Process Biochemistry 47 (2012) 1042–1048

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

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

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ARTICLE INFO

Article history:
Received 27 December 2011
Received in revised form 22 February 2012
Accepted 14 March 2012
Available online 6 April 2012

Keywords:
Asymmetric reduction
(*S*)-(4-Chlorophenyl)-(pyridin-2-yl)methanol
Aqueous two-phase system
Betahistine
Biocatalysis
Kluyveromyces sp.

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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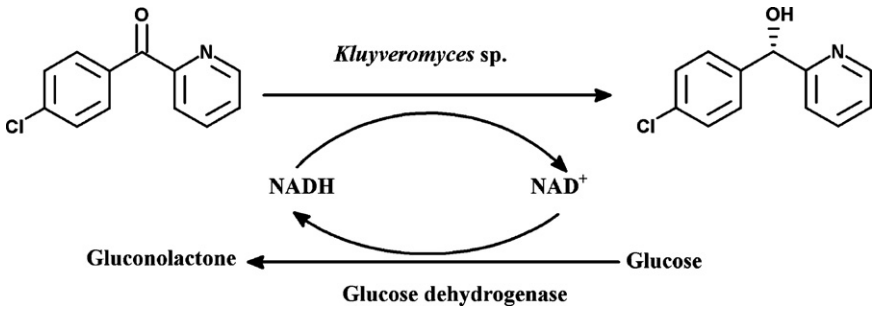
http://dx.doi.org/10.1016/j.procbio.2012.03.022



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