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Enantioselective Hydrolysis of Racemic and *Meso*-Epoxides with Recombinant *Escherichia coli* Expressing Epoxide Hydrolase from *Sphingomonas* sp. HXN-200: Preparation of Epoxides and Vicinal Diols in High *ee* and High Concentration

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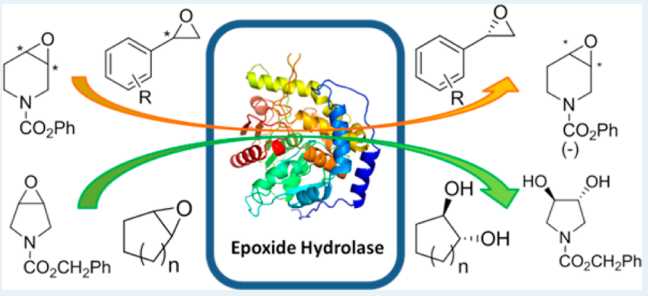
Supporting Information

ABSTRACT: A unique epoxide hydrolase (SpEH) from *Sphingomonas* sp. HXN-200 was identified and cloned based on genome sequencing and expressed in *Escherichia coli*. The engineered *E. coli* (SpEH) showed the same selectivity and substrate specificity as the wild type strain and 172 times higher activity than *Sphingomonas* sp. HXN-200 for the hydrolysis of styrene oxide **1**. Hydrolysis of racemic styrene oxide **1**, substituted styrene oxides **3**, **5**−**7**, and *N*-phenoxycarbonyl-3,4-epoxypiperidine **8** (200−100 mM) with resting cells of *E. coli* (SpEH) gave (*S*)-epoxides **1**, **3**, **5**−**7** and (−)-**8** in 98.0−99.5% enantiomeric excess (*ee*) and 37.6−46.5% yield. Hydrolysis of cyclopentene oxide **9**, cyclohexene oxide **10**, and *N*-benzyloxycarbonyl-3,4-epoxypyrrolidine **11** (100 mM) afforded the corresponding (*R*, *R*)-vicinal *trans*-diols **12**−**14** in 86−93% *ee* and 90−99% yield. The *ee* of (1*R*, 2*R*)-cyclohexane-1,2-diol **13** was improved to 99% by simple crystallization. These biotransformations showed high specific activity (0.28−4.3 U/mg cdw), product concentration, product/cells ratio, and cell-based productivity. Hydrolysis at even higher substrate concentration was also achieved: (*S*)-**1** was obtained in 430 mM (51 g/L_{org}) and 43% yield; (1*R*, 2*R*)-**13** was obtained in 500 mM (58 g/L) and >99% yield. Gram-scale preparation of epoxides (*S*)-**1**, (*S*)-**3**, (*S*)-**6** and diols (1*R*, 2*R*)-**12**, (1*R*, 2*R*)-**13**, (3*R*, 4*R*)-**14** were also demonstrated. *E. coli* (SpEH) cells showed the highest enantioselectivity to produce (*S*)-**1** (*E* of 39) among all known EHs in the form of whole cells or free enzymes and the highest enantioselectivities to produce (*S*)-**3**, **5**, **6**, **7**, (−)-**8**, and (*R*, *R*)-**14** (*E* of 36, 35, 28, 57, 22, and 28) among all known EHs. The easily available and highly active *E. coli* (SpEH) cells are the best biocatalysts known thus far for the practical preparation of these useful and valuable enantiopure epoxides and vicinal diols via hydrolysis.



KEYWORDS: *biocatalysis, epoxide hydrolase, enantioselective hydrolysis, epoxide, vicinal diol, high product concentration, whole-cell biotransformation*

INTRODUCTION

Epoxide hydrolase (EH)-catalyzed enantioselective hydrolysis of racemic and *meso*-epoxides provides a simple and green method for the syntheses of enantiopure epoxides and vicinal diols that are useful and valuable synthons and chiral pharmaceutical intermediates.[1−4] EHs are cofactor independent, relatively stable, and widely spread in nature. Many microbial EHs have been discovered, cloned, and engineered for organic syntheses.[1−4] Nevertheless, it is still a significant challenge to develop easily available, highly active, and highly enantioselective EH for the practical production of desired epoxides and vicinal diols in high enantiomeric excess (*ee*), high yield, and high concentration at low catalyst loading within short reaction time.

We are interested in developing a novel EH and engineering a powerful biocatalyst for the practical preparation of useful and valuable chiral epoxides (*S*)-**1**−**7**, (−)-**8**, and vicinal diols (*R*, *R*)-**12**−**14** (Scheme 1). (*S*)-styrene oxide **1** is an intermediate for the synthesis of nematocide,[5] anticancer agent[6] Levamisole, and anti-HIV agent (−)-hyperolactone C.[7] (*S*)-2-, 3-, and 4-chlorostyrene oxides **2**−**4** are useful in the preparation of antiviral agents EMI39.3, EMI40.1, and EMI37.1, respectively.[8] In addition, (*S*)-**3** is an intermediate for the preparation of IGF-1R kinase inhibitor BMS-536924.[9] (*S*)-4-fluorostyrene oxide (*S*)-**6** and (*S*)-3-bromostyrene oxide (*S*)-**7** are useful for synthesizing several potential bioactive compounds,[10,11] while (−)-*N*-phenoxycarbonyl-3,4-epoxypiperidine **8** is an intermediate for the preparation of a single enantiomer of antidepressant

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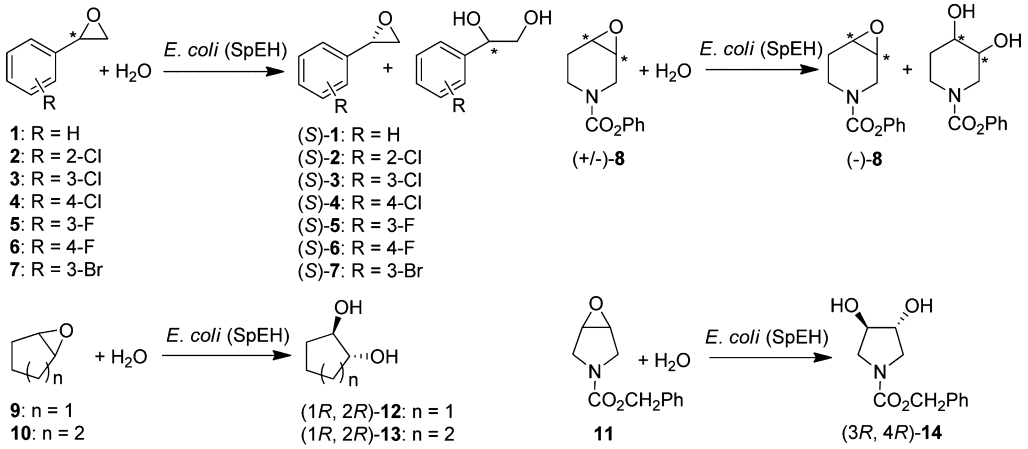
Scheme 1. Enantioselective Hydrolysis of Racemic and *Meso*-Epoxides with Recombinant *E. coli* (SpEH) expressing EH from *Sphingomonas* sp. HXN-200



Ifoxetine sulfate[12] and prokinetic agent Cisapride hydrate.[13] (1*R*, 2*R*)-cyclopentane-1,2-diol **12** is useful for the preparation of hepatitis C virus NS3-NS4A protease inhibitors[14] and chiral ligands of enantioselective catalysts.[15] (1*R*, 2*R*)-cyclohexane-1,2-diol **13** is a chiral auxiliary[16] and also a starting material in the synthesis of several anticancer compounds.[17,18] (3*R*, 4*R*)-3,4-dihydroxypyrrolidine **14** is an intermediate for the preparation of antibiotics,[19] Sialyl Lewis X mimetics,[20] and aza-sugars.[21]

Many EHs have been reported for the syntheses of these target compounds. EHs from *Aspergillus niger* exhibited an enantioselectivity factor (*E*) of 16,[22] 5,[23] 2,[23] 80,[24] 14,[25] and 2[23] for the hydrolysis of racemic epoxides to give (*S*)-**1**−**4**, **6**, and **7**, respectively; EH from *Agrobacterium radiobacter* showed an *E* of 16,[26] 16,[26] 10,[26] and 15[26] for the hydrolysis to give (*S*)-**1**−**4**, respectively; and EH from *Rhodotorula glutinis* gave an *E* of 15[27] for the hydrolysis of racemic **1** to give (*S*)-**1**. With the exception of EH of *A. niger* for producing (*S*)-**4**, the enantioselectivities of these EHs are not satisfactory. Recently, we discovered *Sphingomonas* sp. HXN-200 containing a novel EH giving an *E* of 29, 12, 41, and 11 for the hydrolysis of racemic epoxide **1**−**4** to give (*S*)-**1**−**4**, respectively.[28,29] This EH is more enantioselective than other known EHs for producing (*S*)-**1** and (*S*)-**3**. It is also the only known EH for the hydrolysis of racemic **8** to give (−)-**8** with good selectivity (*E* = 21).[30] Regarding the enantioselective hydrolysis of *meso*-epoxides, EH from *R. glutinis* produced vicinal diols (1*R*, 2*R*)-**12** in 98% *ee* and (1*R*, 2*R*)-**13** in 90% *ee*, respectively;[31] EH from *A. radiobacter* gave (1*R*, 2*R*)-**13** in 99% *ee*;[32] and engineered EH BD10721, BD9883, and BD9884 afforded (1*R*, 2*R*)-**12** in 90% *ee*, (1*R*, 2*R*)-**13** in 96% *ee*, and (3*R*, 4*R*)-**14** in 93% *ee*, respectively.[33] Interestingly, our recently discovered EH from *Sphingomonas* sp. HXN-200 also catalyzed the enantioselective hydrolysis of *meso*-epoxides, giving (3*R*, 4*R*)-**14** with higher *ee* (95% *ee*).[30,34]

Thus far, there are only a few reported examples of using EH to prepare enantiopure epoxides and diols in high concentration.[24,35−40] In the best two known examples, immobilized EH from *A. niger* (20 g/L) was used to prepare 500 mM (*S*)-**1** in 99% *ee* and 50% yield,[40] and crude EH preparation from *A. niger* (256 g/L) was applied to produce 940 mM (*S*)-**4** in 99% *ee* and 47% yield.[24] From a practical point of view, the use of easily available and low-cost whole cell biocatalysts is a much more economical alternative. However, in the only reported example of whole-cell based biotransformation with high product concentration, the recombinant cells expressing EH from *R. glutinis* were used at 92 g cdw/L for the hydrolysis of racemic **1** at 4 °C for 24 h to give 719 mM (*S*)-**1** in 98% *ee* and 41% yield.[39] Obviously, the activity of the cells is too low, and the required cell density is too high for practical application. For the EH from *Sphingomonas* sp. HXN-200, the whole-cell activity of the wild type strain was also too low.[28−30] Moreover, the growth of *Sphingomonas* sp. HXN-200 on *n*-octane is difficult. Here we report the identification and cloning of the EH from *Sphingomonas* sp. HXN-200, the engineering of recombinant *E. coli* cells expressing the EH as a highly active, easily available, and low-cost catalyst for the enantioselective hydrolysis of racemic and *meso*-epoxides, and the whole cell-based preparation of epoxides (*S*)-**1**−**7**, (−)-**8** and vicinal *trans*-diols (*R*, *R*)-**12**−**14** in high concentration and high *ee* at low catalyst loading within a short reaction time.

RESULTS AND DISCUSSION

Genetic Engineering, Cell Growth, and Specific Activity of Recombinant *E. coli* Strain Expressing SpEH. Genome information is a valuable source to identify powerful enzymes from microbes.[41,42] To identify and clone the EH, the whole genome of *Sphingomonas* sp. HXN-200 was sequenced. Bioinformatic analysis suggested four putative EH genes from a total of 4544 possible open reading frames (genes) in the genome. Primers were designed to amplify the four possible EHs' genes from the genome by polymerase chain reaction (PCR); the amplified products were digested with their respective restriction enzymes, the products were ligated into pRSFduet vector, and the resulting plasmids were transformed into chemical competent *E. coli* cells. All four putative EHs were heterologously expressed in *E. coli*; however, only one EH (SpEH) was found to be active for the hydrolysis of racemic styrene oxide **1** to give the corresponding diol.

The gene of SpEH consists of 1146 bp encoding a 381 amino acids polypeptide with a calculated molecular weight of 43.04 kDa. A BLASTP search against the NCBI protein database reveals that the most related protein is a putative EH from marine gamma *proteobacterium* HTCC2148, which shares 54% amino acid identity. The low identity suggests that SpEH is unique. Multiple alignments of SpEH and other known EHs (Supporting Information, Figure S1) showed that SpEH shares the conserve motifs (H-G-X-P and G-X-Sm-X-S/T), the

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catalytic trial (D-H-D/E), and two conserve Y229 and Y294 with other known EHs, such as EHs from *A. radiobacter*[43] and *A. niger*.[44] This indicates a similar reaction mechanism of SpEH to these EHs.

The recombinant *E. coli* (SpEH) grown easily in TB medium, and the expression of SpEH was induced by adding IPTG. As shown in Figure 1, a cell density of 4.0−4.5 g cdw/L was

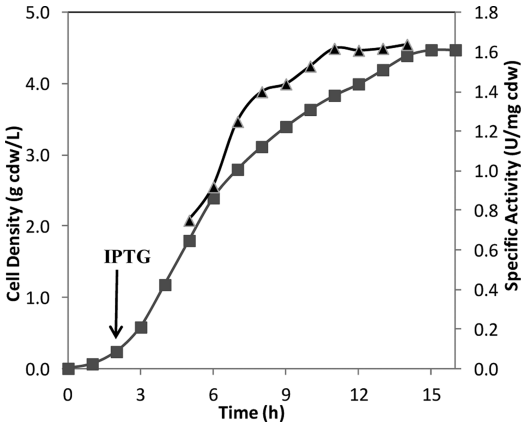

Figure 1. Cell growth and specific activity for the hydrolysis of (±)-**1** of *E. coli* (SpEH). ■: cell density; ▲: specific activity. Cells were initially grown at 37 °C, induced at 2 h by the addition of IPTG (0.5 mM), and then grown at 25 °C.

achieved at 12−15 h. Cells taken at different time points showed different activity toward the hydrolysis of racemic styrene oxide **1**. The highest specific activity was observed for the cells grown at 11−14 h at the late exponential growing phase. The expressing of SpEH was clearly shown in the SDS-PAGE of the cell-free extracts (CFE) of the *E. coli* cells taken at 14 h (Figure 2, Lane 3). The specific activity of the *E. coli* cells

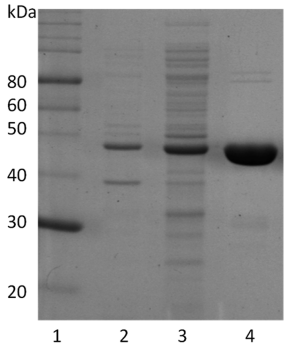

Figure 2. SDS-PAGE. Lane 1: Marker; Lane 2: cell debris of *E. coli* (SpEH); Lane 3: cell free extract of *E. coli* (SpEH); Lane 4: purified His-tagged SpEH.

reached 1.6 U/mg cdw which is 172 times higher than that of the cells of the wild-type strain *Sphingomonas* sp. HXN-200 (9.3 U/g cdw).[28] These results demonstrated clearly that *E. coli* (SpEH) cells are highly active and easily available biocatalyst for epoxide hydrolysis. The use of the *E. coli* cells as catalyst is of economic advantage over the use of CFE or purified enzyme. In addition to the higher specific activity, *E. coli* cells gave also cleaner reaction than the wild-type cells which contain the EH at low level and also other enzymes for side reactions.

Kinetics of Enantioselective Hydrolysis of Styrene Oxide 1 with the Recombinant SpEH. An *E. coli* strain expressing SpEH with His-tagged at the C-terminal was genetically engineered to facilitate the purification of SpEH. Cells were produced and broken, and the CFE was subjected to affinity chromatography with a Ni-NTA column. His-tagged SpEH was purified to apparent homogeneity as indicated in SDS-PAGE (Figure 2, Lane 4), with the molecular weight of 46 kDa. This value is very close to the calculated one of 43.9 kDa. The specific activity for the hydrolysis of styrene oxide **1** was determined to be 16 U/mg protein for His-tagged SpEH. A set of hydrolysis reactions was performed with SpEH (1 μg/mL) at various concentrations (0.2−8 mM) of (*S*)-**1** and (*R*)-**1**, respectively, to determine the kinetic data. The initial velocities at different substrate concentrations were used for a Lineweaker−Burk plot (1/v vs 1/[S]). The following data were obtained from the plot: K_m of 1.24 mM, V_{max} of 10.3 μmol min^{-1} mg^{-1}, k_{cat} of 7.4 s^{-1}, and k_{cat}/K_m = 6.0 mM^{-1} s^{-1} for the hydrolysis of (*S*)-**1**; K_m of 0.15 mM, V_{max} of 19.8 μmol min^{-1} mg^{-1}, k_{cat} of 14.2 s^{-1}, and k_{cat}/K_m of 97.3 mM^{-1} s^{-1} for the hydrolysis of (*R*)-**1**. The enantioselectivity factor *E* was calculated from $(k^R_{cat}/K^R_m)/(k^S_{cat}/K^S_m)$ as 16 for the hydrolysis of racemic styrene oxide **1**. This value is slightly lower than the *E* value of 21−23 established previously by the kinetic resolution of **1** with resting cells of *Sphingomonas* sp. HXN-200 in the same single aqueous phase system.[28]

Enantioselective Hydrolysis of Racemic Epoxides 1−8 with Resting Cells of *E. coli* (SpEH). Resting cells of *E. coli* (SpEH) were explored for the enantioselective hydrolysis of racemic epoxides **1**−**8** in a two-phase system consisting of *n*-hexane/aqueous buffer (1:1). In such a system, *n*-hexane acts as a reservoir of the substrate, which reduces the nonenzymatic hydrolysis as well as the toxic effect of the epoxide on cells; the maintaining of diol products in aqueous phase allows for easy separation of epoxides and diols.[45] As listed in Table 1, substrate concentration of 200−100 mM and cell density of 0.5−1.0 g cdw/L were used. (*S*)-**1** was produced in 99.1% *ee* and 41.6% yield after 2.5 h reaction, with a specific activity of 1.4 U/mg cdw. The enantioselectivity factor *E* reached 30, similar to that obtained with the cells of the wild-type stain *Sphingomonas* sp. HXN-200 in the same two-liquid phase system.[28]

Furthermore, hydrolysis of epoxide **3**, **5**−**7** gave (*S*)-**3**, (*S*)-**5**−**7** in 98.0−99.4% *ee* and 42.3−46.5% yield within 1.5−5.5 h. The *E* values of 36, 35, 28, and 57 represent the highest enantioselectivities among all the known EHs for the preparation of (*S*)-**3**,[23,46] (*S*)-**5**, (*S*)-**6**,[23,25] and (*S*)-**7**,[23] respectively. The specific activities of 2.9−0.88 U/mg cdw for the *E. coli* cells were also very high. Compared to the epoxides with a substitution at the *para* or *ortho* position (**2**, **4**, **6**), the epoxides with a substitution at the *meta* position (**3**, **5**, **7**) were hydrolyzed faster (higher specific activity) and more selectively (higher *E*). This preference is significantly different from other well-known EHs (such as EH from *A. niger*[23,25]), indicating the unique substrate specificity and special synthetic application of SpEH.

In addition, *E. coli* (SpEH) cells showed an *E* value of 22 for the hydrolysis of 100 mM *N*-phenoxycarbonyl epoxypiperidine **8** to give (−)-**8** in 99.5% *ee* and 37.6% yield. The specific activity reached 4.3 U/g cdw, and the resolution was completed within only 25 min.

In the view of even more practical applications, the enantioselectivity of SpEH could be further improved. Currently, we are working on the directed evolution[47] of this

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Table 1. Enantioselective Hydrolysis of Racemic Epoxides 1−8 with Resting Cells of *E. coli* (SpEH)[a]

sub.	conc. (mM)[b]	cell density (g cdw/L)[c]	time (min)	activity (U/mg cdw)[d]	prod.	*ee* (%)[e]	yield (%)[e]	*E*[f]
1	200	0.5	150	1.4	(*S*)-**1**	99.1	41.6	30
2	200	1.0	420	0.28	(*S*)-**2**	99.3	19.9	7
3	200	0.5	330	2.2	(*S*)-**3**	98.0	44.3	36
4	200	0.5	480	0.92	(*S*)-**4**	98.1	35.1	14
5	200	0.5	90	2.5	(*S*)-**5**	99.4	42.3	35
6	200	0.5	280	0.88	(*S*)-**6**	98.3	42.3	28
7	150	0.5	180	2.9	(*S*)-**7**	98.0	46.5	57
8	100	1.0	25	4.3	(−)-**8**	99.5	37.6	22

[a]The reaction was performed in a two-phase system consisting of Tris−HCl buffer (50 mM, pH 7.5) and *n*-hexane (1:1). [b]Concentration was based on the volume of organic phase. [c]Cell density was based on the volume of aqueous phase. [d]Specific activity was determined at 30 min. [e]*ee* value and yield were determined by chiral HPLC analysis. [f]*E* value was calculated by $E = \ln[(1 - c)(1 - ee_s)]/\ln[(1 - c)(1 + ee_s)]$.

Table 2. Enantioselective Hydrolysis of *Meso*-Epoxides 9−11 with Resting Cells of *E. coli* (SpEH)[a]

sub.	conc. (mM)	cell density (g cdw/L)	time (min)	activity (U/mg cdw)[b]	prod.	*ee* (%)[c]	yield (%)[d]	*E*[e]
9	100	1.0	360	0.34	(1*R*, 2*R*)-**12**	88	99	16
10	100	1.0	120	0.81	(1*R*, 2*R*)-**13**	86	99	13
11	100	1.0	60	2.3	(3*R*, 4*R*)-**14**	93	90	28

[a]The reaction was performed in Tris−HCl buffer (50 mM, pH 7.5). [b]Specific activity was determined at 30 min. [c]*ee* value was determined by chiral GC analysis. [d]Yield was determined by GC analysis. [e]*E* value was calculated by $E = (1 + ee_p)/(1 - ee_p)$.

EH to enhance its enantioselectivity toward the hydrolysis of racemic epoxides **1**−**8**.

Enantioselective Hydrolysis of *Meso*-Epoxides 9−11 with Resting Cells of *E. coli* (SpEH). Biotransformation of *meso*-epoxides **9**−**11** at 100 mM were carried out with resting cells of *E. coli* (SpEH) at a cell density of 1.0 g cdw/L. Aqueous buffer was used as a single phase for the reaction, since the nonenzymatic hydrolysis rates were low for these cyclic epoxides. As shown in Table 2, the corresponding (*R*, *R*)-vicinal *trans*-diols **12**−**14** were produced in 86−93% *ee* and 90−99% yield, respectively. Although the *ee* of (1*R*, 2*R*)-**12** is lower than that obtained with EH from *R. glutinis*, the specific activity of 0.34 U/mg cdw is much higher than that of the *R. glutinis* cells.[31] The *ee* of (1*R*, 2*R*)-**13** is also lower than that obtained with EH from *A. radiobacter*;[32] however, a simple crystallization in ethyl acetate improved the *ee* to 99%. The high specific activity of 0.81 U/mg cdw makes the application of the *E. coli* (SpEH) cells for the preparation of (1*R*, 2*R*)-**13** practical. The *E. coli* (SpEH) cells showed also very high activity (2.3 U/mg cdw) for the hydrolysis of *meso*-epoxide **11** to (3*R*, 4*R*)-**14**, allowing for the completion of the reaction within 1 h. The enantioselectivity for this substrate is the best among known examples.[33]

Synthesis of (*S*)-Styrene Oxide 1 in High Concentration by Enantioselective Hydrolysis of Racemic Epoxide 1 with Resting Cells of *E. coli* (SpEH). The easily available and highly active *E. coli* (SpEH) cells were examined for the hydrolysis at even higher substrate concentration. For demonstration, styrene oxide **1** was chosen as a model substrate. One M (120 g/L_{org}) racemic **1** was hydrolyzed in a simple two-phase system consisting of *n*-hexane:aqueous buffer (1:1) at a density of *E. coli* (SpEH) cells of 5.0 g cdw/L_{aq}. As shown in the time course of the biotransformation (Figure 3), the decrease of (*R*)-**1** is linear during the first 40 min, and the reaction finished within 1 h to give (*S*)-**1** >99% *ee* and 43% yield. The *E* value at this high substrate concentration was 39, which is the highest among all EHs in the form of free enzyme, cell extracts, or whole cells.[22,28,39,48−50] The product concentration reached 430 mM in organic phase (51 g/L_{org}), and the

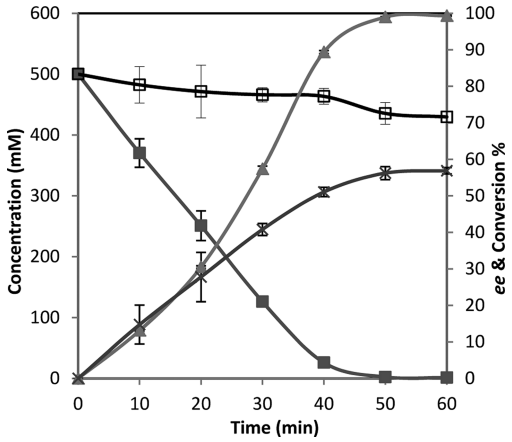

Figure 3. Time course of the enantioselective hydrolysis of 1 M racemic styrene oxide **1** with resting cells of *E. coli* (SpEH) (5 g cdw/L) in a two-phase system consisting of Tris−HCl buffer (50 mM, pH 7.5) and *n*-hexane (1:1). □: (*S*)-**1** concentration; ■: (*R*)-**1** concentration; ▲: *ee* of remaining **1**; × : conversion of **1**. The error bar represents standard deviation of three independent experiments.

overall space-time-yield amounted to 26 g/L/h. The cell-based specific productivity reached 10.3 g/h/g cdw. This value is 542 times higher than that (0.019 g/h/g dry weight of cell-free extracts) with *Sphingomonas* sp. HXN-200.[28] It is also 264 times higher than that (0.039 g/h/g cdw) achieved in recombinant *R. glutinis* EH-catalyzed kinetic resolution of 1.8 M **1** at a cell density of 92 g cdw/L for 24 h.[39] Obviously, *E. coli* (SpEH) cells are highly productive catalyst for the resolution of styrene oxide **1** to prepare (*S*)-**1**.

Synthesis of (1*R*, 2*R*)-Cyclohexane 1,2-Diol 13 in High Concentration by Enantioselective Hydrolysis of *Meso*-Epoxide 10 with Resting Cells of *E. coli* (SpEH). There is no report on EH-catalyzed enantioselective hydrolysis of *meso*-epoxides in high concentration. The *E. coli* (SpEH) cells were thus examined for the hydrolysis of cyclohexene oxide **10**, as a mode *meso*-epoxide, in high concentration. The reaction was carried out in aqueous buffer. Because of the limited solubility

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of **10**, a second phase was formed initially at high substrate concentration, and it was emulsified into aqueous buffer during the course of biotransformation. Various substrate concentrations and cell densities were tested, while their ratio was fixed at 100 mM: 1 g cdw/L. As shown in Figure 4, the higher

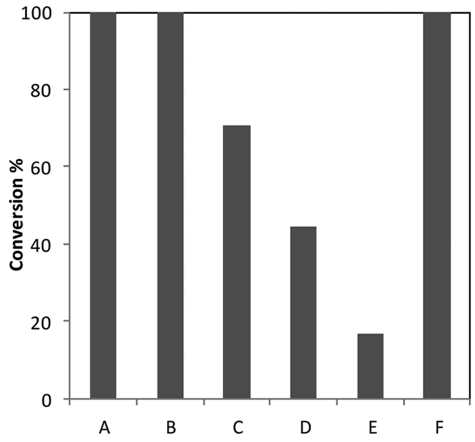

Figure 4. Conversion of the enantioselective hydrolysis of cyclohexene oxide **10** with resting cells of *E. coli* (SpEH) in Tris−HCl buffer (50 mM, pH 7.5) at various substrate concentration (mM), cell density (g cdw/L), and reaction time. A: 200 mM substrate, 2 g cdw/L cells, 2 h; B: 400 mM substrate, 4 g cdw/L cells, 3 h; C: 600 mM substrate, 6 g cdw/L cells, 3 h; D: 800 mM substrate, 8 g cdw/L cells, 3 h; E: 1000 mM substrate, 10 g cdw/L cells, 3 h; F: 500 mM substrate, 10 g cdw/L cells, 1 h.

substrate concentration, the lower the conversion. While 200 mM and 400 mM **10** were fully converted to (1*R*, 2*R*)-diol **13** in 86% *ee* in 2 and 3 h, respectively, 600 mM, 800 mM, and 1 M substrate were transformed to (1*R*, 2*R*)-**13** in only 70%, 45%, and 17%, respectively, after 3 h reactions. Obviously, substrate at these concentrations can become toxic to the cells and inhibit the catalytic activity. To achieve high conversion at higher substrate concentration, higher cell density was applied. For instance, at the cell density of 10 g cdw/L, 500 mM **10** was transformed to diol **13** in >99% yield (58 g/L) in 1 h. To our knowledge, this is the first example of EH-catalyzed enantioselective hydrolysis of *meso*-epoxide to give high product concentration (>30 g/L).

Preparation of (*S*)-1, (*S*)-3, and (*S*)-6 with Resting Cells of *E. coli* (SpEH). Enantioselective hydrolysis of styrene oxide **1** was performed on a 10-g scale. Biotransformation was carried out in 200 mL mixture of Tris−HCl buffer and *n*-hexane (1:1) containing 1 M (120 g/L_{org}) styrene oxide **1** in the organic phase and 5.0 g cdw/L_{aq} of *E. coli* (SpEH) cells. Ten milliliters of cell suspension (5 g cdw/L) was added at 1 h, and the reaction was stopped at 70 min. (*S*)-**1** remained in 42% with >99% *ee*. The space-time yield reached 18 g/L/h, which is higher than those of known bioprocesses for the preparation of (*S*)-**1**, including the asymmetric epoxidation of styrene.[51] Workup and flash chromatography gave 4.338 g of enantiopure (*S*)-**1** in 36.4% isolated yield (Table 3). Gram scale resolution of **3** (200 mM, 30 g/L_{org}) and **6** (500 mM, 69 g/L_{org}) was also achieved with the resting cells of *E. coli* (SpEH) under nonoptimized conditions within 80−90 min. Enantiopure epoxides (*S*)-**3** and (*S*)-**6** were obtained in 37.9% and 31.3% isolated yields, respectively (Table 3). In addition to the high product concentrations, the product/cells ratios (7.9−11.7 g/g cdw) as well as cell-based specific productivities (5.7−8.8 g/h/g cdw) of these three preparative biotransformations are also high. These results suggested that *E. coli* (SpEH) is a powerful catalyst for the practical preparation of these useful and valuable (*S*)-epoxides. When *E. coli* (SpEH) was compared with the well-known Jacobsen's Co(salen) catalyst[52] for the preparation of (*S*)-styrene oxide **1**, the product/catalyst ratio is similar; but *E. coli* (SpEH) showed higher catalyst-based specific productivity (6.8 g product/h/g cells) than Co(salen) catalyst (0.2 g product/h/g cat); moreover, *E. coli* (SpEH) cells are greener and cheaper than Co(salen) catalyst.

Preparation of (*R*, *R*) Vicinal *trans*-Diols 12, 13, and 14 with Resting Cells of *E. coli* (SpEH). Practical syntheses of vicinal *trans*-diols from *meso*-epoxides were also demonstrated. Preparation of (1*R*, 2*R*)-1,2-cyclohexanediol **13** was performed in 200 mL of aqueous buffer containing 500 mM (49.1 g/L) cyclohexene oxide **10** and 10 g cdw/L of resting cells of *E. coli* (SpEH). A conversion of >99% was achieved at 2 h. The space-time yield reached 26 g/L/h, which is attractive for industrial application and also the highest one reported for the biocatalytic synthesis of (1*R*, 2*R*)-**13**.[31−34] Simple workup afforded 10.284 g (89% isolated yield) (1*R*, 2*R*)-**13** in 86% *ee* (Table 4). Very importantly, the *ee* value was improved to 99% by simple crystallization in ethyl acetate, with an overall isolated yield of 68.5% starting from substrate **10**. Other two useful vicinal *trans*-diols (1*R*, 2*R*)-**12** and (3*R*, 4*R*)-**14** were also synthesized by the enantioselective hydrolysis of *meso*-epoxides **9** (200 mM, 17 g/L) and **11** (200 mM, 44 g/L) with resting cells of *E. coli* (SpEH) (4.0 g cdw/L), respectively (Table 4). After >99% conversion was reached at 3 and 2 h, respectively, simple workup afforded (1*R*, 2*R*)-**12** in 87% *ee* and 70.4% isolated yield and (3*R*, 4*R*)-**14** in 93% *ee* and 94.1% isolated yield, respectively. Moreover, the product/cells ratios (3.6−11.1 g/g cdw) and the cell-based specific productivities (1.2−5.6 g/h/g cdw) of these three preparations are also attractive

Table 3. Preparation of (*S*)-1, (*S*)-3, and (*S*)-6 by Enantioselective Hydrolysis of the Corresponding Racemic Epoxides with Resting Cells of *E. coli* (SpEH)

								isolated yield			
sub.	scale (mL)[a]	sub. conc. (mM)[b]	cell density (g cdw/L)[c]	time (min)	prod.	prod. conc. (g/L)[d]	*ee* (%)[e]	(g)	(%)	prod./ cells (g/g cdw)	productivity (g/h/g cdw)
1	210	1000	5.0	70	(*S*)-**1**	50.4	>99	4.338	36.4	7.9	6.8
3	80	200	1.0	80	(*S*)-**3**	12.7	>99	0.468	37.9	11.7	8.8
6	40	500	2.5	90	(*S*)-**6**	26.1	>99	0.432	31.3	8.6	5.7

[a]The reaction was performed in a two-phase system consisting of Tris−HCl buffer (50 mM, pH 7.5) and *n*-hexane (1:1), and the scale was referred to the total volume. [b]Concentration was based on the volume of the organic phase. [c]Cell density was based on the volume of the aqueous phase. [d]Product concentration was determined by chiral HPLC analysis and based on the volume of the organic phase. [e]*ee* was determined by chiral HPLC analysis.

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Table 4. Preparation of (*R*, *R*) Vicinal *trans*-Diols 12, 13, and 14 by Enantioselective Hydrolysis of the Corresponding *Meso*-Epoxides with Resting Cells of *E. coli* (SpEH)

								isolated yield			
sub.	scale (mL)[a]	sub. conc. (mM)	cell density (g cdw/L)	time (min)	prod.	prod. conc. (g/L)[b]	*ee* (%)[c]	(g)	(%)	prod./ cells (g/g cdw)	productivity (g/h/g cdw)
9	100	200	4.0	180	(1*R*,2*R*)-**12**	20.4	87	1.436	70.4	3.6	1.2
10	200	500	10.0	120	(1*R*,2*R*)-**13**	58.1	86	10.28	88.6	5.1	2.6
							99[d]		68.5[d]		
11	5	200	4.0	120	(3*R*,4*R*)-**14**	47.4	93	0.223	94.1	11.1	5.6

[a]The reaction was performed in Tris−HCl buffer (50 mM, pH 7.5). [b]Product concentration was determined by GC analysis. [c]Product *ee* value was determined by chiral GC analysis. [d]Product *ee* value and yield were obtained after crystallization in ethyl acetate.

for practical syntheses. The facile preparation of these useful vicinal *trans*-diols in high *ee*, high concentration, and high yield demonstrated the great application potential of the *E. coli* (SpEH).

CONCLUSION

The epoxide hydrolase (SpEH) of *Sphingomonas* sp. HXN-200 was successfully identified, cloned, and expressed in *E. coli* as an easily available, low-cost, and highly active biocatalyst. Hydrolysis of racemic epoxides **1**, **3**−**8** (200−100 mM; two-phase system) and *meso*-epoxides **10** and **11** (100 mM; one-phase system) with resting cells of *E. coli* (SpEH) gave the corresponding epoxides (*S*)-**1**, (*S*)-**3**−**7**, and (−)-**8** in 99.5−98.0% *ee* and 46.5−35.1% yield and vicinal *trans*-diols (*R*, *R*)-**13**−**14** in 86−93% *ee* and 90−99% yield, respectively. These biotransformations showed very high specific activity (0.81−4.3 U/mg cdw), high product concentration, high product/cells ratio, and high cell-based productivity, representing the best results known thus far for the production of these enantiopure epoxides and vicinal diols via hydrolysis and demonstrating the potential for industrial application. *E. coli* (SpEH) cells showed the highest enantioselectivity for producing (*S*)-**1** (*E* of 39) among all known EHs in the form of whole cells or free enzymes as well as the highest enantioselectivities for producing epoxides (*S*)-**3**, **5**, **6**, **7**, and (−)-**8** (*E* of 36, 35, 28, 57, and 22) and diol (*R*, *R*)-**14** among all known EHs. Higher product concentrations [(*S*)-**1**, 430 mM, 51 g/L_{org}; (1*R*, 2*R*)-**13**, 500 mM, 58 g/L] were also achieved, and gram-scale preparations were successfully performed. The *ee* of (1*R*, 2*R*)-**13** was easily improved to 99% by simple crystallization.

EXPERIMENTAL SECTION

Engineering of *E. coli* (SpEH) Expressing Epoxide Hydrolase from *Sphingomonas* sp. HXN-200. The wild type cell *Sphingomonas* sp. HXN-200 was grown in E2 medium and *n*-octane vapor as described previously.[28] The genomic DNA of *Sphingomonas* sp. HXN-200 was extracted by using Qiagen Blood and Tissue Kit, and then sent to Beijing Genome Institute for *de novo* bacterial genome sequencing (platform: Illumina Highseq2000). The whole genome of *Sphingomonas* sp. HXN-200 was about 4.75 Mb with 4,544 open reading frames (genes) predicted by Glimmer 3.0 software and functional annotated by searching against the KEGG, COG, SwissProt, TrEMBL, NR databases. There are four possible EHs in the genome: Sp154 (SpEH), Sp990, Sp4354, and Sp3368. All of these putative EHs were amplified by PCR (by using Phusion DNA polymerase) with appropriate primers (Sp154-F: ATCG CAT ATG ATG AAC GTC GAA CAT ATC CGC CC and Sp154-R: ATCG CTC GAG TCA AAG ATC CAT CTG TGC AAA GGC C for SpEH, PCR program: initial denaturation at 98 °C for 3 min, denaturation at 98 °C for 10 s, annealing at 70 °C for 30 s, extension at 72 °C for 45 s, total 32 cycles, final extension at 72 °C for 7 min). The PCR product was subjected to double digestion with appropriate enzymes (NdeI/XhoI for SpEH) and ligated to pRSFduet plasmid (Novagen). The ligation products were transformed into T7 Express Competent *E. coli* cell (New England Biolabs) to yield *E. coli* (SpEH) for recombinant protein expression and activity test.

Cell Growth and Specific Activity of *E. coli* (SpEH). The *E. coli* (SpEH) was grown in LB medium (2 mL) containing kanamycin (50 mg/L) at 37 °C for 7−10 h and then inoculated into TB (terrific broth) medium (50 mL) containing kanamycin (50 mg/L). When OD600 reached 0.6 (around 2 h), IPTG (0.5 mM) was added to induce the expression of protein. The cells continued to grow for 10−12 h at 25 °C and the cell density will reach >4 g cdw/L. Then the cells were harvested by centrifuge (5000 g, 5 min) and resuspended in Tris buffer (50 mM, pH 7.5) for activity testing or asymmetric hydrolysis.

Activity test: freshly prepared *E. coli* (SpEH) cells were diluted by Tris buffer (50 mM, pH 7.5) to 2 mL with 0.5 g cdw/L cell density. And then a second phase *n*-hexane (2 mL) containing styrene oxide **1** (200 mM) was added to the reaction system in the flask. The reaction mixture was shaken (250 rpm) in an incubator at 30 °C for the 30 min. 300 μL aliquots were taken out at 0, 10, 20, and 30 min for HPLC analysis.

General Procedure for Enantioselective Hydrolysis of Racemic Epoxides with Resting Cells of *E. coli* (SpEH). Freshly prepared *E. coli* (SpEH) cells were diluted by Tris buffer (50 mM, pH 7.5) to a 5 mL system with required cell density (as indicated in Table 1) in a flask (100 mL). And then a 5 mL second phase of *n*-hexane containing appropriate amount of epoxides (as indicated in Table 1) was added to the reaction system in the flask. The reaction mixture was shaken (250 rpm) in an incubator (New Brunswick Scientific) at 30 °C for the appropriate time. 200 μL aliquots were taken out at different time points for HPLC analysis. Analytic samples were prepared by centrifugation, and then organic phases (50 μL) were separated and diluted with *n*-hexane (containing 2 mM ethyl benzene as internal standard) before HPLC analysis for quantification of *ee* and concentration of the epoxides.

General Procedure for Enantioselective Hydrolysis of *Meso*-Epoxides with Resting Cells of *E. coli* (SpEH). Freshly prepared *E. coli* (SpEH) cells were diluted by Tris

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buffer (50 mM, pH 7.5) to multiple 1 mL systems with required cell density (as indicated in Table 2) in 50 mL flasks. Next, an appropriate amount of epoxides (as indicated in Table 2) was added to the reaction systems in the flasks. The reaction mixture was shaken (250 rpm) in an incubator (New Brunswick Scientific) at 30 °C for the appropriate time. One flask was taken out at different time points and totally extracted by adding ethyl acetate (2 mL). Analytic samples were prepared by centrifugation, and then 300 μL of organic phases were separated, diluted with ethyl acetate (containing 2 mM *n*-dodecane as internal standard), and dried over Na_2SO_4 before GC quantification of *ee* and concentration of the epoxides.

Preparation of (*S*)-Styrene Oxide 1, (*S*)-2-(3-Chlorophenyl)oxirane 3, and (*S*)-2-(4-Fluorophenyl)-oxirane 6 by Enantioselective Hydrolysis of the Corresponding Racemic Epoxides with Resting Cells of *E. coli* (SpEH). Freshly prepared *E. coli* (SpEH) cells were diluted by Tris buffer (50 mM, pH 7.5) to a 20−110 mL system with required cell density (as indicated in Table 3) in a 250−1000 mL flask. And then a second phase *n*-hexane (20−100 mL) containing appropriate amount of epoxides (as indicated in Table 3) was added to the reaction system in the flask. The reaction mixture was shaken (250 rpm) in an incubator at 30 °C for the appropriate time. The reaction was monitored by HPLC, and terminated by cooling down on ice once the *ee* value of residual epoxide reached 99%. The reaction system was then immediately extracted three more times by *n*-hexane (3 × 20−100 mL), and all the organic phases were combined. After drying over Na_2SO_4, the solvents were removed by evaporation. The crude product was then purified by flash chromatography on a silica gel column with *n*-hexane:ethyl acetate = 50:1 (R_f = 0.3 for all the three products).

(*S*)-**1**: Obtained as a colorless liquid after column chromatography; yield: 36.4%; > 99% *ee*. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ = 7.26−7.38 (m, 5H, ArH), 3.85−3.88 (dd, *J* = 4.0, 2.8 Hz, 1H), 3.14−3.16 (dd, *J* = 5.2, 4.0 Hz, 1H), 2.79−2.82 (dd, *J* = 5.6, 2.8 Hz, 1H).

(*S*)-**3**: Obtained as a light yellow liquid after column chromatography; yield: 37.9%; > 99% *ee*. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ = 7.26−7.28 (m, 3H, ArH), 7.16−7.18 (m, 1H, ArH), 3.82−3.84 (dd, *J* = 4.0, 2.4 Hz, 1H), 3.13−3.16 (dd, *J* = 5.6, 4.0 Hz, 1H), 2.75−2.77 (dd, *J* = 5.6, 2.4 Hz, 1H).

(*S*)-**6**: Obtained as a colorless liquid after column chromatography; yield: 36.4%; > 99% *ee*. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ = 7.22−7.27 (m, 2H, ArH), 7.00−7.06 (m, 2H, ArH), 3.83−3.85 (dd, *J* = 4.0, 2.4 Hz, 1H), 3.12−3.15 (dd, *J* = 5.6, 4.0 Hz, 1H), 2.75−2.77 (dd, *J* = 5.2, 2.4 Hz, 1H).

Preparation of (1*R*, 2*R*)-1,2-Cyclopentanediol 12, (1*R*, 2*R*)-1,2-Cyclohexanediol 13, and (3*R*, 4*R*)-*N*-Benzyloxycarbonyl-3,4-dihydroxypyrroli-dine 14 by Enantioselective Hydrolysis of the Corresponding *Meso*-Epoxides with Resting Cells of *E. coli* (SpEH). Freshly prepared *E. coli* (SpEH) cells were diluted by Tris buffer (50 mM, pH 7.5) to a 5−200 mL system with required cell density (as indicated in Table 4) in a 100−1000 mL flask. And appropriate amount of epoxides (as indicated in Table 4) was added to the reaction system in the flask. The reaction mixture was shaken (250 rpm) in an incubator at 30 °C for the appropriate time. The reaction was monitored by GC, and terminated by cooling down on ice once the conversion of *meso*-epoxides reached 99%. The reaction system was immediately saturated with NaCl and extracted four times by ethyl acetate (4 × 5−200 mL), and then all the organic phases were combined. After drying over Na_2SO_4, the solvents were removed by evaporation. The crude product was then purified by flash chromatography on a silica gel column with ethyl acetate (for **14**, R_f = 0.3) or crystallization in ethyl acetate (dissolved at 60 °C and slowly cooling down to 4 °C, for **13**).

(**1*R*, 2*R*)-12**: Obtained as light yellow oil; yield: 70.4%; 88% *ee*. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ = 3.93−3.98 (m, 2H), 3.18 (s, 2H, OH), 1.94−2.04 (m, 2H), 1.67−1.74 (m, 2H), 1.47−1.56 (m, 2H).

(**1*R*, 2*R*)-13**: Obtained as a white crystal after crystallization; yield: 88.6% before and 68.5% after crystallization; 99% *ee* after crystallization. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ = 3.32−3.34 (m, 2H), 3.08 (br s, 2H, OH), 1.95−1.96 (m, 2H), 1.68−1.69 (m, 2H), 1.24−1.30 (m, 4H).

(**3*R*, 4*R*)-14**: Obtained as light yellow syrup; yield: 94.1%; 93% *ee*. ^{1}H NMR (400 MHz, $CDCl_3$, TMS): δ = 7.24−7.36 (m, 5H, ArH), 5.07 (s, 2H), 4.08−4.09 (m, 2H), 3.62−3.66 (m, 2H), 3.62−3.66 (m, 2H), 3.25 (s, 2H).

■ ASSOCIATED CONTENT

Supporting Information

Chemicals; strains and biochemicals; chemical synthesis of epoxides **5**, **7**, **8**, and **11**; analytical methods; kinetic study; amino acid sequence of SpEH; chiral HPLC chromatograms; chiral GC chromatograms; and ^{1}H NMR spectra of biotransformation products. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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