Biocatalytic Resolution of *para*-Nitrostyrene Oxide by Resting Cells of Different *Aspergillus niger* Strains

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Biocatalytic resolution of racemic *para*-nitrostyrene oxide was accomplished by employing the epoxide hydrolases from the whole cells of several *Aspergillus niger* (*A. niger*) strains. In the cases investigated, excellent selectivity was achieved with such strains as *A. niger* 5450, *A. niger* 5320.

Keywords Epoxide hydrolase, kinetic resolution, chiral epoxide, chiral vicinal diol

Introduction

Enantiomerically pure epoxides and vicinal diols are important chiral building blocks in asymmetric synthesis and can be used as key intermediates in the synthesis of more complex bioactive compounds. In recent years many intensive efforts have been devoted to production of such useful chirons, most of which were achieved by chemo-catalytic methods.[1-5] There are also some biochemo-catalytic methods.[6] One of the most promising biocatalytic approaches is using the cofactor-independent enzyme, epoxide hydrolases (EHs).

Epoxide hydrolases are widely distributed in the nature. Most of those epoxide hydrolases that have been studied are of mammalian origin[7,8] with limited availability. This seriously hampered their applications on the preparative scales. Recently, EHs from microbes, such as bacteria, yeast and fungi[9-11] have been identified. Some of them show excellent enantio- and regio-selectivity when they enantiospecifically hydrolyze different types of epoxides. Because the organisms can be cultured on large scales, these EHs are readily available and have become one of the most useful tools for asymmetric synthesis.

In this work, 15 strains of *Aspergillus niger* (*A. niger*) collected from several soil samples were screened for EHs. Some of the them showed remarkable enantioselectivity, when using the *para*-nitrostyrene oxide as the substrates (Scheme 1).

Results and discussion

In 1993, Furstoss group reported that the fungal *A. niger* LCP 521 showed high enantioselectivity[11] in hydrolysis of some racemic epoxides. They also found that the fungus *A. niger* LCP 521 could enantioselectively hydrolyze a range of *p*-substituted styrene oxides,[12,13] leading to the (*S*)-epoxides and the (*R*)-diols of high enantiomeric excess.

In the present work, *para*-nitrostyrene oxide was selected as substrate for screening because it is very stable in neutral and weak basic conditions. Almost no chemical hydrolysis could be found after 24 h at pH 7—8. This property eliminated the interference of chemical hydrolysis and secured the reliability of the results.

The activity of EH in *A. niger* depends on the growing medium. Two kinds of culture media were used in this work. Medium **A**, NH_4NO_3 (1.5 g), KH_2PO_4

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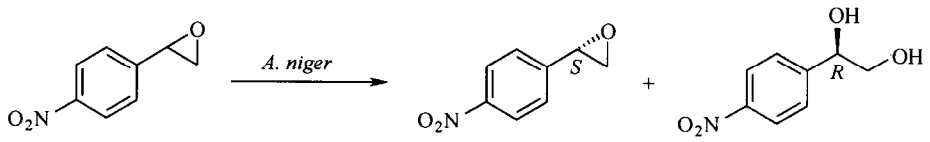
Scheme 1 Enantio- and regio-resolution of racemic *para*-nitrostyrene oxide by *A. niger*



(+/-)-1 (*S*)-1 (*R*)-2

(1.0 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), sucrose (30 g/L), pH 6.8—7.0 (Czapek culture); Medium **B**,[14] KH_2PO_4 (1.0 g/L), KCl (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), fructose (10 g/L), corn steep (15 g/L), pH 6.8—7.0.

As shown in Table 1, when medium **A** was used, the EH activity was low. Even after 3 h the resolution was not finished yet. Switching to medium **B** led to much better activity as shown by the results after about 45 min, which suggested that medium **B** was more favorable for *A. niger* to generate epoxide hydrolase.

Table 1 Influence of growing media on *A. niger* **5450** and *A. niger* **5332**

Strain	Culture medium	Yield (%) of epoxide	*ee* (%) of epoxide	Yield (%) of diol	*ee* (%) of diol	Reaction time (min)
A. niger **5450**	**A**	58	46	28	91	180
A. niger **5450**	**B**	46	100	30	75	48
A. niger **5332**	**A**	50	77	41	88	180
A. niger **5332**	**B**	49	100	42	69	45

Fifteen strains of *A. niger* were then collected to screen for the highly enantioselective epoxide hydrolases. All of them were cultured in the medium **B** for about three days at 30℃. Most of them grew well except four strains.

The cells of *A. niger* were harvested and washed twice with 100 mL of phosphate buffer (0.1 mol/L, pH 8.0). After filtration the wet biomass of different strains could be directly used for screening of the highly active and enantioselective epoxide hydrolases.

Table 2 Resolution of racemic *para*-nitrostyrene oxide by resting cells of 11 strains of *A. niger*[a]

Strain	Reaction time (min)	Yield (%) of epoxide	*ee* (%) of epoxide[b] (*S*)	Yield (%) of diol	*ee* (%) of diol[b] (*R*)
A. niger **5450**	48	46	100	30	75
A. niger **5332**	45	49	100	42	69
A. niger **5394**	720	62	39	33	75
A. niger **5381**	720	55	25	37	80
A. niger **5320**	40	40	100	51	73
A. niger **5805**	120	32	100	53	60
A. niger **5798**	138	47	100	43	56
A. niger **5714**			to convert to diol within 30 min		
A. niger **5858**	80	43	63	34	89
A. niger **5844**	90	45	100	44	53
A. niger **5785**			slight conversion after 12 h		

[a] The course of the bioconversion was monitored by TLC. When an appropriate degree of conversion was reached, the reaction was stopped by adding 50 mL of ethyl acetate. [b] The *ee* values of the remaining epoxide and vicinal diol were determined by chiral HPLC. The absolute configuration was assigned *via* chiral HPLC analysis by comparison with authentic samples.

In the screening studies of Table 2, we found that 8 out of 11 (*A. niger* **5450**, *A. niger* **5714**, *A. niger* **5332**, *A. niger* **5320**, *A. niger* **5805**, *A. niger* **5798**, *A. niger* **5844**, *A. niger* **5858**) strains of *A. niger* showed high EH activity. All of these biohydrolysis could be stopped within 3 h, when an appropriate degree of conversion was reached. It was found that most of strains of *A. niger* (except *A. niger* **5858** and *A. niger* **5714**) showed remarkable enantioselectivity (the unreacted *S*-epoxide **1** could be obtained with about 100% *ee*), especially for *A. niger* **5450** (the unreacted *S*-epoxide **1** was obtained with 100% *ee* and the product of *R*-diol **2** was obtained with > 70% *ee*). There are also some strains with low activities of epoxide hydrolase, which could not complete the resolution even after 12 h.

Further work with strains of *A. niger* **5450** and *A. niger* **5320** is in progress.

Conclusion

The results of this study indicate that most strains of *A. niger* examined in this work appear to possess epoxide hydrolase. Some of them (*A. niger* **5450**, *A. niger* **5320**) showed high epoxide hydrolases activity and remarkable enantio- and regio-selectivity.

Experimental

General methods

The strains of *A. niger* used in this work were collected from the natural sources. All melting points are uncorrected. EI mass spectra (MS) were run on an HP-5989A mass spectrometer. ^{1}H NMR spectra were recorded on a Varian EM-390 (90 MHz) or a Bruker AMX-300 (300 MHz) spectrometer with tetramethylsilane as the internal standard. HPLC was carried out using chiral OD and OJ columns. TLC and column chromatography were carried out on HSG F_{254} silica gel plates and silica gel (200—400 mesh) respectively.

Synthesis of oxide and its corresponding diol

(±)-*p*-Nitrostyrene oxide was synthesized according to the literature[13] as a yellow solid, m. p. 84—85℃. ^{1}H NMR (90 MHz, CCl_4) δ: 2.6 (dd, J = 5, 2 Hz, 1H), 3.0 (dd, $J_1 = J_2$ = 4.5 Hz, 1H), 3.6—3.8 (m, 1H) 7.4 and 8.1 (AB, J = 7.5 Hz, 4H); MS *m/z* (rel. intensity): 166 ($[M+1]^+$, 2), 165 (M^+, 3), 164 (6), 148 (47), 118 (68), 91 (30), 89 (100), 77 (12), 65 (21), 63 (47), 51 (21).

(±)-1-(4-Nitrophenyl)-1,2-ethanediol was synthesized according to the literature[13] as a light yellow solid, m.p. 78—79℃. ^{1}H NMR (300 MHz, CD_3COCD_3) δ: 3.46 (bs, 2H) 3.58 (dd, J = 11.1, 6.7 Hz, 1H), 3.66 (dd, J = 11.1, 4.9 Hz, 1H), 4.88 (dd, J = 6.6, 5.0 Hz, 1H), 7.69 and 8.18 (AB, J = 8.7 Hz, 4H); MS *m/z* (rel. intensity): 184($[M+1]^+$, 100), 182 (2) 166 (17), 152 (63), 136 (26), 122 (16), 106 (43), 94 (35), 91 (11), 78 (44).

General procedure for the growth of different strains of A. niger

All the strains of *A. niger* were maintained on agar slants and grown in medium **B**. For large scale growth of cultures, a two-stage process was used. 100 mL of medium **B** (in a 500 mL of flask) was first inoculated and then cultured for 20 h. In the second stage, 1000 mL of medium B (in a 5 L of flask) was inoculated with 5% of the first stage culture. The second stage lasted for 60 h. In all stages, cultures were grown on a shaker maintained at 30℃ and 100 rpm. *A. niger* cells were harvested by centrifugation (HITACHI, CR20B2) at 7,000 rpm, 5℃ for 30 min. The cells were washed twice with 100 mL of 0.1 mol/L (pH 8.0) phosphate buffer. *A. niger* cells were harvested by filtration. The wet biomass of *A. niger* could be stored at 4℃ for 15 days without any discernible loss of the activity of EH.

General process of biohydrolysis of racemic para-nitrostyrene oxide by different strains of A. niger

Biomass 26 g (wet) of the fungal *Aspergillus niger* was suspended in a 500 mL three-necked, round-bottomed flask which was filled with 200 mL of sodium phosphate buffer 0.1 mol/L (pH 8) equipped with a mechanical stirrer and a water bath (30℃). 130 mg of epoxide (±)-**1** was added to the suspension as a solution in DMF (3 mL). The medium was stirred at about 600 rpm.[15,16] The course of the bioconversion was monitored by TLC. The reaction was stopped by adding 50

mL of ethyl acetate when an appropriate degree of conversion was reached. The biomass was filtered off. The fungal cake was washed with 20 mL of ethyl acetate. The aqueous phase was saturated with sodium chloride before being extracted 3 times with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and the solvent was removed. Flash chromatography (petroleum ether/EtOAc, gradient from 4:1 to 1:1) of the residue gave (*S*)-**1** and (*R*)-**2**. All of them were analyzed with chiral HPLC.

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References

1 Johnson, R. A.; Sharpless, K. B. In "*Catalytic Asymmetric Synthesis*," Ed.: Ojima, I., Verlag Chemie, New York, **1993**, p.103.

2 Kolb, H. C.; van Nieuwenhze, M. S.; Sharpless, K. B. *Chem. Rev.* **1994**, *94*, 2483.

3 Katsuki, T. J. *Synth. Org. Chem. Jpn.* **1995**, *53*, 940.

4 Jacobsen, E. S.; Kakiuchi, F.; Fonsler, R. G.; Larrow J. F.; Tokunaga, M. *Tetrahedron Lett.* **1997**, *38*, 773.

5 Larrow, J. F.; Schauss, S.E.; Jacobsen, E. N. *J. Am. Chem. Soc.* **1996**, *50*, 249.

6 Archelas, A.; Furstoss, R. *Annu. Rev. Microbiol.* **1997**, *51*, 491.

7 Arand, M.; Grant, D. F.; Beetham, J. K.; Friedberg, T.; Oesch, F.; Hammock, B. D. *FEBS Lett.* **1994**, *338*, 251.

8 Arand, M.; Wagner, H.; Oesch. F. *J. Biol. Chem.* **1996**, *271*, 4223.

9 Faber, K.; Mischitz, M.; Kroutil, W. *Acta Chem. Scand.* **1996**, *50*, 249.

10 Weijers, C. G. A. M. *Tetrahedron: Asymmetry* **1997**, *8*, 639.

11 Chen, X. J.; Archelas, A.; Furstoss, R. *J. Org. Chem.* **1993**, *58*, 5528.

12 Moreau, P.; Moussou, C.; Baratti, J. C.; Zylber, J.; Archelas, A.; Furstoss, R. *Tetrahedron* **1997**, *53*, 9707.

13 Moussou, P.; Morisseau, C.; Zylber, J.; Archelas, A.; Baratti, J.; Furstoss, R. *J. Org. Chem.* **1996**, *61*, 7402.

14 Morisseau, C.; Venturi, G.; Moussou, P.; Baratti, J. *Biotechnol. Tech.* **1998**, *12*, 805.

15 Morisseau, C.; Nellaiah, H.; Archelas, A.; Furstoss, R.; Baratti, J. C. *Enzyme Microb. Technol.* **1997**, *20*, 446.

16 Pedragosa-Moreau, S.; Moussou, C.; Baratti, J.; Zylber, J.; Archelas, A.; Furstoss, R. *Tetrahedron* **1997**, *53*, 9707.

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