Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 27 (2004) 155–159
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Biocatalytic resolution of nitro-substituted phenoxypropylene oxides with *Trichosporon loubierii* epoxide hydrolase and prediction of their enantiopurity variation with reaction time

Yi Xu, Jian-He Xu*, Jiang Pan, Liang Zhao, Si-Liang Zhang

Laboratory of Biocatalysis and Bioprocessing, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

Received 30 June 2003; received in revised form 11 November 2003; accepted 11 November 2003

Abstract

Biocatalytic resolution of 3-(2′-nitrophenoxy)propylene oxide (**1a**), 3-(3′-nitrophenoxy)propylene oxide (**1b**) and 3-(4′-nitrophenoxy)propylene oxide (**1c**) were exploited by using lyophilized cells of yeast *Trichosporon loubierii* ECU1040 with epoxide hydrolase (EH) activity, which preferentially hydrolyzes (*S*)-enantiomers of the epoxides (**1a–c**), yielding (*S*)-diols and (*R*)-epoxides. The activity increased as the nitro group in the phenyl ring was shifted from 4′-position (**1c**) to 2′-position (**1a**). When the substrate concentration of **1a** was increased from 10 to 80 mM, the *E*-value increased at first, until reaching a peak at 40 mM, and then decreased at higher concentrations (>40 mM). The optically active epoxide (*R*)-**1a** was prepared at gram-scale (97% ee, 41% yield). Furthermore, a simple method was developed to predict the enantiomeric excess of substrate (ee_s) at any time of the whole reaction course based on the ee_s value determined at a certain reaction time at a relatively lower substrate concentration. This will be helpful for terminating the reaction at a proper time to get both higher optical purity and higher yield of the remaining epoxides.

Keywords: Nitro-substituted phenoxypropylene oxide; *Trichosporon loubierii*; Epoxide hydrolase; Kinetic resolution; Theoretical prediction; Enantioselective hydrolysis

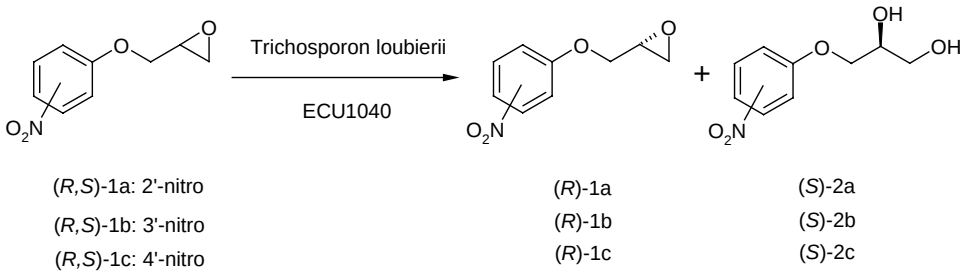
1. Introduction

Chiral epoxides and vicinal diols play an important role in the preparation of various bioactive products in pharmaceutical and agrochemical industries, due to their chemical versatility and high reactivity [1,2]. Both chemical and biological methods have been explored for the synthesis of enantiopure epoxides and diols. Heavy metal-based catalysts have been developed to synthesize the epoxide in enantiopure form via either direct epoxidation of an olefin or kinetic resolution of racemic epoxides [3,4]. Meanwhile, a biochemical approach using epoxide hydrolases (EHs) has also drawn much attention in recent years for its mild condition and environmental friendliness [5–10]. Moreover, EHs are cofactor independent enzymes. Depending on the substrate, these microbial enzymes may exhibit high activity and enantioselectivity. This will allow organic chemists to prepare enantiopure epoxides in a simple way from cheap racemic epoxides.

Aryl glycidyl ethers and their related compounds are potentially useful intermediates for the synthesis of chiral amino alcohols [9] and β-blockers [11]. Nevertheless, for this kind of compounds, only very few were investigated regarding their kinetic resolution by EHs [12,13]. A bacterial strain *Bacillus megaterium* ECU1001, which was previously isolated in our laboratory, could preferentially hydrolyze the (*R*)-enantiomer of phenyl glycidyl ether [14]. Recently, a yeast strain (*Trichosporon loubierii* ECU1040) with opposite enantioselectivity was also discovered, which could preferentially hydrolyze the (*S*)-enantiomers of phenyl glycidyl ether and its analogs, yielding (*R*)-epoxides and (*S*)-diols with good selectivity [15]. This paper focuses on the kinetic resolution of 3-(nitrophenoxy)propylene oxides (**1a–c**) using the whole cells of *T. loubierii* ECU1040 (Scheme 1). In addition, a simple equation has been established for successful prediction of the time-dependent changes in enantiomeric excess of the substrate (ee_s) during the reaction course.

* Corresponding author. Tel.: +86-21-6425-2498; fax: +86-21-6425-2250.
E-mail address: jianhexu@ecust.edu.cn (J.-H. Xu).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2003.11.006



Scheme 1. The enantioselective hydrolysis of racemic nitro-substituted phenoxypropylene oxides (**1a–c**) catalyzed by lyophilized cells of *T. loubierii* ECU1040.

2. Experimental

2.1. General

NMR spectra were recorded in $CDCl_3$ at 500 MHz. Chemical shifts (δ) are reported in ppm from tetramethylsilane (TMS) as an internal standard. All the chemicals were obtained commercially and of analytical grade.

2.2. Chemical synthesis of racemic epoxides (*1*)

To a solution of nitrophenol (0.2 mol, 27.8 g) in 1.0 mol (∼79 ml) of epichlorohydrin in a round-bottom flask, was added a solution of 50 mmol sodium hydroxide in 4 ml deionized water. The reaction mixture was magnetically stirred at 85 °C for 2 h, and then filtrated after addition of 50 ml chloroform. The organic layer was washed serially by 5% NaOH solution (40 ml, 2×) and water (40 ml, 2×). After the organic phase was dried over anhydrous Na_2SO_4, the solvent was removed under reduced pressure. The resulting crude product was recrystallized in hot ethanol. The epoxide was isolated as a light yellow solid. For **1a** (13.8 g, 35% yield), ^{1}H NMR (500 MHz, $CDCl_3$, TMS) (δ, ppm): 2.87 (dd, 1H, $J_1 = 4.8$ Hz, $J_2 = 2.7$ Hz), 2.93 (dd, 1H, $J_1 = J_2 = 4.5$ Hz), 3.39–3.41 (m, 1H), 4.15 (dd, 1H, $J_1 = 11.3$ Hz, $J_2 = 5.0$ Hz), 4.42 (dd, 1H, $J_1 = 11.2$ Hz, $J_2 = 2.8$ Hz), 7.06–7.14 (m, 2H), 7.52–7.54 (m, 1H), 7.84–7.86 (m, 1H) [16]. For **1b** (9.0 g, 23% yield), ^{1}H NMR (500 MHz, $CDCl_3$, TMS) (δ, ppm): 2.77 (dd, 1H, $J_1 = 4.8$ Hz, $J_2 = 2.7$ Hz), 2.93 (dd, 1H, $J_1 = J_2 = 4.5$ Hz), 3.35–3.38 (m, 1H), 3.97 (dd, 1H, $J_1 = 11.0$ Hz, $J_2 = 6.0$ Hz), 4.35 (dd, 1H, $J_1 = 11.0$ Hz, $J_2 = 2.6$ Hz), 7.24–7.28 (m, 1H), 7.40–7.46 (m, 1H), 7.73–7.74 (m, 1H), 7.82–7.84 (m, 1H). For **1c** (7.9 g, 20% yield), ^{1}H NMR (500 MHz, $CDCl_3$, TMS) (δ, ppm): 2.79 (dd, 1H, $J_1 = 4.8$ Hz, $J_2 = 2.6$ Hz), 2.95 (dd, 1H, $J_1 = J_2 = 4.5$ Hz), 3.38–3.41 (m, 1H), 4.01 (dd, 1H, $J_1 = 11.1$ Hz, $J_2 = 6.0$ Hz), 4.39 (dd, 1H, $J_1 = 11.1$ Hz, $J_2 = 2.8$ Hz), 6.99–7.02 (m, 2H), 8.20–8.23 (m, 2H).

The racemic diols were prepared by acid-catalyzed hydrolysis of the corresponding epoxides. For **2a**, ^{1}H NMR (500 MHz, $CDCl_3$, TMS) (δ, ppm): 3.80–3.89 (m, 2H), 4.12–4.16 (m, 1H), 4.21 (dd, 1H, $J_1 = 9.2$ Hz, $J_2 = 5.9$ Hz), 4.27 (dd, 1H, $J_1 = 9.2$ Hz, $J_2 = 4.0$ Hz), 7.07–7.12 (m, 2H), 7.55–7.58 (m, 1H), 7.90–7.92 (m, 1H).

For **2b**, ^{1}H NMR (500 MHz, $CDCl_3$, TMS) (δ, ppm): 3.72 (dd, 1H, $J_1 = 11.3$ Hz, $J_2 = 5.2$ Hz), 3.81 (dd, 1H, $J_1 = 11.3$ Hz, $J_2 = 4.5$ Hz), 4.05–4.10 (m, 3H), 7.18–7.20 (m, 1H), 7.36–7.40 (m, 1H), 7.69–7.70 (m, 1H), 7.78–7.80 (m, 1H).

For **2c**, ^{1}H NMR (500 MHz, $CDCl_3$, TMS) (δ, ppm): 3.75 (dd, 1H, $J_1 = 11.3$ Hz, $J_2 = 5.1$ Hz), 3.85 (dd, 1H, $J_1 = 11.4$ Hz, $J_2 = 3.5$ Hz), 4.10–4.16 (m, 3H), 6.95–6.97 (m, 2H), 8.17–8.18 (m, 2H).

2.3. Cultivation of yeast ECU1040 and preparation of lyophilized cells

The yeast ECU1040 was grown in a 5-l jar fermenter with 3.0 l of the following medium (per liter): glucose 2 g, glycerol 5 g, peptone 4 g, yeast extract 2.7 g, $MgSO_4{\cdot}7H_2O$ 0.45 g, NH_4Cl 0.67 g, NaCl 0.45 g, KH_2PO_4 0.95 g, K_2HPO_4 2.53 g, phenyl glycidyl ether 1.67 g in pH 5.2. In the late exponential growth phase (28–32 h), the cells were harvested by centrifugation (5000 × *g*), resuspended in phosphate buffer (50 mM, pH 7.0), centrifuged again and lyophilized. Typical yields of lyophilized cells ranged from 8 to 12 g l^{-1} culture.

2.4. General procedure for the enantioselective hydrolysis of *1a–c*

Lyophilized yeast cells (3 g) were rehydrated in sodium phosphate buffer (90 ml, 100 mM, pH 7.0) for 30 min on a shaker (160 rpm, 30 °C). Then 10 ml DMSO containing 500 mg of the substrate was added and the mixture was agitated at 30 °C. The reaction progress was monitored by TLC or HPLC. After the degree of hydrolysis was around 50%, the mixture was centrifuged. The pellet of cells were washed three times with ethyl acetate while the aqueous phase was saturated with NaCl and then extracted three times with ethyl acetate. The combined organic phase was dried over Na_2SO_4 and condensed under reduced pressure. Purification from the crude product by flash chromatography with ethyl acetate/hexane (3:2) afforded (*R*)-epoxides and (*S*)-diols.

2.5. Effect of substrate concentration on kinetic resolution of epoxide **1a**

Lyophilized cells (100 mg) were suspended in 1.8 ml of sodium phosphate buffer (100 mM, pH 7.0) and pre-incubated on a shaker (160 rpm, 30 °C) for 10 min. A 0.2 ml of DMSO containing different concentrations of epoxide **1a** was added, giving final substrate concentrations of 10, 20, 40, 60 and 80 mM. The reactions were stopped after 40 min of incubation (160 rpm, 30 °C). The conversion ratio and ee value of the substrate was monitored by HPLC.

2.6. Analytical methods

The substrate concentration was determined by HPLC using a reverse phase C_{18} column and methanol/water (60/40 v/v) as the mobile phase (0.8 ml min^{-1}). α-Naphthol was used as the internal standard. The enantiomeric excesses of the epoxide and diol were also determined by HPLC using a chiral column (Chiralcel OD or Chiralcel OJ, 250 mm × ∅ 4.6 mm, Daicel, Japan) and the sample was detected at 254 nm. Prior to Chiral HPLC analysis, the diols (**2a**–**c**) were derived into its acetate, as described below.

A 10 ml of CH_2Cl_2 solution containing 5 mmol of a diol and 6 mmol of pyridine, acetic anhydride (200 mmol) was added dropwise. Then the resulting solution was stirred for 4 h at room temperature. After filtration with micropore membrane, the resultant sample was subjected to Chiral HPLC analysis using Chiralcel OD column for **2a** and **2c** (the mobile phase is hexane/isopropanol, 90/10 (v/v), 1.0 ml min^{-1}) or Chiralcel OJ column for **2b** (the mobile phase is hexane/isopropanol, 80/20 (v/v), 1.0 ml min^{-1}).

The ee value of epoxide **1a** was determined directly by HPLC analysis using Chiralcel OD column. The mobile phase was hexane/isopropanol 90/10 (v/v) at a flow rate of 1.0 ml min^{-1}. The ee values of epoxide **1b** and **1c** were determined by HPLC analysis of the corresponding methoxy derivatives using Chiralcel OJ column eluted with hexane/isopropanol 80/20 (v/v), 0.8 ml min^{-1}). The methoxy derivative was made in two steps: Firstly, the epoxide (**1b** or **1c**) was reacted with NaOMe (15 equivalents) in methanol at 30 °C (ca. 5–6 h), then the resultant mixture was extracted by CH_2Cl_2 and acetylated using pyridine and acetic anhydride, as described above.

3. Results and discussion

3.1. Kinetic resolution of 3-(2′-nitrophenoxy) propylene oxide (**1a**) by ECU1040

The general reaction condition was firstly investigated with the enantioselective hydrolysis of racemic 3-(2′-nitrophenoxy) propylene oxide (**1a**) by lyophilized cells of ECU1040. The highest activity was shown at pH 7.0 when pH was varied in the range of 4–10. The lyophilized cells showed the highest activity at 30–35 °C and the activity was lost completely when the temperature was higher than 45 °C. Owing to the low solubility of the epoxide in the aqueous solution, various co-solvents including DMSO, methanol, acetonitrile, ethanol, isopropanol and tetrahydrofuran were tested. Karboune et al. [17] reported that the activity reached its maximum at 8% (v/v) of DMF during the enantioselective hydrolysis of *para*-nitrostyrene oxide by an epoxide hydrolase from *Aspergillus niger*. While the lyophilized cells of ECU1040 showed the best activity when the buffer contained 10% of DMSO. Fig. 1 shows typical curves of conversion and ee_s versus time based on the conditions mentioned above. It can be seen from Fig. 1 that the reaction rate became slower when the conversion of compound **1a** reached around 50%, at the same time the ee_s was close to 100%. This phenomenon implies a high enantioselectivity of ECU1040 cells toward (*S*)-enantiomer of compound **1a**.

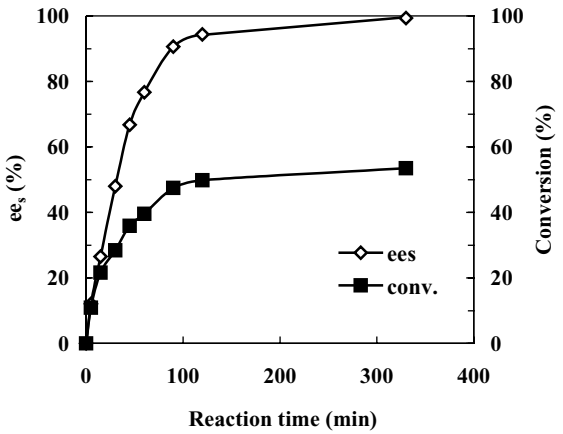


Fig. 1. Time course of biocatalytic production of (*R*)-**1a**. Reaction conditions: substrate concentration, 10 mM; lyophilized cells, 100 g l^{-1}; temperature, 30 °C. Symbols: (■) conversion; (◇) enantiomeric excess of substrate.

3.2. Effect of substrate concentration

Because substrate concentration is a sensitive parameter influencing the catalytic performance of fungal epoxide hydrolase, it was optimized, as shown in Fig. 2. A three fold selectivity-enhancement was observed when increasing substrate concentration from 10 to 40 mg ml^{-1}. However, an additional increase in substrate concentration caused a decrease in enantioselectivity. Hellström et al. [8] also found a 1.6-fold selectivity-enhancement upon doubling the substrate concentration from 17 to 33 mM using lyophilized cells of *Rhodococcus rubber* with an epoxide hydrolase activity. They explained it as interfacial enzyme activation. On the contrary, Genzel et al. [18] found the *E*-value decreased as the substrate concentration increased from 5 to 80 mM in the hydrolysis of racemic pyridyloxirane by an epoxide

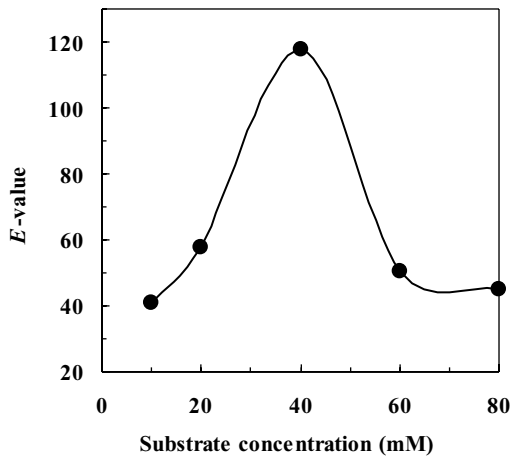


Fig. 2. Effect of substrate (**1a**) concentration on the enantioselectivity. Lyophilized cells, 50 g l^{-1}; reaction temperature, 30 °C.

hydrolase from *A. niger* and a substrate inhibition was found to be the reason. So it might be presumed that interfacial enzyme activation occurred with increasing of substrate concentration. While at high substrate concentrations, the inhibition of substrate will become prominent, leading to a decrease in the *E*-value.

3.3. Preparation of (R)-epoxides from their racemates (*1a–c*)

A preparative scale experiment was carried out with 500 mg of (*R,S*)-epoxide (**1a–c**) and 3.0 g of lyophilized cells suspended in 100 ml of 100 mM sodium phosphate buffer solution containing 10% of DMSO. The mixture was magnetically stirred at 30 °C for appropriate time. Both the residual epoxide and the produced diol were extracted by ethyl acetate, and then separated by flash chromatography. The results were summarized in Table 1.

From Table 1, it can be concluded that the epoxide hydrolase activity of lyophilized ECU1040 cells increased when the position of the nitro group at the benzene ring was shifted from 4′-position (**1c**) to 2′-position (**1a**). So a much longer time was needed to get a high optical purity for **1c** as compared to **1a** under current experimental conditions.

Table 1
The biocatalytic resolution of nitro-substituted phenoxypropylene oxides (**1a–c**) by lyophilized cells of *T. loubierii* ECU1040

Substrate	Reaction time (h)	(*R*)-epoxide		(*S*)-diol	
		yield (%)	ee (%)	yield (%)	ee (%)
± **1a**	7.5	41.0	97.2	41.8	74.0
± **1b**	24	28.2	67.2	29.4	90.4
± **1c**	48	33.1	58.7	34.2	85.7

Reaction conditions: 500 mg of (*R,S*)-epoxide (**1a–c**) and 3.0 g of lyophilized cells suspended in 100 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 10% DMSO. The mixture was magnetically stirred at 30 °C and stopped at different times for different substrates.

3.4. Prediction of the time-dependant changes in enantiomeric excess of substrate ($ee_s \sim t$)

According to Chen et al. [19] and Lu et al. [20], for a simple irreversible kinetic resolution, the *E*-value is shown to be:

$$E = \frac{\ln(A/A_0)}{\ln(B/B_0)} \tag{1}$$

This indicates that the discrimination between two competing enantiomers (*A* and *B*) by an enzyme is equal to a constant *E*. Eq. (1) can be re-written as:

$$\frac{\ln(A/A_0)}{\ln(B/B_0)} = \frac{-kt}{-kt/E} \tag{2}$$

Then Eq. (2) can be derived to:

$$A = A_0 \exp(-kt) \tag{3}$$

$$B = B_0 \exp\left(\frac{-kt}{E}\right) \tag{4}$$

At a low initial substrate concentration according to Lu's derivation and Michaelis–Menten equation (if the substrate concentration is low enough relative to K_m, the reaction is first order). Here A_0 and B_0 are initial concentrations of the fast- and slow-reacting enantiomers and *k* is the rate constant for the fast-reacting enantiomer. For the kinetic resolution of a racemate ($A_0 = B_0 = 0.5S_0$), it is known that:

$$ee_s = \frac{B - A}{B + A} \tag{5}$$

By substituting Eq. (3) and (4) into Eq. (5), we can write:

$$\ln\left(\frac{B}{A}\right) = k\left(1 - \frac{1}{E}\right)t = \ln\left(\frac{1 + ee_{s2}}{1 - ee_{s2}}\right) \tag{6}$$

Considering that both *k* and *E* are constant, we can get:

$$\frac{1}{t_1}\ln\left(\frac{1 + ee_{s1}}{1 - ee_{s1}}\right) = \frac{1}{t_2}\ln\left(\frac{1 + ee_{s2}}{1 - ee_{s2}}\right) \tag{7}$$

where ee_{s1} and ee_{s2} are ee_s values at t_1 and t_2, respectively. Eq. (7) can be written as:

$$ee_{s2} = \frac{[(1 + ee_{s1})/(1 - ee_{s1})]^{t_2/t_1} - 1}{[(1 + ee_{s1})/(1 - ee_{s1})]^{t_2/t_1} + 1} \tag{8}$$

It can be concluded from Eq. (8) that if we know ee_{s1} at t_1, then we can theoretically predict the ee_s value at another time (t_2) in the same reaction mixture in a low substrate concentration range. If the substrate concentration is relatively high, Eq. (3) and (4) will no longer valid. So Eq. (8) does not fit to high substrate concentration and in that case the result of prediction based on Eq. (8) may deflect the experiment data. From Fig. 3A, one can see that both of the theoretical curves (the curves were plotted according to Eq. (8) and ee_s values at 30 and 60 min, respectively) fit the experimental data quite well. This enables one to stop the reaction at a

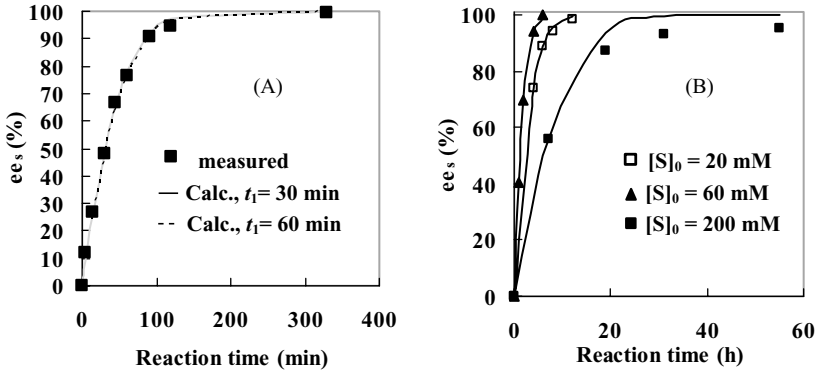


Fig. 3. Variation of ee_s in the resolution course of racemic **1a** by lyophilized cells of *T. loubierii* ECU1040 (100 g l^{-1}) at different substrate concentrations. Symbols: (A) (■) measured; (— ---) calculated with the ee_s at $t = 30$ min (10 mM) and 60 min (10 mM), respectively; (B) (■, □, ▲)measured; (—) calculated, respectively, with the ee_s at $t = 1$ h (20 mM), $t = 4$ h (60 mM) and $t = 7$ h (200 mM).

proper time (e.g. $ee_s \geq 98\%$) to get both high optical purity and high yield of the epoxide. This will also simplify the work of measurement.

The test was also performed at different substrate concentrations from 10 to 200 mM. Three typical curves were shown in Fig. 3B. It was found that the theoretical curves fit the data quite well at low substrate concentrations and a slight deflection was found at a substrate concentration of 60 mM. When the substrate concentration was further increased to 200 mM, the deflection of the theoretical curve from the experimental data was more obvious. While the deflection can be minimized if the time point used for prediction (t_1) is near the time point to be predicted (t_2), because the change of k value is minimized in a shorter time interval. In order to minimize errors, it is recommended to use data sets of ee_s within a range of 25–75% for the calculation.

The effort in deriving an equation for the ee_p (ee value of product) at a certain time was also tried, but failed unfortunately. The present method is not applicable to a reversible reaction and the validity of the equation (Eq. (8)) for other biocatalytic systems needs to be further proved.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant Nos. 20272013 & 203900506) and the Ministry of Science and Technology, P.R. China (No. 2003 CB 716008).

References

[1] A. Steinreiber, K. Faber, Curr. Opin. Biotechnol. 12 (2001) 552.
[2] A. Archelas, R. Furstoss, Curr. Opin. Chem. Biol. 5 (2001) 112.
[3] J.M. Ready, E.N. Jacobsen, J. Am. Chem. Soc. 123 (2001) 2687.
[4] K. Konishi, K. Oda, K. Nishida, T. Aida, S. Inoue, J. Am. Chem. Soc. 114 (1992) 1313.
[5] Y. Genzel, A. Archelas, Q.B. Broxterman, B. Schulze, R. Furstoss, J. Mol. Catal. B: Enzymatic 16 (2002) 217.
[6] I. Osprian, W. Kroutil, M. Mischitz, K. Faber, Tetrahedron: Asymmetry 8 (1997) 65.
[7] M. Cleij, A. Archelas, R. Furstoss, Tetrahedron: Asymmetry 9 (1998) 1839.
[8] H. Hellström, A. Steinreiber, S.F. Mayer, K. Faber, Biotechnol. Lett. 23 (2001) 169.
[9] A. Kamal, Y. Damayanthi, M.V. Rao, Tetrahedron: Asymmetry 3 (1992) 1357.
[10] H.M. Krieg, J.C. Breytenbach, K. Kiezer, J. Membr. Sci. 180 (2000) 69.
[11] H.Y. Aboul-Enein, I.W. Wainer (Ed.), The Impact of Stereochemistry on Drug Development and Use, Wiley, New York, 1997.
[12] J.H.L. Spelberg, R. Rink, R.M. Kellogg, D.B. Janssen, Tetrahedron: Asymmetry 9 (1998) 459.
[13] W.J. Choi, E.C. Huh, H.J. Park, E.Y. Lee, C.Y. Choi, Biotechnol. Tech. 12 (1998) 225.
[14] Y.F. Tang, J.H. Xu, Q. Ye, B. Schulze, J. Mol. Catal. B: Enzymatic 13 (2001) 61.
[15] J. Pan, J.H. Xu, Enzyme Microb. Technol. 33 (2003) 527.
[16] Z.Z. Liu, H.C. Chen, S.L. Gao, R.T. Li, Synth. Commun. 24 (1994) 833.
[17] S. Karboune, L. Amourache, H. Nellaiah, C. Morisseau, J. Baratti, Biotechnol. Lett. 23 (2001) 1633.
[18] Y. Genzel, A. Archelas, Q.B. Broxterman, B. Schulze, R. Furstoss, J. Org. Chem. 66 (2001) 538.
[19] C.S. Chen, Y. Fujimoto, G. Girdaukas, C.J. Sih, J. Am. Chem. Soc. 104 (1982) 7294.
[20] Y. Lu, X. Zhao, Z.N. Chen, Tetrahedron: Asymmetry 6 (1995) 1093.