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# Isolation, identification and characterization of marine bacteria exhibiting complementary enantioselective epoxide hydrolase activity for preparing chiral chlorinated styrene oxide derivatives

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ABSTRACT

Marine bacteria possessing epoxide hydrolase (EH) activity were isolated from the oil-spilled foreshore of South Korea. While the isolated *Rhodococcus* sp. YSMI04 and YSNA32 showed different enantioselectivities to racemic styrene oxide (SO), two strains exhibited same enantiopreference to 3-chlorostyrene oxide (3-CSO) and 4-CSO. In the case of 3-CSO, *Rhodococcus* sp. YSMI04 and YSNA32 exhibited (*R*)-3-CSO preferred hydrolysis activity, whereas *Roseobacter* sp. TSBP12 showed a preference for (*S*)-3-CSO. In the case of 4-CSO, *Rhodococcus* sp. YSMI04 and YSNA32 showed enantiopreference to (*S*)-4-CSO, *Roseobacter* sp. TSBP12 exhibited (*R*)-4-CSO preferred activity. (*S*)-4-CSO was obtained with 35% yield (theoretically 50%) using *Roseobacter* sp. TSBP12.



## Introduction

Pharmaceutical compounds possessing chiral carbon have been developing as single enantiomers because one specific enantiomer generally exhibits pharmacological activity [1]. Chiral intermediates such as chiral epoxides and diols play a key role in the production of chiral pharmaceuticals [2]. Chiral epoxides can be produced using epoxide hydrolase (EH, EC 3.3.2.9 or EC 3.3.2.10)-catalyzed kinetic resolution of cheap racemic epoxides [3]. EH catalyzes enantioselective hydrolysis of the reactive three-membered epoxide ring. EH is an easy-to-use enzyme because troublesome NADH regeneration is not required and recombinant whole cells expressing EH can be used as a biocatalyst [4]. Immobilized EHs have been employed for the production of chiral epoxides [5–7].

At present, chiral epoxide production using EH has not yet been implemented in industry. In order to produce specific chiral epoxides, various types of EHs need to be developed for customized production of target chiral epoxides. Enantioselectivity of EH is the key feature for the customized biocatalyst. Molecular engineering for the modulation of the enantioselectivity and activity of EHs based on protein engineering and directed evolution have been investigated [8,9]. As a practical approach for recruiting EHs with desired enantioselectivities, isolation of microbial strains possessing various enantioselectivities can be considered. There is still much chance for isolation of positive microbial strains from various sites, although various EHs have been identified from various microorganisms, animals and plants [10–12]. Isolation of novel microorganisms from marine environments and unique contaminated sites has been increasingly investigated [13,14].

Recently, we isolated and characterized a novel marine microbial bacterium with enantioselective EH activity from gasoline and polycyclic aromatic hydrocarbon (PAH) [15]. In this study, we isolated and identified marine microbial strains possessing enantioselective EH activity for styrene oxide and its chlorinated derivatives from the oil-spilled foreshore of South Korea (Byeonsan, Gunsan, Taean, and Yeosu) after enrichment with PAH. Enantioselectivity of the isolated microbial strains was analyzed, and batch kinetic resolution of racemic styrene oxide and its chlorinated derivatives was investigated.

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## Experimental

### Chemicals

Culture medium components were purchased from Difco Co. (USA). Styrene oxide (SO) and 4-chlorostyrene oxide (4-CSO) were purchased from Aldrich Co. (St. Louis, MO, USA). 2-Chlorostyrene oxide (2-CSO) and 3-chlorostyrene oxide (3-CSO) were purchased from RStec Co. (Daejeon, Korea).

### Isolation of microbial strains from the oil-spilled foreshore and cell culture for preparation of whole cells biocatalyst

Marine broth medium (Difco) and mineral salt medium (MSM) ($NaNO_3$, 4 g/L; $KH_2PO_4$, 1.5 g/L; $FeCl_3{\cdot}6H_2O$, 0.005 g/L; $MgSO_4$, 0.2 g/L; $CaCl{\cdot}2H_2O$, 0.01 g/L; $Na_2HPO_4$, 0.5 g/L; pH 7.2) were used for isolation and culture of the strains. Various microbial strains were isolated from the oil-spilled foreshore of South Korea (Byeonsan, Gunsan, Taean and Yeosu) and then enriched with PAH as a carbon source.

The isolated microbial strains with EH activity were cultured in marine broth medium at 25 °C for 3–4 days in a shaking incubator (180 rpm). The cells were harvested, washed twice with 100 mM Tris–HCl buffer (pH 8.0), and used for the enantioselective hydrolysis reaction.

### Enantioselective hydrolysis of racemic styrene oxide derivatives and chiral GC analysis of the reaction products

Enantioselective hydrolysis of 4 mM racemic SO, 2-CSO, 3-CSO and 4-CSO was conducted using dried whole cells (10 mg) in 10 mL vials containing 1 mL of 100 mM Tris–HCl (pH 8.0) in a shaking incubator (25 °C, 200 rpm). The residual epoxides were extracted with 2 mL hexane, and the organics phase was analyzed using chiral GC with cyclodextrin β-DEX 120 (30 m length, 0.25 mm ID, and 0.25 μm film thickness; Supelco, USA) and FID detector. The temperature of injector and detector was 180 °C. Enantiomeric excess (*ee*) and yield were determined as follows: *ee* (%) = {(*S*)-enantiomer − (*R*)-enantiomer}/{(*S*)-enantiomer + (*R*)-enantiomer} × 100, yield (%) = [{residual (*S*)-enantiomer}/{initial racemic (*S*, *R*)-enantiomer}] × 100 (theoretical yield = 50%), and $E = \ln\{(1-c)(1-ee_s)\}/\ln\{(1-c)(1+ee_s)\}$].

### Nucleotide sequence submission and strain deposition

The nucleotide sequences of 16S rRNA genes of the three strains (*Rhodococcus* sp. YSMI04, *Rhodococcus* sp. YSNA32 and *Roseobacter* sp. TSBP12) were submitted to the GenBank database with accession numbers of JX522530, JX522531 and JX522532, respectively. All strains isolated in this study were deposited to Korean Collection for Type Cultures (KCTC) (*Rhodococcus* sp. YSMI04, KCTC 12263BP; *Rhodococcus* sp. YSNA32, KCTC 12260BP; *Roseobacter* sp. TSBP12, KCTC 12261BP).

## Results and discussion

### Isolation, strain identification and evaluation of marine microbial strains possessing epoxide-degrading activity from oil-spilled foreshore

We have isolated various marine microbial strains from the oil-spilled foreshores of South Korea (Byeonsan, Gunsan, Taean, and Yeosu) [15]. For enrichment experiment, PAH was used as the sole carbon substrate. Among the isolated strains, microbial strains possessing epoxide-degrading activity were further screened using 10 mg dry cells and racemic SO substrate in 100 mM Tris–HCl (pH 8.0). When the residual SO extracted with hexane from the reaction mixtures were analyzed, two strains (YSMI04 and YSNA32) showed different enantioselectivity toward two enantiomers of SO. Interestingly, the isolated strain TSBP12 completely degraded both of the enantiomers of racemic SO in 2 h (Table 1), and 80% of the substrate was degraded within 1 h, indicating that TSBP12 might possess highly active EH although the EH has no enantiopreference for SO. In contrast, at least 16 h was required to completely degrade one of the enantiomers of racemic SO by strain YSMI04 and YSNA32 (data not shown). Although the EH did not show any enantioselectivity for SO, we further characterized the strain TSBP12 due to its high degrading activity.

The strain YSMI04, YSNA32, and TSBP12 were identified as *Rhodococcus* sp., *Rhodococcus* sp., and *Roseobacter* sp., respectively, based on 16S rRNA sequence analysis. Phylogenetic trees were constructed based on 1358–1434 base pairs of 16S rRNA of the strain YSMI04 and YSNA32. The strain YSMI04 and YSNA32 showed 99.93 and 99.77% sequence similarity with *Rhodococcus* sp. M1 5-2 (1414 bp, AY762055), respectively (Supplementary Fig. 1). A phylogenetic tree of the strain TSBP12 was constructed from 1399 base pairs of 16S rRNA (Supplementary Fig. 2), and the strain TSBP12 showed 99.92% sequence similarity with *Roseobacter* sp. 204Z-13 (1435 bp, GU584169) and *Celeribacter* sp. L-6 (1423 bp, HM997022). From the phylogenetic analysis, we designated the strains of YSMI04, YSNA32, and TSBP12 as *Rhodococcus* sp. YSMI04 (GenBank accession number, JX522530), *Rhodococcus* sp. YSNA32 (JX522531), and *Roseobacter* sp. TSBP12 (JX522532), respectively. Herein, *Roseobacter* sp. TSBP12 was identified as a strict marine microorganism.

### Enantioselectivity analysis of Rhodococcus sp. YSMI04, Rhodococcus sp. YSNA32, and Roseobacter sp. TSBP12 for various styrene oxide derivatives

The enantioselectivity of *Rhodococcus* sp. YSMI04, *Rhodococcus* sp. YSNA32, and *Roseobacter* sp. TSBP12 toward SO, 2-CSO, 3-CSO, and 4-CSO was investigated (Fig. 1). Interestingly, *Rhodococcus* sp. YSMI04 and *Rhodococcus* sp. YSNA32 showed different enantiopreferences for SO even though they are included in same genus. *Rhodococcus* sp. YSMI04 exhibited (*R*)-styrene oxide specificity, while *Rhodococcus* sp. YSNA32 showed (*S*)-styrene oxide enantiopreference (Table 1).

In the case of 2-CSO, all of three strains did not show any enantiopreference (Table 1). When 3-CSO was supplied, *Roseobacter* sp. TSBP12 had (*S*)-3-CSO-preferred activity, whereas both of *Rhodococcus* sp. YSMI04 and *Rhodococcus* sp. YSNA32 showed (*R*)-3-CSO-specific enantiopreference. In the case of 4-CSO, *Rhodococcus* species and *Roseobacter* species showed reverse enantioselectivity, compared to those for 3-CSO. *Roseobacter* sp. TSBP12 showed enantioselectivity toward (*R*)-4-CSO, whereas both of *Rhodococcus* sp. YSMI04 and *Rhodococcus* sp. YSNA32 exhibited (*S*)-4-CSO-preferred enantioselectivity.

**Table 1**
Enantioselective epoxide hydrolase activity of the isolated strains from oil-contaminated sediments toward styrene oxide and its derivatives.

| Strains | Hydrolysis rate ($\times 10^{-2}$) mg/h | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | SO | | 2-CSO | | 3-CSO | | 4-CSO | |
| | (*R*) | (*S*) | (*R*) | (*S*) | (*R*) | (*S*) | (*R*) | (*S*) |
| YSMI04[a] (*Rhodococcus* sp. YSMI04) | 2.48 | 2.38 | 0.42 | 0.43 | 1.71 | 0.28 | 1.33 | 1.74 |
| YSNA32[a] (*Rhodococcus* sp. YSNA32) | 1.45 | 1.67 | 0.03 | 0.07 | 1.04 | 0.44 | 1.13 | 1.66 |
| TSBP12[b] (*Roseobacter* sp. TSBP12) | 36.84 | 36.05 | 0.26 | 0.20 | 0.95 | 1.79 | 1.64 | 0.30 |

[a] Using whole cells (dry weight, 10 mg) and reaction time for 16 h.
[b] Using whole cells (dry weight, 10 mg) and reaction time for 2 h.

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