Biocatalytic Preparation of Chiral Epichlorohydrins Using Recombinant *Pichia pastoris* Expressing Epoxide Hydrolase of *Rhodotorula glutinis*

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Abstract The use of enantioselective hydrolysis for preparing chiral epichlorohydrins was investigated using recombinant *Pichia pastoris* with the enantioselective epoxide hydrolase of *Rhodotorula glutinis*. The rate of the recombinant epoxide hydrolase-catalyzed enantioselective hydrolysis of racemic epichlorohydrins was enhanced by the addition of 5% (v/v) Tween 20. Enantiopure (*R*)-epichlorohydrins with an enantiopurity of 100% *ee* and a yield of 26% were obtained within 5 min from 50 mM racemates.

Keywords: enantioselective hydrolysis, epichlorohydrin, epoxide hydrolase, *Rhodotorula glutinis*, *Pichia pastoris*

Enantiopure epoxides are important chiral synthons for producing optically active compounds [1]. Various chemocatalytic and biocatalytic methods have been developed for preparing chiral epoxides [2,3]. Among the biocatalytic methods, the kinetic resolution of racemic epoxides *via* an enantioselective hydrolysis reaction by an epoxide hydrolase (EH) is a very promising method since enantiopure epoxides with a high optical purity can be obtained from cheap and readily available racemic epoxides [4,5].

The enantioselective hydrolysis of racemic epoxides by yeasts has previously been investigated for several aryl and alkyl epoxides using the EH activity of *Rhodotorula glutinis* [6], which exhibits good enantioselectivity towards aliphatic epoxides such as 1,2-epoxyhexane, 1,2-epoxyoctane, and 1,2-epoxyoctene. Yet, most yeast EH-catalyzed resolutions of racemic epoxides have been performed using whole cells or EH enzymes, which have certain commercial limitations, as the whole cells of wild-type yeast only include small amounts of EHs. Purification of the enzyme to homogeneity is time-consuming and the overall yield is low due to enzyme instability during the purification procedure. In addition, the purified membrane-associated EH of yeast has a significantly lower activity, especially the EH from *R. glutinis* and *R. toruloides* [7]. Therefore, from a practical point of view, more efficient production of enantiopure epoxides can be achieved using recombinant cells expressing highly active EH. A number of microbial EHs have already been cloned and characterized for developing recombinant biocatalysts [2,8-10]. However, the current study used a highly active recombinant *Pichia pastoris* with the enantioselective EH of *R. glutinis* as a biocatalyst for preparing (*R*)-epichlorohydrin. The effect of adding various detergents was investigated and the reaction conditions for enantioselective hydrolysis characterized for preparing (*R*)-epichlorohydrin.

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The recombinant *P. pastoris* was cultured at 30°C on a BMGY medium containing 10 g yeast extract/L and 10 g peptone/L [10,11]. The expression of EH was induced by the periodic addition of 1%(v/v) methanol over 72 h. The harvested cells were washed with a Tris·HCl buffer (100 mM, pH 8) and stored at 4°C.

Next, the cultured cells were suspended in 10 mL of a 100 mM KH_2PO_4 buffer (pH 8) in a 50-mL screw-cap bottle sealed with a rubber septum. The enantioselective hydrolysis was initiated with the addition of 20 mM racemic epichlorohydrin in a shaking incubator at 30°C and 250 rpm. The reaction was then stopped by extraction with an equal volume of cyclohexane. The progression of the enantioselective hydrolysis was analyzed based on samples withdrawn periodically from the reaction mixtures. The spontaneous chemical degradation of epichlorohydrin throughout recombinant cell-catalyzed enantioselective hydrolysis was negligible within short reaction time below 10 min. The enantioselective hydrolysis activity of wild-type *P. pastoris* was not detected by a chiral GC analysis.

Various detergents were added to the hydrolysis reaction vials at given concentrations and the mixtures preincubated for 5 min. Then, 20 mM racemic epichlorohydrin and 12.5 mg (dry cell weight) of cells were added and the progression of the batch kinetic resolutions ana-

Table 1. Effect of detergents on initial hydrolysis rate and initial enantioselectivity of recombinant *P. pastoris*[a]

Detergent (5%(v/v))	Initial hydrolysis rate (nmol mg^{-1} min^{-1})	E[b]
Control[c]	453	1.2
18-Crown-6 ether	412	1.2
Triton X-100	961	1.6
Tween 20	1024	2.2
Tween 80	728	1.3

[a] The 12.5 mg (dry cell weight) of cells were used for the enantioselective hydrolysis of racemic epichlorohydrin.
[b] Initial enantiomeric ratio ($E = \ln(R_0/R)/\ln(S_0/S)$) was determined from concentration data after 1 min.
[c] Enantioselective hydrolysis of (*S*)-epichlorohydrin was performed in absence of detergents.

lyzed periodically by chiral GC.

The enantiomeric excess (*ee* = 100 × (*R*-*S*)/(*R* + *S*)) and yield (yield = 100 × residual (*R*)-epoxide concentration/initial racemic epoxide concentration) of chiral epichlorohydrin were determined by a chiral GC analysis. The reaction mixture was extracted with an equal volume of cyclohexane, and the organic solvent layer analyzed by GC using a fused silica capillary beta-DEX-250 column (60 m length, 0.25 mm ID, and 0.25 μm film thickness, Sulpelco Inc.) and FID detector. The temperatures of the column, injector, and detector were 100, 220, and 220°C, respectively.

The stimulatory effect of the detergents on the enantioselective hydrolysis of the racemic epoxides prompted further investigation of the effect of adding detergents on the initial hydrolysis rate and enantioselectivity of the epoxide hydrolase-catalyzed resolution of racemic epichlorohydrins [12]. Enhanced hydrolysis rates and enantioselectivity were obtained when non-ionic detergents, Triton X-100, Tween 20 and Tween 80, were added (Table 1). In comparison to the control experiments in the absence of detergents, the initial rate and enantioselectivity were enhanced 2.3-fold by the addition of 5%(v/v) Tween 20. Varying the Tween 20 concentrations from 0.1 to 10%(v/v) was found to produce a similar level of enhancement in the initial hydrolysis rates (data not shown). However, more detailed research on the mechanism of the stimulatory effects of detergents on the enantioselective hydrolysis of racemic epoxides is still needed along with the identification of a cheaper detergent to reduce overall costs.

The effect of varying the recombinant cell concentrations on the reaction time and yield was investigated to maximize the yield and minimize the reaction time. The reaction time needs to be decreased to reduce the spontaneous hydrolysis of epichlorohydrins in an aqueous buffer. Normally, 10 mM epichlorohydrins are completely degraded within 5 h even in the absence of cells or enzymes [5]. As shown in Fig. 1, the reaction times required to reach 98% *ee* decreased when the cell concentration was increased, while the yield only decreased slightly with an increasing cell concentration. Therefore, the reaction time

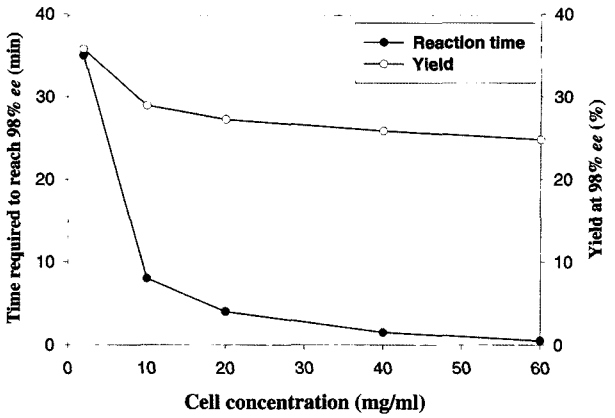

Fig. 1. Effect of cell concentrations on yield and reaction time required for 99% *ee* in enantioselective hydrolysis of 20 mM racemic epichlorohydrin by recombinant *P. pastoris*.

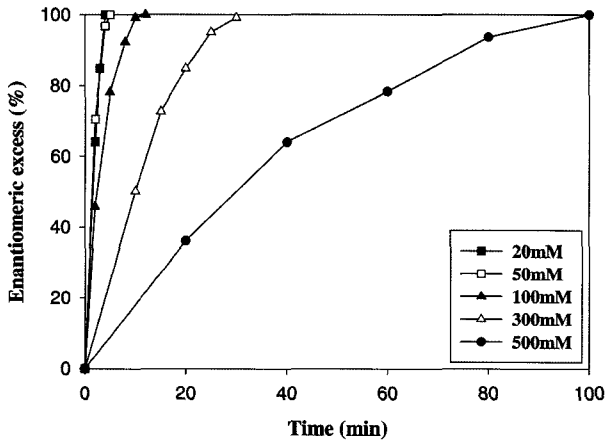

Fig. 2. Effect of initial substrate concentrations on enantiomeric excess of enantioselective hydrolysis of racemic epichlorohydrins by recombinant *P. pastoris*. The 12.5 mg cells were used as a biocatalyst.

could be readily decreased with a relatively low reduction in the yield by increasing cell concentration. The yields of enantiopure (*R*)-epichlorohydrin fluctuated between 24.8 and 35.8% when varying the cell concentration within a range of 2~60 mg/mL, and the optimal cell concentration range for a batch resolution was 10~20 mg/mL. The effect of the initial substrate concentrations on the enantiomeric excess was also studied (Fig. 2), and an enantiopurity of more than 98% *ee* was obtained with an initial substrate concentration of up to 500 mM in the batch resolution of racemic epichlorohydrin. However, the yield decreased (data not shown) and the reaction time required for 98% *ee* increased with an increase in the initial substrate concentrations.

The enantioselective resolution of 50 mM racemic epichlorohydrins by recombinant *Pichia* was performed under optimal reaction conditions. The time-course of the batch kinetic resolution is shown in Fig. 3. The enan

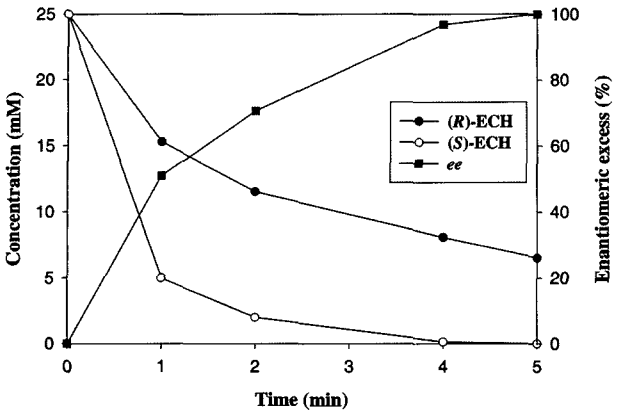

Fig. 3. Kinetic resolution of 50 mM racemic epichlorohydrins by recombinant *P. pastoris*-catalyzed enantioselective hydrolysis. The 12.5 mg cells were used as a biocatalyst.

tiopurity of the remaining epichlorohydrins increased from 0 to 99% *ee* after 5 min, and the 50 mM racemic epichlorohydrins were resolved with an enantiopurity of almost 100% *ee* and 26% yield (theoretical yield = 50%). In conclusion, commercially important epoxides, including chiral epichlorohydrins, can be readily obtained with a high optical purity when using recombinant *P. pastoris* expressing enantioselective EH of *R. glutinis*.

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