Biotechnol Lett (2008) 30:1219–1225
DOI 10.1007/s10529-008-9668-7

ORIGINAL RESEARCH PAPER

Enantioconvergent bioconversion of *p*-chlorostyrene oxide to (*R*)-*p*-chlorophenyl-1,2-ethandiol by the bacterial epoxide hydrolase of *Caulobacter crescentus*

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Received: 7 January 2008 / Revised: 25 January 2008 / Accepted: 28 January 2008 / Published online: 13 February 2008
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Abstract The enantioselective hydrolysis of eight racemic styrene oxide derivatives has been investigated by using the recombinant cell containing epoxide hydrolase (EH) of *Caulobacter crescentus*. Some styrene oxide derivatives were hydrolyzed via enantioconvergent manner so that enantiopure diol products could be prepared with a 100% theoretical yield. The recombinant cell containing *C. crescentus* EH exhibited an ability to hydrolyze racemic *p*-chlorostyrene oxide the most enantioconvergently, thus affording the formation of the corresponding (*R*)-diol with enantiomeric excess (*ee*) as high as 95% and a 72% yield in preparative-scale (16.8 g/l) bioconversion.

Keywords *Caulobacter crescentus* · Enantioconvergent hydrolysis · Epoxide hydrolase · *p*-Chlorostyrene oxide

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Introduction

Because of the safety and regulation pressure on the chirality of biologically active compounds by US FDA, the commercial need of chiral intermediates for the synthesis of chiral pharmaceuticals is tremendously increasing (Breuer et al. 2004). Enantiopure epoxides and their corresponding vicinal diols (*vic*-diols) are valuable synthetic building blocks for the synthesis of chiral pharmaceuticals (Kasai et al. 1998; Monterde et al. 2004).

Many biocatalytic conversions have been developed to prepare chiral epoxides and diols (Breuer et al. 2004; Lee and Shuler 2007). One of the promising biocatalytic approaches is the kinetic resolution of racemic epoxides via enantioselective hydrolysis reaction catalyzed by epoxide hydrolase (EH, EC 3.3.2.3) (Orru et al. 1998). Even though kinetic resolution has been proved to be commercially important, it has an intrinsic limitation that the theoretical yield cannot exceed 50%. To overcome the limitation of kinetic resolution, many efforts have been paid to develop so-called enantioconvergent processes leading to a 100% yield and 100% enantiopurity from a racemic mixture as the substrate (Lutje Spelberg et al. 1998; Steinreiber et al. 2001; Strauss et al. 1999). We can set up an enantioconvergent process by the combined use of two different EHs possessing complementary enantio- and regioselectivities (Manoj et al. 2001; Genzel et al. 2002). In some cases, it is possible to enantioconvergently

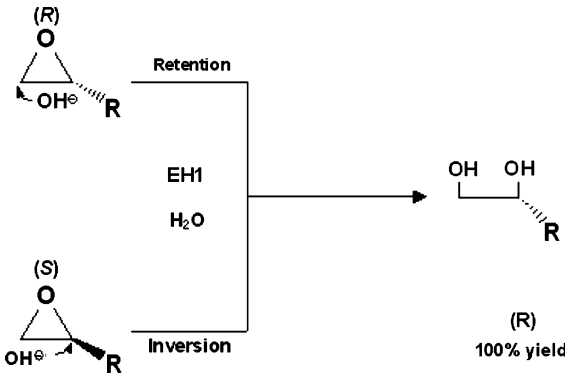

Fig. 1 Reaction schemes of the enantioconvergent hydrolysis of racemic epoxides using the recombinant cell containing *C. crescentus* EH. Given yields are theoretical values

hydrolyze racemic epoxides by using single EH biocatalyst based on 'stereochemical flexibility' of some EHs (Fig. 1) (Moussou et al. 1998; Steinreiber et al. 2001).

The plant EH from potato was employed to catalyze the enantioconvergent bioconversion of racemic styrene oxide derivatives (Monterde et al. 2004). This is only one report on the enantioconvergent hydrolysis of styrene oxide derivatives using single biocatalyst, and no enantioconvergent process for the preparation of enantiopure diol using heavy metal-based chemocatalysts has been reported so far, to the best of our knowledge. Recently, we have reported enantioconvergent bioconversion of racemic styrene oxide to prepare (*R*)-phenyl-1,2-ethanediol by employing a bacterial EH from *Caulobacter crescentus* and a marine fish EH from *Mugil cephalus* (Kim et al. 2008). The bacterial EH of *C. crescentus* exhibited interesting activity that some racemic epoxides were converted into the corresponding diols in enantiomerically enriched way. In this paper, the hydrolysis of eight styrene oxide derivatives was investigated to achieve an enantioconvergent conversion by using single cell containing *C. crescentus* EH (Fig. 2). The enantioconvergent conversion of *p*-chlorostyrene oxide was optimized and preparative-scale preparation was also carried out.

Materials and methods

Media and culture conditions

The recombinant *E. coil* BL21(DE3) harboring the EH gene of *C. crescentus* was grown in 1 LB broth

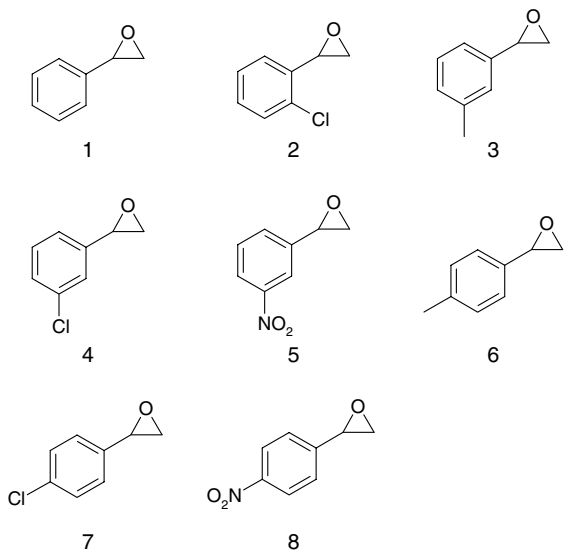

Fig. 2 Various epoxides used as substrates for enantioconvergent hydrolysis by the recombinant cell containing *C. crescentus* EH (1, styrene oxide; 2, *o*-chlorostyrene oxide; 3, *m*-methylstyrene oxide; 4, *m*-chlorostyrene oxide; 5, *m*-nitrostyrene oxide; 6, *p*-methylstyrene oxide; 7, *p*-chlorostyrene oxide; 8, *p*-nitrostyrene oxide)

containing 50 μg kanamycin/ml at 37°C (Hwang et al. 2005). When the OD_{600} reached 0.6, 1 mM IPTG was added to the culture broth. After induction, the cells were incubated at 20°C overnight with shaking at 250 rpm. The cells were harvested by centrifugation (1,000*g*, 10 min, 4°C), washed with 50 ml 20 mM phosphate buffer (pH 8.0). The harvested cells were freeze-dried for the bioconversion reactions.

General procedure for the synthesis of racemic epoxides

To a stirred solution of various styrene derivatives (1 equiv.) in dry methylene dichloride (13 ml/mmol), a solution of *m*-CPBA (1.2 equiv.) in dry methylene dichloride (13 ml/mmol) was added at room temperature. The reaction was monitored by TLC. After the reaction was finished, the reaction mixture was washed with $NaHSO_3$ 40% (w/v) and then saturated with aqueous $NaHCO_3$ solution. The organic layer was dried over Na_2SO_4, filtered, and then concentrated in vacuum.

Substrate screening

The freeze-dried recombinant whole cells (0.27 U/ml) were preincubated in 20 mM phosphate buffer (pH 8.0) at 25°C. Enantioselective hydrolysis reaction was initiated by adding 10 mM styrene oxide derivatives (see Fig. 2). The reaction was periodically monitored by withdrawing the samples from the reaction mixture. The samples were extracted with the same volume of ethyl acetate containing decane as an internal standard for GC analysis. The styrene oxide derivatives and their corresponding diols (see Fig. 3) in the ethyl acetate phase were analyzed by chiral GC and HPLC (Table 1).

Determination of regioselectivity coefficient

The freeze-dried recombinant whole cells were preincubated in 20 mM phosphate buffer (pH 8.0) at 25°C. The reaction was initiated by adding 10 mM *p*-chlorostyrene oxide. The enantiopurities of the remaining epoxide and the formed diol were measured by chiral GC and HPLC, respectively. Regioselectivity coefficients were calculated by the computer program made by Faber and Kroutil (2002).

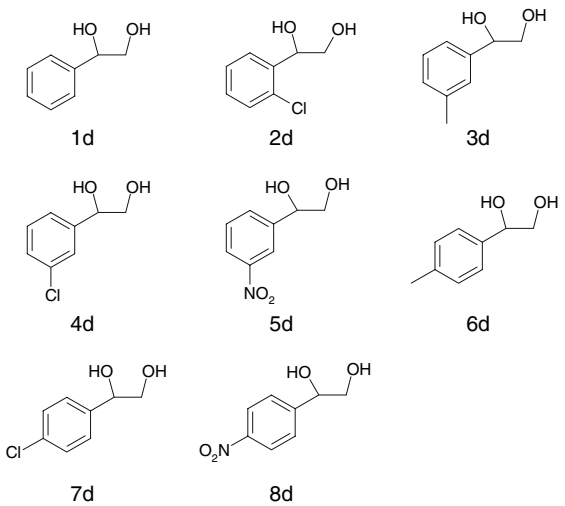

Fig. 3 Diols as products for enantioconvergent hydrolysis by the recombinant cell containing *C. crescentus* EH (1, phenyl-1, 2-ethanediol; 2, *o*-chlorophenyl-1,2-ethanediol; 3, *m*-methylphenyl-1,2-ethanediol; 4, *m*-chlorophenyl-1,2-ethanediol; 5, *m*-nitrophenyl-1,2-ethanediol; 6, *p*-methylphenyl-1,2-ethanediol; 7, *p*-chlorophenyl-1,2-ethanediol; 8, *p*-nitrophenyl-1,2-ethanediol)

Table 1 Analysis conditions for determination of enantiopurity and yield of enantiopure epoxides

Entry	Instrument	Column	Analysis conditions
1	GC	*β*-dex 250	Oven temperature: 120°C
1**d**	LC	Chiracel OD	Hexane: 2-propanol = 9:1 (v/v)
2	GC	*β*-dex 120	Oven temperature: 110°C
3	GC	*β*-dex 120	Oven temperature: 130°C
4	GC	*β*-dex 120	Oven temperature: 105°C
5	GC	*β*-dex 120	Oven temperature: 140°C
6	GC	*β*-dex 120	Oven temperature: 110°C
7	GC	*β*-dex 250	Oven temperature: 140°C
7**d**	LC	Chiracel OD	Hexane: 2-propanol = 9:1 (v/v)
8	GC	*β*-dex 120	Oven temperature: 130°C
8**d**	LC	Chiracel OD	Hexane: 2-propanol = 9:1 (v/v)

Co-solvent effect on enantioconvergent biohydrolysis

The freeze-dried recombinant whole cells (2 U/ml) were preincubated in 20 mM phosphate buffer (pH 8.0) containing 10% (v/v) various co-solvents at 25°C. The reaction was initiated by adding *p*-chlorostyrene oxide to 10 mM final concentration. The sample was periodically withdrawn to analyze the enantiopurity of the remaining epoxide and the formed diol.

Preparative-scale enantioconvergent hydrolysis of 4-chlorostyrene oxide

In a 300 ml reactor, 1g rac-**7** was dissolved in 60 ml phosphate buffer (pH 8.0). The freeze-dried recombinant whole cells (400 U) were added, and then the mixture was vigorously stirred at 25°C. After the reaction, the diol product was extracted with ethyl acetate three times from the mixture, dried over $MgSO_4$, concentrated in vacuum, and purified with flash chromatography (hexane:ethyl acetate = 2:1, by vol.).

Results and discussion

Substrate screening for enantioconvergent hydrolysis

The enantioselective hydrolysis of racemic styrene oxide derivatives (substrates **1–8)** was investigated to evaluate the enantioselectivity of *C. crescentus* EH (Table 2). Among the eight styrene oxide derivatives, the cell containing *C. crescentus* EH exhibited enantioselectivity toward *rac*-**1, 2, 7** and **8** substrates. Among these substrates, the hydrolysis of *rac*-**7** occurred at a higher hydrolysis rate. After about 120 min, the hydrolysis of *rac*-**7** (*p*-chlorostyrene oxide: *p*CSO) reached a 99% conversion ratio (Fig. 4). The *C. crescentus* EH hydrolyzed (*S*)-*p*CSO in preference to the other enantiomer, which is confirmed by comparison to authentic samples. The enantiomeric ratio (E) value, determined on the basis of the calculation equation suggested by Faber and Kroutil (2002), was about 30. The resulting vicinal diol **7d** was shown to have more than 90% enantiopurity analyzed by the chiral HPLC. The formed diol **7d** appeared to be also (*R*)-configuration determined by polarimeter ($[\alpha]^{14}_{D} = -52.16$, c = 1.02, $CHCl_3$). These results represent that *C. crescentus* EH has enantioconvergent hydrolysis activity toward racemic *p*CSO.

Determination of regioselectivity coefficient

To obtain enantiopure diol with more than 90%*ee* via a hydrolysis of racemic *p*CSO, each enantiomer should be hydrolyzed in an enantioconvergent manner. As an epoxide substrate can be attacked at either carbon atom of an epoxide ring, we determined the regioselectivity for the (*R*)-and (*S*)-enantiomer of *p*CSO. When only (*S*)-enantiomer of *rac*-**7** was supplied, (*R*)-diol was formed as the main product with the inversion of stereochemistry (Table 3). Hence, we could expect that *C. crescentus* EH mainly attacked at the benzylic carbon instead of terminal carbon of the epoxide ring of (*S*)-enantiomer. The (*R*)-diol was also formed from (*R*)-enantiomer of *rac*-**7**. The (*R*)-enantiomer of *rac*-**7** was attacked at the less hindered carbon atom, resulting in the formation of (*R*)-**7d** with unchanged configuration. Hence, we determined the regioselectivity of *C. crescentus* EH toward *p*CSO as shown in Table 3. The retention-inversion ratio (RI ratio) and regioselectivity (α) were determined based on the program proposed by Faber and Kroutil (2002). More than 94% of (*S*)-**7** was attacked at the benzylic position, whereas 99% of (*R*)-**7** was attacked at the terminal carbon atom by *C. crescentus* EH.

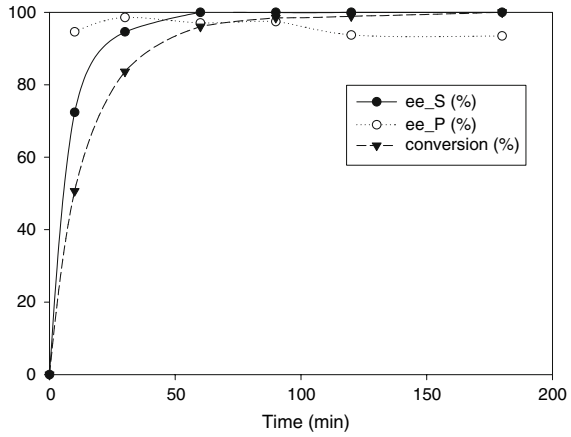

Fig. 4 Biohydrolysis of 10 mM *rac*-**7** using the recombinant cell containing *C. crescentus* EH (The figure legends, ee_S and ee_P, represent the enantiomeric excess of substrate and product, respectively)

Table 2 Reaction parameters of the biohydrolysis of **1–8** (10 mM, 25°C) using the *C. crescentus* EH

Substrate	Reaction time (min)	Conversion (%)	$ee_{epoxide}$ (%)	ee_{diol} (%)
rac-**1**	120	93	~99	90
rac-**2**	60	75	35	–
rac-**3**	120	40	7	–
rac-**4**	120	36	6	–
rac-**5**	NR	–	–	–
rac-**6**	NR	–	–	–
rac-**7**	120	~99	~99	96
rac-**8**	120	80	92	80

Table 3 Regioselectivity of *C. crescentus* EH toward *p*-chlorostyrene oxide

$RI_{(S)}$	$RI_{(R)}$	α_S (%)	α_R (%)
0.06	100.58	5.88	99.00

$RI_{(S)} = k_1/k_2$, $RI_{(R)} = k_4/k_3$, $RI_{(S)} = k_1/k_2$, $\alpha_S = k_1/(k_1 + k_2)$, $\alpha_R = k_4/(k_3 + k_4)$

In conclusion, the cell biocatalyst containing *C. crescentus* EH appeared to possess a good to excellent enantioconvergency. The enantioconvergent bioconversion of *rac*-**7** was further investigated to optimize the reaction condition for an enhanced enantiopurity and yield.

Effect of substrate concentration and co-solvent on enantioconvergent reaction

In order to set up an efficient process for the enantioconvergent biohydrolysis of *rac*-**7**, the effects of substrate concentrations on enantiopurity and yield were studied. As shown in Fig. 5, we could obtain more than 90% enantiopurity with up to 100 mM substrate although the reaction rate decreased and conversion ratio could not reach the 100% in the prolonged reaction time for the high substrate concentration. Reaction retardation at high substrate concentrations seemed to be due to product inhibition. To evaluate the product inhibition, hydrolysis reaction (5 mM initial substrate concentration) in the presence of various *rac*-**7d** concentrations was carried out. The hydrolysis of *p*-chlorostyrene oxide using cell containing *C. crescentus* EH was inhibited in the presence of above 40 mM *rac*-**7d** concentration (Fig. 6).

The effect of cosolvent addition on enantioconvergent biohydrolysis of *rac*-**7** was examined since the organic solvent might be required to increase the solubility of *rac*-**7**. Various water-miscible organic solvents were tested in hydrolysis of 40 mM *rac*-**7** at 25°C. The values of conversion ratios and **7d** enantiopurity were measured. Except DMSO and glycerol, all water-miscible organic solvents tested inhibited the biohydrolysis rates of *rac*-**7**, and decreased the enantiopurity of the formed **7d**. DMSO and glycerol did not affect on any positive/negative effect on hydrolysis of *rac*-**7** (data not shown).

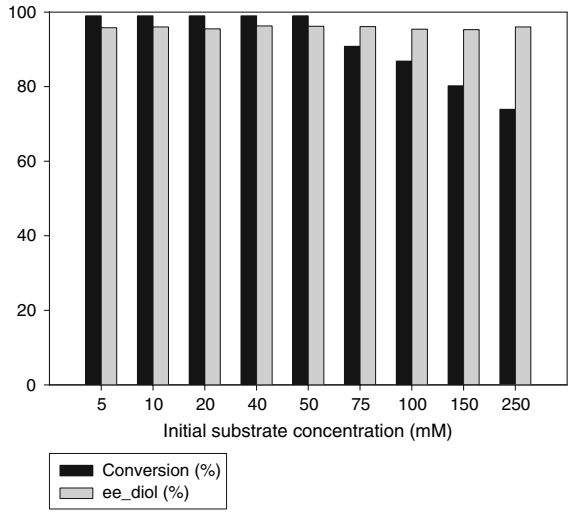

Fig. 5 Effect of initial substrate concentration. The cell activity/substrate ratio was kept constant (0.2 U/mM for *rac*-**7**). The figure legend, ee_diol, represents the enantiomeric excess of diol product

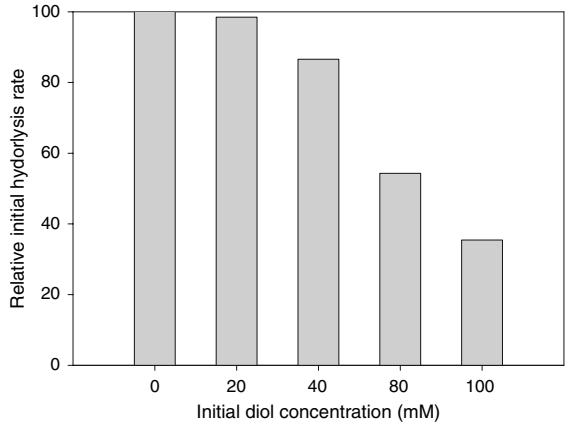

Fig. 6 Influence of the initial **7d** concentration on the biohydrolysis rate. The initial hydrolysis rate in the absence of the diol was 0.45 μmol/min mg of cell

Preparative scale experiments

At the most appropriate experimental conditions for the biohydrolysis of *rac*-**7**, a preparative-scale batch reaction was carried out. In a 300 ml reactor, 400 U of cell biocatalyst containing *C. crescentus* EH was preincubated with 60 ml phosphate buffer (pH 8.0) at 25°C, and then, 1 g *rac*-**7** was added, which corresponded to 16.8 g/l concentration. Due to low solubility of *rac*-**7** in water, the reaction was done

at the biphasic system where the substrate constitutes one phase by itself. The time course of the reaction was monitored by using chiral GC analysis. After 3 h, the reaction reached to the final conversion and then the aqueous solution containing the product **7d** was extracted with ethyl acetate in order to isolate the resulting diol **7d**. We could obtain 0.87g (*R*)-**7d** (overall yield = 78%) with an enantiopurity of 95%*ee* by using single cell containing EH of *C. crescentus*. To our best knowledge, it is the first report that the enantioconvergent biohydrolysis of racemic styrene oxide derivatives has been accomplished by using single bacterial EH. Recently, similar study on an enantioconvergent hydrolysis of racemic styrene oxide derivatives was carried out by using an isolated plant EH enzyme (Monterde et al. 2004). The bacterial EH has advantages over the plant EH in an enantioconvergent biohydrolysis since the bacterial EH can be efficiently expressed in *E. coli*. Hence, we could carry out the enantioconvergent hydrolysis reaction efficiently by using the whole-cell biocatalyst containing *C. crescentus* EH. The EH gene of *C. crescentus* was readily expressed in *E. coli* up to 75% of the total protein, corresponding to about 85% of the soluble protein estimated by densitometric analysis of SDS-PAGE gel (Hwang et al. 2006).

Conclusion

The enantioselective hydrolysis of eight racemic styrene oxide derivatives was investigated by using the recombinant cell containing *C. crescentus* EH. *C. crescentus* EH exhibited an enantioconvergent biohydrolysis activity on racemic *p*-chlorostyrene oxide, thus resulting in the formation of the corresponding (*R*)-diol with high enantiopurity up to 95%*ee* and a yield more than 50%. Each enantiomer of racemic *p*-chlorostyrene oxide was hydrolyzes with complementary enantioselectivity and regioselectivity. Enantiopure (*R*)-*p*-chlorophenyl-1,2-ethanediol with 72% preparative yield and enantiopurity as high as 95%*ee* was obtained via enantioconvergent biohydrolysis of racemic *p*-chlorostyrene oxide in preparative scale (16.8 g/l) by using single cell biocatalyst containing *C. crescentus* EH. Since enantiopure styrene oxide derivatives and their corresponding diols are valuable synthetic intermediates for pharmaceuticals, single recombinant cell biocatalyst-catalyzed enantioconvergent biotransformation can have industrial applications.

Acknowledgments This work was supported by the Marine and Extreme Genome Research Center Program, Ministry of Marine Affairs and Fisheries, Republic of Korea.

References

Breuer M, Ditrich K, Habicher T, Hauer B, Kesseler M, Sturmer R, Zelinski T (2004) Industrial methods for the production of optically active intermediates. Angew Chem Int Ed 43:788–824

Faber K, Kroutil W (2002) Stereoselectivity in biocatalytic enantioconvergent reactions and a computer program for its determination. Tetrahedron Asymmetry 13:377–382

Genzel Y, Archelas A, Broxterman QB, Schulze B, Furstoss R (2002) Microbiological transformation 50: selection of epoxide hydrolases for enzymatic resolution of 2-, 3- or 4-pyridyloxirane. J Mol Catal B: Enzym 16:217–222

Hwang SH, Hyun HJ, Lee BJ, Park YS, Choi CY, Han J, Joo H (2005) Screening from the genome databases: novel epoxide hydrolase from *Caulobacter crescentus*. J Microbiol Biotechnol 16:32–36

Hwang S, Hyun H, Lee B, Park Y, Lee EY, Choi C (2006) Purification and characterization of recombinant *Caulobacter crescentus* epoxide hydrolase. Biotechnol Bioprocess Eng 11:282–287

Kasai N, Suzuki T, Furukawa Y (1998) Chiral C3 epoxides and halohydrins: Their preparation and synthetic application. J Mol Catal B: Enzym 4:237–252

Kim HS, Lee OK, Hwang S, Kim BJ, Lee EY (2008) Biosynthesis of (*R*)-phenyl-1,2-ethanediol from racemic styrene oxide by using bacterial and marine fish epoxide hydrolases. Biotechnol Lett 30:127–133

Lee EY, Shuler ML (2007) Molecular engineering of epoxide hydrolase and its application to asymmetric and enantioconvergent hydrolysis. Biotechnol Bioeng 98:318–327

Lutje Spelberg JH, Rink R, Kellogg RM, Janssen DB (1998) Enantioselectivity of a recombinant epoxide hydrolase from *Agrobacterium radiobacter*. Tetrahedron Asymmetry 9:459–466

Manoj KM, Archelas A, Barati J, Furstos R (2001) Microbiological transformations 45. A green chemistry preparative scale synthesis of enantiopure building blocks of Eliprodil: elaboration of a high substrate concentration epoxide hydrolase-catalyzed hydrolytic kinetic resolution process. Tetrahedron 57:695–701

Monterde MI, Lombard M, Archelas A, Cronin A, Arand M, Furstoss R (2004) Enzymatic transformations. Part 58: Enantioconvergent biohydrolysis of styrene oxide derivatives catalysed by the *Solanum tuberosum* epoxide hydrolase. Tetrahedron Asymmetry 15:2801–2805

Moussou P, Archelas A, Baratti J, Furstoss R (1998) Microbiological transformations. Part 39: determination of the regioselectivity occurring during oxirane ring opening by epoxide hydrolases: a theoretical analysis and a new

method for its determination. Tetrahedron Asymmetry 9:1539–1547

Orru RVA, Archelas A, Furstoss R, Faber K (1998) Epoxide hydrolases and their synthetic applications. Adv Biochem Eng Biotechnol 63:145–167

Steinreiber A, Mayer SF, Saf R, Faber K (2001) Biocatalytic asymmetric and enantioconvergent hydrolysis of trisubstituted oxiranes. Tetrahedron Asymmetry 12:1519–1528

Strauss UT, Felfer U, Faber K (1999) Biocatalytic transformation of racemates into chiral building blocks in 100% chemical yield and 100% enantiomeric excess. Tetrahedron Asymmetry 10:107–117