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Biocatalytic preparation of (*S*)-phenyl glycidyl ether using newly isolated *Bacillus megaterium* ECU1001

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Abstract

A bacterial strain (ECU1001) capable of utilizing phenyl glycidyl ether as sole carbon source and energy source was isolated from soil samples through two steps of screening and was identified as a *Bacillus megaterium*. The epoxide hydrolase from *Bacillus megaterium* ECU1001 was biosynthesized in parallel with cell growth and a maximum activity of 31.0 U/l was reached after 30 h of culture when the biomass (DCW) was 9.1 g/l. A temperature of 35°C and pH 8.0 were optimal for the bioconversion. The lyophilized whole cells of *Bacillus megaterium* ECU1001 could preferentially hydrolyze the (*R*)-enantiomer of phenyl glycidyl ether, yeilding (*S*)-epoxide and (*R*)-diol with high enantioselectivity ($E = 47.8$). The (*S*)-enantiomer of the epoxide remained in the reaction mixture with >99.5% ee (enantiomeric excess) at a conversion of 55.9%. The substrate concentration could be increased up to 60 mM without affecting the ee and (*S*)-phenyl glycidyl ether could be obtained with an optical purity of 100% ee and 25.6% yield. Therefore, the method is potentially useful for the preparative resolution of epoxides.

Keywords: Phenyl glycidyl ether; *Bacillus megaterium*; Epoxide hydrolase; Kinetic resolution; Enantioselective hydrolysis

1. Introduction

Enantiopure epoxides and their corresponding vicinal diols are extensively employed as useful chiral building blocks for synthesis of various biologically active products in the pharmaceutical and agrochemical industries, due to their high ability to react with a broad variety of nucleophiles. Therefore, a great interest exists in the development of methods for the synthesis of enantiopure epoxides and diols. Various chemical and biological methods for synthesis of enantiopure epoxides and diols have been reviewed [1–5]. One of the most promising ways for the synthesis of such chiral synthons under environmentally gentle conditions is the enantioselective hydrolysis of racemic epoxides using cofactor-independent epoxide hydrolases [EC 3.3.2.X] [6]. Although enzymes from mammalian sources, such as liver tissue, have been investigated in great detail during detoxification studies [7], biotransformations on a preparative scale are hampered by the limited supply of enzyme and they rarely surpass the millimole range. It was only in the nineties that microbial sources from either bacteria [8–17] or fungi [18–25] with highly enantioselective

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epoxide hydrolases were identified. This made available an unlimited supply of these enzymes for preparative-scale applications. Most of these strains were obtained by screening from culture collections [8–10,19,21,22,25]. Not always did this approach result in obtaining biocatalysts with sufficiently high enantioselectivity for resolution of a broad range of epoxides. Thus, we think it is also a valuable alternative to isolate from a broader diversity of microorganisms as it is found in soil samples. Previous isolation from soil samples [11–13,15,26] concentrated on utilization of alicyclic and terminal aliphatic epoxides as sole carbon sources. It would therefore be instructive if aryl epoxides were used as carbon sources for the isolation from nature.

Aryl epoxide, phenyl glycidyl ether (PGE) is a potentially useful compound for the synthesis of chiral amino alcohols [27] and bioactive compounds such as β-blockers [28]. No suitable biocatalyst with sufficiently high enantioselectivity ($E > 20$) for the kinetic resolution of this compound has previously been found among bacteria and fungi. Recent reports [25,29] suggested that the yield of residual (*R*)-phenyl glycidyl ether had to be decreased to as low as 26–28% (analytical yield) for achieving an ee of 99%. This prompted us to screen epoxide hydrolase-producing microorganisms with higher enantioselectivity towards phenyl glycidyl ether from soil samples.

In this paper, we describe the isolation, characterization and preliminary application of an aryl epoxide utilizing bacterium.

2. Experimental

2.1. Materials and basal medium

Phenyl glycidyl ether (PGE, 97.6% purity including 2.4% corresponding diol) was purchased from ACROS Co. Ltd. All other chemicals were also obtained commercially and were of analytical grade.

Mineral salts medium (MSM, in g/l): $(NH_4)_2SO_4$ 1.0, $K_2HPO_4{\cdot}3H_2O$ 6.0, KH_2PO_4 3.0, NaCl 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, $CaCl_2$ 0.05.

Fermentation medium (in g/l): glucose 20.0, yeast extract 4.0, peptone 4.0, NH_4Cl 2.0, $K_2HPO_4{\cdot}3H_2O$ 6.0, KH_2PO_4 3.0, NaCl 0.5, $MgSO_4{\cdot}7H_2O$ 0.5.

2.2. Isolation of PGE-utilizing strains with highly enantioselective epoxide hydrolase activity

Bacteria with epoxide hydrolase activity were isolated from soil samples through two steps of screening: the first screening for epoxide hydrolase activity and the second screening for enantioselective epoxide hydrolase activity. After enrichment culture of soil samples, collected from different locations in Shanghai and Guangzhou, China, in 50 ml of mineral salts medium supplemented with 2.5 ml of 2% (v/v) PGE in dibutylphthalate as sole carbon source at 30°C for 1 week and one successive transfer into fresh medium, samples of the cultures were plated onto agar plates with gaseous PGE as sole carbon source. Colonies that developed were picked up and inoculated individually to MSM with 1% glucose, 1% peptone and 0.1% yeast extract. Two days later, 1% (v/v) PGE was added. After 1 day of conversion, the remaining epoxide and the produced diol were analyzed by HPLC. About 1–2 g (wet weight) of washed cells with obvious epoxide hydrolase activity were suspended in 19 ml of 100 mM potassium phosphate buffer (KPB, pH 8.0) and then 1 ml of 200 mM PGE in DMSO was added to give a final concentration of 10 mM. The mixture was incubated at 30°C for 12 h. Samples (2 ml) of the reaction mixture were withdrawn for ee_s and ee_p determination.

2.3. Culture conditions

The isolate, *Bacillus megaterium* ECU1001, was grown for 36 h in 3.0 l of a fermentation medium supplemented with 50 μl/l of silicone antifoam in a 5 l fermentor at 30°C with vigorous agitation (500 rpm) and with an aeration rate of 0.33 vvm. The medium was inoculated with 200 ml of a preculture in flasks for 11.5 h. At time intervals, 20 ml of culture was taken, the cells were harvested by centrifugation and the epoxide hydrolase activity was assayed. The dry mass of cells was measured after drying the wet cells at 50°C until constant weight. Glucose concentration was measured by using a commercial reagent kit.

2.4. Enzyme assay

To a test tube containing 1.8 ml of 100 mM KPB (pH 8.0), the cells harvested from 10 ml of culture were added. The mixture was vortexed and pre-incubated

on a shaker (120 rpm, 30°C) for 5 min. Then 0.2 ml of 200 mM racemic PGE in ethanol was added, giving a final concentration of 20 mM. After 10 min of incubation, the reaction was stopped by addition of 6 ml methanol solution containing *para*-cresol as internal standard (2.8 mM). After centrifugation, the supernatant was directly subjected to HPLC analysis to determine the quantity of diol formed. One unit of epoxide hydrolase activity is defined as the amount of enzyme catalyzing the formation of 1.0 μmol of diol per minute under the above conditions.

2.5. Kinetic resolution of PGE by lyophilized cells of *Bacillus megaterium*. ECU1001

The isolate, *Bacillus megaterium* ECU1001 was cultured in a fermentation medium as above. At the late exponential growth phase (30 h) the cells were harvested and lyophilized. Lyophilized microbial cells (0.30 g) were suspended in 38 ml of 100 mM KPB (pH 8.0) and pre-incubated for 30 min. Then 2 ml of 200 mM PGE in DMSO was added to give a final concentration of 10 mM and the mixture was shaken at 120 rpm and 30°C. The time-course of the bioconversion was monitored by withdrawing 3 ml of each samples at time intervals, 2 ml for determination of ee_s and ee_p, and 1 ml for determination of product and substrate concentrations.

2.6. Optima of temperature and pH

Lyophilized cell aliquots (25 mg) were added into 1.9 ml of 50 mM KPB (pH 8.0), and then 0.1 ml of 200 mM PGE in DMSO was added. The mixtures were respectively incubated for 10 min at different temperatures and enzyme activity was measured as described above. The pH optimum was determined at 30°C in buffers of various pHs using aliquots of the lyophilized cells as described above for the determination of temperature optimum.

2.7. Effect of initial PGE concentration on kinetic resolution of its racemates

Lyophilized microbial cells (60 mg) were suspended in 1.9 ml of 100 mM KPB (pH 8.0) and 0.1 ml of different concentration of PGE in DMSO was added, giving final concentrations of 10, 20, 40, 60, 80 and 100 mM. Sampling becomes a problem due to the insolubility of the epoxide, therefore different reaction vessels were used for different times. After workup, ee_s and ee_p were determined by chiral HPLC.

2.8. Preparative biohydrolysis experiment

Lyophilized microbial cells (6 g) were suspended in 190 ml of 100 mM KPB (pH 8.0) and 10 ml of 1.2 M PGE in DMSO was added, giving a final concentration of 60 mM. The mixture was shaken at 120 rpm and 30°C. After incubation for 16 h, the mixture was centrifuged. The cells were separately washed three times with ethyl acetate. The aqueous phase was saturated with NaCl and then extracted three times with ethyl acetate, the combined organic layers were dried (Na_2SO_4) and evaporated in vacuum. Purification from the crude product by silica gel chromatography with ethyl acetate-hexane (3:2) afforded (*S*)-**1** and (*R*)-**2**.

2.9. Analytical methods

The concentrations of product and substrate were determined by HPLC using a reverse phase column (Lichrosorb RP-18, Merck, Germany, 200 mm × ∅5.0 mm, 10 μm). The cells in 1 ml of the reaction mixture were removed by centrifugation at 7500 × *g* for 5 min and 0.25 ml of the supernatant was added to 0.75 ml of methanol solution containing *para*-cresol as internal standard (2.8 mM). After centrifuging again, 20 μl of the solution was injected into the HPLC column which was eluated with methanol/water (60/40, v/v, 1.0 ml/min) and detected at 254 nm. The retention times were 4.9, 7.2, 8.6 min for the diol, internal standard and epoxide, respectively.

The relative amount of each enantiomer of the phenyl glycidyl ether and its corresponding diol were analyzed by HPLC using a chiral column (Chiralcel OD, Daicel, Japan, 250 mm × ∅4.6 mm). The cells in 2 ml of sample of the reaction mixture were centrifuged (15,000 × *g*, 10 min) and the clear supernatant was extracted with 2 ml of ethyl acetate after saturation with NaCl. The organic extract was dried over Na_2SO_4 and filtered through a 0.45 μm micro-filtration membrane prior to injection (20 μl) into the chiral column. The mobile phase was

hexane/2-propanol (90/10, v/v) and its flow rate was 1.0 ml/min. Detection was made by measuring absorbance at 254 nm. The retention times for (*R*)-epoxide, (*S*)-epoxide, (*R*)-diol and (*S*)-diol were 6.8, 10.8, 15.2 and 31.8 min, respectively.

3. Results and discussion

3.1. Isolation and preliminary identification of PGE-utilizing strains with enantio-selective epoxide hydrolase activity

In the first screening, the relative activity of epoxide hydrolase (EH) versus chemical auto-hydrolysis was expressed by a ratio of $(X_p - X_o)/X_o$, where X_p represents total conversion of the substrate, while X_o is conversion of the substrate caused by chemical auto-hydrolysis which was measured in the control experiments. It is observed that the most probable errors of assay experiments were within 25% $[(X_p - X_o)/X_o = \pm 0.25]$. Therefore, a value of 0.3 of $(X_p - X_o)/X_o$ was regarded as a reasonable criterion for judging whether a strain had the EH activity or not. Accordingly, about 90 strains among about 400 strains of bacteria obtained in the first screening were considered to be significantly active $[(X_p - X_o)/X_o > 0.5]$ towards the epoxide (PGE). These strains were subjected to secondary examination with respect to their enantioselectivities in bioconversion of the epoxide substrate.

As shown in Fig. 1, the values of ee (ee_s or ee_p) of about 20 strains were larger than 50%. The highest ee_s and ee_p once obtained were 94.9 and 91.3% with two strains. However, when these strains were determined repeatedly, only one strain showed relatively stable values of enantioselectivity ($E > 10$), while other strains showed fluctuating E values in different experiments under similar conditions. It is worth noting that unknown by-products other than diol were observed, although the underlying reasons for the instability of these strains are not yet clear. It is presumed that the by-products are aldehydes or ketones formed via epoxide isomerase catalyzed rearrangement of the oxirane [10]. The strain with consistently high enantioselectivity was chosen for further study.

Taxonomic studies of this strain indicated that it belongs to the genus *Bacillus* because the Gram-positive rods were aerobic, spore-forming, motile, catalase-positive, egg yolk-positive, and lysozyme-resistant and they formed acid on glucose [30]. This strain is presently preserved in the East China University of Science and Technology and designated as *Bacillus megaterium* ECU1001.

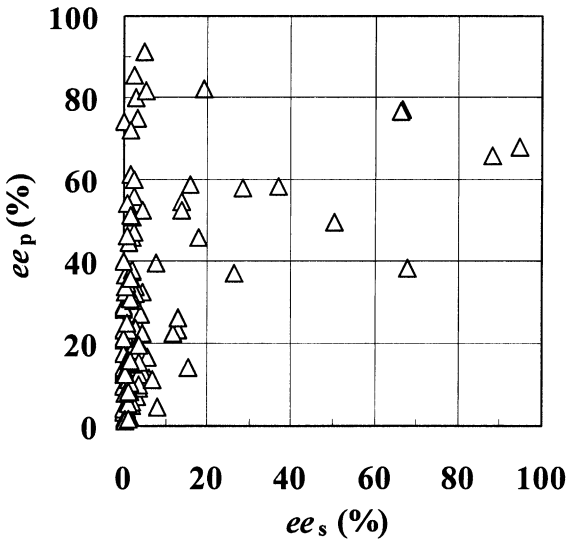


Fig. 1. Enantioselectivities of epoxide hydrolases produced by some isolated bacteria.

3.2. Growth and EH activity of Bacillus megaterium ECU1001 on a fermentation medium

The kinetics of the epoxide hydrolase production was investigated at first by cultivating *Bacillus megaterium* ECU1001 in a 5 l fermentor using the fermentation medium (Fig. 2). Exponential growth was observed from 2 to 10 h with a specific growth rate of 0.33 h^{-1}. The enzyme activity increased to 21.3 U/l in parallel with cell growth. The maximum activity of 31.0 U/l was reached after 30 h of fermentation when the biomass (DCW) was 9.1 g/l. No activity could be detected in the extracellular fraction (culture broth) within the whole fermentation period. Therefore, the epoxide hydrolase of *Bacillus megaterium* ECU1001 is a cell bound activity. Glucose was nearly consumed after 24 h. The detection of high epoxide hydrolase activity (31.0 U/l) after growth on glucose indicates a constitutive basal level of expression. However, higher levels of activity (60.0 U/l) were induced after growth

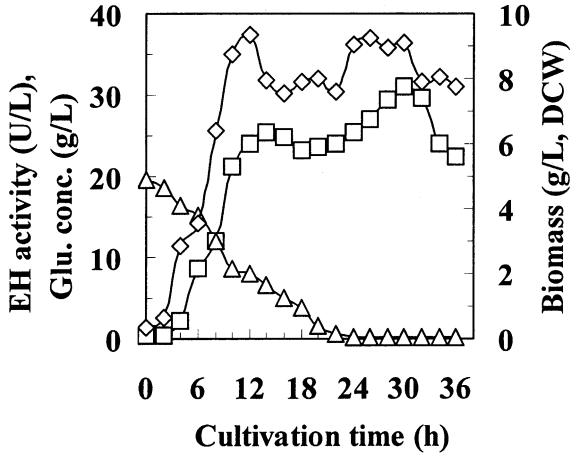


Fig. 2. Growth and epoxide hydrolase production by *Bacillus megaterium* ECU1001 in a 5 l fermentor. Symbols: (◇) biomass; (□) EH activity; (△) residual glucose.

on phenyl glycidyl ether (11.8 mM), the substrate used for its isolation (data not shown). Whether this represents further induction of the constitutive enzyme or a second epoxide hydrolase remains to be established.

3.3. Kinetic resolution of PGE by lyophilized cells of Bacillus megaterium. ECU1001

The kinetics of biocatalytic resolution of racemic phenyl glycidyl ether was examined using lyophilized whole cells of *Bacillus megaterium* ECU1001 as a biocatalyst. The change in the concentrations and ee of the substrate and product, with time, is shown in Fig. 3A. The initial reaction rate calculated over 0.5 h was 11.6 nmol/min/mg (lyophilized cells). The ee of the remaining epoxide increased from 0 to 99.5% after 4 h, while the ee of the diol decreased slightly from 87.3 to 80.6%. After 4 h of reaction, the residual epoxide concentration was 4.5 mM (analytical yield, 41.7%; ee, 99.5%) and the diol concentration was 6.3 mM (analytical yield, 58.3%; ee, 80.6%). From these data, enantiomeric ratio (*E*) of 47.8 was obtained using the equation [31]:

$$E = \ln\left[\frac{(1-c)(1-\mathrm{ee_s})}{(1-c)(1+\mathrm{ee_s})}\right]$$

where $\mathrm{ee_s}$ is the enantiomeric excess of epoxide, $\mathrm{ee_p}$ the enantiomeric excess of diol, and c the corrected

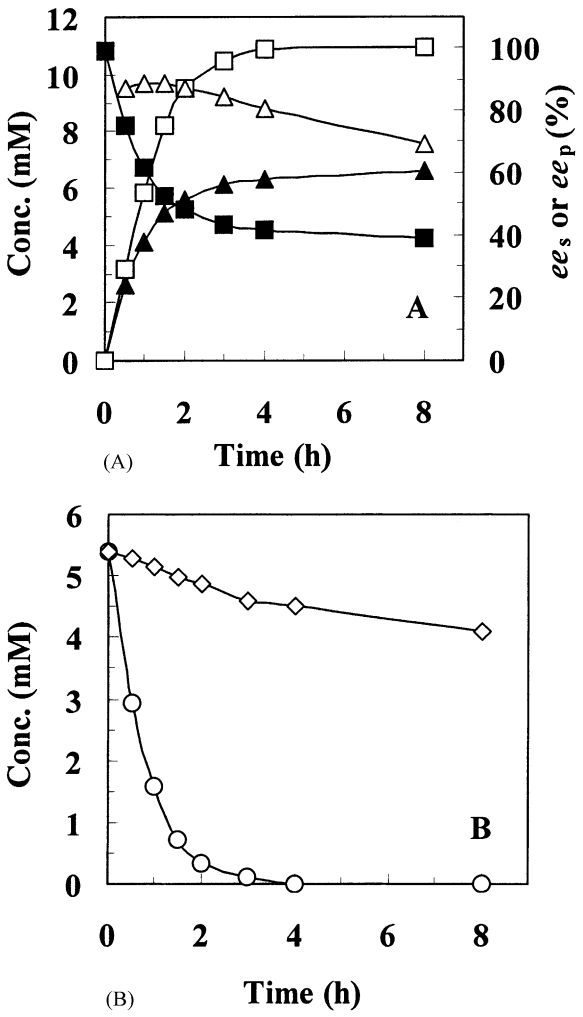


Fig. 3. Kinetic resolution of racemic phenyl glycidyl ether (10.8 mM) by lyophilized cells of *Bacillus megaterium* ECU1001 (7.5 g/l, weight of lyophilized cells). (A) Variation in concentrations of epoxide (■) and diol (▲) and ee of epoxide (□) and diol (△) with time. (B) Variation in concentrations of each enantiomer of the epoxide with time: (*S*)-PGE (◇); (*R*)-PGE (○).

conversion ratio which was calculated from the apparent conversion by subtracting the initial concentration (2.4%) of diol in commercial epoxide.

From the $\mathrm{ee_s}$ and the total concentration of the residual epoxide (C_s), the individual concentrations of (*R*)- and (*S*)-epoxides were calculated using the following equations:

$$[R] = \frac{C_s(1-\mathrm{ee_s})}{2}, \qquad [S] = \frac{C_s(1+\mathrm{ee_s})}{2}$$

The results are shown in Fig. 3B. The concentration of the (*R*)-enantiomer decreased rapidly while that of the (*S*)-enantiomer decreased very slowly. From these data, the initial rates (calculated over 0.5 h) of hydrolysis were estimated to be 11.0 and 0.53 nmol/min/mg (lyophilized cells) for the (*R*)- and (*S*)-enantiomers, respectively; therefore, the (*R*)-epoxide was hydrolyzed 20.8 times faster than the (*S*)-epoxide.

3.4. Optimization of reaction conditions for production of (S)-phenyl glycidyl ether

The initial rate of hydrolysis of phenyl glycidyl ether in a reaction mixture containing 5% (v/v) DMSO was examined at temperatures ranging from 10 to 50°C and at pHs ranging from 4 to 10. The results are shown in Fig. 4 and Fig. 5. The temperature optimum of the enzyme was 35°C. The enzyme had a pH optimum, peaking at around pH 8.0. Below pH 5.0, the activity could no longer be measured accurately, as chemical hydrolysis of the substrate overtook the biological hydrolysis rate.

Since PGE is known to be a toxic chemical to microbial cells, the initial concentration of PGE which gives least substrate inhibition needs to be determined. Fig. 6 shows the ee values for (*S*)-PGE in the enantioselective hydrolysis of racemic PGE. The kinetic resolution was successfully carried out with 100% ee up to 60 mM PGE. However, when the initial PGE concentration was higher than 80 mM,

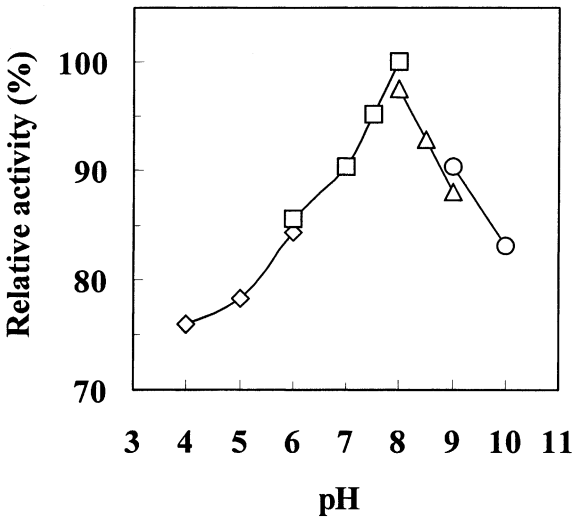


Fig. 5. Effect of pH on activity of epoxide hydrolase produced by *Bacillus megaterium*. The enzyme activity was determined at 30°C. Buffers used: (◇) 50 mM citrate; (□) 50 mM potassium phosphate; (△) 50 mM Tris–HCl; (○) 50 mM glycine-NaOH.

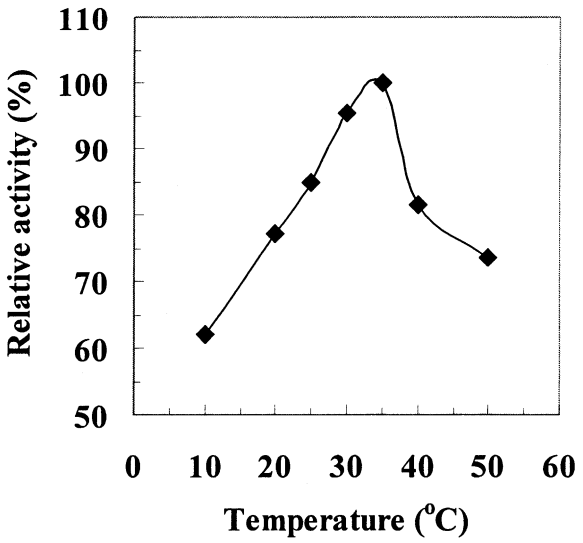


Fig. 4. Effect of temperature on activity of epoxide hydrolase produced by *Bacillus megaterium* The enzyme activity was determined in 50 mM potassium phosphate buffer (pH 8.0).

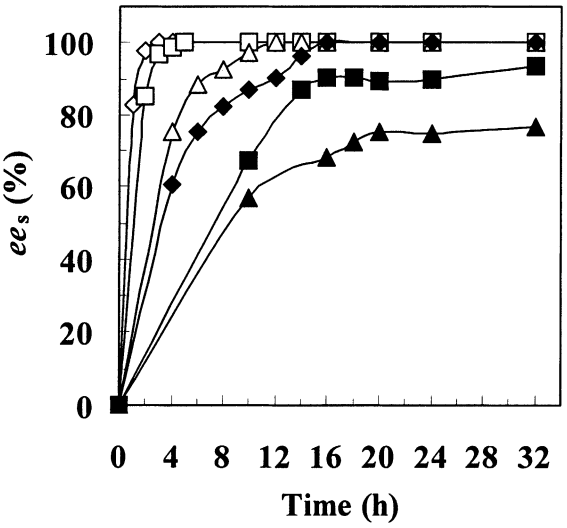
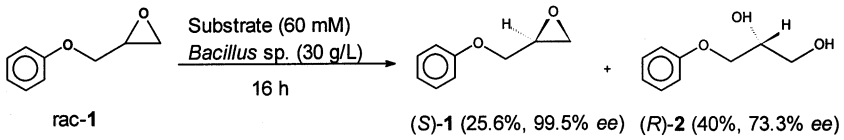


Fig. 6. Effect of substrate concentration on the ee_s during the hydrolysis of racemic substrate by *Bacillus megaterium* (30 g/l, weight of lyophilized cells). Symbols: (◇) 10 mM; (□) 20 mM; (△) 40 mM; (◆) 60 mM; (■) 80 mM; (▲) 100 mM.



Scheme 1.

the concentrations of substrate and diol leveled off at around 38 and 42 mM after 16 h, respectively. It should be noted that the half-life for the enzyme was around 22 h in the absence of substrate (data not shown); thus, the cease of reaction should not be caused by a complete inactivation of the enzyme. Most probably the reaction stopped due to product (diol) inhibition, instead of substrate inhibition, since the residual substrate at 16 h was less than half of its initial concentration while concentration of the accumulated diol exceeded 40 mM. Therefore, excessive product should be removed continuously from the reaction system for efficient conversion of the substrate at higher concentrations (>60 mM).

Finally, preparative hydrolysis was performed with lyophilized cells (30 g/l) in 200 ml of 100 mM KPB (pH 8.0) containing 5% (v/v) DMSO and 60 mM PGE. The result is shown in Scheme 1. Following a 16 h reaction, enantiopure (*S*)-PGE **1** (0.46 g, 25.6% yield, >99.5% ee) was obtained as a colorless oil, $[\alpha]_D^{33} + 9.8$ (*c* 1.00, ethanol) and the (*R*)-3-phenoxy-1, 2-propanediol **2** (0.80 g, 40% yield, 73.3% ee) as a white solid, mp 60–62°C; $[\alpha]_D^{33} - 8.6$ (*c* 1.00, ethanol).

4. Conclusions

The selective isolation of bacterial strains is commonly achieved by the introduction of an inoculum into a medium in which a substrate is used as sole carbon source. With an epoxide as the substrate for isolation of epoxide hydrolase producers, problems arise due to the chemical lability of the substrate and its toxicity against most microorganisms. Therefore we adopted a biphasic isolation system [15] to reduce substrate toxicity. Despite the problems inherent with the isolation of epoxide-degrading microorganism, a phenyl glycidyl ether-degrading bacterium, *Bacillus megaterium* ECU1001, was successfully isolated from enriched cultures through two steps of screening. This is, to our knowledge, the first isolate which was able to grow with an aryl epoxide (phenyl glycidyl ether) as sole carbon and energy source.

The epoxide hydrolase of *Bacillus megaterium* ECU1001 was synthesized in parallel with cell growth and a maximum activity of 31.0 U/l was reached after 30 h when the biomass (DCW) was 9.1 g/l using glucose as carbon source. The intracellular enzyme could preferentially hydrolyze (*R*)-enantiomer of racemic phenyl glycidyl ether with high enantioselectivity ($E = 47.8$), much higher than those reported recently [25,29]. The substrate concentration could be increased up to 60 mM and the (*S*)-epoxide could be obtained with an optical purity of 100% ee and a yield of 25.6%. It seems to be a potentially applicable method for the production of optically pure (*S*)-phenyl glycidyl ether.

Acknowledgements

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