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ORIGINAL PAPER

Burkholderia cenocepacia: a new biocatalyst for efficient bioreduction of ezetimibe intermediate

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Abstract Ezetimibe is a selective acyl-coenzyme A: cholesterol acyltransferase (ACAT) inhibitor used in hypercholesterolemia. Synthesis of ezetimibe requires enantiopure 3-[5-(4-fluorophenyl)-5(*S*)-hydroxypentanoyl]-4(*S*)-4-phenyl-1,3-oxazolidin-2-one (FOP alcohol) as a crucial intermediate which is produced by reduction of the corresponding prochiral ketone (FOP dione). A new biocatalyst from acclimatized soil was screened for bioreduction of the above prochiral ketone. The microorganism was identified by 16S mRNA sequencing, as *Burkholderia cenocepacia*. Various physicochemical conditions were optimized to increase cellmass and enzyme activity. The overall increase in cellmass concentration and enzyme activity was 2.06 and 1.85-fold, respectively. Various reaction conditions, for example pH, temperature, agitation, and cellmass concentration, were optimized for maximum product yield (chiral alcohol) with excellent enantioselectivity. Best reduction was achieved in phosphate buffer (50 mM, pH 8.0) at 40°C (200 rpm) and the yield of enantiopure alcohol from the corresponding prochiral ketone was 54%. This biocatalyst was also used for the reduction of various other prochiral ketones.

Keywords Ezetmibe intermediate · *Burkholderia cenocepacia* · Bioreduction · Medium optimization · Enzyme activity

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Introduction

Chiral alcohols are used as important building blocks in the synthesis of pharmaceuticals with high enantiomeric purity [1]. Application of microbial dehydrogenases for reduction of ketones to the corresponding chiral alcohols has gained popularity over chemical routes [2]. Higher *chemo*, *enantio*, and *regio* selectivity of these enzymes have provided opportunities for development of novel chemical synthesis. Therefore, current research in enantioselective bioreduction is gaining momentum toward the development of less expensive, more efficient, and scalable strategies for synthesis of chiral intermediates. Several bioreductions have been reported for synthesis of chiral drugs and drug intermediates [3].

Ezetimibe (SCH 58235, 1-(4-fluorophenyl)-3-(*R*)-[3-(4-fluorophenyl)-3-(*S*)-hydroxypropyl]-4-(*S*)-(4-hydroxyphenyl)-2-azetidinone), a selective inhibitor of acyl-coenzyme A: cholesterol acyltransferase (ACAT), reduces plasma LDL cholesterol level and increases plasma HDL level [4–6]. Synthesis of ezetimibe involves reduction of 1-(4-fluorophenyl)-5-(2-oxo-4-phenyl-oxazolidin-3-yl)-pentane-1,5-dione (FOP dione) to crucial homochiral intermediate 3-[5-(4-fluorophenyl)-5-(*S*)-hydroxypentanoyl]-4(*S*)-4-phenyl-1,3-oxazolidin-2-one (FOP alcohol), which is further processed to synthesize ezetimibe (Fig. 1) [7, 8]. Enantioselective reduction of FOP dione to (*S*)-FOP alcohol has been achieved mainly by use of chemical catalysts, usually with chiral boron catalysts [9, 10]. One of the main drawbacks of the chemical synthetic strategy is the formation of the diol by-product which is difficult to separate because of its instability. Moreover, these boron catalysts are less enantioselective (<99%) and are costly, non-degradable, and hence pose a threat to the environment. Oxidoreductases may be better alternatives, because of their high selectivity, their

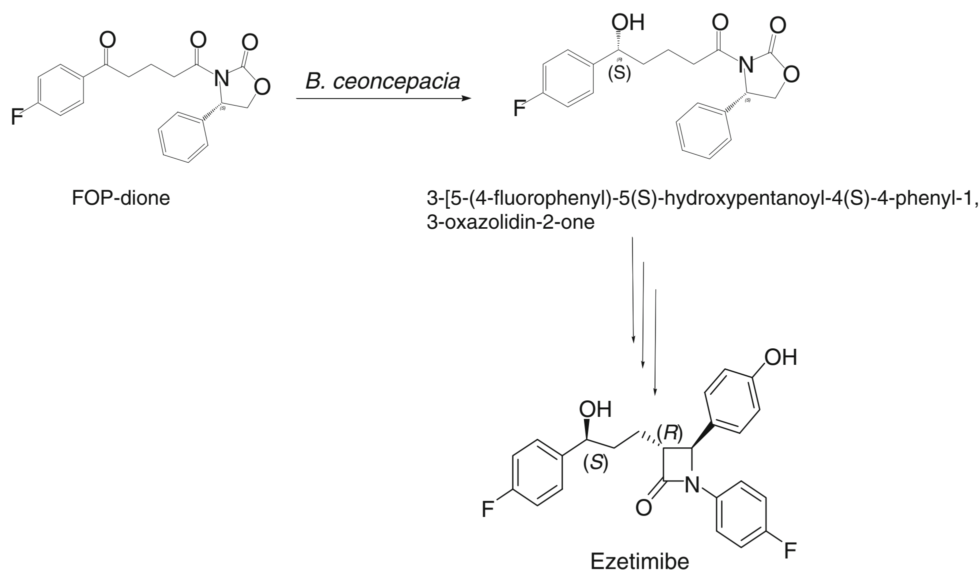

Fig. 1 Schematic diagram of ezetimibe synthesis

stability toward molecular oxygen, the absence of side reaction, and the mild operation conditions required. Only one microbial catalyst has been reported for this reduction—use of *Schizosaccharomyces octosporus* ATCC 2479, for which product yield is 33% [11]. We have isolated and screened various microbial cultures for reduction of FOP dione to (*S*)-FOP alcohol with high enantiomeric excess and found *Burkholderia cenocepacia* to be the best catalyst. Further the enzyme activity of the catalyst was improved by optimizing various environmental and nutritional conditions. This paper reports the bioreduction of FOP dione to (*S*)-FOP alcohol, an intermediate for ezetimibe synthesis using *B. cenocepacia* as biocatalyst. Various other ketones, for example acetophenone and 4′-halo-substituted acetophenones, were also reduced using *B. cenocepacia*.

Materials and methods

Chemicals

FOP dione was a kind gift from Ind-Swift Pharmaceutical (Chandigarh, India). Growth media components were procured from Hi-Media (Mumbai, India). Solvents used for HPLC were purchased from Mallinckrodt Baker (Phillipsburg, USA). Inorganic and buffer salts were obtained from Qualigens (Mumbai, India). All other chemicals used were of analytical grade and obtained from standard companies. Racemic FOP alcohol was synthesized by reduction of FOP dione by BH_3:THF in dichloromethane solvent. Authentic (*S*)-FOP alcohol (*ee* 67%) was prepared by chemical reduction of FOP dione, as reported in the literature [9].

Microorganisms and growth medium

Using the soil acclimatization technique, morphologically different cultures were isolated from soil and grown in minimum salt media (MSM) containing the substrate (FOP dione). Cultures which showed good growth in MSM media were selected and screened for the bioreduction of the ketone. Each culture was grown in nutrient broth containing (g l^{-1}): peptone, 5; yeast extract, 1.5; beef extract, 1.5; and NaCl, 5, at 30°C for 24 h. Cells were harvested by centrifugation, washed with distilled water and cell suspension of 200 mg ml^{-1} was prepared in phosphate buffer (pH 7.0) and used for bioreduction of FOP dione. Aliquots of 1 ml were withdrawn at 2-h intervals and extracted with ethyl acetate. The organic layer was separated, dried by rotary evaporation, and analyzed by HPLC. Various environmental (time of growth, temperature, medium pH, agitation rate, etc.) and nutritional (carbon source, nitrogen source, metal ions, inducers, etc.) conditions were optimized to increase the cellmass concentration and enzyme activity of *B. cenocepacia*.

Analytical procedures

Quantitative bioreduction of ketone was estimated by high-performance liquid chromatography (HPLC) using a C_{18} column (4.0 mm × 250 mm, 5 μm, Waters, The Netherlands). For FOP dione and FOP alcohol the mobile phase was 55:45 acetonitrile–sodium biphosphate buffer, pH 4.0, at a flow rate of 1 ml min^{-1}; the eluent was monitored at 215 nm. The product alcohol and substrate ketone were eluted at 8.1 and 13.6 min, respectively. For acetophenones and 4′-haloacetophenones the mobile phase was 50:50

acetonitrile–water and absorbance was monitored at 205 nm.

The purity of the product alcohol was determined by use of a Chiralcel OD-H column (0.46 mm × 250 mm, 5 µm, Diacel Chemical industries, New Jersey, USA). For FOP alcohol the mobile phase was 75:25 hexane containing 0.1% trifluoroacetic acid–ethyl alcohol at a flow rate of 0.5 ml min^{-1}. Absorbance was monitored at 215 nm. The (*S*) and (*R*)-alcohols were eluted at 24.27 and 27.34 min, respectively. A 90:10 mixture of hexane and isopropyl alcohol was used as mobile phase to check the purity of phenyl ethanols.

Enzyme assay

Carbonyl reductase activity was measured by reduction of 4′-fluoroacetophenone using whole cells of *B. cenocepacia*. The reduced product (alcohol) was measured by HPLC on a C_{18} column. The reaction mixture (5 ml) contained 1 g wet cell mass, 4′-fluoroacetophenone (2 mM) as substrate in phosphate buffer (50 mM, pH 8). The reaction mixture was incubated at 35°C for 4 h (200 rpm) and then centrifuged to remove the cellmass. The aqueous layer was extracted with ethyl acetate, dried by rotary evaporation, and analyzed by HPLC. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 µmol 4′-fluoroacetophenone per minute under standard assay conditions.

Results and discussion

From the acclimatized soil, 89 morphologically different cultures were isolated. Among these, strain no. 20 was found to be most suitable for bioreduction because it gave best conversion (46%) with excellent enantioselectivity (>99%). Strain no. 20 was identified and characterized as *Burkholderia cenocepacia* by 16S mRNA sequencing in Microbial Type Culture Collection and Gene Bank, at the Institute of Microbial Technology, Chandigarh, India, and was given the accession no. MTCC 5427.

Effect of environmental and nutritional conditions on growth and enzyme production

The effect of physicochemical conditions on growth and enzyme production was studied in nutrient broth medium. Inoculum of age 12 h (5% v/v) and growth time 10 h was optimum for enzyme production. It was found that the *B. cenocepacia* was able to grow and produced sufficient enzyme through a broad range of pH (5–10). However, maximum cellmass and enzyme activity was obtained when the initial pH of the medium was set at 8 [cellmass 5.72 ± 0.102 g l^{-1}, specific activity 3.59 ± 0.017 (µmol/min g) × 5 × 10^{-3}]. Beyond this pH, both cellmass and enzyme activity decreased. The effect of temperature on growth and enzyme activity was examined by growing the microorganism at different temperatures ranging from 20 to 40°C. Maximum cellmass (5.62 ± 0.102 g l^{-1}) and enzyme activity [3.20 ± 0.024 (µmol/min g) × 5 × 10^{-3}] were obtained at 35°C. Both above and below this temperature, cellmass and enzyme activity decreased. To study the effect of mixing on growth of, and enzyme production by, *B. cenocepacia*, the culture was incubated (35°C) at different shaking speeds (150, 175, 200, 225, 250 rpm). It was observed that both growth and enzyme activity increased as the shaking speed was increased to 200 rpm; thereafter it decreased. The maximum cellmass (8.17 ± 0.283 g l^{-1}) and enzyme activity [4.58 ± 0.008 (µmol/min g) × 5 × 10^{-3}] were observed at 200 rpm.

Effect of carbon source

Both inorganic (sodium succinate, sodium citrate, sodium potassium tartrate, etc.) and organic (glucose, fructose, fructose, mannitol, sorbitol, glycerol, lactose, etc.) carbon sources were tried (10 g l^{-1}) to increase the cellmass and enzyme activity of *B. cenocepacia*. Sucrose, fructose, and mannitol increased cellmass production whereas other carbon sources had no effect on growth. However, most of the carbon sources, except sodium citrate and sodium succinate, improved enzyme activity (Table 1). Maximum

Table 1 Effect of carbon source on growth of, and enzyme production by, *B. cenocepacia*

Carbon source	Cellmass (g l^{-1})	Specific activity (µmol/min g) × 5 × 10^{-3}
Sucrose	9.4 ± 1.55	4.61 ± 0.086
Glucose	5.9 ± 0.07	4.09 ± 0.059
Fructose	8.01 ± 0.14	5.07 ± 0.028
Mannitol	7.75 ± 0.78	4.34 ± 0.04
Sorbitol	5.81 ± 0.14	4.32 ± 0.026
Glycerol	5.75 ± 0.35	3.59 ± 0.072
Lactose	5.50 ± 0.14	3.92 ± 0.06
Citrate	6.32 ± 0.71	2.19 ± 0.079
Succinate	6.43 ± 0.32	3.22 ± 0.092
Tartrate	6.33 ± 0.71	4.63 ± 0.095
Control	6.42 ± 0.57	3.97 ± 0.012

Carbon sources were added at a final concentration of 10 g l^{-1} in nutrient broth medium (pH 8) and cells were grown at 35°C (200 rpm)

Cells were grown for 10 h, harvested by centrifugation, and suspended in phosphate buffer (pH 8) at a final concentration of 200 mg ml^{-1}

B. cenocepacia was used for bioreduction of 4′-fluoroacetophenone and the extent of bioreduction was determined by RP-HPLC

enzyme activity [5.07 ± 0.028 (μmol/min g) × 5 × 10^{-3}] with good cellmass (8.01 ± 0.14 g l^{-1}) was observed with fructose. Fructose concentration was optimized and 2% (w/v) fructose was found to be optimum for maximum enzyme activity [5.73 ± 0.029 (μmol/min g) × 5 × 10^{-3}] (data not shown).

Effect of nitrogen source

The effect of different organic and inorganic nitrogen sources (beef extract, meat extract, yeast extract, peptone, soyapeptone, tryptone, ammonium sulfate, ammonium chloride, ammonium nitrate and urea) was examined for the cellmass and carbonyl reductase production at a final concentration of 5 g l^{-1}. No cell growth was observed in production media containing inorganic nitrogen sources except ammonium sulfate, for which cell growth was observed, but less than in medium without nitrogen source (control). All organic nitrogen sources supported cellmass production; the maximum was observed with tryptone (8.70 ± 0.57 g l^{-1}); enzyme activity did not improve over control, however (Table 2). Hence no extra nitrogen source was added to the medium.

Effect of metal ions

Effect of various metal ions ($CaCl_2$, $MgSO_4$, $FeCl_3$, K_2HPO_4, NiCl, $ZnSO_4$, and $CuSO_4$) on the growth and enzyme production was studied by adding the metal ions in the medium separately (final concentration 1 mM) (Table 3). Cu^{2+}, Zn^{2+} and K^+ did not support cellmass production and had an inhibitory effect on enzyme activity. Ca^{2+} and Mg^{2+} had a very mild effect whereas Ni^+ was detrimental for both the cellmass production and enzyme activity. Fe^{3+} was favourable for growth (11.56 ± 0.864 g l^{-1}) and enzyme activity [5.82 ± 0.028 (μmol/min g) × 5 × 10^{-3}] and selected as a medium component. Further, optimization of Fe^{3+} concentration revealed that 1.2 mM was optimum for enzyme production [5.92 ± 0.058 (μmol/min g) × 5 × 10^{-3}] by *B. cenocepacia* (data not shown). To further enhance enzyme activity, various inducers (4′-fluoroacetophenone, acetophenone, 4′-chloro-acetophenone, FOP dione, acetone, etc.) were also tried in the medium (1 mM). No significant change in enzyme activity was observed with inducers, indicating non-inducible nature of the enzyme (data not shown).

Table 2 Effect of nitrogen source on growth of, and enzyme production by, *B. cenocepacia*

Nitrogen source	Cellmass (g l^{-1})	Specific activity (μmol/min g) × 5 × 10^{-3}
Ammonium sulfate	6.85 ± 0.21	2.19 ± 0.026
Beef extract	8.41 ± 0.42	3.57 ± 0.028
Meat extract	8.55 ± 0.64	3.08 ± 0.025
Peptone	8.82 ± 0.42	3.34 ± 0.014
Soya peptone	7.74 ± 0.28	3.4 ± 0.022
Tryptone	9.20 ± 0.57	3.63 ± 0.023
Yeast extract	8.44 ± 0.12	3.14 ± 0.018
Urea	nd	nd
Ammonium chloride	nd	nd
Ammonium nitrate	nd	nd
Control	7.54 ± 0.56	5.01 ± 0.036

nd not determined

Nitrogen sources were added at a final concentration of 5 g l^{-1} in nutrient broth media (pH 8) and cells were grown at 35°C (200 rpm)

Cells were grown for 10 h, harvested by centrifugation, and suspended in phosphate buffer (pH 8) at a final concentration of 200 mg ml^{-1}

B. cenocepacia was used for bioreduction of 4′-fluoroacetophenone and the extent of bioreduction was determined by RP-HPLC

Reduction of FOP dione

Effect of pH

In order to discover the optimum pH for bioreduction of FOP dione, cells of *B. cenocepacia* were suspended (200 mg ml^{-1}) in acetate, phosphate, and tris buffer of different pH ranging from 5.0 to 10.0. It was observed that good conversion and *ee* was obtained in the pH range 7.0–9.0, beyond that pH range % conversion decreased (Fig. 2). Because there is no ionizable group in FOP dione, it was probably unaffected by the change in pH. The pH range

Table 3 Effect of metal ions on the growth of, and enzyme production by, *B. cenocepacia*

Metal ions	Cellmass (g l^{-1})	Specific activity (μmol/min g) × 5 × 10^{-3}
Ca^{2+}	9.15 ± 0.354	4.88 ± 0.021
Mg^{2+}	9.70 ± 0.283	4.96 ± 0.049
Fe^{3+}	11.56 ± 0.864	5.82 ± 0.028
K^+	8.75 ± 0.495	4.83 ± 0.057
Ni^+	nd	nd
Zn^{2+}	8.30 ± 0.424	2.71 ± 0.071
Cu^{2+}	8.25 ± 0.212	3.50 ± 0.094
Control	8.64 ± 0.376	5.14 ± 0.074

nd not determined

Metal ions were added at a final concentration of 1 mM in nutrient broth media (pH 8) and cells were grown at 35°C (200 rpm)

Cells were grown for 10 h, harvested by centrifugation, and suspended in phosphate buffer (pH 8) at a final concentration of 200 mg ml^{-1}

B. cenocepacia was used for bioreduction of 4′-fluoroacetophenone and the extent of bioreduction was determined by RP-HPLC

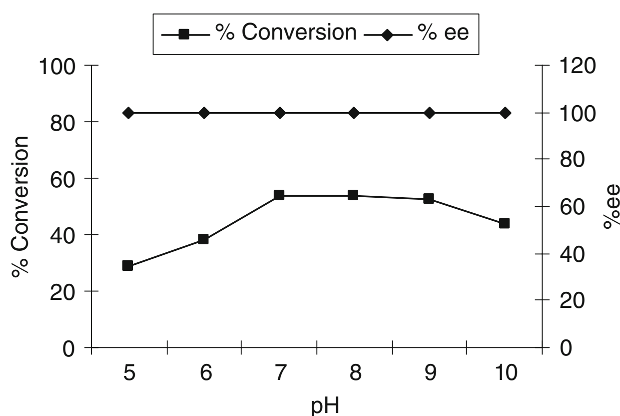

Fig. 2 Effect of pH on the reduction of FOP dione by *B. cenocepacia*. Cellmass concentration 200 mg ml^{-1}, temperature 35°C, agitation 200 rpm

7.0–9.0 may be more favourable for *B. cenocepacia* cells, hence good reduction was observed in this pH range only. Because growth of and enzyme production by *B. cenocepacia* were maximum at pH 8.0, this pH was selected for both growth and enzyme production and bioreduction experiments.

Effect of temperature

The effect of temperature on the bioreduction was examined by setting up reaction at a temperatures ranging from 25 to 50°C. Percentage bioreduction increased with increasing temperature up to 40°C, thereafter, it decreased (Fig. 3). The increase may be because of increased solubility of the substrate at higher temperature and greater availability of the substrate (FOP dione) to the microbial cells. This increased the rate of enzymatic reaction. It was also found that a temperature of 35–37°C was most favourable for the growth and enzyme production by *B. cenocepacia*. Above 40°C, enzyme inactivation resulted in less bioreduction.

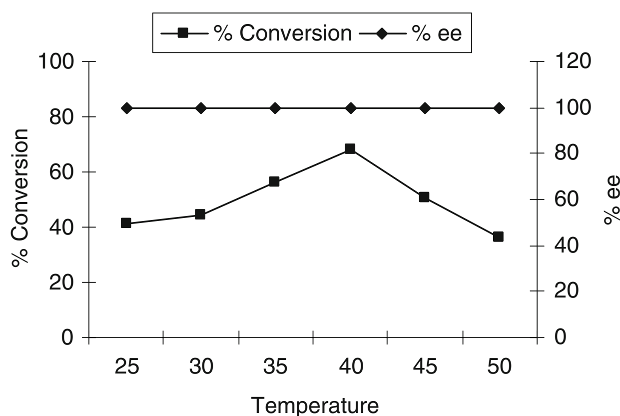

Fig. 3 Effect of temperature on the reduction of FOP dione by *B. cenocepacia*. Cellmass concentration 200 mg ml^{-1}, phosphate buffer pH 8.0, agitation 200 rpm

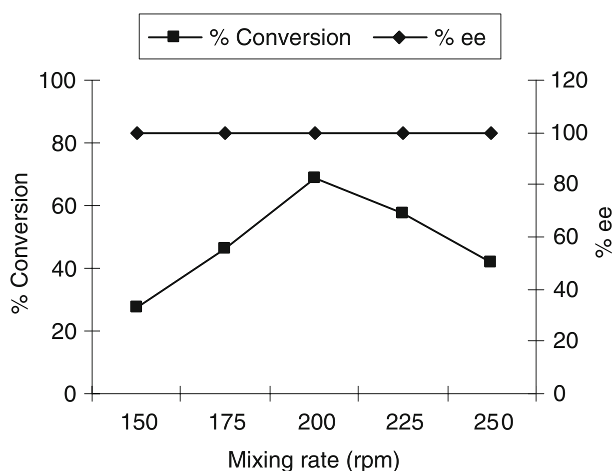

Fig. 4 Effect of mixing on the reduction of FOP dione by *B. cenocepacia*. Cellmass concentration 200 mg ml^{-1}, phosphate buffer pH 8.0, temperature 40°C

Effect of mixing

Mass-transfer plays a significant role in biotransformation reaction. An adequate rate of agitation ensures proper mass transfer in the biocatalytic reaction. The optimum rate of agitation for bioreduction of FOP dione by *B. cenocepacia* was determined by setting up reactions at different agitation rates (150, 175, 200, 225, 250 rpm). Product concentration increased with increasing mixing up to 200 rpm, thereafter; it decreased (Fig. 4). The enantioselectivity of the bioreduction reaction remained unchanged (>99%) in all the experiments.

Effect of cellmass concentration

Reactions with different cell concentrations (150, 200, 250, 300 mg ml^{-1}) were set up to find out its optimum value for reduction of FOP dione at pH 8.0 and 40°C (200 rpm). Percentage reduction increased with increasing cellmass and maximum conversion was achieved with a cell concentration of 250 mg ml^{-1} (54% product yield with *ee* >99%) (Fig. 5). Further increase in cellmass concentration did not increase percentage reduction. Experiments to improve the product yield with higher substrate concentration are in progress in our laboratory.

Reduction of other prochiral ketones

Reduction of various aryl and heteroaryl ketones to the corresponding chiral alcohol was tried with *B. cenocepacia* as biocatalyst. No reduction was achieved with heteroaryl ketones and naphthyl ketones. Acetophenone and 4′ substituted chloro, fluoro, bromo, and aminoacetophenones were found to be suitable substrates (Table 4). Highest reduction was observed with chloro-substituted acetophenones,

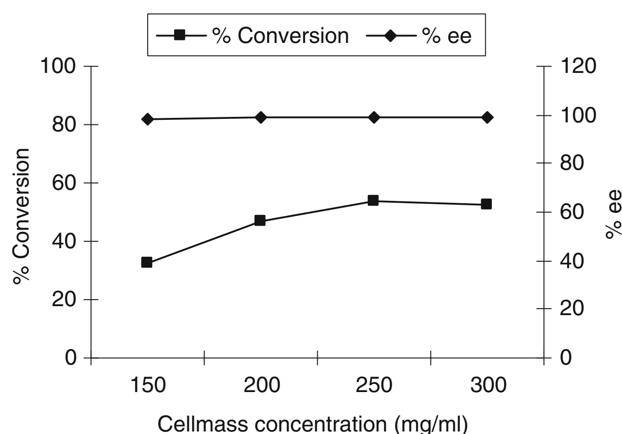

Fig. 5 Effect of cellmass concentration on the reduction of FOP dione by *B. cenocepacia*. Phosphate buffer pH 8.0, temperature 40°C, agitation 200 rpm

Table 4 Reduction of various ketones using *B. cenocepacia* as biocatalyst

Ketone	Bioreduction (%)	*ee* (%)
Acetophenone	68	62 (*S*)
4′-Fluoroacetophenone	92	78 (*S*)
4′-Chloroacetophenone	98	48 (*S*)
4′-Bromoacetophenone	76	62 (*S*)
4′-Aminoacetophenone	23	30 (*S*)
1′-Acetonaphthone	No reduction	nd
4′-Acetylpyridine	No reduction	nd
4′-Acetylthiophene	No reduction	nd

nd not determined

Cells were grown for 10 h at 35°C (200 rpm), harvested by centrifugation, and suspended in phosphate buffer (pH 8) at a final concentration of 200 mg ml^{-1}

Various substrates (ketones) were added (2 mM final concentration) to the cell suspension and incubated at 35°C (200 rpm)

Extent of bioreduction and enantiomeric excess of the product formed were determined by RP and chiral HPLC, respectively

followed by fluoro and bromoacetophenone. Very little bioreduction was observed with amino-substituted acetophenone. This may be because of an effect of the electronegativity of 4′-substituents. Electron-releasing amino substituents decreased the reduction, whereas electron-withdrawing substituents (F, Cl, and Br) enhanced the bioreduction.

Conclusion

The crucial homochiral ezetimibe intermediate (*S*)-FOP alcohol is synthesized by chiral reduction of FOP dione with different chemical catalysts. These chemical catalysts can be replaced by microbial catalysts which are environment friendly and more selective. *B. cenocepacia* was found to be a potential biocatalyst for reduction of FOP dione. Bioreduction was maximum at pH 8.0 and 40°C (200 rpm). Bioreduction of FOP dione yielded 54% chirally pure alcohol with >99% enantioselectivity. A patent (application no. 1909/del/2008) has been filed for bioreduction of FOP dione by *B. cenocepacia*. Cellmass and enzyme production by *B. cenocepacia* were improved by optimizing environmental and nutritional conditions. Overall enzyme activity and cellmass production were enhanced 1.85 and 2.06-fold, respectively, compared with unoptimized media. Various aryl and heteroaryl ketones were reduced using *B. cenocepacia* as biocatalyst, and aryl ketones were found to be suitable substrates for this enzyme.

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