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Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 17 (2006) 47–52

Enantioselective hydrolysis of styrene oxide with the epoxide hydrolase of *Sphingomonas* sp. HXN-200

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Received 6 October 2005; accepted 17 November 2005
Available online 9 January 2006

Abstract—The soluble bacterial epoxide hydrolase (EH) from *Sphingomonas* sp. HXN-200 catalyzed the enantioselective hydrolysis of racemic styrene oxide to give (*S*)-styrene oxide with an enantiomeric ratio (*E*) of 21–23 in aqueous buffer, better than any reported native EHs. The ring opening of the styrene oxide with this EH was only at the terminal position for the (*S*)-enantiomer and at the terminal and benzylic position in an 87:13 ratio for the (*R*)-enantiomer. Enzymatic hydrolysis of the styrene oxide in a two-liquid phase system significantly reduced autohydrolysis, thus improving the *E* to 26–29. Hydrolysis of 160 mM styrene oxide with cell-free extract (CFE) of *Sphingomonas* sp. HXN-200 (10 mg protein/mL) in aqueous buffer and *n*-hexane (1:1) for 30.7 h afforded 39.2% (62.7 mM) of (*S*)-styrene oxide in >99.9% ee. The lyophilized CFE was proven to be stable, while the rehydrated lyophilized CFE powder was successfully used for the hydrolysis of 320 mM styrene oxide in the two-liquid phase system, yielding 40.2% (128.6 mM) of (*S*)-styrene oxide in >99.9% ee after 13.8 h. No inhibitory effect of the diol product on the hydrolysis was observed when the diol concentration was lower than 476 mM, suggesting a straightforward process for the hydrolysis of up to 1 M styrene oxide.

1. Introduction

Epoxide hydrolase (EH) catalyzes the enantioselective hydrolysis of either racemic or meso-epoxides, providing a useful and green method for the preparation of enantiopure epoxides or vicinal diols that are useful synthons and pharmaceutical intermediates. EH is cofactor independent and relatively stable, and many EHs have been discovered from various microorganisms for synthetic applications.[1] A very interesting target reaction for EH is the enantioselective hydrolysis of racemic styrene oxide, since the product, enantiopure styrene oxide, is a valuable and very useful synthon for the preparation of different types of enantiopure compounds.[2] Moreover, (*S*)-styrene oxide is a useful intermediate for the synthesis of Levamisole, a nematocide[3a] and anticancer agent.[3b] Several EHs have so far been reported for the hydrolysis of styrene oxide.[4–12] Among them, EHs from *Beauvaria sulfurescens* ATCC7159[4] and *Streptomyces Antibioticus* Tü 4[5] demonstrated (*S*)-enantioselectivity in the hydrolysis giving (*R*)-styrene oxide in high ee. A higher enantioselectivity was observed for the latter EH with an enantiomeric ratio (*E*) of 31, but the activity was low.[5] On the other hand, some EHs showed an (*R*)-enantioselectivity for the hydrolysis affording enantiopure (*S*)-styrene oxide (Table 1). These include the EHs from microsome (rabbit liver mEH[6]), yeasts (*Rhodotorula glutinis* CIMW147[7] and *Rhodosporidium kratochvilovae* SYU-08[8]), fungi (*Aspergillus niger* LCP521[4] and new *Aspergillus niger* isolate[9]), and a bacterium (*Agrobacterium radiobacter* AD1[10]). The bacterial EH of *A. radiobacter* AD1 showed the best enantioselectivity, with an *E* of 14–16. Methods for improving the enantioselectivity of the existing EHs have also been developed: the EH of *A. radiobacter* AD1 was engineered to create the Y215F mutant, which gave an *E* of 30–32;[11] and immobilization of the multimeric EH of *A. niger* LCP521 onto Eupergit C/CDE increased also the enantioselectivity.[12] These approaches could have great synthetic potential. Herein, we report a novel soluble bacterial EH from *Sphingomonas* sp. HXN-200[13] for the hydrolysis of styrene oxide with higher (*R*)-enantioselectivity than any other known native EHs and the application of this EH in a two-liquid phase system to prepare enantiopure (*S*)-styrene oxide.

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doi:10.1016/j.tetasy.2005.11.018

Table 1. Enantioselective hydrolysis of styrene oxide with known native epoxide hydrolases for the preparation of (*S*)-styrene oxide

Epoxide hydrolase	Ref.	Cat. form	Cat. conc. (g/L)[b]	(±)-**1** conc. (mM)	Temp. (°C)	pH	Time (h)	(*S*)-**1** ee (%)	Yield/ Conv. (%)	*E*[a]
Rabbit liver mEH	6	Enzyme	5	5	37	7.4	n.i.[c]	98	22	7
Rhodotorula glutinis CIMW147	7a	Cells	50–75	10	35	7.5	0.8	98	18	6
Rhodotorula glutinis CIMW147	7b	Cells	21	526	4	8.0	16	98	36	15
Rhodosporidium kratochvilovae SYU-08	8	Cells	n.i.[c]	80	35	8.0	2.7	99	19	7
Aspergillus niger LCP521	4	Cells	8	8	27	8.0	2	99	28	10
Asperginus niger isolate	9	Cells	75[d]	10	30	8.0	6	100	32	13[e]
Agrobacterium radiobacter AD1	10a	Enzyme	0.025–0.125	5	30	9.0	0.8	99	33	16

[a] Enantiomeric ratio *E* was calculated by $E = \ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$,[15] *c*: conversion, ee_s: ee of substrate.
[b] g cell dry weight/L and g protein/L for whole cells and enzyme preparation, respectively.
[c] No information.
[d] g wet cell weight/L.
[e] Calculated with an ee of 99%.

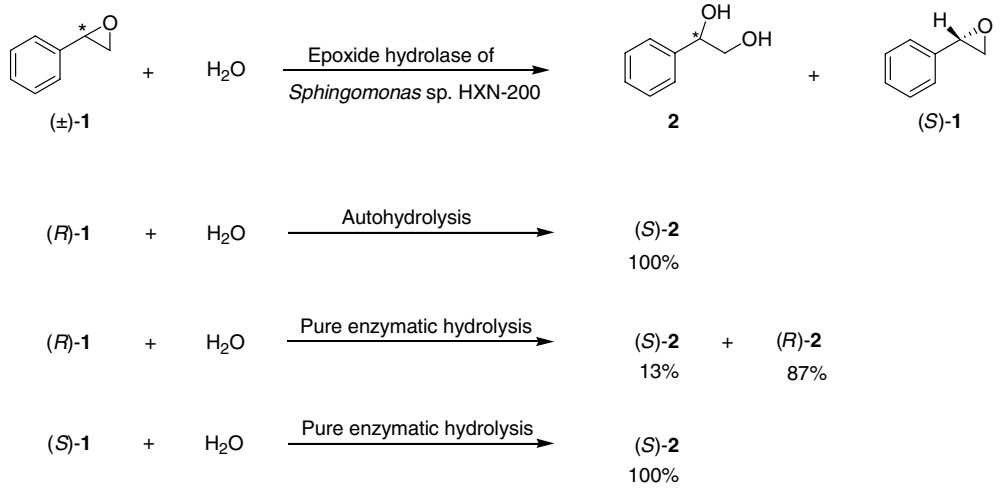
2. Results and discussion

We recently discovered that *Sphingomonas* sp. HXN-200 contains a novel soluble EH, which catalyzes the enantioselective hydrolysis of meso-alicyclic epoxides[13a] as well as racemic alicyclic epoxides.[13b] For the examination of hydrolysis of styrene oxide with this EH, the cells of *Sphingomonas* sp. HXN-200 were prepared by growing on *n*-octane in E2 medium.[14] The cell free extract (CFE) was prepared by passing the cell suspension in 50 mM Tris–HCl buffer (pH = 7.5) through a French press and removing the cell debris by ultracentrifugation. The resulting CFE was used in different concentrations, determined by Bradford protein assay,[16] for the hydrolysis experiment.

2.1. Hydrolysis of styrene oxides (*S*)-1, (*R*)-1, and racemic 1 with CFE of *Sphingomonas* sp. HXN-200 in aqueous buffer

To examine the selectivity for the hydrolysis, pure styrene oxide (*R*)-**1** (9.5 mM) and (*S*)-**1** (9.4 mM) were used as the substrates. Biotransformations were performed with CFE (9.8 g protein/L) in 10 mL 50 mM Tris–HCl buffer (pH = 7.5) at 25 °C and 300 rpm. The conversion and ee of diol **2** were analyzed by HPLC with a C18 column and a chiralpark AS column, respectively. In the hydrolysis of epoxide (*R*)-**1**, the reaction was complete after 2 h, with a specific activity of 9.3 U/g protein (U = μmol/min) over the first hour. This gave 83% of (*R*)-diol **2** and 17% of (*S*)-diol **2**. For the hydrolysis of epoxide (*S*)-**1**, the reaction rate was slower, with an activity of 5.6 U/g protein. Styrene oxide (*S*)-**1** was completely converted to diol **2** after 4 h. After 2 h, a conversion of 72% was reached with the formation of diol (*S*)-**2** and (*R*)-**2** in a 94:6 ratio. To examine non-enzymatic hydrolysis, 10 mL CFE (9.8 mg/mL) in buffer (pH = 7.5) was first boiled for 45 min and then used for the hydrolysis of 11.9 mM epoxide (*R*)-**1**. This gave diol (*S*)-**2** as the sole product, confirming the ring opening of styrene oxide at the benzylic position for non-enzymatic hydrolysis; 4.2% of diol was formed after 2 h. The non-enzymatic hydrolysis suggested that (*S*)-epoxide **1** was enzymatically opened only at the terminal position, while (*R*)-epoxide **1** was enzymatically opened at the terminal and benzylic position in a 87:13 ratio (Scheme 1).

To explore the enzymatic resolution of styrene oxide with this EH, 20 mM racemic **1** was hydrolyzed with CFE (10 g protein/L) under the same conditions as described above. As shown in Figure 1, the hydrolysis



Scheme 1.

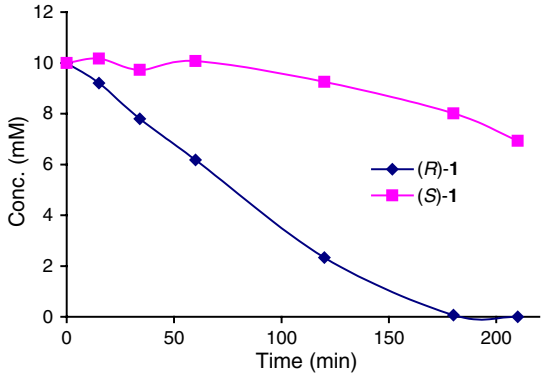


Figure 1. Enantioselective hydrolysis of 20 mM racemic styrene oxide **1** with a cell-free extract of *Sphingomonas* sp. HXN-200 (10 g protein/L) in 50 mM Tris–HCl buffer (pH = 7.5).

of the (*R*)-enantiomer is much faster than that of the (*S*)-enantiomer, leaving 40% of the unreacted epoxide (*S*)-**1** in 98.4% ee at 3 h and 34.6% in >99.9% ee at 3.5 h. From these data, an *E* of 23 was calculated, which is higher than that of any native EHs for this reaction reported thus far. As described above, the difference between the reaction rates for the hydrolysis of pure epoxide (*R*)-**1** and (*S*)-**1** is not very big. Thus, K_m of (*R*)-**1** should be much smaller than that of (*S*)-**1**.

To further explore the synthetic potential of this EH, higher substrate concentration was examined. As listed in Table 2, a similar enantioselectivity ($E = 21$) was obtained with 40 mM (±)-**1**, leading to the formation of 38.4% of epoxide (*S*)-**1** in 98.8% ee after 5.8 h reaction. Increasing the substrate concentration to 160 mM resulted in a significant decrease in enantioselectivity. After 23.7 h, 37.2% of epoxide (*S*)-**1** was obtained in 93.8% ee, corresponding to an *E* of 13. The decrease in the enantioselectivity with higher substrate concentration at a given CFE concentration was expected while the velocity of real enzymatic hydrolysis remains the same. The velocity of the autohydrolysis increased with substrate concentration.

2.2. Hydrolysis of racemic styrene oxide 1 with CFE of *Sphingomonas* sp. HXN-200 in a two-phase system

To reduce the rate of autohydrolysis, a two-liquid phase system containing buffer and *n*-hexane (1:1) was applied for the biotransformation. The non-enzymatic hydrolysis in this system was first examined. Tris–HCl buffer (50 mM; pH = 7.5) containing CFE (10 g protein/L) was boiled for 45 min. The solution was then mixed with an equal volume of *n*-hexane containing 160 mM racemic styrene oxide **1**, followed by shaking at 25 °C and 300 rpm. After 68.5 h, 7.0% of diol **2** was formed, which corresponds to an autohydrolysis rate of 0.1% per hour. This is a significantly reduced rate compared to that in buffer. Hydrolysis of 160 mM racemic epoxide **1** was performed with 10 mL buffer containing CFE (10 g protein/L) and 10 mL *n*-hexane. As shown in Figure 2, the reaction rates for both enantiomers were slightly slower in the two-phase system than in the aqueous buffer. This was possibly due to the decreased autohydrolysis rates. The enantioselectivity in the two-phase system was much higher ($E = 26$–29) than that in buffer ($E = 13$). Finally epoxide (*S*)-**1** was formed in >99.9% ee and 39.2% (62.7 mM).

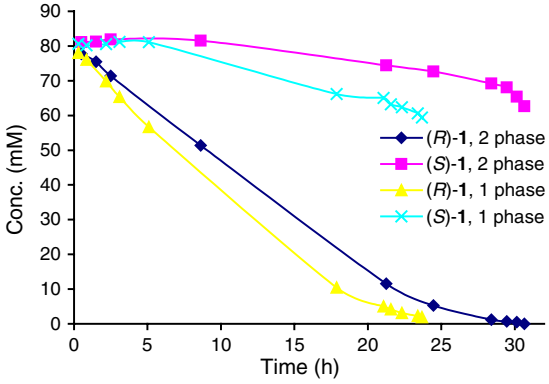


Figure 2. Enantioselective hydrolysis of 160 mM racemic styrene oxide **1** with a cell-free extract of *Sphingomonas* sp. HXN-200 (10 g protein/L) in 50 mM Tris–HCl buffer (pH = 7.5) and in buffer/*n*-hexane (1:1), respectively.

Table 2. Preparation of (*S*)-styrene oxide **1** by enantioselective hydrolysis of racemic styrene oxide (±)-**1** with EH of *Sphingomonas* sp. HXN-200

(±)-**1** (mM)	CFE (g prot./L)	Lyophilized CFE (g powder/L)	Phase	Activity[a] (U/g p.)	Time (h)	(*S*)-**1** ee (%)	(*S*)-**1** Conv. (%)	*E*
20	10		One[b]	6.2	3.0	98.4	40.0	23
40	10		One[b]	6.2	5.8	98.8	38.4	21
160	10		One[b]	7.4	23.4	92.8	37.9	13
					23.7	93.8	37.2	13
160	10		Two[c]	5.7	28.4	96.6	43.3	29
					29.5	97.9	42.6	29
					30.1	98.6	40.9	26
					30.7	>99.9	39.2	
320		60	Two[d]	5.0	10.7	87.7	47.4	52
					12.1	96.2	44.9	38
					13.1	98.8	41.6	29
					13.8	>99.9	40.2	

[a] Average activity during the first hour; in U/g protein.
[b] In 10 mL cell-free extract in 50 mM Tris–HCl (pH = 7.5).
[c] In 10 mL buffer and 10 mL *n*-hexane.
[d] In 2 mL buffer and 2 mL *n*-hexane.

2.3. Hydrolysis of racemic styrene oxide 1 with lyophilized/rehydrated CFE of *Sphingomonas* sp. HXN-200 in a two-phase system

The soluble EH of *Sphingomonas* sp. HXN-200 was proven to be stable. Lyophilization of the CFE of this strain resulted in a catalyst powder, which could be easily stored and transported. The lyophilized powder was rehydrated and then used for the hydrolysis of styrene oxide in a higher concentration: 2 mL buffer containing 120 mg lyophilized CFE was mixed with 2 mL *n*-hexane containing 320 mM (±)-**1**. This gave a hydrolysis activity of 5.0 U/g powder, similar to the activity of 5.7 U/g protein obtained with CFE in the same two-phase system. As shown in Figure 3, the reaction velocities for (*R*)- and (*S*)-enantiomers were nearly constant and the former is much bigger than the latter during 11 h, indicating a great enantioselectivity ($E = 52$ at 10.7 h). Similar to the hydrolysis with CFE, as described before, hydrolysis became less enantioselective at the later stages. Nevertheless, styrene oxide (*S*)-**1** was obtained in 41.6% and in 98.8% ee after 13.1 h with an E of 29. Continuing the reaction for 13.8 h afforded (*S*)-**1** in >99.9% ee and 40.2% yield (128.6 mM). This provided us with a useful method for the preparation of (*S*)-styrene oxide.

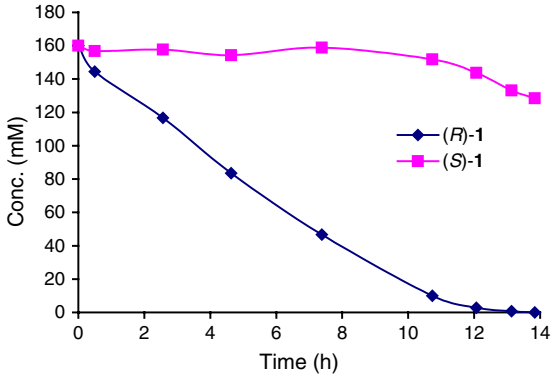


Figure 3. Enantioselective hydrolysis of 320 mM racemic styrene oxide **1** with a lyophilized cell-free extract of *Sphingomonas* sp. HXN-200 (60 g powder/L) in 50 mM Tris–HCl buffer (pH = 7.5) and *n*-hexane (1:1).

2.4. Inhibitory effect of diol 2 on the enzymatic hydrolysis of racemic styrene oxide 1 with CFE of *Sphingomonas* sp. HXN-200

It was known that hydrolysis product diol **2** could inhibit the enzymatic hydrolysis of epoxide **1**. To investigate this effect, racemic diol **2** at different concentrations was added to a CFE suspension (15 g protein/L) in 50 mM Tris–HCl buffer (pH = 7.5). The mixture was then used for the hydrolysis of 15 mM racemic epoxide **1**. As shown in Figure 4, there was no inhibitory effect of the diol on the hydrolysis activity when the concentration of diol **2** was lower than 476 mM. Increasing diol concentration to 521 mM decreased the hydrolysis activity by one third. Thus, it was possible to use as high as 1 M styrene oxide for the enzymatic resolution without diol inhibition. Beyond this substrate concentration, it was necessary to remove diol **2** from the enzyme-containing aqueous phase for retaining high activity.

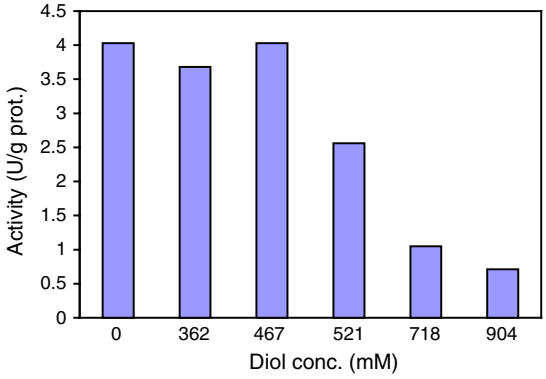


Figure 4. Effect of the concentration of diol **2** on the activity of enantioselective hydrolysis of 15 mM racemic styrene oxide **1** with a cell-free extract (15 g protein/L) of *Sphingomonas* sp. HXN-200 in 50 mM Tris–HCl buffer (pH = 7.5) in the presence of diol **2**. Activity is given as average activity in the first hour.

3. Conclusion

The soluble bacterial epoxide hydrolase (EH) from *Sphingomonas* sp. HXN-200 demonstrated the highest enantioselectivity among the known native EHs for the enantioselective hydrolysis of racemic styrene oxide giving (*S*)-styrene oxide, with an enantiomeric ratio (E) of 21–23 in aqueous buffer and 26–29 in a two-liquid phase system. Hydrolysis of 320 mM styrene oxide with rehydrated lyophilized CFE of *Sphingomonas* sp. HXN-200 in buffer/*n*-hexane (1:1) gave 40.2% (128.6 mM) of (*S*)-styrene oxide in >99.9% ee, providing a practical synthesis of this useful and valuable pharmaceutical intermediate. Further improvement of the productivity could be achieved by the development of recombinant strain overexpressing this novel EH.

4. Experimental

4.1. Analytical methods

Concentrations of styrene oxide **1** and 1-phenyl-1,2-ethanediol **2** in an aqueous sample were analyzed by high performance liquid chromatography (HPLC) with a Hewlett Packard 1040M Series II instrument. Column: Hypersil BDS-C18 (4 × 125 mm). Eluent: 10 mM KH_2PO_4 (pH = 7)/acetonitrile (80:20). Flow rate: 1 mL/min. DAD detection: 210 nm. Retention time: 1.9 min for **2**; 2.7 min for benzyl alcohol (internal standard); 9.7 min for **1**.

Concentrations and ees of styrene oxide **1** and 1-phenyl-1,2-ethanediol **2** in an organic sample were analyzed with a Merck Hitachi L-500 HPLC on a Chiralpak AS column. Eluents: *n*-hexane/isopropanol (95:5). Flow rate: 1.0 mL/min. UV detection: 210 nm. Retention times: 5.3 min for (*R*)-**1**; 5.8 min for (*S*)-**1**; 20.2 min for (*S*)-**2**; 20.6 min for (*R*)-**2**.

The enantiomeric excess of 1-phenyl-1,2-ethanediol **2** in organic sample was also analyzed with a Merck Hitachi D-7000 HPLC on a Chiralpak AS column. Eluents: *n*-hexane/isopropanol (98:2). Flow rate: 0.6 mL/min. DAD detection: 210 nm. Retention times: 77.8 min (*S*)-**2**, 86.4 min for (*R*)-**2**.

4.2. Materials

Racemic styrene oxide **1** (97%) was purchased from Janssen. Styrene oxide (*R*)-**1** (⩾98%) and (*S*)-**1** (⩾98%) were obtained from Fluka. 1-Phenyl-1,2-ethanediol racemic-**2** (97%), (*S*)-**2** (99%) and (*R*)-**2** (99%) were purchased from Aldrich.

4.3. Growth of *Sphingomonas* sp. HXN-200

Sphingomonas sp. HXN-200 was grown on E2-medium agar plate under *n*-octane vapor. It was then inoculated into 20 mL LB-medium (1 L LB-medium contains 10.0 g tryptone, 5.0 g yeast extract, and 10.0 g NaCl; pH = 7.0) and incubated at 30 °C and 300 rpm to an optic density at 450 nm (OD_{450}) of 6.0. The culture was transferred into a 100 mL LB-medium and shaken at 30 °C and 300 rpm to an OD_{450} of 6.0. The preculture was inoculated into 2 L E2 medium[17] containing 2 mL 1 M $MgSO_4$, 100 μL polypropylene glycol P2000, and 2 mL standard 1000*MT solution (2.78 g $FeSO_4$*7H_2O, 1.98 g $MnCl_2$*4H_2O, 2.81 g $CoSO_4$*7H_2O, 1.47 g $CaCl_2$*2H_2O, 0.17 g $CuCl_2$*2H_2O, and 0.29 g $ZnSO_4$*7H_2O in 1 L 1 N HCl) in a 3-L bioreactor. The bioreactor was stirred at 1500 rpm and at 30 °C, and air saturated with *n*-octane vapor (at 20 °C) was introduced at 1 L/min. The pH was maintained at 7.0–7.2 by using 25% NH_4OH and 25% phosphoric acid. Cells were harvested at OD_{450} of 24 at 62 h. The cell pellet was washed with 5 mM KH_2PO_4 buffer (pH = 7.5) and stored at −80 °C.

4.4. Preparation of the cell free extract (CFE) of *Sphingomonas* sp. HXN-200

Frozen cells of *Sphingomonas* sp. HXN-200 were thawed and then suspended in 50 mM Tris–HCl buffer (pH = 7.5) to an OD_{450} of 140. Cells were broken by passing three times through a French press. Ultracentrifugation at 245,000*g* and 4 °C for 45 min gave the supernatant as the CFE. The protein concentration of the CFE was determined by a Bradford protein assay.[16]

4.5. Hydrolysis of styrene oxide (*R*)-1 or (*S*)-1 with CFE of *Sphingomonas* sp. HXN-200 in aqueous buffer

To a 10 mL 50 mM Tris–HCl buffer (pH = 7.5) containing CFE (10 g protein/L) of *Sphingomonas* sp. HXN-200 was added either enantiopure (*R*)-**1** (9.5 mM) or (*S*)-**1** (9.4 mM). The biotransformation was performed at 25 °C and 300 rpm, and samples (500 μL) were taken at different time points for the analysis. After centrifugation, 200 μL supernatant was mixed with 800 μL MeOH containing 4 mM benzyl alcohol as internal standard for the analysis of the conversion of diol **2** by HPLC on C-18 column; another 200 μL supernatant was extracted with 800 μL ethyl acetate, the organic phase was separated, dried, and mixed with *n*-hexane in 1:4, and the sample was analyzed by HPLC on Chiralpak AS column with *n*-hexane/isopropanol (98:2) to determine the ee of diol **2**.

4.6. General procedure for hydrolysis of racemic styrene oxide 1 with CFE of *Sphingomonas* sp. HXN-200 in aqueous buffer

To a 10 mL 50 mM Tris–HCl buffer (pH = 7.5) containing CFE (10 g protein/L) of *Sphingomonas* sp., HXN-200 was added racemic **1** (20–160 mM), and the mixtures shaken at 25 °C and 300 rpm. Aliquots (200 μL) were taken at different time points, mixed with 800 μL ethyl acetate, and centrifuged. The organic phase was dried over $MgSO_4$ and mixed with *n*-hexane in a 1:1–19 ratio depending on the substrate concentration. Samples were analyzed by HPLC on Chiralpak AS column with *n*-hexane/isopropanol (95:5) to determine the concentration and ee of styrene oxide **1** and diol **2**. The activity was calculated as the following: average activity at 1 h (U/g cdw) = product concentration (μM)/[60 (min) ∗ cell density (g cdw/L)].

4.7. Hydrolysis of racemic styrene oxide 1 with CFE of *Sphingomonas* sp. HXN-200 in a two-liquid phase system

To a 10 mL 50 mM Tris–HCl buffer (pH = 7.5) containing CFE (10 g protein/L) of *Sphingomonas* sp. HXN-200 was added 10 mL *n*-hexane containing racemic **1** (160 mM), and the mixtures shaken at 25 °C and 300 rpm. Aliquots (200 μL) were taken at different time points, centrifuged, and separated into aqueous and organic phase. Analytic samples from aqueous phase were prepared by extraction with ethyl acetate, as described above. An analytic sample from the organic phase was prepared by mixing with *n*-hexane in a 1:79 ratio. All samples were analyzed by HPLC on Chiralpak AS column with *n*-hexane/isopropanol (95:5) to determine the concentration and ee of styrene oxide **1** and diol **2**. The reaction was stopped at 30.7 h, giving 39.2% (62.7 mM) of (*S*)-styrene oxide **1** in >99.9% ee.

4.8. Hydrolysis of racemic styrene oxide 1 with rehydrated lyophilized CFE of *Sphingomonas* sp. HXN-200 in a two-liquid phase system

To 2 mL of 50 mM Tris–HCl buffer (pH = 7.5) was added the lyophilized CFE powder (120 mg) of *Sphingomonas* sp. HXN-200 and 2 mL *n*-hexane containing racemic-**1** (320 mM). The mixtures were shaken at 25 °C and 300 rpm, and the aliquots (200 μL) taken at different time points for the analysis of the concentration and ee of styrene oxide **1** and diol **2**, as described above. After 13.8 h, (*S*)-styrene oxide **1** was formed in 40.2% (128.6 mM) and in >99.9% ee.

4.9. General procedure for hydrolysis of racemic styrene oxide 1 with CFE of *Sphingomonas* sp. HXN-200 in the presence of diol 2 in aqueous buffer

To 5 mL 50 mM Tris–HCl buffer (pH = 7.5) containing CFE (16 g protein/L) of *Sphingomonas* sp. HXN-200

was added 1-phenyl-1,2-ethanediol **2** (0–904 mM). Racemic styrene oxide **1** was added to a concentration of 15.0 mM, and the mixtures shaken at 25 °C and 300 rpm. Aliquots (200 μL) were taken at different time points, mixed with 800 μL ethyl acetate, and centrifuged. The organic phase was dried over $MgSO_4$ and mixed with *n*-hexane in 1:1. Samples were analyzed by HPLC on Chiralpak AS column with *n*-hexane/isopropanol (95:5) to determine the concentration and ee of styrene oxide **1**.

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