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Enantioselective epoxide hydrolase activity of a newly isolated microorganism, *Sphingomonas echinoides* EH-983, from seawater

Hee Sook Kim [a], Ok Kyung Lee [a], Soo Jung Lee [a], Seungha Hwang [b], Sang Jin Kim [c], Sung-Hyun Yang [c], Sunghoon Park [d], Eun Yeol Lee [a,*]

[a] *Department of Food Science and Technology, Kyungsung University, Busan 608-736, Republic of Korea*
[b] *School of Chemical and Biological Engineering, Seoul National University, Seoul 151-742, Republic of Korea*
[c] *Marine Biotechnology Research Centre, Korea Ocean Research & Development Institute, Ansan 426-755, Republic of Korea*
[d] *Department of Chemical and Biochemical Engineering, Pusan National University, Busan 609-735, Republic of Korea*

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Abstract

A marine microorganism, *Sphingomonas echinoides* EH-983, which possesses epoxide hydrolase (EH) activity was isolated from seawater and characterized. The EH of *S. echinoides* EH-983 preferentially metabolized (*R*)-enantiomer when the racemic styrene oxides were supplied as substrates. The optimal pH and temperature for the enantioselective hydrolysis by whole-cells of *S. echinoides* EH-983 were 7.0 and 20 °C, respectively. When kinetic resolution was conducted with a racemic mixture of styrene oxides at an initial concentration of 40 mM, enantiopure (*S*)-styrene oxide was obtained in 180 min with a yield of 21.3%. To our best knowledge, *S. echinoides* EH-983 is the first marine microorganism that is reported to have EH activity.

Keywords: Enantioselective hydrolysis; Epoxide hydrolase; *Sphingomonas echinoides* EH-983; Marine microorganism

1. Introduction

Biocatalysts are being used increasingly in synthetic chemistry [1,2]. One of the most interesting applications of biocatalysts is enantioselective hydrolysis of racemic epoxides using an epoxide hydrolase (EH) [3]. The EH catalyzes the addition of a water molecule to the oxirane ring to form the corresponding diol [4]. The EH is cofactor-independent, easy to use and stable, thus has a great potential in industrial applications. Enantiopure epoxides are useful building blocks in organic synthesis due to their ability to react with a broad variety of nucleophiles [5]. Using EHs, enantiopure epoxides can be prepared from racemic epoxides that are cheap and readily available [6].

Mammalian EHs have been studied extensively for several decades for their important physiological roles [7–9]. In mammalian cells, EHs are essential in the cellular detoxification of xenobiotics, in particular aromatic compounds. In bacteria, EHs are related with carbon assimilation and the metabolism of secondary metabolites [10,11]. Due to different possible sources and availability in a large quantity, microbial EHs have been widely explored for organic synthesis [12–15]. In many cases, they are constitutively expressed at a high level even when the microorganisms are grown under non-optimized conditions in the absence of any inducer.

The EHs are ubiquitous in microbial world. To date, numerous microbial EHs have been identified from soil bacteria, yeast and fungi. Some of these EHs have been cloned, produced in a large quantity, and even applied for the preparative-scale production of enantiopure epoxides [3–6,13,15]. However, no EH has been reported from marine microorganisms [16]. Hence, it is of a great interest to isolate a new EH with novel characteristics in enantioselectivity and/or regioselectivity from marine microorganisms to expand the potential uses of EH in industrial biocatalysis. In this paper, we describe the discovery of a new EH activity from a microorganism isolated from marine environment. The microorganism was identified by 16S rDNA analysis. Kinetic resolutions were examined using racemic styrene oxides as model substrates. The substrate specificity on other aromatic

* Corresponding author. Tel.: +82 51 620 4716; fax: +82 51 622 4986.
E-mail address: eylee@ks.ac.kr (E.Y. Lee).

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epoxides as well as the effects of environmental conditions on the EH activity were also studied.

2. Experimental

2.1. Isolation of microorganisms possessing EH activity from marine environment

The screening of the EH activity in various marine microorganisms was conducted using colorimetric assay methods. The cell suspensions in 100 mM KH_2PO_4 buffer (pH 8.0) were incubated in 96 well microtiter plates for 2 h at 30 °C with racemic styrene oxides, and the degradation of epoxides and the formation of diols were monitored by using 4-(*p*-nitrobenzyl)pyridine (NBP) assay or periodate-coupled fluorogenic assay as described in the literatures [17,18]. For the isolates showing the EH activity, the enantioselectivity was examined by chiral GC analysis. The hydrolysis reaction mixtures for the positive isolates were extracted with equal volume of diethyl ether, and the organic layers were analyzed by chiral GC.

2.2. 16S rDNA analysis and identification of the isolate

Genomic DNA extraction, PCR and sequencing of 16S rDNA gene were conducted by the procedures described in the previous works [19,20]. The 16S rDNA was amplified from genomic DNA by PCR using the bacterial primers 27f (5′-AGA GTT TGA TCM[C:A] TGG CTC AG-3′; 20mer) and 1518r (5′-AAG GAG GTG ATC CAN[A:C:G:T] CCR[A:G] CA-3′; 20mer). The PCR products were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). Alignment gaps and unidentified base positions were not taken into consideration for the calculations. The nearly complete 1381 bp nucleotide sequences of 16S rDNA were used for phylogenetic analysis. The 16S rDNA gene sequences were compared with the sequence data in GenBank database by using the BLAST algorithm. The related sequences were preliminarily aligned with the default settings of CLUSTAL X. The complete alignments were performed using PHYDIT and manual comparison to secondary structures. The phylogenetic analysis was performed with PHYLIP. Phylogenetic trees were inferred using the Fitch-Margoliash, maximum-likelihood, maximum-parsimony and neighbor-joining algorithms. The robustness of the topology in the phylogenetic trees was evaluated by bootstrap analysis of the neighbor-joining method based on 1000 re-sampling.

2.3. Culture conditions

S. echinoides EH-983 was cultured on a MB medium [19,20] for 36 h. The optimal temperature range for cell growth was determined by using a temperature gradient incubator (TVS126MA, Advantec). The effect of pH on cell growth was studied by adjusting the pH of the MB medium using the following buffers (Sigma) at 20 mM: 2-morpholinoethanesulfonic acid (MES) buffer for pH 4.5–5.5; piperazine-*N*,*N*′-bis (2-ethanesulfonic acid) (PIPES) buffer for pH 6–6.5; *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer for pH 7 and 7.5; 3-(1,1dimethyl-2hydroxylethyl)-amino-2-hydroxy propanesulfonic acid (AMPSO) buffer for pH 8, 9, and 10. The requirement of NaCl was tested using the modified MB that contains 5 g/l bactopeptone, 1 g/l yeast extract, 0.01 g/l $FePO_4{\cdot}4H_2O$ and different concentration of NaCl. To analyze the effects of culture temperature, specific growth rates were determined at early growth phase of batch cultures at given ranges of temperatures from 10 to 45 °C.

2.4. Enantioselective hydrolysis reaction and determination of optimal reaction conditions

Fully grown cells were harvested and suspended in 100 mM KH_2PO_4 buffer at 40 mg DCW (dry cell weight)/ml otherwise specified in the text. The cell suspension of 5 ml was placed in a 10 ml screw-cap bottle sealed with a rubber septum, and added with racemic styrene oxides at 10 mM to start the reaction. The reaction was performed in a shaking incubator at 250 rpm. To stop the reaction, 5 ml of diethyl ether was added to the reaction mixture. The progression of enantioselective hydrolysis was monitored by the analysis of samples withdrawn periodically from the reaction mixture in a 10 ml bottle with a given cell mass as described in Ref. [13].

To analyze the effects of pH, temperature, and initial substrate concentrations, enantioselective hydrolysis reactions were carried out as described above at given ranges of the variables. The pH ranged from 6 to 9, and the temperature ranged from 15 to 37 °C. The initial concentrations of racemic styrene oxide were in the range from 10 to 80 mM.

2.5. Analyses

Cell concentration was measured by a spectrometer at 600 nm (UV1240, Shimazhu, Japan). Enantiopure epoxides were analyzed by a chiral GC with a fused silica capillary beta-DEX 120 column (30 m length, 0.25 mm i.d., and 0.25 μm film thickness, Supelco Inc., USA) and a FID detector. The temperatures of the column, injector, and detector were 100, 220, and 220 °C, respectively. Enantiomeric excess (e.e. = $100 \times (S-R)/(S+R)$) and yield for enantiopure styrene oxide were determined by chiral GC analysis. The absolute configuration of the remaining epoxides and diols were analyzed by chiral GC and HPLC analysis, and assigned by comparison with reference epoxides and diols [10].

3. Results and discussion

3.1. Isolation of a marine microorganism possessing enantioselective EH activity

Two colorimetric assay methods were employed to isolate microorganisms possessing an EH activity from marine environment. One assay method is based on the formation of blue color between epoxide and NBP. If a strain possesses EH activity, the concentration of epoxide in a reaction mixture decreases

and the intensity of blue color also decreases [17]. The second assay method is based on the change in a maximal absorption of UV spectra of the reaction mixture during the oxidation of the diol product to the corresponding aldehyde by periodate [18]. Both methods were developed originally for the enzymatic activity of EH and modified to detect whole-cell EH activity in the present study [21]. Seawater samples were taken from the costal area of Mokpo North Harbor, Korea. From agar plate culture, four strains were identified to have the colorimetric activity with both NBP and periodate. Among them, the isolate EH-983 was selected for further investigation since it showed the highest activity in the periodate oxidation assay.

The enantioselectivity of the EH activity of the isolate EH-983 was studied with racemic styrene oxides as substrates. The cells were grown in MB medium for 36 h, harvested, and then resuspended in 100 mM phosphate buffer containing racemic epoxide substrates. When incubated with the EH-983, the (*R*)-enantiomer was preferentially degraded, leaving most of the (*S*)-enantiomer undegraded. This indicates that the EH of the isolate EH-983 possesses an enantiopreference toward (*R*)-styrene oxide. The substrate specificities of the EH on other epoxides have also been investigated (Table 1). It was observed that the enantioselectivity of the EH of EH-983 varied significantly depending on the structure of the epoxide. For epichlorohydrin that is a short aliphatic epoxide, (*S*)-enantiopreference was

Table 1
Substrate specificity of the isolated marine microorganism possessing EH activity

Substrate	Hydrolysis activity[a]	Enantiomeric excess[b]
Styrene oxide	+++	+++
p-Nitrostyrene oxide	++	+++
p-Chlorostyrene oxide	++	+++
Epichlorohydrin	+	++
Glycidol	–	–

[a] Hydrolysis activity denoted as: (+++) excellent [relative hydrolysis rate = 100%]; (++) good [relative hydrolysis rate = 80–100%]; (+) moderate [relative hydrolysis rate = 50–80%]; (−) low [relative hydrolysis rate = 0–50%].

[b] Enantiomeric excess denoted as: (+++) excellent [relative e.e. = 100%]; (++) good [relative e.e. = 80–100%]; (+) moderate [relative e.e. = 50–80%]; (–) low [relative e.e. = 0–50%].

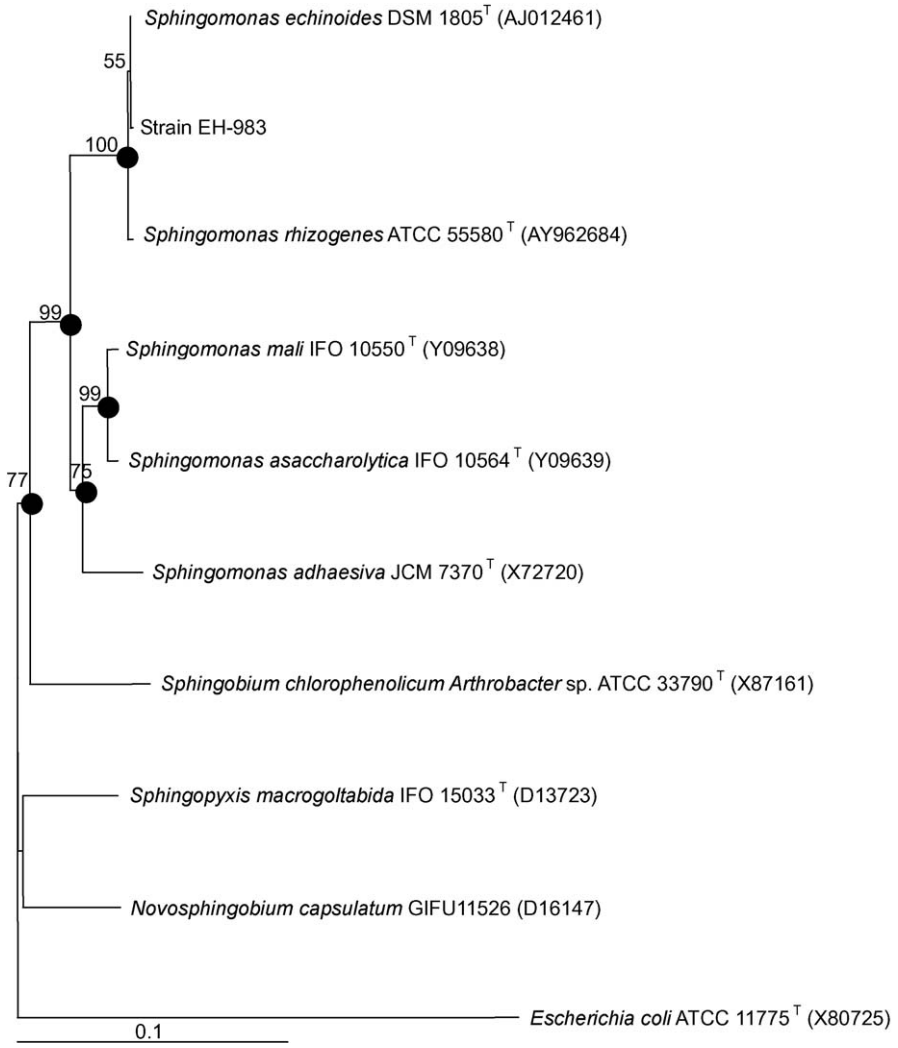

Fig. 1. Neighbor-joining tree showing the phylogenetic position of the strain EH-983 and related taxa based on 16S rDNA gene sequences. Numbers at the nodes are the levels of bootstrap support based on neighbor-joining analyses of 1000 re-sampled data sets; only values over 50% are given. Solid circles indicate clades that were also supported by the Fitch-Margoliash, maximum-parsimony, and maximum-likelihood trees. The scale bar indicates 0.1 substitutions per nucleotide position.

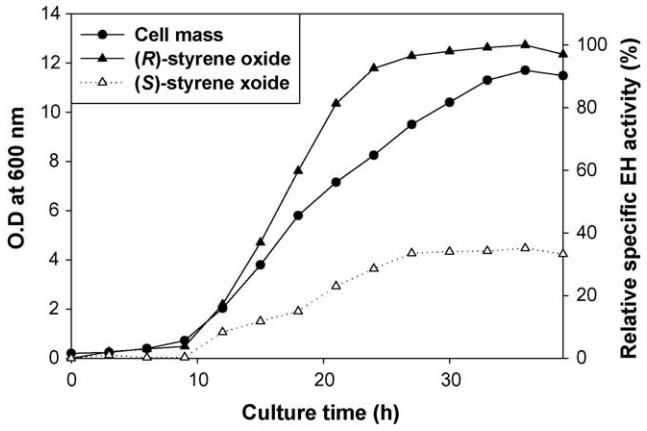

Fig. 2. Time course of cell growth and relative EH activity of *Sphingomonas echinoides* EH-983. Relative EH activity was calculated by dividing the value of the EH activity at the given time by that of the best result.

observed. For aromatic epoxides such as *p*-nitrostyrene oxide and *p*-chlorostyrene oxide, on the other hand, (*R*)-specific activity was observed. In case of glycidol, the EH showed no hydrolysis activity.

3.2. 16S rDNA analysis and identification of the isolate

To study the phylogenetic affiliation of the isolate, the nearly complete 16S rDNA (1381 nucleotides) was sequenced. The subsequent BLAST analysis on sequence similarity indicated that the closest relatives of the strain EH-983 were *Sphingomonas echinoides* DSM 1805^T (99.9%), *Sphingomonas rhizogenes* ATCC 55580^T (99.7%) and *Sphingomonas mali* IFO 10550^T (96.7%). Fig. 1 shows the phylogenetic tree constructed using the neighbor-joining method based on the comparative sequence analysis of 16S rDNA. On the basis of phylogeny analysis it was concluded that the strain EH-983 belongs to the genus *Sphingomonas*, and was designated as *S. echinoides* EH-983.

3.3. Cell growth and EH activity

Microbial EH is usually associated with the metabolism of secondary metabolites or carbon substrates assimilation, thus the expression and the activity of the EH depend on the growth stage of the cell [22–24]. In order to study the relationship between cell growth and EH activity, batch experiment was conducted with *S. echinoides* EH-983. As shown in Fig. 2, the EH activity of *S. echinoides* EH-983 increased with culture time, and then reached the maximum specific activity of 2.6 nmol/min mg DCW at the early stationary growth phase. The EH activity remained constant after the cells entered a stationary growth phase for 10 h and then decreased down, indicating that cells should be harvested at the stationary phase about 30 h.

We investigated the effects of culture temperature and pH on the specific growth rate of *S. echinoides* EH-983. As shown in Fig. 3A, *S. echinoides* EH-983 grew well between 16 and 42 °C with an optimal growth at 35 °C. *S. echinoides* EH-983 could grow in a wide range of pH between 4.5 and 8.5 with an optimal growth between 5.5 and 7.0 (Fig. 3B).

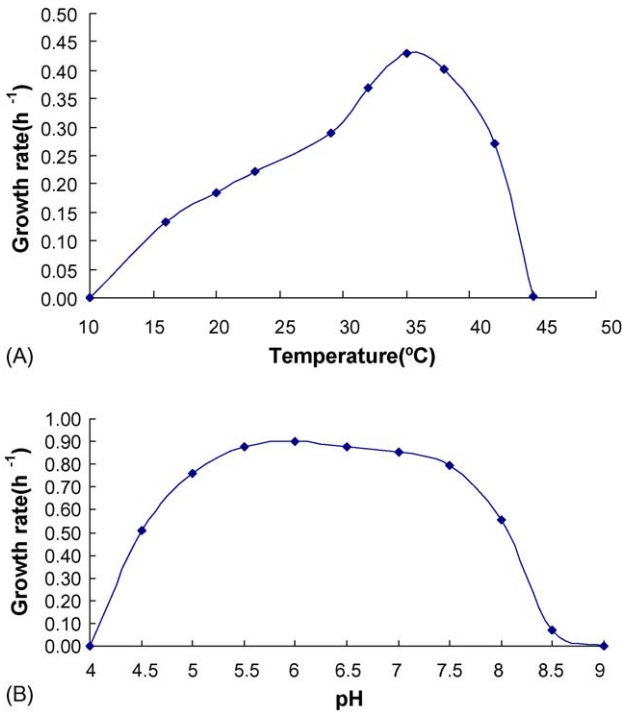

Fig. 3. Effect of culture temperature (A) and culture pH (B) on the growth rate of *S. echinoides* EH-983.

3.4. Characterization of the whole-cell EH activity of S. echinoides EH-983

The effects of pH, temperature and initial substrate concentration on the hydrolysis rate and enantioselectivity of the whole-cell EH activity of *S. echinoides* EH-983 were investigated. Racemic styrene oxide was used as a substrate. The pH dependence was studied in the range of pH 6–9. The EH activity of *S. echinoides* EH-983 showed pH optimum of 7.0 (data not shown). In case of reaction temperature, the EH of *S. echinoides* EH-983 appeared to be active at a broad range of temperature (Fig. 4). The EH activity at low temperature, 20 °C, exhibited same level of activity at 30 °C, whereas most EHs from soil microorganisms have temperature optima between 30 and 40 °C. Reaction temperature has a significant influence on kinetic resolution of racemic epoxides by EHs [13,25]. It has been reported in many cases that yield and enantioselectivity of biocatalytic reactions increase as reaction temperature decreases [25]. Therefore, it is of advantage to carry out kinetic resolutions at low temperature using EHs exhibiting a high activity at low temperature. The favorable effect of temperature on enantioselectivity can be readily analyzed by measuring the *E* ratio, defined as $E = (\ln R_0/R)/(\ln S_0/S)$ where S_0 and R_0 represent concentrations at time 0, and estimated from the slope in the plot of $(\ln R_0/R)$ against $(\ln S_0/S)$. In case of *S. echinoides* EH-983, although the initial hydrolysis rates at 20 and 30 °C were similar each other, the *E*-value of 3.4 at 20 °C was 30.7% higher than that at 30 °C.

Enantioselective hydrolysis by *S. echinoides* EH-983 was carried out at various initial substrate concentrations ranging from 10 to 80 mM. All of the reactions were performed at pH 7.0 and temperature 20 °C. Enantiopurity of more than 99% e.e.

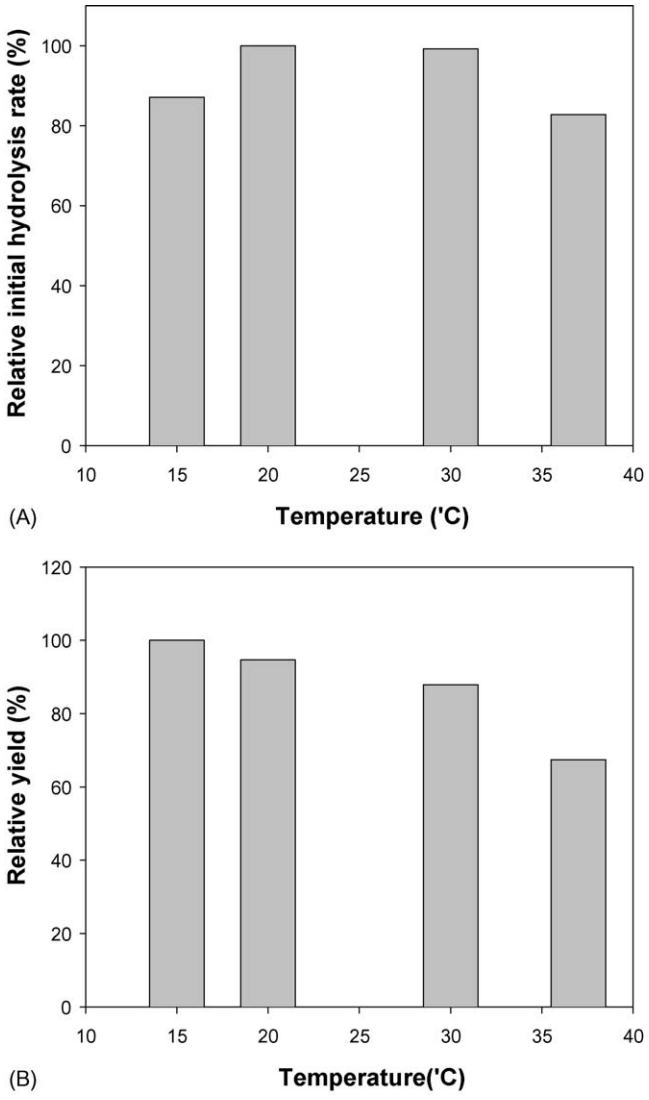

Fig. 4. Effect of reaction temperature on the relative hydrolysis rate (A) and the relative yield (B) of racemic styrene oxide in the enantioselective hydrolysis by whole cells of *S. echinoides* EH-983. The relative hydrolysis rate and yield were calculated by dividing the values of the hydrolysis rate and yield at the given time by those of the best result, respectively.

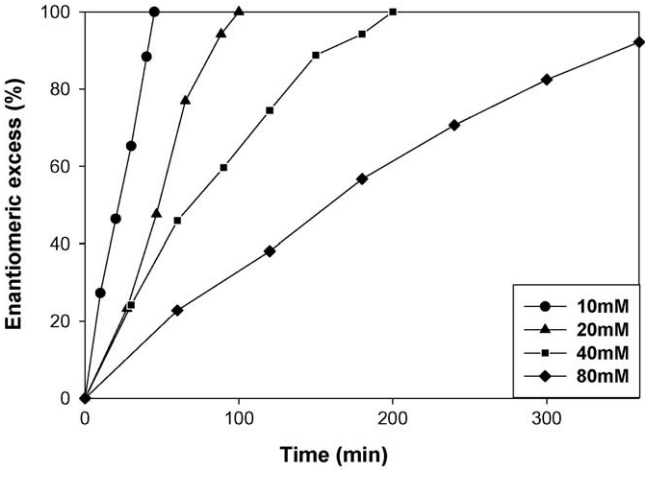

Fig. 5. Time course of enantiomeric excess for different substrate concentrations of the hydrolysis of styrene oxide by whole cells of *S. echinoides* EH-983.

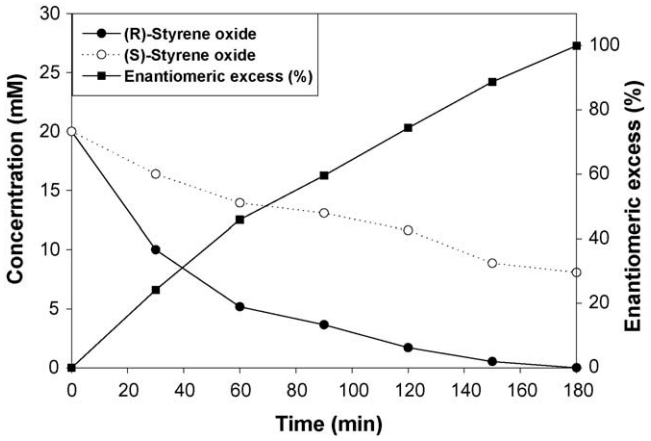

Fig. 6. Batch kinetic resolution of 40 mM racemic styrene oxide to produce (*S*)-styrene oxide using enantioselective hydrolysis activity of whole cells of *S. echinoides* EH-983.

was obtained for up to 40 mM initial concentrations (Fig. 5). In the case of 40 mM, the enantiopurity of (*S*)-styrene oxide increased from 0 to 99% after 180 min with the final yield of 21.3% (Fig. 6), demonstrating that EH activity of *S. echinoides* EH-983, a microorganism isolated from marine environment, can be successfully applied as a biocatalyst to prepare enantiopure epoxides such as styrene oxides.

4. Conclusion

A marine microorganism was isolated and characterized to identify EH activity from seawater samples. Based on the comparative sequence analysis of 16S rDNA, the isolate was designated as *S. echinoides* EH-983, the first isolated marine microorganism exhibiting EH activity from seawater, to our best knowledge. We demonstrated the enantioselective resolution of racemic epoxides by EH activity of *S. echinoides* EH-983. Enantiopure (*S*)-styrene oxide was prepared from its racemate, with a yield of 21.3% from an initial concentration of 40 mM using the whole-cells of *S. echinoides* EH-983 as a biocatalyst. EH of *S. echinoides* EH-983 is under investigation in more detail in the enantioselective resolution of a wide range of racemic epoxides and molecular basis of EH gene.

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