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Synthesis of both enantiomers of ethyl-4-chloro-3-hydroxbutanoate from a prochiral ketone using *Candida parapsilosis* ATCC 7330

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ABSTRACT

Candida parapsilosis ATCC 7330 when grown in a medium containing glycerol reduced ethyl-4-chloro-3-oxobutanoate to (*R*)-ethyl-4-chloro-3-hydroxybutanote (ee >99%, yield: 94%) while glucose and sucrose grown cells yielded (*S*)-ethyl-4-chloro-3-hydroxybutanote (ee >99%, yield: 96%). The activity of ethyl-4-chloro-3-oxobutanoate reductase was higher in glucose-grown cells (160 U/g protein) when compared to sucrose (158 U/g protein) and glycerol (22 U/g protein). Both the enantiomers of ethyl-4-chloro-3-hydroxybutanoate (ee >99%) can thus be obtained using *Candida parapsilosis* ATCC 7330 by altering the carbon source in the growth medium.

1. Introduction

Enantiomerically pure ethyl-4-chloro-3-hydroxybutanoate is a useful chiral building block for the synthesis of pharmaceuticals and other bioactive molecules. (*R*)-Ethyl-4-chloro-3-hydroxybutanoate is a precursor of L-carnitine[1] and (*S*)-ethyl-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] is a key chiral intermediate in the synthesis of Slagenins B and C,[2] HMG-CoA reductase inhibitors and 1,4-dihydropyridine type β-blockers.[3] These chiral synthons can be obtained via resolution of the racemate or the asymmetric reduction of the prochiral ketone.[4] Asymmetric reduction of prochiral ethyl-4-chloro-3-oxobutanoate (COBE) has an advantage over resolution because asymmetric reduction can give 100% yield, unlike resolution, where the maximum yield is limited to 50%. The asymmetric reduction of ethyl-4-chloro-3-oxobutanoate (COBE) by chemical catalysts has already been reported on.[5,6] The biocatalytic asymmetric reduction of COBE to CHBE has been reported using Baker's yeast [rate 3.4 mmol/lh, ee (*S*) 97%], *Candida magnoliae* [rate 9 mmol/lh, ee (*S*) 96%], *Aureobasidium pullulans* [ee (*S*) 97%], *Lactobacillus kefir* [rate 85.7 mmol/lh, ee (*S*) 99.5%], *Sporobolomyces salmonicolor* [ee (*R*), rate 8.2 mmol/lh] and *Lactobacillus fermentum* [rate 0.5 mmol/lh, ee (*R*) 98%].[4,7–14]

Microbial asymmetric reductions have become increasingly attractive due to the easy availability of the biocatalysts, and mild reaction conditions in addition to being relatively inexpensive. However, due to the presence of multiple dehydrogenases in the whole cells, the enantiomeric purity of the products of the asymmetric reduction is not always high.[3] The stereoselectivity of the microbial reductions can be controlled by screening of the microorganisms,[15,16] the addition of the substrate,[17,18] modifying the substrate,[1] addition of specific inhibitors[11,19–23] and thermal treatment.[11]

Changes in cultivation conditions have also been shown to affect the enantioselectivity and specific activity of whole cell catalysed reductions. Ushio et al. demonstrated that resting-cell suspensions of two methylotrophic yeasts could be persuaded to reduce β-keto esters (including methyl 4-chloroacetoacetate) to a higher level of enantioselectivity when grown on a methanol- rather than a glucose-supplemented medium.[24] In the reduction of methyl acetoacetate by *Candida parapsilosis* DSM 70125, the highest specific rate was achieved using cells grown on a medium supplemented with decanoic acid rather than glycerol or glucose; a marked effect on enantioselectivity was also observed.[16] Growing *Pichia capsulata* with xylose instead of glucose as the major carbon source for growth resulted in an eight-fold increase in the specific rate of ethyl (*R*)-4-chloro-3-hydroxybutanoate production. The enantioselectivity was slightly reduced (41% ee) as compared to that achieved with glucose-grown cells (61% ee).[25]

Recently, we reported an efficient method for the asymmetric reduction of COBE to (*S*)-CHBE (ee >99%) using *C. parapsilosis* ATCC 7330 under optimized culture and reaction conditions.[26] Herein we report the synthesis of both enantiomers of ethyl-4-chloro-3-hydroxybutanote in the homochiral form via a *C. parapsilosis* ATCC 7330 mediated asymmetric reduction and by changing the carbon source in the growth medium.

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2. Results and discussion

2.1. Effect of carbon sources in the biocatalyst preparation (*C. parapsilosis* ATCC 7330) on the asymmetric reduction of COBE

Carbon sources in the growth medium can markedly influence the enantioselectivity and activity of the yeast mediated asymmetric reduction.[16,24] The effect of various carbon sources such as glucose, sucrose, maltose, mannitol, starch or glycerol, during the growth of *C. parapsilosis* ATCC 7330 on the asymmetric reduction of COBE was studied. The organism accepted all of the carbon sources for growth, with the exception of starch (Table 1). The asymmetric reduction of COBE was observed in cultures grown in sucrose, glucose and glycerol resulting in the formation of enantiomerically pure CHBE with an excellent enantiomeric excess (>99%, Table 1). Maltose and mannitol supported the growth of *C. parapsilosis* ATCC 7330 but the enantiomeric excess (ee) of the product CHBE was 55% (*S*) and 97% (*S*), respectively. The *C. parapsilosis* ATCC 7330 grown in glucose exhibited the highest rate of asymmetric reduction of COBE. (*S*)-CHBE (anti-Prelog) was the product formed by *C. parapsilosis* ATCC 7330 for all of the carbon sources tested, except in the case of glycerol where (*R*)-CHBE (Prelog product) was observed in excellent enantiomeric excess of >99%. It should be noted that the *C. parapsilosis* DSM 70125 grown in glycerol, when used for the reduction of acetoacetic acid methyl ester showed no stereoselectivity (ee 0%).[16] Our observations led us to speculate that an (*S*)-specific COBE reductase (anti-Prelog enzyme) is expressed during the growth of *C. parapsilosis* ATCC 7330 with glucose and sucrose as carbon sources, whereas an (*R*)-specific COBE reductase (Prelog enzyme) is expressed when glycerol is used as the carbon source.

2.2. Regulation of COBE reducing enzyme(s) in *C. parapsilosis* ATCC 7330

In an attempt to better understand the COBE reducing enzymes in *C. parapsilosis* ATCC 7330, experiments were carried out with the cell free extracts of *C. parapsilosis* ATCC 7330 grown in glucose, sucrose and glycerol. The COBE reducing enzyme from glucose and sucrose grown cells preferred NADH, whereas those from glycerol grown cells accepted both NADH and NADPH. The COBE reduction by glycerol grown cells gave the (*R*)-alcohol irrespective of the cofactor.

2.2.1. Effect of harvest time on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330

The growth of *C. parapsilosis* ATCC 7330 in glucose, sucrose and glycerol is almost identical (specific growth rate 0.33 h^{-1}) (Fig. 1a). Glucose and sucrose grown cells exhibited a maximum COBE reductase activity at the mid log phase (14 h), while glycerol grown cells a recorded maximum activity at the late log phase (24 h). Furthermore, glycerol grown cells exhibited less COBE reductase activity (activity: 22 U/g protein, 27 mmol/lh and yield 94%) compared to cells grown in glucose (activity: 160 U/g protein, rate 163 mmol/lh and yield: 96%) and sucrose (activity: 1 U/g, 160 mmo/lh and yield: 95%) (Fig. 1b). This is in contrast to *C. parapsilosis* DSM 70125 grown in glycerol which exhibited 1.5 times higher reductase activity than when grown in glucose.[16] Furthermore, the glycerol grown cells exhibited maximum enantiomeric excess (>99%) at stationary phase as opposed to the exponential phase (ee 89%), whereas in the case of glucose and sucrose the enantiomeric excess (>99%) did not vary with growth (Fig. 1c). These results suggest that *C. parapsilosis* ATCC 7330 has multiple COBE reductase enzymes, which are differentially expressed with different carbon sources. In contrast, the *Pichia capsulate* grown in glucose exhibited various (43–93% ee) enantioselectivities in the asymmetric reduction of COBE with the harvest time.[25]

2.2.2. Effect of inhibitors on the COBE reductase in *C. parapsilosis* ATCC 7330

The enzymes responsible for the reduction of COBE in yeast are aldehyde reductases,[16] alcohol dehydrogenases[28] and carbonyl reductases.[31] Alcohol dehydrogenase and aldehyde reductase activities were assayed using the cell free extract of *C. parapsilosis* ATCC 7330 grown on glucose, sucrose and glycerol in order to investigate the enzyme responsible for the reduction of COBE. The substrates used for the assay were *p*-NO_2-benzaldehdye and acetaldehyde. Both alcohol dehydrogenase and aldehyde reductase activities were detected in the cell free extract of *C. parapsilosis* ATCC 7330 grown on glucose, sucrose and glycerol (Fig. 2). Cell free extracts from glucose and sucrose grown cells exhibited higher alcohol dehydrogenase activity compared to the cell free extract from glycerol grown cells, whereas aldehyde reductase activity remained high irrespective of the carbon source used. Higher aldehyde reductase activity irrespective of the carbon source, is due to the non-specific reduction of *p*-NO_2-benzaldehdye, which is a substrate used for the aldehyde reductase activity, by other aldo-keto reductase enzymes present in the yeast *C. parapsilosis* ATCC 7330.[30] The aldehyde reductase activity was seven times higher than alcohol dehydrogenase in the glycerol grown cells and this may be due to repression of the alcohol dehydrogenase I by a non-fermentable carbon source (glycerol) in the yeast and induction of the alcohol dehydrogenase by a fermentable carbon source (glucose).[32] The above study indicated the presence of aldehyde reductase enzymes and alcohol dehydrogenase enzymes in *C. parapsilosis* ATCC 7330 but the question of (*S*)- and (*R*)-specific COBE reductases still remained unanswered.

In order to differentiate between the (*S*)-specific reductase in the glucose and sucrose grown cells and the (*R*)-specific reductase from the glycerol grown cells, the effect of *o*-phenanthroline (an alcohol dehydrogenase inhibitor)[32] and quercetin (an aldehyde/carbonyl reductase inhibitor)[27] was studied on the asymmetric reduction of COBE by *C. parapsilosis* ATCC 7330. The asymmetric

Table 1
Effect of carbon sources during the growth of *C. parapsilosis* ATCC 7330 on the asymmetric reduction of COBE

Carbon source	Rate of asymmetric reduction (mmol/lh)	Enantiomeric excess (%)	Absolute configuration
Glucose	45 ± 3	>99	(*S*)
Sucrose	43 ± 2	>99	(*S*)
Maltose	38 ± 3	55	(*S*)
Starch	13 ± 2	65	(*S*)
Mannitol	34 ± 3	98	(*S*)
Glycerol	27 ± 4	>99	(*R*)
Without a carbon source	6 ± 1	>99	(*S*)

Culture conditions: *C. parapsilosis* ATCC 7330 was grown under the optimum conditions (inoculum age 12 h and 4% v/v of inoculum whose cell density is 1.7 g/l) and the biomass was harvested at 22 h. 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.

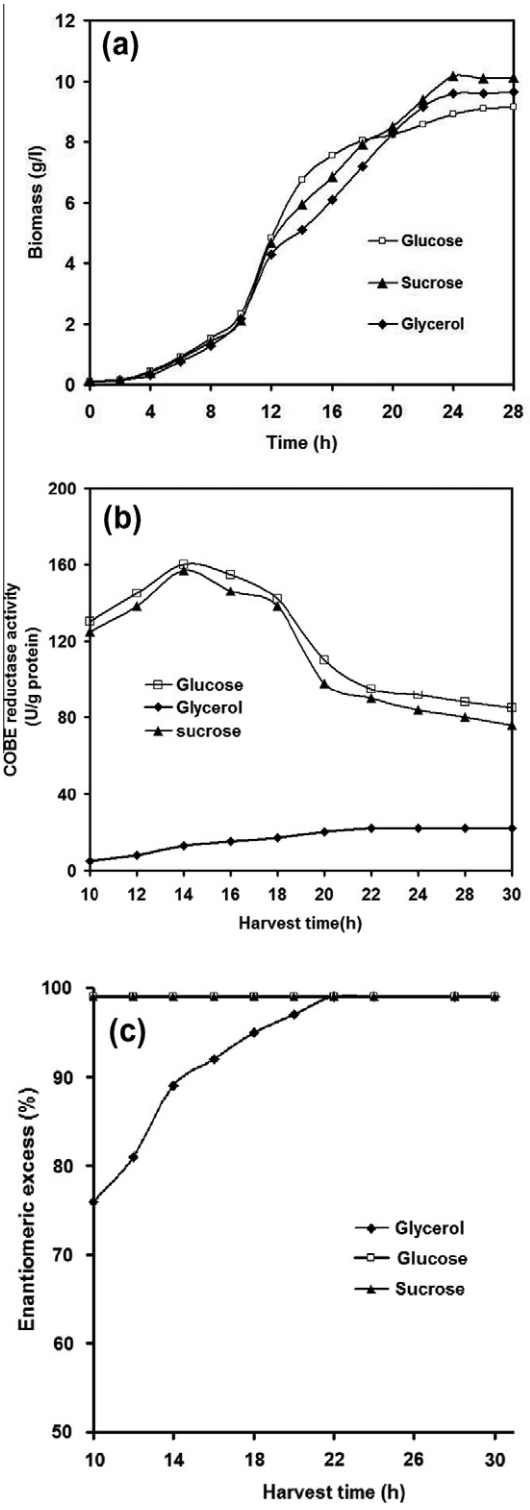

Figure 1. Effect of the harvest time on the asymmetric reduction COBE by *C. parapsilosis* ATCC 7330. Culture conditions: *C. parapsilosis* ATCC 7330 was grown under optimum conditions (inoculum age 12 h and 4% v/v of inoculum whose cell density is 1.7 g/l) and biomass was harvested at different times and their corresponding effect on the growth, COBE reductase activity and enantiomeric excess were determined. COBE reductase activity: The assay mixture contained Tris–HCl buffer (20 mM, pH 7.8), NADH (0.2 mM), COBE (8 mM) and 20 µl of the crude extract in a total volume of 1 ml. The consumption of reduced NADH was followed spectrophotometrically (V-530 UV/vis spectrophotometer) at 334 nm and 25 °C using a molar absorption coefficient of 6180 M^{-1} cm^{-1}.

reduction of COBE by cell free extracts from glucose and sucrose grown cells was completely inhibited by *o*-phenanthroline; in contrast quercetin did not inhibit this reaction (Table 2). The cell free extract from the glycerol grown cells harvested at 24 h was completely inhibited by quercetin but not inhibited by 1,10-phenanthroline (Table 2). The (*S*)-specific reductase from the glycerol grown cells expressed during the exponential growth phase (Fig. 1c) was also inhibited by 1,10-phenanthroline. The results from the inhibitor study indicated that the (*S*)-specific COBE reductase is an alcohol dehydrogenase I while the (*R*)-specific COBE reductase is an aldehyde reductase in *C. parapsilosis* ATCC 7330. In contrast to these results, the (*R*)-specific COBE reductase from *C. parapsilosis* DSM 70125[29] and *C. parapsilosis* IFO 1396[28,11] was inhibited by 1,10-phenathroline. Extending this to the present work, the (*S*)-specific COBE reductase from *C. parapsilosis* ATCC 7330 is an NADH alcohol dehydrogenase since it prefers NADH and is inhibited by 1,10-phenanthroline, which is an alcohol dehydrogenase inhibitor, whereas the (*R*)-specific COBE is an aldehyde reductase since it is inhibited by quercetin[27,32] (Fig. 3). The decrease in activity of the (*S*)-specific reductase in *C. parapsilosis*

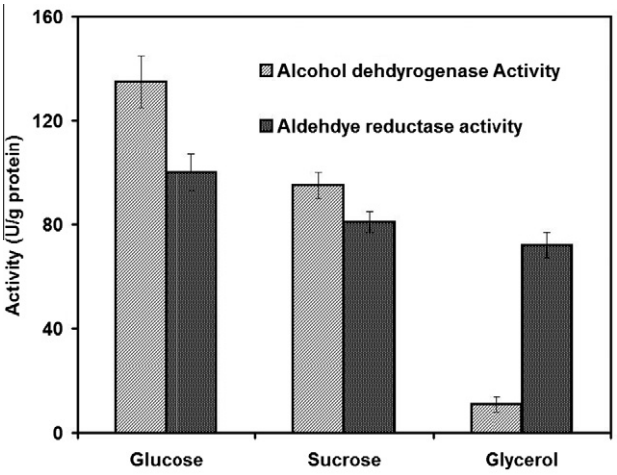

Figure 2. Effect of various carbon source during the growth *C. parapsilosis* ATCC 7330 on the alcohol dehydrogenase and aldehyde reductase activity. Culture conditions: *C. parapsilosis* ATCC 7330 was grown under optimum conditions (inoculum age 12 h and 4% v/v of inoculum whose cell density is 1.7 g/l) and harvested time for a glucose-grown cell is 14 h and for a glycerol grown cell is 24 h. Enzyme assay: The assay mixture contained Tris–HCl buffer (20 mM, pH 7.8), cofactor (0.2 mM), substrate (8 mM) and 20 µl of the crude extract in a total volume of 1 ml. The consumption of reduced NADH was followed spectrophotometrically (V-530 UV/vis spectrophotometer) at 334 nm and 25 °C using molar absorption coefficient of 6180 M^{-1} cm^{-1}. Acetaldehyde and *p*-nitro benzaldehyde were used as a substrate for the alcohol dehydrogenase assay and the aldehyde reductase assay, respectively.

Table 2
Effect of various inhibitors on the COBE reductase from *C. parapsilosis* ATCC 7330

Inhibitors	COBE reductase activity U/g protein Carbon source		
	Glucose	Sucrose	Glycerol
None	160 ± 2	157 ± 3	22 ± 3
EDTA	160 ± 1	157 ± 2	22 ± 1
1,10-Phenanthroline	0	0	21 ± 1
Quercetin	155 ± 1	153 ± 3	0

Culture conditions: *C. parapsilosis* ATCC 7330 was grown under the optimum conditions (inoculum age 12 h and 4% v/v of inoculum whose cell density is 1.7 g/l) and harvested time for glucose-grown cell was 14 h and for glycerol grown cell was 22 h. COBE reductase activity: The assay mixture contained Tris–HCl buffer (20 mM, pH 7.8), NADH (0.2 mM), COBE (8 mM) and 20 µl of the crude extract in a total volume of 1 ml. The consumption of reduced NADH was followed spectrophotometrically (V-530 UV/vis spectrophotometer) at 334 nm and 25 °C using a molar absorption coefficient of 6180 M^{-1} cm^{-1}. Inhibitors and metal ions were added in the cell free extract and incubated for 30 min before the assay.

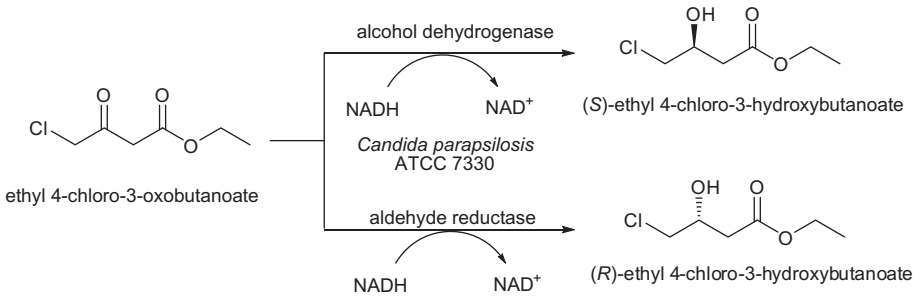

Figure 3. *Candida parapsilosis* ATCC 7330 mediated asymmetric reduction of ethyl-4-chloro-3-oxobutanoate.

ATCC 7330 grown in glycerol may be due to the decrease in functional alcohol dehydrogenase I mRNA in yeast when grown on a non-fermentable carbon source.[31] The (*R*)-specific COBE reductase activity was induced only when *C. parapsilosis* ATCC 7330 was grown in glycerol and was inhibited by the aldehyde reductase inhibitor, thus implying that the (*R*)-specific COBE reductase is the aldehyde reductase (EC.1.1.1.21) involved in the metabolism of glycerol in the yeast.[30]

When compared to the reported whole cell yeast and fungal biocatalytic systems for the asymmetric reduction of COBE,[4,7–14] *Candida parapsilosis* ATCC 7330 as reported herein showed improved enantiomeric excess and rate of asymmetric reduction. Moreover, both enantiomers of ethyl-4-chloro-3-hydroxybutanoate in the homochiral form can be synthesized via asymmetric reduction using *Candida parapsilosis* ATCC 7330 by changing the carbon source in the growth medium. In addition, this method does not require the pretreatment of cells and costly cofactors, such as NADH or NADPH. This method, which uses the same biocatalyst to synthesise both enantiomers of CHBE in the homochiral form with high enantiomeric purity (ee >99%), a high rate of asymmetric reduction [(*S*)-CHBE: 163 mmol/lh[26] and (*R*)-CHBE: 27 mmol/lh] and a high product concentration [(*S*)-CHBE: 230 g/l[26] and (*R*)-CHBE: 4.8 g/l] by simply changing the carbon source in the growth medium, is suitable for further scale-up to an industrial level. This method also provides a common platform for process development, which is simpler than generating recombinant strains and using them for scale-up.

3. Conclusion

C. parapsilosis ATCC 7330 when grown in a medium containing glycerol reduced ethyl-4-chloro-3-oxobutanoate to (*R*)-ethyl-4-chloro-3-hydroxybutanote (ee >99%) whereas glucose and sucrose grown cells yielded (*S*)-ethyl-4-chloro-3-hydroxybutanote (>99%). In *C. parapsilosis* ATCC 7330, the (*S*)-specific COBE is an NADH alcohol dehydrogenase, whereas the (*R*)-specific COBE is an NADH aldehyde reductase which is induced by glycerol. The main advantage of this method is that both enantiomers of CHBE can be prepared directly in high enantiomeric purity by using the same biocatalyst by simply changing the carbon source in the growth medium. This method also provides a common platform for process development which is much simpler than the recombinant techniques and does not require as much care as processes which involve the recombinant strain.

4. Materials and methods

4.1. 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).

4.2. Microorganism

C. parapsilosis ATCC 7330 was obtained from ATCC (Manassas, VA 20108, USA) 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.

4.3. Cultivation of microorganism

C. parapsilosis ATCC 7330 was precultured for 12 h at 25 °C with orbital shaking at 200 rpm in a 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 dry weight/l was transferred to a 250 ml Erlenmeyer flask that contained 48 ml of growth medium (5 g/l peptic digest of animal tissue, 3 g/l malt extract, 3 g/l yeast extract and 10 g/l carbon source). The culture was grown on a rotatory shaker at 25 °C and 200 rpm for 22 h. The cultivated cells were harvested by centrifugation (10,000 rpm, 10 min) at 4 °C and washed three times with distilled water.

4.4. Asymmetric reduction of COBE

Cells harvested by centrifugation were washed and resuspended (19 g cell dry weight/l) in a 10 mM potassium phosphate buffer, pH 6.8. Cell suspension (25 ml) was taken in a 150 ml Erlenmeyer flask capped with a cotton plug and pre-incubated in a water-bath shaker at 200 rpm and 25 °C for 15 min after adding glucose (50 g/l). 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 [for (*R*)-CHBE, the reaction time was 1 h] after the addition of COBE after which the reaction mixture was extracted with ethyl acetate (2 × 100 ml). The organic layer was dried over anhydrous sodium sulfate and the pure product was obtained by silica gel chromatography using hexane/ethyl acetate (9:1) as a mobile phase. In order to determine the rate of formation of CHBE, a 0.5 ml sample was taken every 5 min and centrifuged (10,000 rpm, 10 min) at 4 °C to remove the cells. The supernatant was extracted with ethyl acetate (1 ml) of which 1 μl was injected into the GC to determine the concentrations of COBE and CHBE.

4.5. Preparation of cell free extract

The cell free extract was prepared after lysis of the cell suspension (40 g dry cell/l) in a 20 Mm phosphate buffer pH 6.8 by ultrasonication for 15 min (pulse of 1 s) at 4 °C. Cell debris was removed by centrifugation (10,000 rpm for 30 min at 4 °C). The supernatant (crude extract) was used to determine the enzyme activity.

4.6. COBE reductase activity

The assay mixture contained 20 mM of Tris–HCl buffer pH 7.8, NADH (0.2 mM), COBE (8 mM) and 20 μl of the crude extract in a total volume of 1 ml. The consumption of reduced NADH was followed spectrophotometrically (V-530 UV/vis spectrophotometer) at 334 nm and 25 °C using a molar absorption coefficient of 6180 M^{-1} cm^{-1}. One Unit (U) of COBE reductase is defined as the amount of enzyme that catalyses the reduction of 1 μmol NADH min^{-1} under the conditions specified. The substrate used for alcohol dehydrogenase was acetaldehyde, while for the aldehyde reductase, *p*-nitro benzaldehyde was used.[27]

4.7. Analytical methods

Conversion of the substrate (COBE) into product (CHBE) was determined by GC using a 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 a Jasco PU-1580 HPLC equipped with a PDA detector. The chiral column used was Chiralcel OB-H (Daicel, 4.6 × 250 mm) while the mobile phase used was hexane/isopropanol (95:5) at a flow rate of 0.5 ml/min at 25 °C monitored at 220 nm. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on JEOL GSX400 spectrometers operating at 400 MHz and 100 MHz respectively. Chemical shifts are expressed in ppm values (δ) using TMS as the internal standard. Infrared spectra were recorded on a Shimadzu IR 470 Instrument. Optical rotations were determined on an Autopol® 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 a 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). The specific growth rate was calculated by an exponential growth model.

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