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ORIGINAL PAPER

Asymmetric synthesis of (*S*)-ethyl-4-chloro-3-hydroxybutanoate using *Candida parapsilosis* ATCC 7330

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Abstract Asymmetric reduction of ethyl-4-chloro-3-oxobutanoate to (*S*)-ethyl-4-chloro-3-hydroxybutanoate in aqueous medium by resting cells of *Candida parapsilosis* ATCC 7330 was optimized. The influence of culture parameters (inoculum size, inoculum age and biocatalyst harvest time) and reaction parameters (co-substrate, resting cell, pH and substrate concentrations) on the asymmetric reduction were studied. It was found that these parameters significantly influenced the rate of the asymmetric reduction. Under the optimum conditions, the final concentration of (*S*)-ethyl-4-chloro-3-hydroxybutanoate, enantiomeric excess and the isolated yield of (*S*)-ethyl-4-chloro-3-hydroxybutanoate were 1.38 M (230 g/l), >99 and 95%, respectively. The space time yield was 115 mmol/lh, which is significantly higher than other whole cell biocatalysts reported so far.

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Keywords Asymmetric reduction · Biotransformation · *Candida parapsilosis* ATCC 7330 · Ethyl-4-chloro-3-oxobutanoate · Ethyl-4-chloro-3-hydroxybutanoate

Introduction

(*S*)-Ethyl-4-chloro-3-hydroxybutanoate ((*S*)-CHBE) is a key chiral intermediate in the synthesis of Slagenins B and C [1], HMG-CoA reductase inhibitors and 1, 4-dihydropyridine type β-blockers [2]. The preferred method for the synthesis of optically active ethyl-4-chloro-3-hydroxybutanoate is asymmetric reduction of prochiral ethyl-4-chloro-3-oxobutanoate (COBE) and is known to be carried out by chemical catalysts [3–6] and biocatalysts [7–10]. The intrinsic advantage of the asymmetric reduction is that its yield could be 100%, whereas the yield of resolution can not be more than 50%.

Biocatalytic asymmetric reduction of COBE resulting in (*S*)-CHBE has been reported using Baker's yeast (rate 3.4 mmol/lh, ee 97%), *Candida magnoliae* (rate 9 mmol/lh, ee 96%), *Aureobasidium pullulans* (ee 97%) and *Lactobacillus kefir* (rate 85.7 mmol/lh, ee 99.5%) [7–12]. We previously showed that *Candida parapsilosis* ATCC 7330 is an efficient biocatalyst for the deracemization of aryl substituted β-hydroxy esters by a stereo inversion mechanism to give the optically pure (*S*)-enantiomer in high isolated and optical yields (up to 80% and ee up to >99%) [13, 14]. We now report the asymmetric reduction of COBE (an aliphatic β-keto ester) by *C. parapsilosis* ATCC 7330 (Fig. 1). Asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330 was optimized with respect to culture and reaction conditions to give an efficient and improved rate of formation, yield and enantiomeric excess of (*S*)-CHBE.

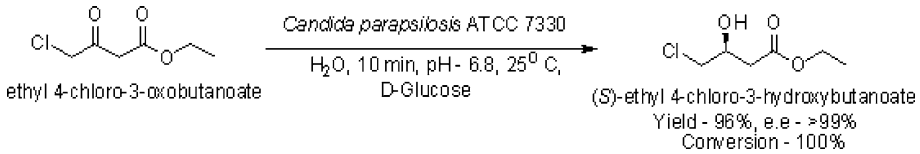

Fig. 1 Asymmetric reduction of COBE by *Candida parapsilosis* ATCC 7330

Materials and methods

Chemicals

COBE was purchased from Lancaster (Morecambe, England) and distilled under vacuum before use. Racemic CHBE was synthesized by sodium borohydride reduction of COBE. All other chemicals were purchased from Merck (India).

Microorganism

Candida parapsilosis ATCC 7330 was obtained from ATCC (Manassas, VA) and maintained at 4°C in yeast malt agar medium that contained 5 g/l peptic digest of animal tissue, 3 g/l malt extract, 3 g/l yeast extract, 10 g/l dextrose and 20 g/l agar.

Cultivation of microorganism

Candida parapsilosis ATCC 7330 was precultured for 12 h at 25°C with shaking at 200 rpm in yeast malt broth medium that contained 5 g/l peptic digest of animal tissue, 3 g/l malt extract, 3 g/l yeast extract and 10 g/l dextrose. The precultured broth, 2 ml [4% (v/v)] with a cell density of 1.7 g/l, was transferred to a 250-ml Erlenmeyer flask that contained 48 ml of yeast malt broth. The culture was grown on rotatory shaker at 25°C and 200 rpm for 14 h. The cultivated cells were harvested by centrifugation (10,000 rpm, 10 min) at 4°C and washed thrice with distilled water.

Asymmetric reduction of COBE

Resting cells, 19 g cell dry weight/l and 50 g/l glucose in 25 ml of potassium phosphate buffer (10 mM, pH 6.8), were pre-incubated in a 150-ml Erlenmeyer flask capped with cotton plug in a water-bath shaker at 200 rpm and 25°C for 15 min. A solution of 0.12 g of COBE in 0.5 ml of ethanol was then added to the reaction flask. Incubation was continued for 15 min after addition of COBE, after which the reaction mixture was extracted with 2 × 100 ml of ethyl acetate. The organic layer was dried with anhydrous sodium sulphate, and the pure product was obtained by silica gel chromatography using hexane/ethyl acetate (9:1) as a mobile phase. To determine the optimum pH for the asymmetric reduction of COBE, the reaction was carried out at different pHs using 10 mM buffers: citrate buffer for pH 5.8, phosphate buffer for a pH range 6.8–7.8 and Tris–HCl for pH 8.8. The enantiomeric excess (ee %) was determined by HPLC. In order to determine the rate of formation of CHBE, 0.5 ml of sample was taken every 5 min and centrifuged (10,000 rpm, 10 min) at 4°C to remove cells. The supernatant was extracted with 1 ml of ethyl acetate, and 1 µl of organic layer was injected into the GC to determine the concentrations of COBE and CHBE.

Asymmetric reduction with intermediate addition of COBE

The mixture consisting of 19 g cell dry weight/l of resting cells and 50 g/l of glucose in 25 ml of potassium phosphate buffer (10 mM, pH 6.8), in a 150-ml Erlenmeyer flask capped with a cotton plug, was pre-incubated in a water-bath shaker at 200 rpm and 25°C for 15 min. Every 15 min, 0.12 g of COBE in 0.5 ml of ethanol was added to the reaction flask. After 90 min, fresh biocatalyst was added to the reaction mixture to increase the resting cell concentration from 19 to 200 g/l, and the reaction was carried out for 12 h with intermediate addition of COBE every 15 min.

Preparative scale asymmetric reduction of COBE

A mixture consisting of 200 g cell dry weight/l of resting cell and 50 g/l glucose (maintained constant through out the biotransformation) in 100 ml of potassium phosphate buffer (10 mmol/l, pH 6.8), in a 500-ml Erlenmeyer flask capped with a cotton plug, was pre-incubated in a water-bath shaker at 200 rpm and 25°C for 15 min. Every 15 min, 0.48 g of COBE was added to the reaction mixture. After 12 h the reaction mixture was extracted with 2 × 100 ml of ethyl acetate. The organic layer was dried with anhydrous sodium sulphate, and the pure product was obtained by silica gel chromatography using hexane/ethyl acetate (9:1) as a mobile phase.

Analytical methods

Concentrations of the substrate (COBE) and product (CHBE) were determined by GC using TC Wax capillary column under the following conditions: oven temperature 130°C, injector and detector at 250°C, carrier gas: He at 1 kg/cm^2, detector: flame ionization detector. Sample injection volume was 1 µl. The enantiomeric excess (ee %) was determined by HPLC analysis on a Jasco PU-1580 liquid chromatograph equipped with a PDA detector. The chiral

column used was Chiralcel OB-H (Daicel, 4.6 × 250 mm). The mobile phase used for the chiral HPLC analysis was hexane/isopropanol (95:5) at a flow rate of 0.5 ml/min at 25°C, and the absorbance was monitored using PDA detector at 220 nm. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on JEOL GSX400 spectrometers operating at 400 and 100 MHz, respectively. Chemical shifts are expressed in ppm values (δ) using TMS as an internal standard. Infrared spectra were recorded on a Shimadzu IR 470 Instrument. Optical rotations were determined on an Autopal® digital polarimeter. Thin layer chromatography was performed on silica gel 60 F-240 precoated silica gel aluminium sheets to monitor the progress of the reaction. The mobile phase used for the thin layer chromatography was 20% ethyl acetate in hexane. Mass spectra were recorded on a Finnigan Mat 8230-GC-MS Spectrometer. Cell density in the medium was monitored by measuring the optical density using Jasco V-530 UV/Vis spectrophotometer at 600 nm. Dry cell weight was calculated from the established calibration (OD_{600} of 1 corresponds to 0.26 g dry cell weight/l). Specific growth rate was calculated by exponential growth model.

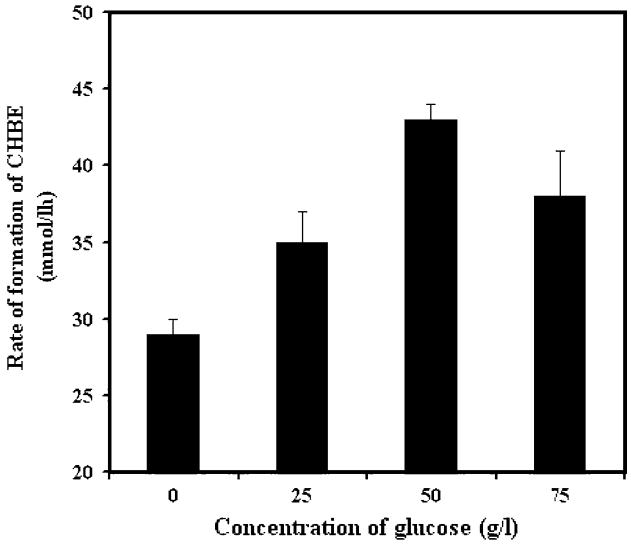

Fig. 2 Effect of glucose (co-substrate) on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330. Culture conditions: inoculum age, inoculum size and biomass harvest time were 14 h, 5% (v/v) and 22 h, respectively. Reaction conditions: 15 g cell dry weight/l, glucose concentration was varied from 0 to 75 g/l, 0.12 mg of COBE in 0.5 ml of ethanol, 25 ml of potassium phosphate buffer (10 mM, pH 6.8), and incubated at 25°C and 200 rpm. All the experiments were performed in duplicates, and each measurement was done twice

Results and discussion

Preliminary asymmetric reduction of COBE (substrate) carried out using *C. parapsilosis* ATCC 7330 resulted in the formation of (*S*)-CHBE (product) in 45% isolated yield and >99% enantiomeric excess in 15 min. The product was characterized by GC, HPLC, NMR, GC-MS, FT-IR and specific rotation in comparison with that reported in the literature [15]. Influence of culture parameters (inoculum size, inoculum age and biocatalyst harvest time) and reaction parameters (co-substrate resting cell, pH and substrate concentrations) were studied to improve the yield and the rate of asymmetric reduction.

Effect of co-substrate (glucose) concentration on the asymmetric reduction of COBE

It is well known that glucose and ethanol have an important role in the reaction medium for the regeneration of cofactors in yeast-mediated asymmetric reduction [16]. Addition of ethanol (1–5% v/v) did not show any significant change on the rate of asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330, but addition of glucose increased the rate of asymmetric reduction of COBE. At a concentration of 50 g/l, glucose showed a maximum asymmetric reduction rate of 43.4 ± 2 mmol/lh with 100% conversion and >99% ee in 30 min (Fig. 2). The rate of reaction was enhanced 1.5-fold in the presence of glucose as co-substrate as compared to the control (no glucose), possibly because glucose is an efficient co-substrate to generate co-factors (as compared to ethanol) for yeast-mediated asymmetric reduction [17].

Optimization of culture conditions

Optimization of culture conditions includes inoculum age, inoculum size and biocatalyst harvest time. Inoculum age and size significantly affected the asymmetric reduction of COBE. It was found that 4% (v/v) (cell density 1.7 g/l) of 12 h inoculum resulted in the maximum rate of asymmetric reduction with an enantiomeric excess of >99% in shake flasks (Fig. 3a, b). The production of carbonyl reductase (enzyme involved in the biocatalytic reduction of prochiral ketones) in *C. viswanathii* was also shown to depend on inoculum age and size for enhanced asymmetric reduction of prochiral ketones [18]. In the present study, maximum activity was achieved with the 12 h inoculum (mid exponential phase), and decrease in enzyme activity and growth was observed with aged inoculum [19]. Growth and enzyme activity reached the maximum at inoculum concentration of 4% (v/v). At lower inoculum concentrations (<4%), the number of yeast cells may not be sufficient to utilize the available amount of nutrients, and at higher concentration, the tremendous growth of yeast results in a nutritional imbalance in the medium, leading to decreased growth and enzyme activity [20]. *C. parapsilosis* ATCC 7330 harvested at mid exponential phase (~14 h) exhibited

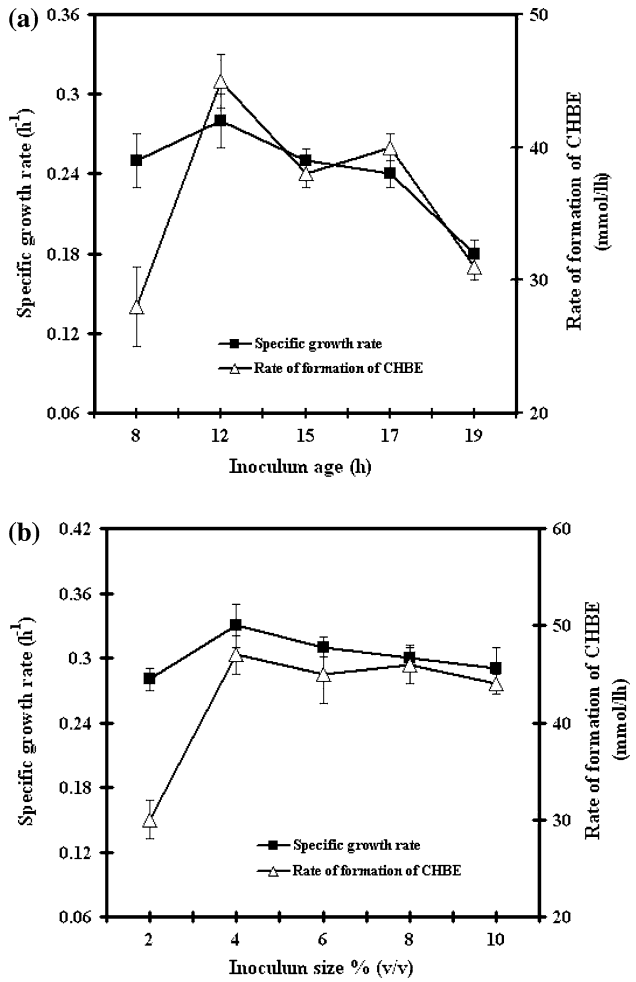

Fig. 3 Effect of culture conditions on the asymmetric reduction of COBE. **a** Effect of inoculum age on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330. Culture conditions: inoculum age was varied from 8 to 19 h, inoculum size and biomass harvest time were 0.03 g dry cell/l and 22 h, respectively. Reaction conditions: 15 g cell dry weight/l, 50 g/l glucose, 0.12 g of COBE in 0.5 ml of ethanol, 25 ml of potassium phosphate buffer (10 mM, pH 6.8), and incubated at 25°C and 200 rpm. **b** Effect of inoculum size on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330. Culture conditions: inoculum size was varied from 2 to 10% (v/v), inoculum age and biomass harvest time were 12 and 22 h, respectively. All the experiments were performed in duplicates, and each measurement was done twice

maximum activity beyond which the activity decreased (Fig. 4), whereas in the case of *C. tropicalis and C. viswanathii*, maximum activity was recorded at the stationary phase [21, 22].

Effect of resting cell concentration on the asymmetric reduction

The effect of resting cell concentration on the asymmetric reduction of COBE revealed that 19 g cell dry weight/l recorded the maximum rate of asymmetric reduction of

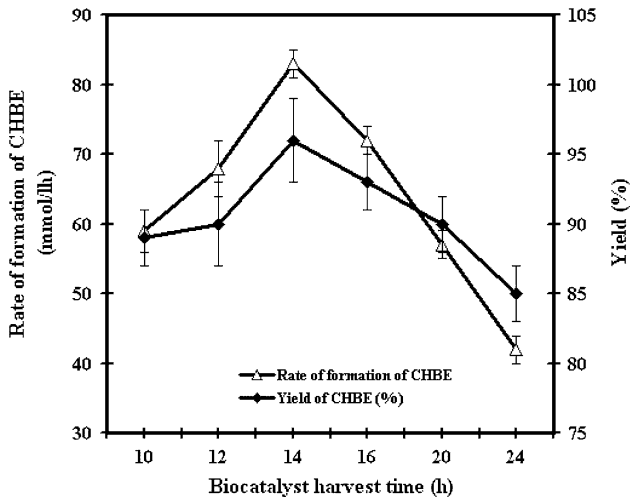

Fig. 4 Effect of biocatalyst harvest time on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330. Culture conditions: *C. parapsilosis* ATCC 7330 was grown under optimum conditions (inoculum age 12 h and 4% v/v of inoculum whose cell density is 1.7 g/l), and biomass was harvested at different time points; their corresponding effect on the rate of asymmetric reduction, yield and enatiomeric excess was determined. Reaction conditions: 15 g cell dry weight/l of resting cell, 50 g/l glucose, 0.12 g of COBE in 0.5 ml of ethanol, 25 ml of potassium phosphate buffer (10 mM, pH 6.8), and incubated at 25°C and 200 rpm. All the experiments were performed in duplicates, and each measurement was done twice

163 mmol/lh with an enantiomeric excess of >99% (Fig. 5). The enantiomeric excess of the product was found to be >99% at all concentrations of resting cells, indicating the high enantiospecificity of the enzyme(s) involved. And as expected, the rate of the asymmetric reduction was significantly affected by the resting cell concentration.

Effect of pH on the asymmetric reduction of COBE

To determine the optimum pH of the biocatalytic asymmetric reduction of COBE, the reaction was carried out in media with different pHs varying from 4.8 to 8.8. The reaction carried out at pH 6.8 recorded the maximum rate of asymmetric reduction (163 mmol/lh). The ee and isolated yield were >99 and 96%, respectively. This substrate, COBE, on asymmetric reduction by *C. magnoliae* and *A. pullulans* (which produces (*S*)-CHBE) also exhibited maximum activity in the range of pH 6.5 to 7.0 [10, 12].

Effect of substrate concentration and substrate feeding on the asymmetric reduction

The effect of initial COBE concentration on the rate of asymmetric reduction, yield and enantiomeric excess is shown in Fig. 6a. Rate of asymmetric reduction (163 mmol/lh) and isolated chemical yield (96%) were found to be maximum at COBE concentration of 4.8 g/l.

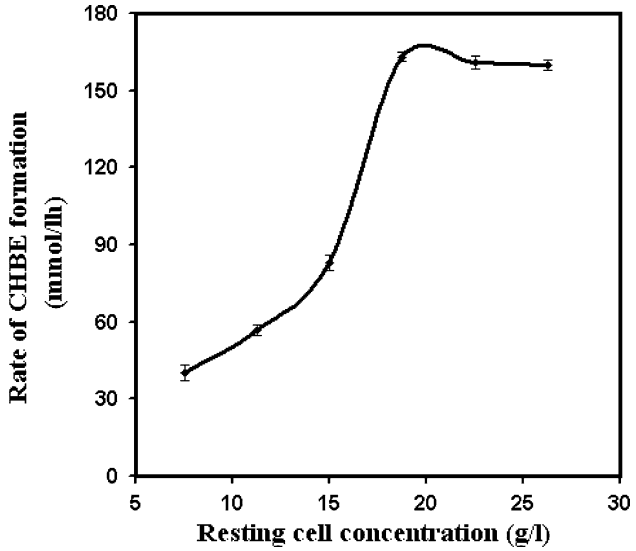

Fig. 5 Effect of resting cell concentration on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330. Culture conditions: *C. parapsilosis* ATCC 7330 was grown under optimum conditions (inoculum age 12 h and 4% v/v of inoculum whose cell density is 1.7 g/l), and biomass was harvested at the 14th h. Reaction conditions: resting cell concentration in the reaction mixture was varied from 7.5 to 26 g cell dry weight/l, 50 g/l glucose, 0.12 g of COBE in 0.5 ml of ethanol, 25 ml of potassium phosphate buffer (10 mM, pH 6.8), and incubated at 25°C and 200 rpm. All the experiments were performed in duplicates, and each measurement was done twice

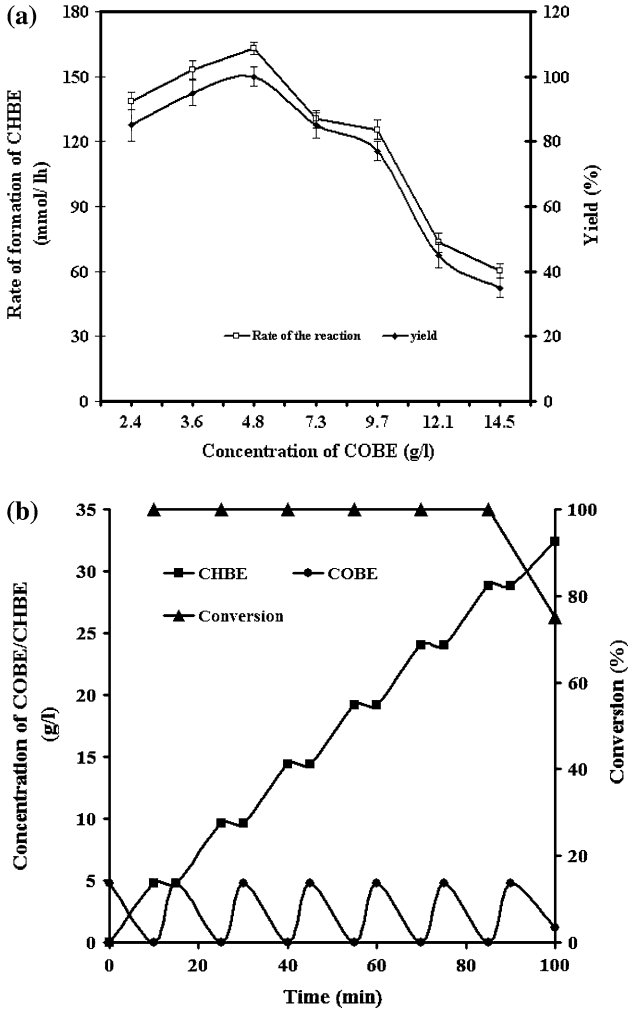

Fig. 6 Effect of substrate concentration and substrate feeding on the asymmetric reduction. **a** Effect of substrate concentration on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330. Culture conditions: *C. parapsilosis* ATCC 7330 was grown under optimum conditions (inoculum age 12 h and 4% v/v of inoculum whose cell density is 1.7 g/l), and biomass was harvested at the 14th h. Reaction conditions: 19 g cell dry weight/l of resting cell, 50 g/l glucose, COBE concentration was varied from 2.4 to 14.5 g/l, 25 ml of potassium phosphate buffer (10 mM, pH 6.8), and incubated at 25°C and 200 rpm. **b** Effect of substrate feeding on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330. Culture conditions: *C. parapsilosis* ATCC 7330 was grown under optimum conditions (inoculum age 12 h and 4% v/v of inoculum), and biomass was harvested at the 14th h. Reaction conditions: 19 g cell dry weight/l of resting cell, 50 g/l glucose, 25 ml of potassium phosphate buffer (10 mM, pH 6.8), 25°C and 200 rpm. Every 15 min 0.12 g of COBE in 0.5 ml of ethanol was added to the reaction mixture. All the experiments were performed in duplicates, and each measurement was done twice

Decrease in the rate and yield of the asymmetric reduction was observed when the COBE concentration was more than 4.8 g/l, indicating substrate inhibition in aqueous medium at higher concentrations of COBE [23]. The product enantiomeric excess was found to be >99% under the range of COBE concentration tested. The problem of substrate inhibition can be avoided by batch conversion with intermediate addition of COBE [24]. In the present study, after every 15 min, 120 mg of COBE was added to the reaction mixture, and the rate, enantiomeric excess and yield were determined. The reaction rate and conversion remained constant up to 6th addition. Successive addition of COBE (4.8 g/l × 6) made it possible to isolate 28.8 g/l of CHBE in 90 min (Fig. 6b). Intermediate addition of COBE in batch conversion resulted in a sixfold increase in CHBE [product] concentration from 4.8 to 28.8 g/l with an enantiomeric excess of >99%. After 90 min the concentration of the biocatalyst was increased from 19 g cell dry weight/l to 200 g cell dry weight/l since there was a drop in conversion from 100 to 75% (Fig. 6b). Addition of fresh biocatalyst after 90 min boosted the conversion to 100% and maintained it for 12 h. This strategy led to a further increase of almost tenfold in product (CHBE) concentration from 28.8 to 230 g/l with an enantiomeric excess of >99% in 12 h. The catalytic productivity (1.15 g CHBE/g dry cell) and rate of asymmetric reduction (115 mmol/lh) were not affected up to 200 g cell dry weight/l, but above this resting cell concentration, a drop in catalytic productivity (0.96 g CHBE/g dry cell) and rate of asymmetric (109 mmol/lh) reduction was observed (data not shown).

Preparative scale asymmetric reduction

Under the optimized conditions, preparative scale (100 ml) asymmetric reduction of COBE was carried out using *C. parapsilosis* ATCC 7330. Every 15 min, 480 mg of COBE was added to the reaction mixture. Asymmetric reduction was carried out at 25°C and 200 rpm. Space time yield, final concentration of (*S*)-CHBE, enantiomeric excess and isolated yield of the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330 were 115 mmol/lh, 230 g/l (1.38 M), >99 and 95%, respectively. These data are seemingly better than all the reported data so far [7–12]. As compared to the reported whole cell, yeast and fungal biocatalytic systems for the asymmetric reduction of COBE [Baker's yeast (ee 97%), *C. magnoliae* (ee 96%) and *A. pullulans* (ee 97.7%)] [8–12], *C. parapsilosis* ATCC 7330 as reported in this paper resulted in improved stereoselectivity, space time yield and final concentration of the (*S*)-CHBE. Asymmetric reduction of COBE with high stereoselectivity (ee >99%), which is comparable to *C. parapsilosis* ATCC 7330, were reported using *L. kefir* and recombinant *E. coli* [7, 8]. However, asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330 in aqueous medium showed an improved final concentration of (*S*)-CHBE and space time yield when compared to the asymmetric reduction of COBE using recombinant *E. coli* (space time yield: 96 mmol/lh, 1.25 M) and *L. kefir* (space time yield: 85 mmol/lh, 1.2 M) [7, 8]. Moreover, asymmetric reduction of COBE by recombinant *E. coli* requires external addition of the expensive cofactor NADP, and *E. coli* cells needed to be permeabilized by pretreatment with Triton X-100 [8]. In this study, the biocatalyst shows high stereoselectivity without any pretreatment. The use of organic solvents in the asymmetric reduction of COBE using *C. parapsilosis* ATCC 7330 is minimal, and the addition of external cofactors is not necessary.

In conclusion, *C. parapsilosis* ATCC 7330 is an efficient biocatalyst for the asymmetric synthesis of (*S*)-CHBE in an aqueous medium. The high space-time yield (115 mmol/lh) and high final concentration of (*S*)-CHBE (230 g/l) with enantiomeric excess (>99%) without pretreatment of the biocatalyst makes it a potential biocatalytic process for large-scale applications.

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References

1. Jiang B, Liu JF, Zao SY (2003) Enantioselective synthesis for the antipodes of slagenins B and C. J Org Chem 68:2376–2384
2. Kita K, Kataoka M, Shimizu S (1999) Diversity of 4-chloroacetoacetate ethyl esters reducing enzymes in yeast and their application to chiral alcohol synthesis. J Biosci Bioengg 88:591–598
3. Imamoto T, Nishimura M, Koide A, Yoshida K (2007) t-BU-QuinoxP ligand: applications in asymmetric Pd-catalysed allylic substitution and Ru catalysed hydrogenation. J Org Chem 72:7413–7416
4. Kitamura M, Ohkuma T, Takaya H, Noyori R (1988) A practical asymmetric synthesis of carnitine. Tetrahedron Lett 29:1555–1556
5. Pai CC, Li YM, Zhou ZY, Chan ASC (2002) Synthesis of new chiral diphosphine ligand (Bisbenzodioxan phos) and its application in asymmetric catalytic hydrogenation. Tetrahedron Lett 43:2789–2792
6. Wan Y, Sun Y, Luo Y, Li D, Zhang Z (2005) Synthesis of a bulky and electron rich derivative of SEG phos and its application in Ru catalyzed enatioselective hydrogenation of beta keto esters. J Org Chem 70:1070–1072
7. Amidjojo M, Weuster-Botz D (2005) Asymmetric synthesis of chiral synthon ethyl-4-chloro-3-hydroxybutanoate using *Lactobacillus kefir*. Tetrahedron Asymmetr 16:899–901
8. Kizaki N, Yasohara Y, Hasegawa M, Wada M, Kataoka M, Shimizu S (2001) Synthesis of optically pure S-ethyl-4-chloro-3-oxobutanoate by *E. coli* transformed cells expressing the carbonyl reductase and glucose dehydrogenase genes. Appl Microbiol Biotechnol 55:590–595
9. Yang ZH, Yao SJ, Lin DQ (2004) Improving the stereoselectivity of asymmetric reduction of 3-oxo esters with pretreatments on Bakers' yeast. Ind Eng Chem Res 43:4871–4875
10. Yashohara Y, Kizaki J, Hasegawa S, Takahashi M, Wada M, Kataoka S (1999) Synthesis of optically active ethyl-4-chloro-3-hydroxybutanoate by microbial reduction. Appl Microbiol Biotechnol 51:847–851
11. Heldge E, Rupert P, Cumther W, Weuster-Botz D (2004) Stereoselective reduction of ethyl-4-chloro acetoacetate with recombinant *Pichia pastoris*. Tetrahedron Asymmetr 15:3591–3593
12. He JY, Sum ZH, Ruan WQ, Xu Yan (2006) Biocatalytic synthesis of ethyl-(*S*)-4chloro-3-hydroxy-butanoate in an aqueous-organic solvent biphasic system using *Aureobasidium pullulans* CGMCC 1244. Process Biochem 41:244–249
13. Padhi SK, Titu D, Pandian NG, Chadha A (2006) Deracemisation of *b*-hydroxy esters using immobilised whole cells of *Candida parapsilosis* ATCC 7330: substrate specificity and mechanistic investigation. Tetrahedron Asymmetr 62:5133–5140
14. Titu D, Chadha A (2008) Preparation of optically pure alkyl 3-(hetero-2-yl)-3-hydroxypropanoates by *Candida parapsilosis* ATCC 7330 mediated deracemisation. J Mol Catal B Enzyme 52:168–172
15. Yoshihiko Y, Noriyuki K, Hasegawa K, Wada M, Kataoka M, Shimizu S (2001) Stereoselective reduction of alkyl 3-oxobutanoate by carbonyl reductase from *Candida mangnoliae*. Tetrahedron Asymmetr 12:1713–1718
16. Ifoeng CJ, Jurgen H, Adrie JJ, Jaap A, Andreas L, Joseph J (2002) Reduction of ethyl 3-oxobutanoate using non-growing baker's yeast in a continuously operated reactor with cell retention. Enzyme Microb Technol 31:665–672
17. Zhang B, Pionnier S (2003) Cofactor recycling mechanism in asymmetric biocatalytic reduction of carbonyl compounds mediated by yeast: which is the efficient electron donor? Chemistry 9:3604–3610
18. Pankaj S, Gandhan SP, Banerjee UC (2006) Optimization of physicochemical parameters for the enhancement of carbonyl reductase production by *Candida viswanathii*. Bioprocess Biosyst Eng 29:149–156
19. Sreenath HK, Chapman TW, Jeffries TN (1986) Ethanol production from D-xylose in batch fermentation with *Candida shehatar*: process variable. Appl Microbiol Biotechnol 24:294–299
20. Shafiq S, Ali S, Ehsan A, Haq I (2003) Optimization of assay condition and inoculum size with kinetic analysis for biosynthesis of invertase by *Sacchromyces cerevisiae*. J Biol Sci 3:191–196

21. Ashwini LK, Pankaj S, Banerjee UC (2005) Biocatalytic synthesis of *S*-(-)-1-(1'-napthyl)-ethanol by a novel isolate of *Candida viswanathii*. J Mol Catal B Enzyme 35:1–6
22. Pankaj S, Banerjee UC (2005) Biotransformations for the production of the chiral drug (*S*)-duloxetine catalyzed by a novel isolate of *Candida tropicalis*. Appl Microbiol Biotechnol 67:771–777
23. Houng JY, Liau JS (2003) Applying slow release biocatalysis to the asymmetric reduction of ethyl-4-chloro-acetoacetate. Biotechnol Lett 25:17–21
24. Shimizu S, Kataoka M, Katoh M, Morikawa T, Miyoshi T, Yamada H (1990) Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by a microbial aldehyde reductase in an organic solvent-water diphaseic system. Appl Environ Microbiol 56:2374–2377