Enantioselective hydrolysis of racemic epichlorohydrin (ECH) leading to enantiopure (R)-ECH using recombinant epoxide hydrolase (EH) was successfully undertaken in organic solvents. The lyophilized recombinant whole-cell *Pichia pastoris* expressing the EH gene of *Rhodotorula glutinis* was used as the biocatalyst; *n*-dodecane containing 2.5 % (v/v) water was employed as the reaction medium to prevent the spontaneous chemical degradation of racemic ECH. Enantiopure (R)-epichlorohydrin (ECH) with 99 % ee was obtained from 20 mM of the racemate with a yield of 28.5 % in *n*-dodecane within 60 min.

**Keywords:** enantioselective hydrolysis, recombinant epoxide hydrolase, (R)-epichlorohydrin, organic solvent, *Rhodotorula glutinis*
Table 1. Effect of Adding Lyoprotectants and Detergents During the Lyophilization of the Recombinant P. pastoris Expressing the EH gene from R. glutinis on the Initial Hydrolysis Rate of (S)-Epichlorohydrin in Cyclohexane and the Enantioselectivity

<table>
<thead>
<tr>
<th>Lyoprotectant or detergent</th>
<th>Initial hydrolysis rate (nmol min(^{-1}) mg(^{-1}) of cell)</th>
<th>(E)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^b)</td>
<td>44.0</td>
<td>1.45</td>
</tr>
<tr>
<td>Tween 20 (5 % (v/v))</td>
<td>70.3</td>
<td>2.70</td>
</tr>
<tr>
<td>Tween 80 (5 % (v/v))</td>
<td>62.2</td>
<td>2.13</td>
</tr>
<tr>
<td>18-Crown-6 (5 % (w/v))</td>
<td>3.0</td>
<td>1.11</td>
</tr>
<tr>
<td>Skim milk (5 % (w/v))</td>
<td>40.5</td>
<td>1.41</td>
</tr>
<tr>
<td>Gelatin (5 % (w/v))</td>
<td>38.3</td>
<td>1.39</td>
</tr>
<tr>
<td>Mannitol (5 % (w/v))</td>
<td>63.2</td>
<td>2.29</td>
</tr>
<tr>
<td>Triton X-100 (5 % (v/v))</td>
<td>48.8</td>
<td>1.68</td>
</tr>
<tr>
<td>Sucrose (10 % (w/v))</td>
<td>40.0</td>
<td>1.52</td>
</tr>
<tr>
<td>Lactose (5 % (w/v))</td>
<td>45.6</td>
<td>1.62</td>
</tr>
<tr>
<td>BSA (5 % (w/v))</td>
<td>41.6</td>
<td>1.56</td>
</tr>
<tr>
<td>Glycerol (5 % (v/v))</td>
<td>30.3</td>
<td>1.38</td>
</tr>
</tbody>
</table>

\(^a\)Enantiomeric ratios, \(E = \ln(S/R)/\ln(R/S)\), determined after 10 min.

\(^b\)Recombinant cells were lyophilized in the absence of lyoprotectants and detergents.

Previously [10,11]. The recombinant P. pastoris strains were cultivated in a BMGY medium containing 10 g yeast extract/L and 10 g peptone/L at 30 °C and 250 rpm [10]. The EH gene expression was induced by the periodic addition of 1 % (v/v) methanol over 72 h. All standard molecular work was performed as described previously [12,13]. The recombinant cells were harvested by centrifugation, washed with distilled water, and lyophilized. Various lyoprotectants and detergents were added during the lyophilization procedure (-70 °C for 6 h) to increase the stability of the lyophilized cells [14]. Racemic ECH and all organic solvents were of analytical grade (Sigma-Aldrich, USA). Batch kinetic resolution experiments were carried out in a 5-mL screw-capped vial sealed with Teflon to investigate the effects that adding various lyoprotectants, the hydrophobicity of organic solvents, and the water content had on the enantioselectivity of the hydrolysis of racemic ECH in organic solvents. Lyophilized cells (10 mg/mL) were suspended in organic solvents with various hydrophobicities, and enantioselective hydrolysis reactions were initiated with the addition of 10 mM racemic ECH in a shaking incubator at 30 °C and 250 rpm. To optimize the water content, 0 ~ 5 % (v/v) of water was added to the organic solvents prior to beginning the enantioselective hydrolysis reactions. The progression of each enantioselective hydrolysis reaction was analyzed through the analysis of organic samples withdrawn periodically from the reaction mixture. The enantiomeric excess [\(ee = 100 \times (R-S)/(R + S)\)] and yield (yield = 100 × residual (R)-epoxide concentration/initial racemic epoxide concentration) of enantiopure ECH were determined using GC analysis. After the lyophilized cells were removed by centrifugation, 1 µL of the organic solvent layer was analyzed using a GC system equipped with a fused silica capillary beta-DEX 120 column (0.25 mm ID × 30 m, 0.25 µm film thickness, Supelco Inc., USA) fitted with a FID detector. The temperatures of the column, injector, and detector were 100, 220, and 220 °C, respectively.

In general, the catalytic activity of whole-cell biocatalysts decreases during a lyophilization procedure. Various lyoprotectants and detergents have been tested to prevent inactivation during lyophilization [14]. To select the most suitable lyoprotectant, the cells were lyophilized in the presence of different concentrations of various lyoprotectants and detergents (Table 1). After the cells were lyophilized at -70 °C for 6 h, the initial hydrolysis rates of (S)-ECH and enantiomeric ratio at 10 min were determined for the lyophilized cells in cyclohexane. The initial hydrolysis rates were 1.4 - 1.6-fold enhanced for the cells lyophilized in the presence of 5 % (v/v) Tween 20 and Tween 80 and 5 % (w/v) mannitol when compared to that of the cells hydrolyzed in the absence of any kind of lyoprotectant or detergent (Table 1). The enantiomeric ratios calculated for the cells lyophilized in the presence of 5 % (v/v) Tween 20 and Tween 80 and 5 % (w/v) mannitol were also improved by 50 to 90 %. Figure 1 shows the time-dependent changes of the concentrations of (R)- and (S)-ECH during the enantioselective hydrolysis reaction of 10 mM racemic ECH when using the recombinant P. pastoris catalysts prepared in the presence and absence of 5 % (v/v) Tween 20. When the lyophi-
of any organic solvent compatibility of the organic solvents containing 20 % (v/v) organic solvents to evaluate the bio-

teraction resistance in the aqueous phase became problem-

viscosity of the aqueous phase became so high that mass-


tation of low-activity wild-type cells, a mixing problem

improve the hydrolysis rate by increasing the concen-

tration of low-activity wild-type cells, a mixing problem

improve the hydrolysis rate by increasing the concen-

tration of low-activity wild-type cells, a mixing problem

water was the other reactant in the hydrolysis reaction

hydrolysis as 100 %

depended on the nature of the organic solvent; the high-

different hydrophilic and hydrophobic characteristics.

EH activity of the recombinant whole cells clearly
depended on the nature of the organic solvent; the high-

dest hydrolysis activities occurred when hydrophobic or-

ganic solvents, including n-dodecane, phthalic acid, and
dioctyl phthalate, were used (Table 2). The EH activity of
the recombinant cells, however, was not directly pro-
portional to the degree of hydrophobicity of the tested or-
ganic solvents, indicating that the recombinant EH activity
depends not only on the hydrophobicity but also on the
nature of the organic solvent. Among the organic sol-

vents tested, n-dodecane was chosen as the reaction me-

dium for the enantioselective resolution based on its

good biocompatibility.

Water was the other reactant in the hydrolysis reaction of racemic ECH by EH. Water was also required to

maintain the activity of the EH enzyme. The effect of the

water content [varying from 0 to 5 % (v/v)] on the enan-
tioselectivity and yield of the kinetic resolution of 10 mM
ECH was investigated to determine the optimal ratio.

The enantiomeric excess and yield at 2.5 % (v/v) water

content were optimal (data not shown).

Batch kinetic resolution of 20 mM ECH using the

lyophilized recombinant P. pastoris in n-dodecane was

performed under the optimized reaction conditions.
The enantioselective hydrolysis reaction was begun by adding

20 mM ECH into the reaction vial containing 10 mg/mL of

lyophilized cells and 2.5 % (v/v) water at 30 °C and

250 rpm. The time-dependent changes in the concen-

tration of (R)- and (S)-ECH and the enantiomeric excess

are shown in Figure 2. After 60 min, enantiopure
(R)-ECH (99 % ee) was obtained in a yield of 28.5 %.

Relative to other kinetic resolutions of ECH by EHs, the

yield and overall productivity were clearly enhanced [7].

In a previous study, (S)-ECH with a yield of only ca. 2

% was obtained from 10 mM racemic ECH using a pow-
der of Aspergillus niger cells in aqueous buffer [6].

When an organic solvent was employed as the reaction

medium in an effort to increase the yield of the kinetic

resolution of racemic ECH when using powdered

Aspergillus niger cells, the yield was enhanced to 20 %
[7]. The reaction time required to reach 99 % ee for an

initial concentration of 60 mM of racemic ECH was pro-

longed to 16 h, which decreased the average prod-

uctivity. In our present study, we employed a recombi-
nant whole-cell biocatalyst possessing enhanced EH ac-
tivity to shorten the reaction time and improve the over-
all productivity. The reaction time could be shortened to

60 min and the overall yield was enhanced to 28.5 %. To

improve the hydrolysis rate by increasing the concen-

tration of low-activity wild-type cells, a mixing problem

of lyophilized cells exists and large amounts of water
must be added. The latter situation causes a spontaneous

degradation of the ECH substrate, probably resulting in a
decreased yield. Gong and Xu also pointed out that the

viscosity of the aqueous phase became so high that mass-

transfer resistance in the aqueous phase became problem-

Table 2. Effects of Various Organic Solvents on the Enantiomeric Excess and Yield of the Enantioselective Hydrolysis of Racemic ECH by the Recombinant P. pastoris Expressing the EH Gene from R. glutinis

| Organic solvents | Hydrolysis rate (nmole min⁻¹ mg⁻¹ of cell) | Relative ee (%)b | Enantiomeric excess |
|------------------|------------------------------------------|------------------|
| Nonec | 221 | 100 |
| Ethanol | 151 | 35 |
| Methanol | 121 | 29 |
| Dimethyl ether | 126 | 32 |
| Phthalic acid | 182 | 53 |
| n-Dodecane | 198 | 60 |
| Dioctyl phthalate | 189 | 55 |

aHydrolysis reactions were performed in solvent system containing 20 % (v/v) organic solvents to evaluate the bio-
compatibility of the organic solvents
bRelative ee calculated based on the value of enantioselective hydrolysis in aqueous buffer as 100 %
cEnantioselective hydrolysis in aqueous buffer in the absence of any organic solvent

Figure 2. Batch Kinetic Resolution of 20 mM Racemic ECH by the Recombinant P. pastoris Expressing the EH Gene from R. glutinis in N-Dodecane. (Symbol: (R)-Epichlorohydrin (●), (S)-Epichlorohydrin (○), Enantiomeric Excess (■)).
atic, resulting in a decrease in the reaction rate when large amounts of bacterial cells (higher than 15 mg DCW/mL) were used in aqueous/organic two-phase systems [8]. In conclusion, unstable enantiopure epoxides, such as ECH, can be obtained through recombinant EH-catalyzed kinetic resolution of their racemic epoxides in organic solvents.

Acknowledgment

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References
