w)/Na_2HPO_4 (16%, w/w). Besides, it was advantageous that most of the products were accumulated in the upper PEG4000 solution, according to the high *K* values of 5.93–6.85 (Table 1).

Based on the phase diagram of PEG4000/Na_2HPO_4 we measured (data not shown), Na_2HPO_4 of 8–22% (w/w) and PEG4000 of appropriate content are sufficient to form ATPs. Our result indicates that around 86% *ee* and 92% yield could be attained in ATPs containing 14–24% (w/w) of PEG4000 and 12% (w/w) of Na_2HPO_4 (data not shown). As illustrated in Table 1, product CPMA is enriched in the upper PEG4000 phase. To achieve high product recovery at relatively low PEG content, 20% (w/w) PEG4000 was used to compose the ATPs in the bioreduction. Salt bottom phase is the main locus where the bioconversion takes place. For Na_2HPO_4 content of 10–18% (w/w), a decrease in both *ee* and yield was noticed when Na_2HPO_4 was higher than 14% (data not shown). Thus, PEG4000 (20%, w/w)/Na_2HPO_4 (14%, w/w) was determined as the suitable composition of ATPs for the bioreduction in this study.

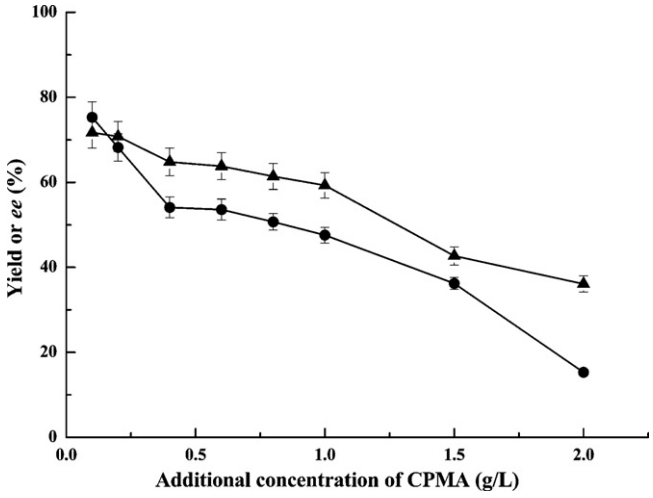

Fig. 6. Effect of product inhibition on the asymmetric reduction of CPMK in aqueous system. Yield (●), *ee* (▲). Reaction conditions: 1 g wet cells, 3% (w/v) glucose, 2 g/L of CPMK, various concentrations (0.1–2.0 g/L) of racemic CPMA, in 10 mL 0.2 M pH 7.0 PBS, at 30 °C for 48 h.

3.5.2. Effect of substrate concentration in ATPs

ATPs could be used to mitigate the inhibitory effect of substrate/product on the biocatalytic reaction. As shown in Fig. 5B, the product yield fell rapidly at CPMK concentration higher than 6 g/L, and only 23.5% yield was remained at 10 g/L CPMK. On the other hand, the product *ee* was decreased from 86.5% to 79.2% as CPMK concentration was increase from 6 g/L to 10 g/L. Therefore, 6 g/L is a feasible substrate concentration for the bioreduction in ATPs containing PEG4000 (20%, w/w)/Na_2HPO_4 (14%, w/w), which is markedly enhanced compared with 2 g/L in aqueous system (Fig. 5A).

3.6. Comparison of asymmetric reduction in aqueous system and ATPs

The time course of asymmetric reduction in aqueous and ATP systems were compared at 6 g/L CPMK (Fig. 7). The reaction rate in ATPs was much faster than that in aqueous system during the entire reduction process. In aqueous system, the product yield of 13.6% was attained at 12 h and no further increase in yield was

Table 1
Screening of ATPs for the asymmetric bioreduction.

	Mw of PEG	Concentration of PEG (%)	Salts	Concentration of salts (%)	*ee* (%)	Yield (%)	Partition coefficient (*K*)
Control	–	–	–	–	78.1	86.3	–
1	2000	12	Na_2HPO_4	12	85.2	90.7	6.31
2	4000	20	Na_2HPO_4	16	87.3	93.6	6.62
3	6000	16	Na_2HPO_4	20	83.1	91.2	6.43
4	2000	16	KH_2PO_4	12	76.7	76.8	6.85
5	4000	20	KH_2PO_4	16	76.5	83.2	6.73
6	6000	12	KH_2PO_4	20	80.4	70.5	6.82
7	2000	20	Na_2SO_4	12	55.0	45.8	6.42
8	4000	12	Na_2SO_4	16	52.7	30.5	6.37
9	6000	16	Na_2SO_4	20	55.2	42.2	5.93
10	2000	12	$(NH_4)_2SO_4$	12	40.1	21.6	6.58
11	4000	20	$(NH_4)_2SO_4$	16	35.8	30.4	6.33
12	6000	12	$(NH_4)_2SO_4$	20	32.9	32.8	6.38

Reaction conditions: 2 g wet cells, 3% (w/v) glucose, 2 g/L of CPMK in 20 mL various ATPs at 30 °C for 48 h.

observed. Whereas in ATPs, product yield increased almost linearly during the first 24 h, and continue to reach its highest level of 93.7% at 73 h, suggesting ATPs could significantly alleviate the substrate inhibition in aqueous system. Additionally, the optical purity of the product was improved in ATPs (86.7% *ee*) compared with that of aqueous system (77.4% *ee*). The result suggests that the substrate inhibition could affect the activity as well as stereoselectivity of carbonyl reductase, and it also explains the cells could remain active for longer in ATPs than aqueous system. Consequently, ATPs could provide a mild and efficient reaction system for the microbial reduction of CPMK.

3.7. Preparative bioreduction of CPMK and identification of the product

Based on the efficient bioreduction of CPMK in a 20-mL PEG4000/Na_2HPO_4 ATP system, a preparative biocatalytic reaction in ATPs was performed on a 1-L scale (Fig. 8). Similar to Fig. 7, the *ee* value was increased gradually in the first 12 h and stabilized at around 85% *ee* after 24 h; the yield was continuously increased in the first 48 h, and reached its highest level of 90.3% at 72 h. One gram of the crude product was further purified by silica gel column chromatography, around 566 mg of purified product were obtained in 85% *ee* and 99.2% purity as determined by HPLC. The optical rotation of the purified product was measured using a polarimeter at

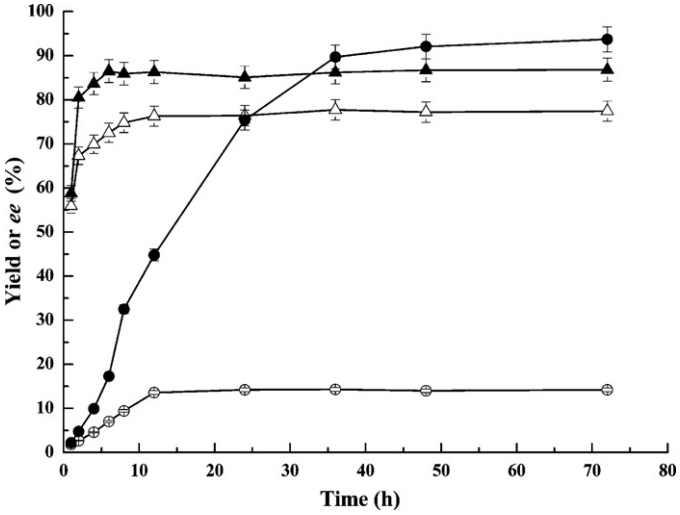

Fig. 7. Comparison of asymmetric reduction of CPMK in aqueous (empty) and ATP (solid) systems. Yield (●, ○), *ee* (▲, △). 2 g wet cells, 3% (w/v) glucose, 6 g/L of CPMK, in 20 mL of 0.2 M, pH 7.0 PBS or PEG4000 (20%, w/w)/Na_2HPO_4 (14%, w/w), at 30 °C for 72 h.

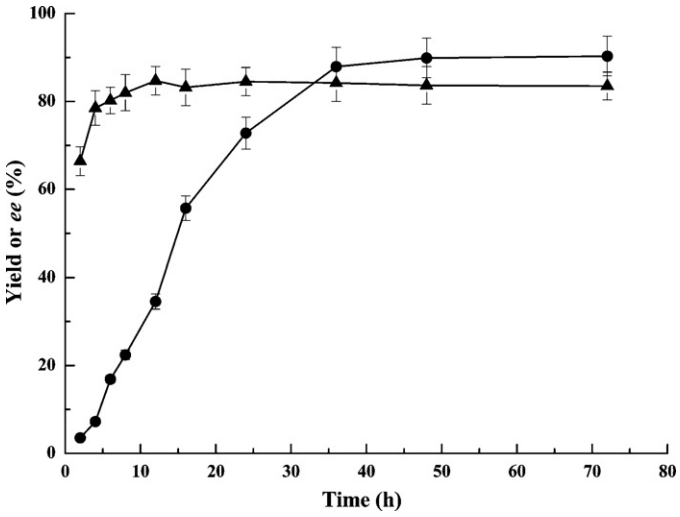

Fig. 8. Preparative bioreduction of CPMK in ATP system. Yield (●), *ee* (▲). 100 g wet cells, 3% (w/v) glucose, 6 g/L of CPMK, in 1 L of PEG4000 (20%, w/w)/Na_2HPO_4 (14%, w/w), at 30 °C for 72 h.

589.3 nm and 30 °C. The product was determined to be positive rotation, $[\alpha]_D^{30} = +0.740$ (85% *ee*). The product was also confirmed to be CPMA by LC–MS.

4. Conclusion

In this work, an effective screening procedure combining enrichment culture and two-step agar plate isolation was established to obtain microorganisms capable of reducing CPMK to (*S*)-CPMA. Among over 400 strains, one strain exhibits high carbonyl reductase activity toward CPMK, and was identified as *Kluyveromyces* sp. At 2 g/L substrate, product of 81.5% *ee* and 87.8% yield were attained in aqueous phase. To mitigate the substrate/product inhibitory effect on the microbial cells, aqueous two-phase system (ATPs) were investigated. In the ATPs consisting of PEG4000 (20%, w/w) and Na_2HPO_4 (14%, w/w), the product reached 86.7% *ee* and 92.1% yield at an elevated substrate concentration of 6 g/L, while only 77.4% *ee* and 14.2% yield were achieved in aqueous system. This study is the first report on the microbial catalyzed production of (*S*)-CPMA, a key chiral intermediate of anti-allergic drug Betahistine.

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