J Ind Microbiol Biotechnol (2006) 33: 274–282
DOI 10.1007/s10295-005-0062-3

ORIGINAL PAPER

Yanbin Liu · Qian Sha · Sheng Wu · Jianjun Wang
Liu Yang · Wanru Sun

Enzymatic resolution of racemic phenyloxirane by a novel epoxide hydrolase from *Aspergillus niger* SQ-6 and its fed-batch fermentation

Received: 30 June 2005 / Accepted: 27 October 2005 / Published online: 1 December 2005

Abstract A microorganism with the ability to catalyze the resolution of racemic phenyloxirane was isolated and identified as *Aspergillus niger* SQ-6. Chiral capillary electrophoresis was successfully applied to separate both phenyloxirane and phenylethanediol. The epoxide hydrolase (EH) involved in this resolution process was (*R*)-stereospecific and constitutively expressed. When whole cells were used during the biotransformation process, the optimum temperature and pH for stereospecific vicinal diol production were 35°C and 7.0, respectively. After a 24-h conversion, the enantiomer excess of (*R*)-phenylethanediol produced was found to be >99%, with a conversion rate of 56%. In fed-batch fermentations at 30°C for 44 h, glycerol (20 g L^{-1}) and corn steep liquor (CSL) (30 g L^{-1}) were chosen as the best initial carbon and nitrogen sources, and EH production was markedly improved by pulsed feeding of sucrose (2 g L^{-1} h^{-1}) and continuous feeding of CSL (1 g L^{-1} h^{-1}) at a fermentation time of 28 h. After optimization, the maximum dry cell weight achieved was 24.5 ± 0.8 g L^{-1}; maximum EH production was 351.2 ± 13.1 U L^{-1} with a specific activity of 14.3 ± 0.5 U g^{-1}. Partially purified EH exhibited a temperature optimum at 37°C and pH optimum at 7.5 in 0.1 M phosphate buffer. This study presents the first evidence for the existence of a predicted epoxide racemase, which might be important in the synthesis of epoxide intermediates.

Y. Liu · Q. Sha · S. Wu · J. Wang · L. Yang · W. Sun (✉)
State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beiyitiao 13, Zhongguancun, Haidian, Beijing, 100080 People's Republic of China
E-mail: sunwr@sun.im.ac.cn
Tel.: +86-10-62587206
Fax: +86-10-62653468

Y. Liu
Graduate School of the Chinese Academy of Sciences, Beijing, People's Republic of China

Keywords *Aspergillus niger* · Epoxide hydrolase · Epoxide racemase · Capillary electrophoresis · Stereoselectivity

Introduction

In the fine chemistry and pharmaceutical industries, enantiopure epoxides and the corresponding vicinal diols are important building blocks in the synthesis of biologically active asymmetric molecules. Chemical hydrolysis of epoxide yields racemic vicinal diol, which requires kinetic resolution to optically pure diol [6]. This procedure makes the process cumbersome. In contrast, enzymatic resolution of epoxide becomes increasingly attractive [2, 6, 36]. Epoxide hydrolases (EHs, EC 3.3.2.3), which are used to catalyze the hydrolysis of epoxides to the corresponding vicinal trans-diols [4, 5, 19, 25, 33], are ubiquitous in nature and occur in many organisms, including mammals [35], insects [21], plants [12] and microorganisms [3, 18, 26, 32, 38]. Recent studies on EH production from *Aspergillus niger* are exciting due to its highly enantioselective nature [1, 3, 22, 29].

Filamentous fungi offer many benefits over other types of cells when used for the production of biologically active molecules and eukaryotic proteins [14]. Unfortunately, submerged cultivation of filamentous fungi is often characterized by high broth viscosity, which can result in a number of problems (e.g., insufficient oxygen mass transfer, high power requirement, formation of nutrient concentration zones) that have the potential to reduce productivity [31]. Bhargava et al. [7, 8] recently proposed a possible solution: "pulsed" addition of the limiting carbon source in an *Aspergillus oryzae* fed-batch fermentation led to significantly reduced broth viscosity and improved oxygen mass transfer efficiency without detrimentally affecting cellular metabolic activity or total secreted protein. The effect of total cycle time for substrate pulsing was also studied

in detail, revealing that high values of cycle time (more than 15 min) showed significantly reduced recombinant enzyme production [10].

The present paper reports the isolation of a novel microorganism, *Aspergillus niger* SQ-6, which shows the ability to stereospecifically convert epoxides to the corresponding vicinal diols. Improvement of (*S*)-phenyloxirane resolution by whole cells was achieved by optimizing the conditions of fed-batch fermentation for cell growth and bioconversion. Some properties of the partially purified EH are also reported.

Materials and methods

Chemicals

Racemic and (*R*)-, (*S*)-phenyloxirane were purchased from Fluka (Buchs, Switzerland). Racemic and (*R*)-, (*S*)-phenylethanediol were purchased from Acros (Geel, Belgium). Triethanolamine was purchased from Merck (Darmstadt, Germany). All other chemicals used in these experiments were of analytical grade.

Microorganisms and cultivation media

Aspergillus niger SQ-6 isolated from soil was used in all experiments. Medium I (used for screening experiments) contained (per liter): 2.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.5 g $Na_2HPO_4 \cdot 12H_2O$, 10 mg $CaCl_2$, 1.5 g $KH_2PO_4 \cdot 3H_2O$, 1.0 mg $FeSO_4 \cdot 7H_2O$, 0.1 mg $ZnSO_4 \cdot 7H_2O$, 0.1 g yeast extract, 0.1 g corn steep liquid (CSL), and was adjusted to pH 7.0; 20 mL racemic phenyloxirane passed through a 0.22 μm filter membrane was added to medium I after autoclaving. Salt solution contained (per liter): 140.0 g KH_2PO_4, 90.0 g $K_2HPO_4 \cdot 3H_2O$, 10.0 g KCl, 10.0 g $MgSO_4 \cdot 7H_2O$; pH 4.0. Trace elements solution contained (per liter): 11.0 g H_3BO_3, 5.0 g $MnCl_2 \cdot 4H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 1.6 g $CoCl_2 \cdot 6H_2O$, 1.1 g $(NH_4)Mo_7O_{24} \cdot 4H_2O$, 5.0 g EDTA; pH 4.0. Medium II (used for small-scale production of mycelia) contained (per liter): 20.0 g sucrose, 20.0 g CSL, 10 mL salt solution; pH 4.0. Medium III (used for submerged fermentation) contained (per liter): 20.0 g glycerol, 30.0 g CSL, 10.0 mL salt solution, 1.0 mL trace elements solution; pH 4.0.

Screening methods

Liquid samples from soil were inoculated into 30 mL medium I in 250-mL flasks and cultivated at 30°C for 72 h. One milliliter culture was diluted 100 times with distilled water and spread on agar Petri dishes of Czapek's medium. Supernatants of fermentation were investigated for the presence of phenylethanediol by thin-layer chromatography (TLC). Microorganisms that can hydrolyze phenyloxirane were confirmed using biocatalytic assays of whole cells and grown in Luria–Bertani (LB) medium, then re-isolated on agar medium until a pure colony was obtained.

Analytical methods

Mycelia were harvested by vacuum filtration (Whatman filter paper no. 4; Whatman, Maidstone, UK) and washed three times with distilled water and three times with 0.1 M phosphate potassium (PK) buffer, pH 8.0. Biomass (dry cell weight, DCW) was estimated after drying the mycelia at 105°C to constant weight. The residual sugar concentration was measured with the first-time filtrate as described previously [17].

TLC was used for qualitative determination of phenylethanediol. Samples were extracted with ether and the water phase was concentrated by vacuum distillation and developed on the silica gel plate with a developing solvent system of benzene:benzinum:acetic acid (50:35:15, v/v).

For quantitative determination of the phenylethanediol formed and the phenyloxirane remaining in the bioconversion broth, the filtrate was diluted with three volumes of methanol and analyses were performed on a HPLC apparatus (HP1050, Agilent, Wilminton, DE) with UV–Vis spectrophotometric detection using a C18 column (Diamosil C18, Dikman Technologies, China). Analysis conditions: column, spherisorb, 5 μm, ϕ 4.6×250 mm; mobile phase, methanol/water (3/1 v/v); flow speed, 1.0 mL min^{-1}; detected wavelength, 280 nm.

Assay of whole-cell biotransformations

Aspergillus niger SQ-6 was cultivated in medium II at 30°C for 36 h. Filtered mycelia were washed twice with 0.1 M PK buffer (pH 7.0) and re-suspended in 1.0 mL of the same buffer containing 4 mM phenyloxirane. Reactions were carried out at 37°C for 20 min and the supernatants were analyzed by HPLC. The phenylethanediol production activities of whole cells were expressed in units per milligram of DCW (U mg^{-1}). One unit was defined as the number of micromoles of phenylethanediol formed per hour.

Product isolation and identification

Enantiomeric excess was determined by chiral capillary electrophoresis (CE) [37]. CE was carried out with a P/ACE MDQ CE instrument (Beckman Coulter, Fullerton, CA) equipped with an on-line UV detector and a fused silica capillary tube (length 37 cm, effective length 30 cm, I.D. 75 μm); The capillary tube was successively flushed with 0.1 M NaOH for 2 min, double deionized water for 2 min, followed by running buffer (10 mM phosphoric acid containing 30 g L^{-1} sulfated β-cyclodextrin as chiral selective agent, adjusted with

triethanolamine to pH 2.5 for phenylethanediol or pH 4.0 for phenyloxirane) for 2 min. Samples were dissolved in methanol to 0.1 mg mL^{-1} and introduced hydrodynamically into the anode end with 0.1 psi for 1 s, then separated. The tube temperature was set at 25°C, and the working voltage was 12 kV for phenylethanediol or 20 kV for phenyloxirane. Detection was by UV detector at 214 nm.

Culture conditions in shake flasks

After inoculating with *A. niger* SQ-6, PDA slants were incubated at 30°C for 5 days, spores were transferred to a 500 mL Erlenmeyer flask containing 100 mL medium II at 5×10^8 spores mL^{-1}. The flask was incubated on a rotary shaker (250 rpm) at 30°C for 48 h. Samples were taken at the end of fermentation to determine biomass and EH activity.

Optimization of culture conditions in fed-batch fermentation

Shake flask culture was carried out in a 1,000-mL flask containing 200 mL medium II and incubated with shaking (250 rpm) at 30°C for 36 h. After transfer of the above 150 mL culture, a 5.0 L jar fermenter (Bioflow 3000, New Brunswick Scientific, Edison, N.J.) was used with 3.0 L medium III. Antifoam was added to a final concentration of 0.05%. The aeration rate, agitation speed and temperature were held at 0.5 vvm, 500 rpm and 30°C, respectively. When necessary, pH was controlled at 4.0 by using 0.5 M NaOH and 0.5 M H_2SO_4. The level of dissolved oxygen (DO) in the fermentation broth was measured by a polarographic DO sensor (New Brunswick Scientific). The readings were expressed as a percentage of the initial level of saturation.

Enzyme preparation and purification

The purification procedure was based on the method of Morisseau et al. [28]. During purification, the temperature was maintained at 4°C with all chromatographic columns and the FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden). For preparation of EH, filtered mycelia were suspended in 20 mM Tris–HCl buffer, pH 8.0, 2 mM β-mercaptoethanol (buffer A) and disrupted using a French Press. After centrifugation (14,000 *g*, 20 min), the crude enzyme extract was loaded on a HiPrep-16/10-DEAE column equilibrated with buffer A and eluted with a linear gradient of NaCl (0–1.0 M). Active fractions were pooled, $(NH_4)_2SO_4$ added to a final concentration of 0.25 M, and the sample was loaded onto a HiPrep-16/10-Phenyl-hs column equilibrated with buffer A containing 0.25 M $(NH_4)_2SO_4$. Elution was performed with a linear gradient of ethylene glycol (0–56%). Active fractions were pooled and stored at −20°C.

During purification, enzyme activities were rapidly assayed by the method of Bhatnagar et al. [11]. One EH unit was defined as the amount of enzyme catalyzing the formation of 1 μmol diol per minute under assay conditions.

Results

Isolation and identification of microorganisms producing EH

Twenty-six strains able to produce diol were isolated from soil by the enrichment culture technique. Of these 26 strains, 8 showed stereoselectivity and were isolated. Strain SQ-6, which had the highest stereoselectivity (ee value 92%) and relatively higher conversion (35%) among these isolates, was selected for further study and identified as *A. niger*. The optimum temperature and pH for growth of *A. niger* SQ-6 were confirmed as 30°C and pH 4.0, respectively (data not shown).

Isolation and identification of reaction products

To investigate the stereoselectivity of our EH, the following procedure was used: 100-mg lyophilized mycelia were re-suspended in 1.0 mL 0.1 M PK buffer (pH 7.0) containing 4 mM phenyloxirane as substrate. After incubating the reaction mixture at 37°C for 24 h, the absolute configuration of phenylethanediol produced and the unconsumed phenyloxirane unconsumed were shown to be (*R*)-phenylethanediol and (*S*)-phenyloxirane when investigated by chiral CE. Figure 1 shows a chiral chromatography of the racemic phenylethanediol and phenyloxirane standard, the (*R*)-phenylethanediol produced and the unconsumed (*S*)-phenyloxirane in the reaction. The produced phenylethanediol or unconsumed phenyloxirane had the same retention time (28.5 or 10.8 min) as the standard (*R*)-phenylethanediol or (*S*)-phenyloxirane, respectively, indicating the absolute configuration of the consumed substrate and product as both (*R*)-enantiomer without configuration diversion. After transformation, the ee value of (*R*)-phenylethanediol was found to be >99%, and the conversion rate 56%. From these results, the EH expressed in *A. niger* SQ-6 was shown to be (*R*)-stereospecific.

Effects of temperature and pH on whole-cell biotransformation

To evaluate the effects of different temperatures on whole-cell biotransformation, the temperature range was varied from 20 to 50°C. With a rise in temperature, EH activity increased; optimum activity was recorded at 35°C (Fig. 2a). With a further increase in temperature, activity decreased. To determine the effects of pH on whole-cell biotransformation, the pH of suitable buffers

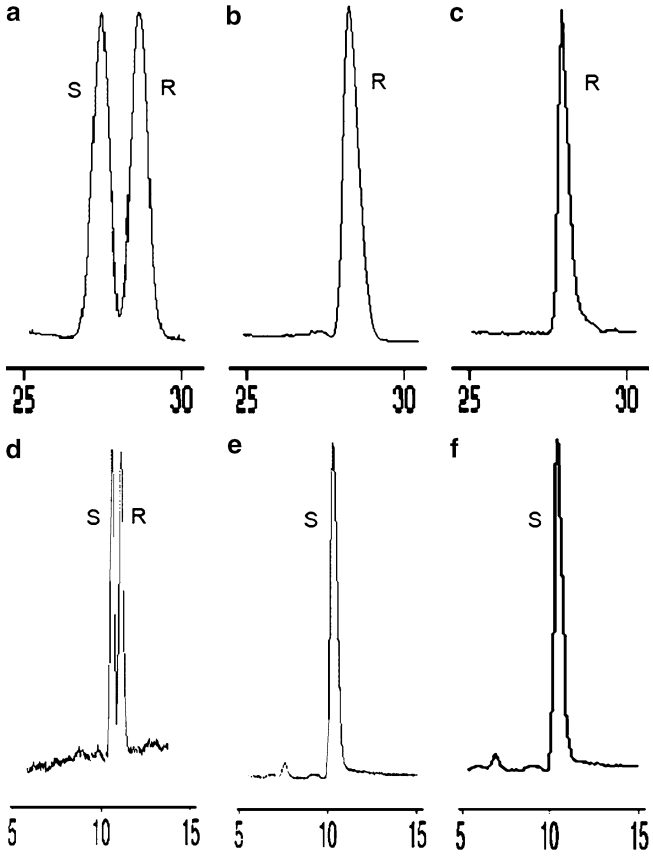

Fig. 1 Chiral capillary electrophoresis (CE) of racemic phenylethanediol (**a**), (*R*)-phenylethanediol standard (**b**), phenylethanediol produced by *Aspergillus niger* SQ-6 (**c**), racemic phenyloxirane (**d**), (*S*)-phenyloxirane (**e**), and remaining phenyloxirane in the *A. niger* SQ-6 reaction mixture (**f**)

was varied from 5 to 9.5. The mycelia showed EH activity in different buffers, with the optimum activity being recorded at pH 7.0 (Fig. 2b).

Effects of different carbon and nitrogen sources

Different carbon and nitrogen sources at a final concentration of 10.0 g L^{-1} for organic nutrients and 5.0 g L^{-1} for inorganic nutrients were added to medium containing 10 mM salt solution, and their effects on cell growth and enzyme synthesis were investigated. Addition of inorganic nutrients had a negative effect on both cell growth and enzyme synthesis, moreover lowering the ratio of phenyloxirane to phenylethanediol. In the case of organic nutrients, sucrose showed the highest ee value (99%) of all carbon sources tested. However, the poor conversion rate and, especially, the low biomass achieved on sucrose made us choose glycerol as the preferred carbon source (Table 1). As a structural enzyme, EH productivity should be enhanced together with increased biomass. According to this principle, CSL was selected as the best nitrogen source despite the high conversion rate achieved using tryptone (Table 2).

The effects of initial glycerol and CSL concentrations on cell growth and EH production were investigated in flask cultures. Different concentrations of glycerol (5, 10, 15, 20, 30, 40 g L^{-1}) were tested with a fixed CSL concentration of 5 g L^{-1}. Likewise, a fixed concentration of glycerol was tested with variable CSL concentrations (10, 15, 20, 30, 40 g L^{-1}). When CSL concentration was invariable, the biomass increased remarkably at low glycerol levels (0–15%) but only slightly at high glycerol levels (40–50%) (Fig. 3a); when glycerol concentration was invariable, biomass increased according to the increased concentrations of CSL (Fig. 3a), indicating that CSL concentration has a crucial effect on fungal biomass. Moreover, glycerol concentration acted as the key factor in EH production (Fig. 3b). Finally, 20 g L^{-1} glycerol and 30 g L^{-1} CSL were used for optimal EH production.

Fed-batch fermentation

EH production and biomass were assessed in fungi cultivated in flasks with medium II with 11 different types of nutrient supplementation at 0 and 24 h. The results of biomass, EH activity and relative activity per DCW at 44 h are shown in Fig. 4. CSL supplementation at 24 h increases only biomass (10.5 g L^{-1}), while supplementation with carbon sources such as glucose,

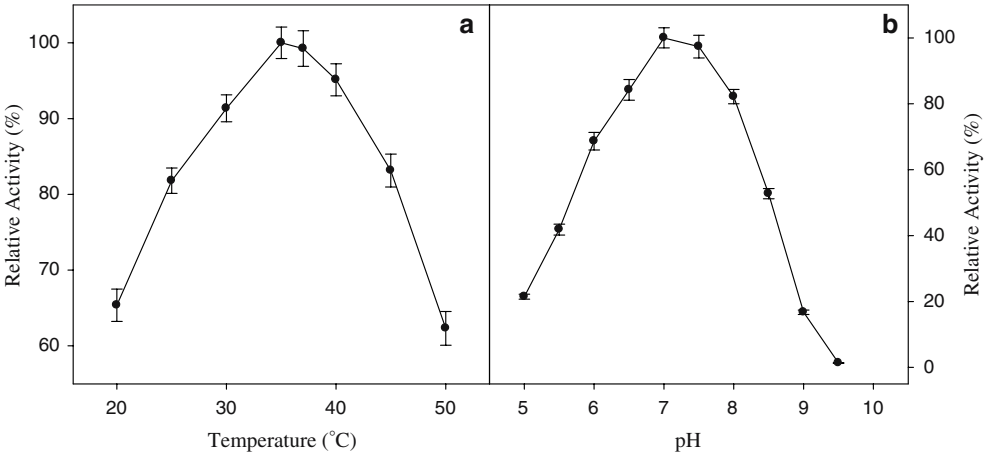

Fig. 2 Effects of temperature (**a**) and pH (**b**) on production of phenylethanediol with whole cells of *A. niger* SQ-6. Mycelia were incubated under standard conditions at the temperatures and pH values indicated: pH 5.0–7.0, 0.1 M phosphate buffer, pH 7.0–9.5, 0.1 M Tris–HCl buffer. *Error bars* Standard deviation from three independent samples

Table 1 Effect of carbon source on cell growth and enzyme activity when using corn steep liquid (CSL; 20.0 g l^{-1}) as nitrogen source

Carbon source	Dry cell weight (g L^{-1})	Conversion (%)	ee value
Glucose	3.54	34	25
Sucrose	3.26	46	99
Lactose	0.86	10	3
Soluble starch	3.50	41	7
Glycerol	4.79	56	92
Dextrin	0.91	6	4
Fructose	4.14	48	56

Table 2 Effect of nitrogen source on cell growth and enzyme activity when using glucose (20.0 g l^{-1}) as carbon source

Nitrogen source	Dry cell weight (g L^{-1})	Conversion (%)	ee value
Yeast extract	0.56	22	5
Fish peptone	3.34	26	11
Soya peptone	1.60	24	16
CSL	3.54	33	64
Beef extract	1.77	10	18
Tryptone	3.50	35	25
$NaNO_3$	0.52	9	14
$(NH_4)_2SO_4$	0.79	14	0
NH_4NO_3	0.91	17	19

sucrose, fructose and glycerol (No. 8–11) at 24 h can improve both biomass and EH productivity. In particular, sucrose supplementation (No. 9) at 24 h markedly improved EH production (111.7 U L^{-1}) and specific activity (11.9 U g^{-1}). Other nutritional supplements showed little effect on either biomass or EH production. EH production may be suppressed due to the negative effects of the presence of analogous EH catalysis products, i.e., phenylethanediol and phenylethanol. In other words, sucrose supplementation can avoid the inhibitory effects of EH biosynthesis, enhancing enzyme yield by nearly 150% under the above conditions. Considering the growth curve of fungal fermentation, 28-h was chosen as the feeding point in fed-batch fermentation (data not shown).

Pulsed feeding of limited carbon sources during fermentation was applied to EH fermentation to reduce the broth viscosity and improve EH productivity. In our case, sucrose (2 g L^{-1} h^{-1}) was added with a feed-pump on for 3 min during each repeated 10-min cycle.

According to the consumption of nitrogen source and maintenance of a DO concentration of 30% during the phase of cell growth and metabolism, a low level of CSL should be fed continuously at the same time as sugar feeding and controlled for a DO of not more than 30% saturation. Figure 5 shows the results of optimized fed-batch cultivations with pulsed feeding of sucrose (2 g L^{-1} h^{-1}) and continuous feeding of CSL (1 g L^{-1} h^{-1}). It was concluded that both cell biomass (24.5 ± 0.8 g L^{-1}) and EH production (351.2 ± 13.1 U L^{-1}) were higher but the specific activity (14.3 ± 0.5 U g^{-1}) varied little from that achieved above. The specific growth rate changed from logarithmic growth phase (0.84 h^{-1}) to enzyme production phase (0.47 h^{-1}), then to negative growth phase (−0.23 h^{-1}). In short, CSL supplementation at a limited level can improve EH productivity by increasing fungal biomass.

Partial physical properties of EH

EH was partially purified by ion exchange chromatography and hydrophobic interaction chromatography and its activity as a function of temperature from 0 to 65°C was measured; activity was highest at 37°C (Fig. 6a). Upon incubation for different times (10–60 min) at varying temperatures (30, 37, and 45°C), the enzyme was found to remain 80% activity for over 50 min at 30°C, for 30 min at 37°C and for 20 min at 45°C (Fig. 6b). The enzyme maintained maximum EH activity at pH 7.5 in 0.1 M phosphate buffer (Fig. 7).

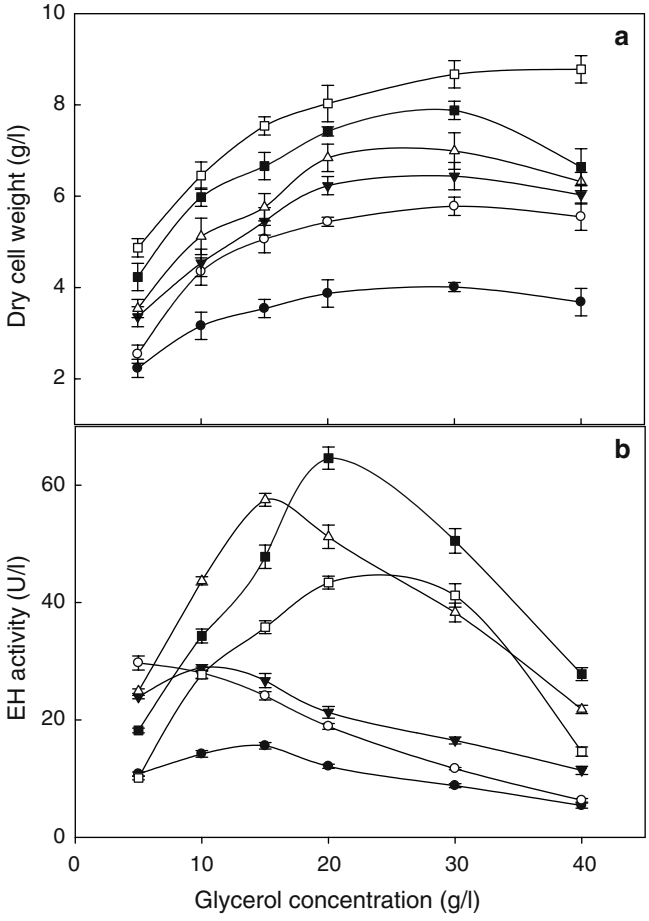

Fig. 3 Effects of initial concentrations of glycerol on biomass (**a**) and epoxide hydrolase (EH) production (**b**) at a fixed corn steep liquid (CSL) concentration of 5 g L^{-1}. CSL concentrations (g L^{-1}): *Filled circles* 5, *open circles* 10, *filled inverted triangles* 15, *open inverted triangles* 20, *filled squares* 30, *open squares* 40. *Error bars* Standard deviations from three independent samples

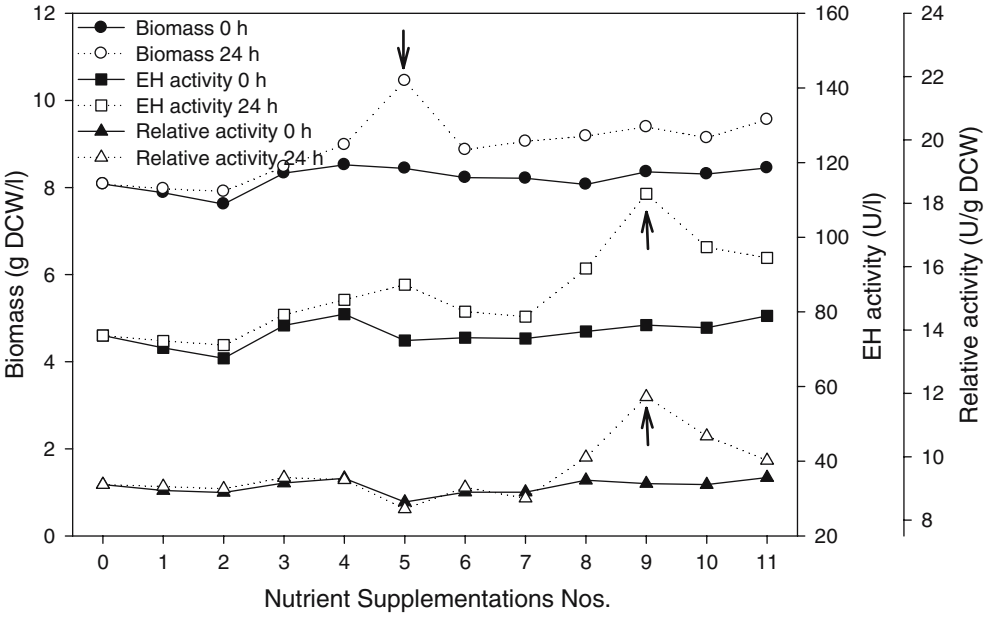

Fig. 4 Effects of various supplementations at different times on cell growth and EH production. Nutrient supplementation Nos.: *0* supplementation control, *1* phenylethanediol (1 g L^{-1}), *2* *β*-phenylethanol (1 g L^{-1}), *3* B vitamins mixture (1 g L^{-1}), *4* amino acid mixture (1 g L^{-1}), *5* CSL (10 g L^{-1}), *6* yeast extract (10 g L^{-1}), *7* fish peptone (10 g L^{-1}), *8* glucose (10 g L^{-1}), *9* sucrose (10 g L^{-1}), *10* fructose (10 g L^{-1}), *11* glycerol (10 g L^{-1}). *White* and *black* symbols addition at 0 and 24 h, respectively. *Black arrows* indicate the maximum values of biomass, EH activity and relative activity

Discussion

Enzymatic hydrolysis of racemic epoxide is catalyzed by the enzyme epoxide hydrolase (EH). The cells of many organisms harbor EH activity; however, regarding enantioselectivity and conversion rate, only a few organisms can be exploited for the resolution of racemic epoxide. Some fungal cells (e.g., from *Aspergillus* and *Beauveria* sp.) show good EH activity towards styrene oxide-type substrates [32]. A novel aspergillus strain able to produce highly enantioselective (*R*)-phenylethanediol and to resolve (*S*)-phenyloxirane from racemic phenyloxirane has now been isolated and identified as *A. niger* SQ-6. Improvement of asymmetric hydrolysis by whole cells was achieved through optimization of the growth medium composition and fermentation mode.

EH enzymes obtained from *A. niger* strains can catalyze hydrolysis of epichlorohydrin, cyclohexene oxide, styrene oxide, nitrostyrene oxides, pyridyloxiranes, and related compounds [13, 15, 16, 20, 23, 30], and have been applied in various syntheses of key, enantiopure, building blocks [27, 34]. Partial purification of EH from *A. niger* SQ-6 suggests that this enzyme shows some similarities with the EH identified in *A. niger* LCP521 [28], including substrate specificity, optimum pH and regioselectivity. However, the carbon source glucose, which was suited for strain LCP521, shows only a weak effect

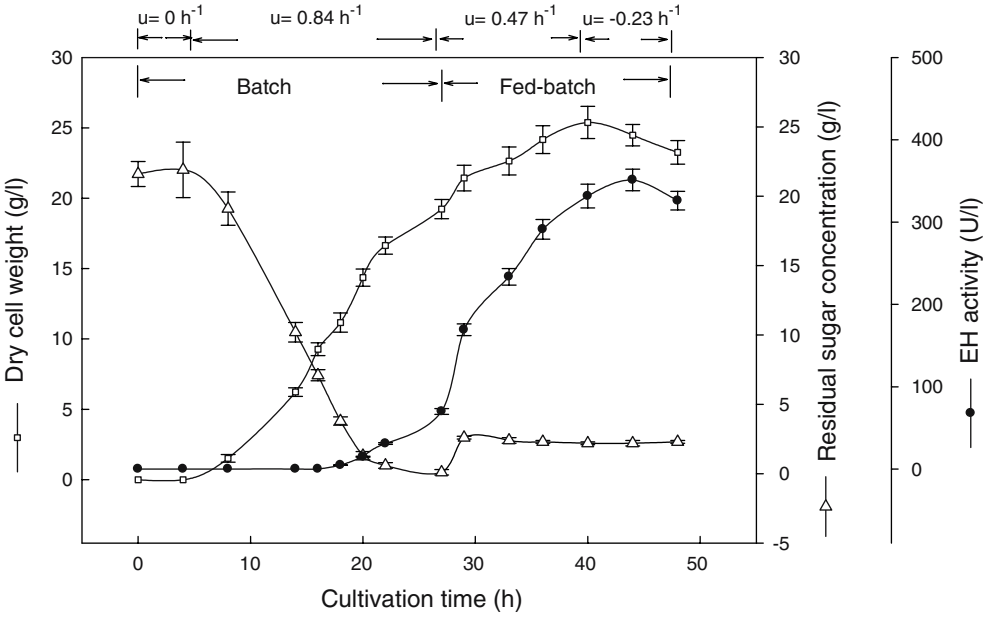

Fig. 5 Kinetics of fungal growth (*open squares*), EH production (*filled circles*) and residual sugar concentration (*open triangles*) in a fed-batch fermentor after optimization with pulsed-fed sucrose and continuous feeding of CSL. *Arrow* Feeding point of sucrose and CSL, *error bars* Standard deviation from three independent samples

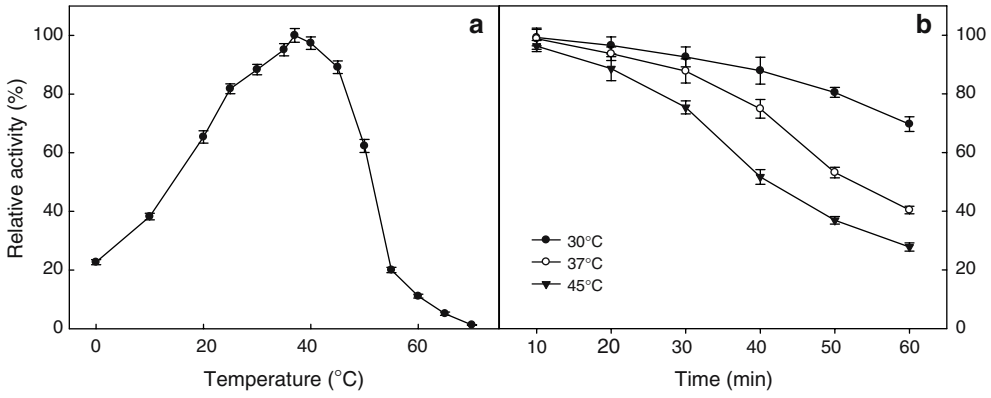

Fig. 6 Effects of temperature on the activity (**a**) and stability (**b**) of partially purified EH from *A. niger* SQ-6. **a** Assays were performed at various temperatures under standard enzyme assay conditions. The relative activity is expressed as the percentage of the maximum activity attained under the experimental conditions. **b** The remaining activity was assayed under enzyme assay conditions after holding the enzyme at the temperatures and times indicated in 0.2 mL 50 mM phosphate buffer, pH 7.5. The relative residual activity is expressed as the percentage of the maximum residual activity attained under the experimental conditions. *Error bars* Standard deviation from three independent samples

on the enantioselectivity of strain SQ-6, and the optimum growth temperatures for strains LCP521 and SQ-6 are different. Also, strain SQ-6 has a higher conversion rate than LCP521. Another newly reported *A. niger* strain, M200, shows EH activity towards *tert*-butyl glycidyl ether-type substrates [24].

In this work, a process of fed-batch fermentation of *A. niger* for production of highly enantioselective EH was demonstrated for the first time. Mycelial morphology, as well as the utilization rate of different nitrogen and carbon sources, and the subsequent changes in the metabolism of the fungus may be important in explaining the effects of the latter on cell growth and EH production. However, EH activity was observed regardless of the presence of an epoxide derivative during cultivation.

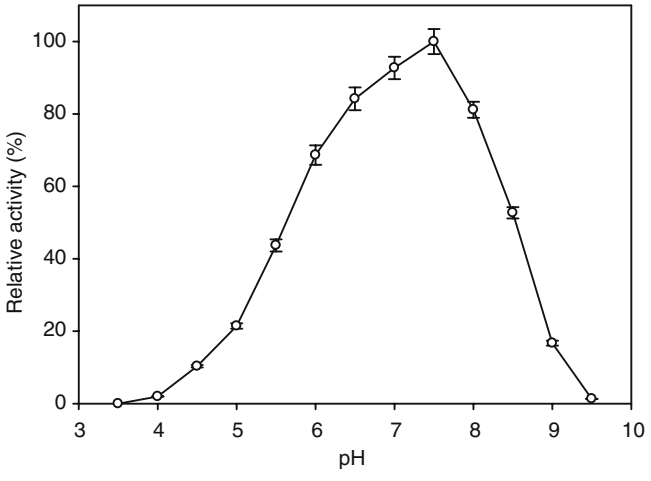

Fig. 7 Effect of pH on the activity of partially purified EH from *A. niger* SQ-6. Enzyme activity was assayed under the enzyme assay conditions except that the following 0.1 M buffers were used: citric acid/sodium citrate buffer pH 3.5–5.5, sodium phosphate pH 6.0–7.5, Tris–Cl pH 8.0–8.5 and glycine/NaOH pH 9.0–9.5. *Error bars* Standard deviation from three independent samples

Pulsed addition of a limiting carbon source to reduce broth viscosity during heterologous protein expression has been studied thoroughly in fed-batch fermentation in bacteria, yeast, and in one filamentous fungus (*A. oryzae* [7–10]). In this paper, this technique was successfully applied in an *A. niger* fed-batch fermentation with excellent results. A cycle time of 10 min was used in *A. niger* fermentation to ensure fungal morphology of small mycelium rather than a pellet. Further optimization of the shear stress and DO tension in the fermentation process might enhance EH productivity.

Common sense tells us that the conversion rate of enzymatic resolution should not be more than 50% (47% for EH from *A. niger* LCP521) [28]. However, in our study, a conversion rate of 56% was apparent during 24-h whole cell catalysis after excluding self hydrolysis. Furthermore, as the EH in this study enantioselectively transforms the *R* configuration, no conversion should occur when using (*S*)-phenyloxirane as substrate during 24-h whole cell catalysis. Surprisingly, however, (*R*)-phenyloxirane was obtained (Fig. 8)! This implies that an unknown epoxide racemase with the ability to racemize epoxide enantiomers might occur in *A. niger* SQ-6. As this predicted epoxide racemase, or vicinal diol isomerase, might decrease the enantiopurity of substrates or products, and the stereoselectivity of EH cannot be changed by alteration of the culture conditions, the ee value catalyzed by the whole cells was an important reason for wanting to inhibition production of the predicted epoxide racemase or vicinal diol isomerase. That was why we chose CSL instead of tryptone as the nitrogen source. However, we failed to purify epoxide racemase, possibly due to the time-consuming enzyme assay method as well as low enzyme activity. Further study on the potential epoxide racemase should focus on a simple enzyme assay method and on adapting fungal fermentation for high expression of epoxide racemase. An *A. niger* SQ-6

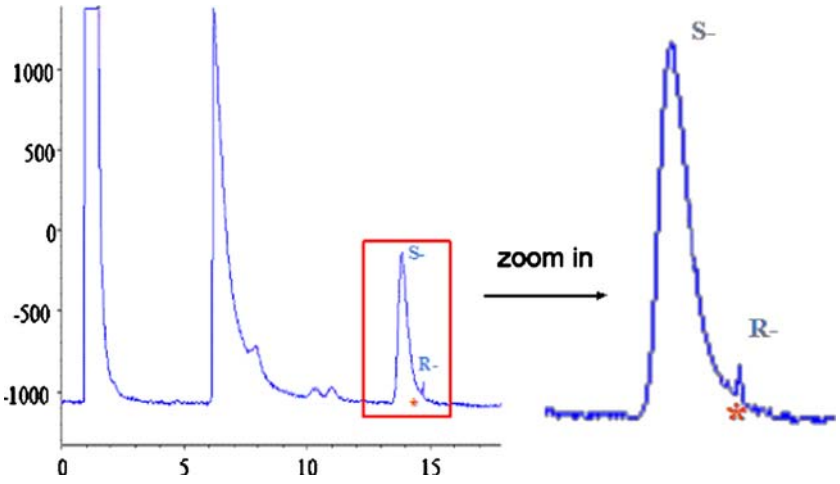

Fig. 8 Evidence of epoxide racemase catalyzing *S*-enantiomer to *R*-enantiomer of phenyloxirane conversion during whole cell catalysis. Analysis method: HP 5890 serial II gas chromatography with flame ionization detector linked to a 3665 HP work station; CHIRALDEX G-TA chiral capillary column of 10 m×250 (I.D.), 0.125 μm (F.T.) (Advanced Separation Technologies, Whippany, N.J.); carrier gas nitrogen, 50 cm s^{-1}; inlet temperature, 160°C; oven temperature, 75°C; detector temperature, 200°C and split ratio of 1:50

cDNA expression library and a high throughput screening method for the unknown epoxide racemase that might yield satisfactory results in the future have been set up in our laboratory.

References

1. Arand M, Hemmer H, Durk H, Baratti J, Archelas A, Furstoss R, Oesch F (1999) Cloning and molecular characterization of a soluble epoxide hydrolase from *Aspergillus niger* that is related to mammalian microsomal epoxide hydrolase. Biochem J 344:273–280
2. Archelas A, Furstoss R (1997) Synthesis of enantiopure epoxides through biocatalytic approaches. Annu Rev Microbiol 51:491–525
3. Archelas A, Furstoss R (1998) Epoxide hydrolases: new tools for the synthesis of fine organic chemicals. Trends Biotechnol 16:108–116
4. Archelas A, Furstoss R (2001) Synthetic applications of epoxide hydrolases. Curr Opin Chem Biol 5:112–119
5. Archer IVJ (1997) Epoxide hydrolases as asymmetric catalysts. Tetrahedron 53:15617–15662
6. Besse P, Veschambre H (1994) Chemical and biological synthesis of chiral epoxides. Tetrahedron 50:8885–8927
7. Bhargava S, Nandakumar MP, Roy A, Wenger KS, Marten MR (2003) Pulsed feeding during fed-batch fungal fermentation leads to reduced viscosity without detrimentally affecting protein expression. Biotechnol Bioeng 81:341–347
8. Bhargava S, Wenger KS, Marten MR (2003) Pulsed addition of limiting-carbon during *Aspergillus oryzae* fermentation leads to improved productivity of a recombinant enzyme. Biotechnol Bioeng 82:111–117
9. Bhargava S, Wenger KS, Marten MR (2003) Pulsed feeding during fed-batch *Aspergillus oryzae* fermentation leads to improved oxygen mass transfer. Biotechnol Prog 19:1091–1094
10. Bhargava S, Wenger KS, Rane K, Rising V, Marten MR (2005) Effect of cycle time on fungal morphology, broth rheology, and recombinant enzyme productivity during pulsed addition of limiting carbon source. Biotechnol Bioeng 89:524–529
11. Bhatnagar T, Manoj KM, Baratti JC (2001) A spectrophotometric method to assay epoxide hydrolase activity. J Biochem Biophys Methods 50:1–13
12. Blee E, Schuber F (1992) Regio- and enantioselectivity of soybean fatty acid epoxide hydrolase. J Biol Chem 267:11881–11887
13. Cagnon JR, Porto ALM, Marsaiolil AJ, Manfio GP, Eguchi SY (1999) First evaluation of the Brazilian microorganisms biocatalytic potential. Chemosphere 38:2237–2242
14. Carlsen M, Nielsen J, Villadsen J (1996) Growth and [alpha]-amylase production by *Aspergillus oryzae* during continuous cultivations. J Biotechnol 45:81–93
15. Choi WJ, Huh EC, Park HJ, Lee EY, Choi CY (1998) Kinetic resolution for optically active epoxides by microbial enantioselective hydrolysis. Biotechnol Tech 12:225–228
16. Choi WJ, Lee EY, Yoon SJ, Yang ST, Choi CY (1999) Biocatalytic production of chiral epichlorohydrin in organic solvents. J Biosci Bioeng 88:339–341
17. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
18. Faber K, Mischitz M, Kroutil W (1996) Microbial epoxide hydrolase. Acta Chem Scand 50:249–258
19. Fretland AJ, Omiecinski CJ (2000) Epoxide hydrolases: biochemistry and molecular biology. Chem Biol Interact 129:41–59
20. Genzel Y, Archelas A, Broxterman QB, Schulze B, Furstoss R (2001) Microbiological transformations. 47. A step toward a green chemistry preparation of enantiopure (*S*)-2-, -3-, and -4-pyridyloxirane via an epoxide hydrolase catalyzed kinetic resolution. J Org Chem 66:538–543
21. Graham SM, Prestwich GD (1994) Synthesis and inhibitory properties of pheromone analogs for the epoxide hydrolase of the Gypsy Moth. J Org Chem 59:2956–2966
22. Grogan G, Rippe C, Willetts A (1997) Biohydrolysis of substituted styrene oxides by *Beauveria densa* CMC 3240. J Mol Catal B 3:253–257
23. Jin H, Li Zy (2002) Enantioselective hydrolysis of *o*-nitrostyrene oxide by whole cells of *Aspergillus niger* CGMCC 0496. Biosci Biotechnol Biochem 66:1123–1125
24. Kotik M, Brichac J, Kyslik P (2005) Novel microbial epoxide hydrolases for biohydrolysis of glycidyl derivatives. J Biotechnol (in press)
25. Meijer J, DePierre JW (1988) Cytosolic epoxide hydrolase. Chem Biol Interact 64:207–249
26. Mischitz M, Kroutil W, Wandel U, Faber K (1995) Asymmetric microbial hydrolysis of epoxides. Tetrahedron Asymmetry 6:1261–1272

27. Monfort N, Archelas A, Furstoss R (2004) Enzymatic transformations. Part 55: Highly productive epoxide hydrolase catalysed resolution of an azole antifungal key synthon. Tetrahedron 60:601–605
28. Morisseau C, Archelas A, Guitton C, Faucher D, Furstoss R, Baratti JC (1999) Purification and characterization of a highly enantioselective epoxide hydrolase from *Aspergillus niger*. Eur J Biochem 263:386–395
29. Morisseau C, Archelas A, Guitton C, Faucher D, Furstoss R, Baratti JC (1999) Purification and characterization of a highly enantioselective epoxide hydrolase from *Aspergillus niger*. Eur J Biochem 263:386–395
30. Nellaiah H, Morisseau C, Archelas A, Furstoss R (1995) Enantioselective hydrolysis of *p*-nitrostyrene oxide by an epoxide hydrolase preparation from *Aspergillus niger*. Biotechnol Bioeng 49:70–77
31. Olsvik E, Kristiansen B (1994) Rheology of filamentous fermentations. Biotechnol Adv 12:1–39
32. Orru RV, Faber K (1999) Stereoselectivities of microbial epoxide hydrolases. Curr Opin Chem Biol 3:16–21
33. Orru RV, Archelas A, Furstoss R, Faber K (1999) Epoxide hydrolases and their synthetic applications. Adv Biochem Eng Biotechnol 63:145–167
34. Pedragosa-Moreau S, Morisseau C, Baratti J, Zylber J, Archelas A, Furstoss R (1997) Microbiological transformations 37. An enantioconvergent synthesis of the [beta]-blocker (*R*)-Nifenalol(R) using a combined chemoenzymatic approach. Tetrahedron 53:9707–9714
35. Seidegard J, DePierre JW (1983) Microsomal epoxide hydrolase. Properties, regulation and function. Biochim Biophys Acta 695:251–270
36. Swaving J, de Bont JAM (1998) Microbial transformation of epoxides. Enzyme Microb Technol 22:19–26
37. Wang JJ, Zheng GJ, Yang L, Sun WR (2001) Enantioselective separation of epoxides by capillary electrophoresis employing sulfated β-cyclodextrin as chiral selector. Analyst 4:438–440
38. Weijers CAGM, de Bont JAM (1999) Epoxide hydrolases from yeasts and other sources: versatile tools in biocatalysis. J Mol Catal B 6:199–214