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# Effect of Mass Transfer Limitations on the Enzymatic Kinetic Resolution of Epoxides in a Two-Liquid-Phase System

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**Abstract:** Optically active epoxides can be obtained by kinetic resolution of racemic mixtures using enantioselective epoxide hydrolases. To increase the productivity of the conversion of sparingly aqueous soluble epoxides, we investigated the use of a two-phase aqueous/organic system. A kinetic model which takes into account interphase mass transfer, enzymatic reaction, and enzyme inactivation was developed to describe epoxide conversion in the system by the epoxide hydrolase from *Agrobacterium radiobacter*. A Lewis cell was used to determine model parameters and results from resolutions carried out in the Lewis cell were compared to model predictions to validate the model. It was found that n-octane is a biocompatible immiscible solvent suitable for use as the organic phase. Good agreement between the model predictions and experimental data was found when the enzyme inactivation rate was fitted. Simulations showed that mass transfer limitations have to be avoided in order to maximize the yield of enantiomerically pure epoxide. Resolution of a 39 g/L solution of racemic styrene oxide in octane was successfully carried out in an emulsion batch reactor to obtain (S)-styrene oxide in high enantiomeric excess (>95% e.e.) with a yield of 30%. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* 73: 44–54, 2001.

**Keywords:** kinetic resolution; two-phase system; epoxide hydrolase; n-octane; Lewis cell; mass transfer limitations

## INTRODUCTION

Epoxides are important intermediates for the production of a wide range of fine-chemical and pharmaceutical products (Furuhashi, 1992). Biological activity of these products often lies with only one enantiomer and the potential toxic side effects of the other enantiomer have made their production in enantiomerically pure form increasingly important (Crosby, 1992).

Some successful chemocatalytic routes for the production of enantiomerically pure epoxides, such as the heavy metal complex catalyzed oxidation by Sharpless and Jacobsen/Katsuki, have been developed. However, these can be ap-

plied only for specific substrate structures (Besse and Veschambre, 1994; Tokunaga et al., 1997). Synthetic biocatalytic methods include alkene epoxidation by monooxygenases and reduction of  $\alpha$ -haloketones to optically pure halohydrins (de Bont, 1993), and are usually cell-mediated processes since cofactors are required.

Enzymatic enantioselective degradation of racemic epoxides, which are relatively cheap to produce, has been recognized as an attractive alternative to these (bio)synthetic routes (Archer, 1997; Pedragosa-Moreau et al., 1996; Weijers and de Bont, 1999). Some epoxide hydrolases are capable of hydrolyzing epoxides enantioselectively to diols. According to a recent review on epoxide degrading enzymes (Swaving and de Bont, 1998), epoxide-hydrolases are the most promising enzymes for biocatalytic production of enantiopure epoxides since no cofactors are involved; they have a broad substrate range; and both the optically pure epoxide and the diol can be used as chiral synthons.

We previously isolated an enantioselective epoxide hydrolase from *Agrobacterium radiobacter* AD1 (Rink et al., 1997), which hydrolyzes a variety of terminal epoxides with a moderate to high enantioselectivity (Lutje Spelberg et al., 1998). Overexpression of epoxide hydrolase in *E. coli* has made this enzyme available in large quantities (Rink et al., 1997), increasing its potential for large-scale enantiopure epoxide preparation.

The productivity of this biocatalytic route is limited, however, by the low aqueous solubility of epoxides, which is typically a few grams per liter, the loss of epoxide due to chemical hydrolysis, and enzyme inhibition at high diol concentrations.

The use of cosolvents has been attempted to increase epoxide aqueous solubilities (Nellaiah et al., 1996). Alternatively, poorly soluble substrates can be applied as two-phase systems with the substrate as the second phase. For example, asymmetric hydrolysis by a fungal epoxide hydrolase, of a solid epoxide (50 g/l) as a suspension in buffer containing 20% (v/v) dimethyl sulfoxide, has been demon-

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strated (Morisseau et al., 1997). However, enzyme inactivation seemed to be a limitation in this system. Substrates can also be applied in two-phase systems dissolved to high concentrations in an apolar solvent. The solvent should not have deleterious effects on enzyme activity and should be chosen so that partitioning of substrate and product over the two phases facilitates downstream separation. If a solvent is toxic to the biocatalyst, a membrane can be employed to separate the biocatalyst from the organic solvent. A resolution of 1,2-epoxyhexane dissolved in dodecane at high concentrations using a recycle membrane reactor has been demonstrated with yeast cells, which allowed highly enantioselective for hydrolysis of the epoxide (Choi et al., 1999).

In this article, we describe the use of a two-liquid-phase system for the resolution of racemic epoxides without using a membrane by selecting a suitable organic solvent. A two-phase system should be advantageous since the organic liquid can act as a reservoir of substrate, increasing bioreactor productivity. Furthermore, loss of epoxide by spontaneous, nonenantioselective chemical hydrolysis may be reduced by partitioning of a large proportion of the epoxide to the organic phase.

In heterogeneous systems of immobilized enzymes, mass transfer limitations through the carrier material can greatly reduce the effective enantioselectivity of a resolution (Barros et al., 2000; Matson et al., 1989). Mass transfer limitations also affect the effective enantiomeric ratio,  $E$ , observed for solid-to-solid kinetic resolutions (Straathof et al., 1998). In our system, styrene oxide was used as the model substrate for which the (R)-enantiomer is preferentially hydrolyzed with an  $E$  value of 16 (Lutje Spelberg et al., 1998). The kinetic mechanism for this substrate has been studied in detail (Rink and Janssen, 1998). Typical of enantioselective hydrolysis of epoxides by this epoxide hydrolase is that the enantiopure substrate (S) is rapidly degraded after the unwanted (R)-enantiomer is hydrolyzed. The same type of sequential conversion kinetics has been observed for the enantioselective hydrolysis of *tert*-butyloxirane and styrene oxide by microsomal epoxide hydrolase (Watabe et al., 1981; Wistuba and Schurig, 1992). Because of these complex kinetics it is expected that the relative rates of substrate interphase mass transfer, enzymatic reaction, and enzyme inactivation are critical factors in determining the effective enantioselectivity of the conversion in the two-liquid-phase system. We therefore studied mass transfer and enzyme inactivation in a Lewis cell (Woodley et al., 1991), which has a well-defined interfacial area. We developed a mathematical model to describe the conversion of racemic epoxide in the system. The results of simulations showed that mass transfer limitations have to be avoided in order to obtain high yields of enantiomerically pure epoxide in short process times. Resolution of a concentrated solution of racemic styrene oxide in octane (39 g/L) was carried out to demonstrate the potential of using this two-phase system.

## Modeling of Two-Phase Enzymatic Hydrolysis

The model describes interphase mass transfer, enzymatic reaction, spontaneous epoxide hydrolysis, and enzyme inactivation. These processes are coupled through mass balances over both the aqueous and organic phases.

It is assumed that the enzymatic reaction takes place in the bulk of the aqueous phase and that mass transfer of the epoxide to the aqueous phase and hydrolysis occur sequentially. Assuming interfacial equilibrium, the (S) and (R)-enantiomer interfacial concentrations are given by:

$$S_i = S/m, \quad (1)$$

$$R_i = R/m, \quad (2)$$

where  $m$  is the partition coefficient.

Since  $m$  is high it is assumed that mass transfer resistances occur only in the aqueous phase. The flux of each enantiomer over the interface,  $J$ , can then be described based on an aqueous phase driving force using the aqueous phase mass transfer coefficient,  $k_a$ , which is assumed constant over the range of operating conditions:

$$J_S = k_a(S_i - S_a), \quad (3)$$

$$J_R = k_a(R_i - R_a). \quad (4)$$

As the (R) and (S)-enantiomers react in the bulk of the aqueous phase, transfer of (R) and (S) occurs from the organic phase to the aqueous phase. The diol is assumed to remain completely in the aqueous phase, therefore the change in diol aqueous concentrations is described by:

$$\frac{dD_S}{dt} = -r_S, \quad (5)$$

$$\frac{dD_R}{dt} = -r_R. \quad (6)$$

The enzymatic conversion of both enantiomers of styrene oxide in the aqueous phase can be described by competitive Michaelis-Menten kinetics and competitive product inhibition, with a term also for spontaneous chemical hydrolysis (Lutje Spelberg et al., 1998):

$$r_S = -\frac{k_{cat}^S[E]S_a}{S_a + \left(\frac{R_a}{K_m^R} + \frac{D_S}{K_i^{DS}} + \frac{D_R}{K_i^{DR}} + 1\right)K_m^S} - k_c S_a, \quad (7)$$

$$r_R = -\frac{k_{cat}^R[E]R_a}{R_a + \left(\frac{S_a}{K_m^S} + \frac{D_S}{K_i^{DS}} + \frac{D_R}{K_i^{DR}} + 1\right)K_m^R} - k_c R_a. \quad (8)$$

Initial rate experiments were performed to determine competitive product inhibition constants for both enantiomers of the diol product. Kinetic constants are summarized in Table I.

Enzyme inactivation is assumed to be described by a first-order process:

**Table I.** Kinetic constants for the conversion of styrene oxide by *A. radiobacter* AD1 epoxide hydrolase.

Enantiomer	$K_m$ ( $\mu\text{M}$ )*	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )*	$k_c$ ( $\text{s}^{-1}$ )	$K_i$ (mM)
(R)-styrene oxide	$0.6 \pm 0.2$	$4.2 \pm 0.3$	$9.54 \times 10^{-6}$	—
(S)-styrene oxide	$25 \pm 5$	$10.5 \pm 1.0$	$9.54 \times 10^{-6}$	—
(R)-1-phenyl-1,2-ethane-diol	—	—	—	$42 \pm 10$
(S)-1-phenyl-1,2-ethane-diol	—	—	—	$29 \pm 5$

\*Rink and Janssen, 1998. The value of  $k_c$  was determined at pH 7.5.

$$\frac{d[E]}{dt} = -k_d[E]. \quad (9)$$

A mass balance over the aqueous phase gives:

$$\frac{dS_a}{dt} = J_S \left( \frac{a}{1 - \Phi} \right) + r_S, \quad (10)$$

$$\frac{dR_a}{dt} = J_R \left( \frac{a}{1 - \Phi} \right) + r_R; \quad (11)$$

and for the organic phase:

$$\frac{dS}{dt} = -J_S \frac{a}{\Phi}, \quad (12)$$

$$\frac{dR}{dt} = -J_R \frac{a}{\Phi}. \quad (13)$$

## MATERIALS AND METHODS

### Materials

Racemic styrene oxide (97%) was obtained from Aldrich Chemical Co. (Milwaukee, WI) and n-octane (p.a. grade) from Fluka (Buchs, Switzerland). All other solvents used were at least reagent grade. (R)-*p*-nitrostyrene oxide (pNSO) was synthesized as described by Westkaemper and Hanzlik (1981). The aqueous phase was a buffer of composition 50 mM Tris-SO<sub>4</sub>, pH 7.5, 1 mM EDTA, 1 mM  $\beta$ -mercaptoethanol.

### Simulation and Parameter Estimation

Model simulations and parameter estimation was carried out using the software program Scientist<sup>™</sup> (MicroMath, Salt Lake City, UT).

### Enzyme Preparation

A partially purified enzyme was used throughout this work. Protein was expressed as described by Rink et al. (1997). Purification was carried out with a DE52 anion exchange column. The partially purified enzyme was dialyzed against TEMAG buffer (25 mM Tris-SO<sub>4</sub>, pH 7.5, 1 mM EDTA, 1

mM  $\beta$ -mercaptoethanol, 0.02% sodium azide, and 10% glycerol) and concentrated to 14 mg/ml (400  $\mu\text{M}$ ). The enzyme was stored at 4°C.

### Analytical Procedures

Epoxide concentrations in the octane phase were analyzed by diluting 100–200  $\mu\text{l}$  samples in 2 ml octane, containing mesitylene (0.05% v/v) as internal standard and analyzed by GC. Epoxide in the water phase was analyzed after extracting 0.5 ml samples with 1 ml diethyl ether (containing mesitylene as internal standard) for 1 min. The top layer was dried on a short column of anhydrous Na<sub>2</sub>SO<sub>4</sub> and then analyzed by GC.

Chiral analysis for Lewis cell experiments was performed with a HP 5890 GC equipped with a FID detector, using a Chiraldex G-TA capillary column, 50 m in length and 0.25 mm internal diameter. Injector and detector temperatures were 250°C and isothermal operation was used at 120°C. Chiral analysis for the emulsion experiment was performed with a HP6890 GC equipped with a FID detector, using a 25 m  $\times$  250  $\mu\text{m}$   $\times$  0.25  $\mu\text{m}$  CP Chirasil-Dex CB column. Injector and detector temperatures were 250°C. The oven was held at 100°C for 5 min after which the temperature was increased at 5°C/min to 120°C, at which it was held for 2 min. Nonchiral analysis was performed with the HP6890 GC, using a 0.2 mm  $\times$  25 m HP5 column. Injector and detector temperatures were 300°C. The oven was held at 50°C for 1 min, after which the temperature was increased at 10°C/min to 110°C, at which it was held for 5 min.

Aqueous phase concentrations of 1-phenyl-1,2-ethanediol were measured by derivatization with 2,2-dimethoxypropane to give a hemiacetal that was analyzed by GC, as described elsewhere (Rink and Janssen, 1998).

### Activity Assays

Enzyme activity assays were performed in a Perkin Elmer Lambda Bio 40 UV/VIS spectrophotometer with a temperature-controlled cell holder by following the hydrolysis of the colorimetric substrate (R)-pNSO to its corresponding diol at 310 nm, for which extinction coefficients are  $\epsilon_{310} = 4,289 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{310} = 3,304 \text{ M}^{-1}\text{cm}^{-1}$ , respectively. Typically, 1 ml of enzyme solution (approx. 0.06  $\mu\text{M}$ ) was placed in a 1 cm quartz cuvette and the reaction was started by adding up to 10  $\mu\text{l}$  of a 10 mM (R)-pNSO solution in acetonitrile. Conversion curves were numerically fitted to Michaelis-Menten kinetics to determine  $k_{\text{cat}}$  and  $K_m$ .

### Solvent Biocompatibility

To qualitatively compare the effect of the organic solvents on epoxide hydrolase activity, 2.5 ml of enzyme solution (approximately 4  $\mu\text{M}$ ) in 50 mM Tris buffer, pH 9, was incubated with 0.6 ml of organic liquid in closed test tubes and rotated end-over-end at a rate of 5 rpm at 30°C. After 1 and 5 h, the phases were allowed to separate and the aque-

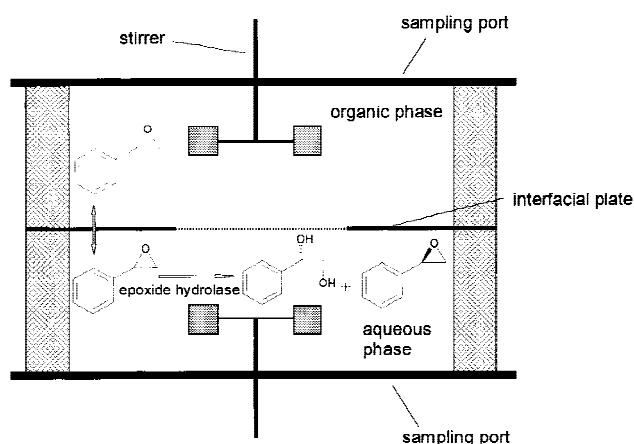
ous layer was assayed for enzyme activity by using pNSO as substrate. In this way, both inactivation by the interface and by dissolved organic solvent are included. To separately determine the effect of dissolved solvent on epoxide hydrolase activity, enzyme was incubated in buffer saturated with the solvents at 30°C, but with no separate solvent phase. The remaining activity was tested after 1 and 5 h of incubation. A control incubation with no solvent was used to measure inactivation of the enzyme alone.

### The Lewis Cell

The Lewis cell is a cylindrical vessel with two compartments separated by an interfacial plate which is attached to four baffles and contains a central hole to allow contact between the two phases (Fig. 1). Interfacial plates with holes of varying sizes were available. In all experiments the Lewis cell was thermostatted at 30°C. Sample ports were present on the top and bottom of the cell to enable samples to be taken from either phase during operation. Each phase was mixed by an independently rotating Rushton impeller mounted in the middle of each compartment. Mixing time experiments (based on acid-base titrations in the presence of an indicator) showed that a stirring rate of 150 rpm was sufficient to maintain homogeneous phases.

### Measurement of Substrate Interphase Mass Transfer Rates and Diol Partitioning

The Lewis cell was filled with 97 ml of the aqueous buffer phase and n-octane was carefully placed on top so that the interface remained flat. Styrene oxide was added to the octane phase to 90 or 100 mM. Stirring in both phases was set to 150 rpm (counter-current). The epoxide aqueous phase concentration was followed in time by taking 0.5 ml samples through a septum in the bottom of the cell, which



**Figure 1.** Diagram of the Lewis cell. Dimensions and operating conditions: height = 4.6 cm, diameter = 7.5 cm, upper and lower volumes = 97 ml each, plate hole interfacial area = 22 cm<sup>2</sup>, turbine diameter = 3.8 cm, blade height = 0.7 cm, blade width = 0.7 cm, baffle width = 0.7 cm, stirrer speed = 150 rpm.

were analyzed as described above. After sampling, fresh buffer was added to replace any volume of the aqueous phase removed for analysis in order to maintain the interface positioned at the interfacial plate. The aqueous phase mass transfer coefficient ( $k_a$ ) and the partition coefficient ( $m$ ) were obtained by fitting the concentration profiles to mass transfer equations (1–4, 10–13), also taking into account spontaneous hydrolysis of the epoxide, using a least-squares minimization procedure of the software program Scientist<sup>TM</sup>. Values are quoted at the 95% confidence level.

The effect of diol present in the aqueous phase on mass transfer and partitioning of epoxide was tested by repeating the same procedure in the Lewis cell as described above, with the aqueous phase initially at various concentrations of diol (10, 22, and 50 mM).

Diol partitioning between the two phases was determined by equilibration experiments. In batch incubations, 3 ml of 10 mM or 100 mM aqueous solutions of diol were added to 3 ml of octane and mixed at 30°C for 24 h. The phases were allowed to separate and the aqueous phase was tested for remaining diol concentration. The organic phase diol concentration after equilibration was calculated from a mass balance.

### Stability of Epoxide Hydrolase

The stability of epoxide hydrolase in buffer was tested by incubating a solution of epoxide hydrolase in buffer (0.11 μM) at 30°C. Periodically, assays were performed with (R)-pNSO to determine the residual enzymatic activity.

To test for molecular toxicity of octane, epoxide hydrolase was incubated in a closed vessel in buffer (0.11 μM) which had previously been saturated with n-octane at 30°C. Periodically, assays were performed with (R)-pNSO to determine the residual enzymatic activity.

The effect of interfacial contact with octane on enzyme activity was tested in the Lewis cell. The Lewis cell was filled as described above but with no substrate present (0.11 μM enzyme concentration). The stirring speed was 150 rpm for both phases with stirrers rotating countercurrently. The interface was stabilized by an interfacial plate, which fixed the contact area at 22 cm<sup>2</sup>. Periodically, assays were performed as follows: 0.5 ml of aqueous phase were withdrawn from the bottom of the Lewis cell and diluted by adding to 0.7 ml of buffer. The activity was tested by a pNSO assay as described. An equal volume of buffer was replaced to the bottom phase directly after each sample was taken. The activities obtained were corrected for this dilution. First order inactivation rate constants were fitted to the inactivation curves with a least-squares minimizing procedure and are quoted at the 95% confidence level.

### Kinetic Resolution of Styrene Oxide in the Lewis Cell and the Emulsion System

Resolution of a racemic mixture of styrene oxide in the Lewis cell was performed by filling the bottom of the cell

with aqueous phase and injecting a known amount of enzyme, then placing the octane on top. Once the stirrers were in motion at 150 rpm, the reaction was initiated by injecting styrene oxide into the top layer to the desired concentration. Periodically, samples were taken of the octane phase and analyzed by chiral GC as described above. Aqueous phase concentrations were not monitored to limit the disturbance of the system.

The 200 ml vessel of the Lewis cell, without an interfacial plate and stirred by only one of the Rushton turbines positioned in the middle of the liquid, was used for the resolutions with an emulsion. The reactor was filled with 120 ml aqueous phase and epoxide hydrolase was added to the desired concentration. With the stirrer in motion, 30 ml ( $\Phi = 0.2$ ) of octane made up to the desired concentration of styrene oxide were injected in the vessel to start the reaction. The stirring speed was set at 200 rpm. Periodically, samples were taken from the organic phase and analyzed by GC on a chiral column as described.

## RESULTS AND DISCUSSION

### Choice of Solvent

Immiscible solvents can cause enzyme inactivation due to dissolved solvent molecules in the aqueous phase interacting with the enzyme (molecular toxicity) and due to direct contact of the enzyme with the interface (interfacial toxicity) (Bar, 1988). Seven solvents were compared for both interfacial and molecular toxicity to epoxide hydrolase by incubating enzyme solutions with each solvent present as a separate phase. The incubation test tubes were rotated slowly end-to-end. All solvents tested caused a decrease in enzymatic activity with time (Table II), whereas the control incubation, with no solvent, showed no change in activity over the tested time period. Octane had the least effect on enzyme activity, which decreased by only 20% after incubation for 5 h. It should be noted that octane and hexane appeared harder to emulsify than the other solvents and with these solvents only relatively small, flat interfaces were created during the gentle mixing, rather than droplets which were formed with all the other solvents. The inactivation

caused by the dissolved part of each solvent was tested by incubating enzyme in buffer saturated with solvent, but with no separate solvent phase present. Dissolved solvent caused complete enzyme inactivation within 5 h for all solvents except hexane and octane. For octanol, the dissolved solvent inactivation effect was slightly greater than when a separate phase was also present. This may be explained if the dissolved octanol concentration did not reach the saturation concentration during the first hour of incubation under such gentle mixing conditions. For hexane and octane, the inactivation was greater when a free interface was present, indicating that molecular and interfacial toxicity effects could be cumulative. The log P of a solvent, the logarithm of the partition coefficient of the solvent in a standard mixture of 1-octanol and water, is a property often used for predicting biocompatibility (Laane et al., 1987), and is usually more indicative of dissolved solvent effects. The relative effects of the solvents correlated well with the biocompatibility criteria since octane and hexane, with log P > 4, had the least effect on enzyme activity, whereas solvents with a log P < 2 had the greatest inactivating effect. The low aqueous solubility of octane (Table II) in water may account for its lower molecular toxicity. Octane was chosen as the best solvent because it had the least effect on enzyme activity. Furthermore, it is miscible with styrene oxide but is a poor solvent for the diol produced. The diol remains mostly in the aqueous phase (see below), with obvious advantages for downstream separation.

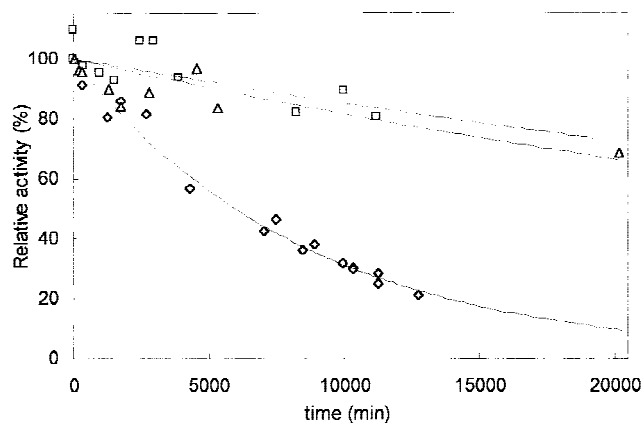
### Stability of Epoxide Hydrolase

Inactivation of epoxide hydrolase could be described by a first-order process. There is no significant difference between the fitted first-order inactivation rate constants found for enzyme incubated in buffer and enzyme incubated in buffer saturated with octane (Fig. 2). The inactivation rate was 6-fold higher when a free interface was present in the Lewis cell, suggesting that, for octane, interfacial toxicity plays a greater role in the inactivation of epoxide hydrolase than molecular toxicity. The effect of shear on enzyme activity caused by mixing at up to 200 rpm in the Lewis cell was tested and no significant inactivation was observed

**Table II.** Properties of organic solvents and their effect on epoxide hydrolase activity.

Solvent	Log P*	Aqueous solubility* at 25°C (M)	Molecular toxicity, % remaining activity after		Molecular and interfacial toxicity, % remaining activity after	
			1 h	5 h	1 h	5 h
i-Amyl acetate	—	—	<1	—	—	0
Diethylether	0.85	0.93	0	—	0	0
n-Butyl acetate	1.7	—	0	—	—	0
1-Hexanol	2.0	0.13	0	—	0	0
1-Octanol	2.8	$4.5 \times 10^{-3}$	5	0	11	0
n-Hexane	4.1	$1.4 \times 10^{-4}$	90	82	80	25
n-Octane	5.2	$6.3 \times 10^{-6}$	99	95	100	80

\*Data from Schwarzenbach, et al. (1993)



**Figure 2.** Inactivation of epoxide hydrolase. Enzyme solutions (0.11  $\mu\text{M}$ ) were incubated in buffer ( $\Delta$ ) ( $k_d = 3.5 \times 10^{-7} \pm 1.7 \times 10^{-7} \text{ s}^{-1}$ ); in buffer saturated with octane but with no separate octane phase ( $\square$ ) ( $k_d = 2.6 \times 10^{-7} \pm 0.9 \times 10^{-7} \text{ s}^{-1}$ ); and in contact with octane in the Lewis cell (150 rpm) with an interfacial contact area of 22  $\text{cm}^2$  ( $\diamond$ ) ( $k_d = 1.9 \times 10^{-6} \pm 0.1 \times 10^{-6} \text{ s}^{-1}$ ); all at pH 7.5 and 30°C. The lines shown are exponential fits of the data.

(data not shown). These results suggest that inactivation may be considerable when much higher interfacial areas are used.

### Interphase Mass Transfer and Partitioning of Substrate and Product

The transfer of substrate from the organic phase to the aqueous phase was followed in the absence of enzymatic reaction, using various concentrations of diol initially present in the aqueous phase. The value of  $k_a$  of  $2.3 \times 10^{-5} \pm 0.3 \times 10^{-5} \text{ m/s}$ , with no diol present, did not vary significantly with increasing concentration of diol in the aqueous phase, but the partition coefficient,  $m$ , decreased with increasing diol concentration. In the range of diol concentrations tested, where  $D_S + D_R \leq 50 \text{ mM}$ , the variation in  $m$  can be taken into account with a linear correlation:

$$m = 44.2 - 0.24(D_S + D_R). \quad (14)$$

This means that as the reaction proceeds and diol accumulates in the aqueous phase, the flux of epoxide to the aqueous phase would increase in accordance with Eqs. 3 and 4.

From the equilibration experiments, the partition coeffi-

cient of diol over the two phases (organic/aqueous) was calculated as 0.03. Because of this low value, the transfer of diol to the organic phase was not taken into account in the model.

### Kinetic Resolution of Styrene Oxide in the Lewis Cell

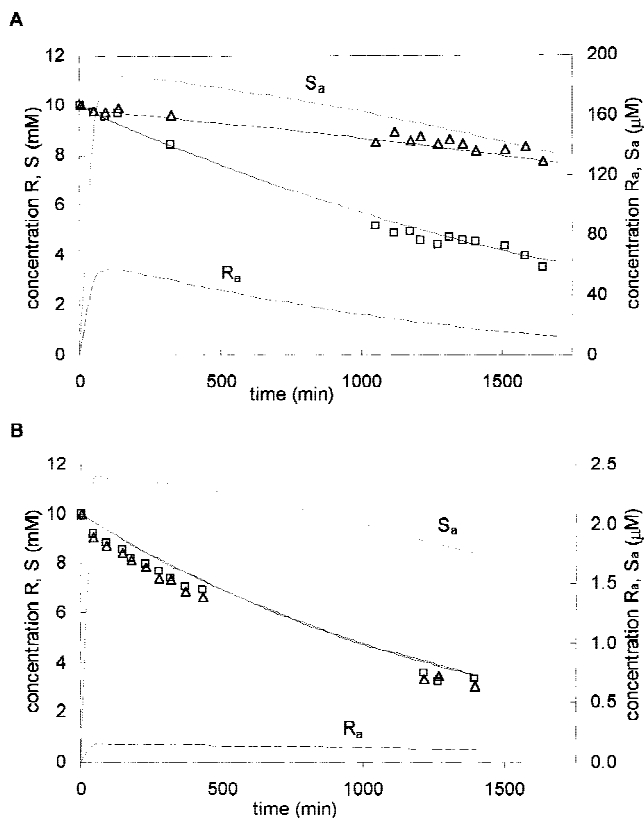
Resolution experiments at various initial enzyme and epoxide concentrations were carried out in the Lewis cell to validate the mathematical model. Experimental conditions and model parameters are summarized in Table III. The enantiomeric excess is a measure of the enantiomeric purity and is defined as  $e.e. = (S - R)/(S + R)$ .

For an initial epoxide concentration of 20 mM, resolution up to 37% e.e. was achieved with an enzyme concentration of 0.022  $\mu\text{M}$  (Fig. 3A). The reaction was terminated before complete conversion had occurred because of the long reaction time. Use of the first-order rate constant found for enzyme inactivation determined with the Lewis cell did not yield satisfactory simulations of the obtained data. The simulation curves shown are obtained by fitting the inactivation rate constant,  $k_d$ . The fitted value of  $k_d$  ( $5.6 \times 10^{-6} \text{ s}^{-1}$ ) is approximately three times higher than the  $k_d$  obtained in the inactivation measurements where no reaction takes place.

When a higher enzyme concentration (0.16  $\mu\text{M}$ ) was used (Fig. 3B), resolution was poor even though epoxide conversion occurred. The concentration of both enantiomers in the octane phase decreased to 3 mM, after which the reaction was terminated due to the long reaction time. Using a  $k_d = 5.6 \times 10^{-6} \text{ s}^{-1}$ , simulated concentrations lie only slightly above the measured ones and the absence of resolution is predicted accurately, with the e.e. remaining below 4%. The enantioselectivity of the conversion of styrene oxide by this epoxide hydrolase is primarily due to the difference in the  $K_m$  values for the two enantiomers (Table I) which results in the sequential conversion of the enantiomers. The (S)-enantiomer, which is converted second, also has a higher  $k_{\text{cat}}$  value and is hydrolyzed faster than the (R)-enantiomer. The poor resolution in this case can be explained by the fact that at the higher enzyme concentration the rate of mass transfer is slow compared to that of

**Table III.** Experimental conditions and modeling parameters of resolution experiments shown in Figures 3–6.

Parameter	Figure 3A	Figure 3B	Figure 4	Figure 6
Initial styrene oxide concentration (mM)	20	20	126	324
$[E]_0$ ( $\mu\text{M}$ )	0.022	0.16	0.054	0.40
$k_d$ (1/s)	$5.6 \times 10^{-6}$	$5.6 \times 10^{-6}$	$7.1 \times 10^{-6}$	$9.5 \times 10^{-6}$
$k_a$ (m/s)	$2.3 \times 10^{-5}$	$2.3 \times 10^{-5}$	$2.3 \times 10^{-5}$	$2.3 \times 10^{-5}$
$A$ ( $\text{m}^2$ )	$2.2 \times 10^{-3}$	$2.2 \times 10^{-3}$	$2.2 \times 10^{-3}$	$4.5 \times 10^{-2}$
Total liquid volume (ml)	194	194	194	150
$\Phi$	0.5	0.5	0.5	0.2

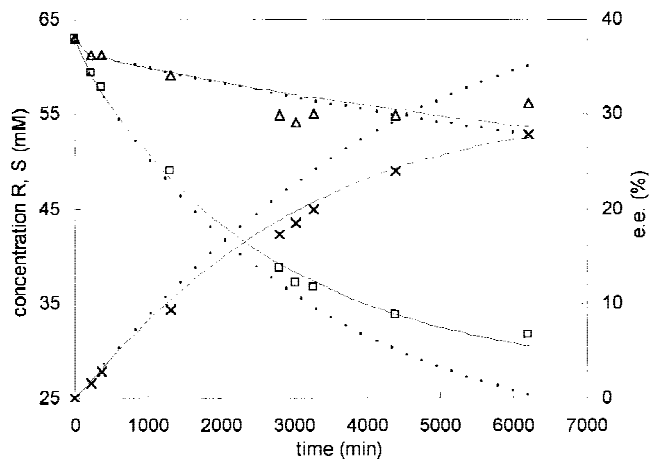


**Figure 3.** Resolution of styrene oxide in the Lewis cell. Profiles of (R) ( $\square$ ) and (S) ( $\triangle$ ) enantiomer conversion at different initial enzyme concentrations in the Lewis cell: (A)  $[E]_0 = 0.022 \mu\text{M}$ , (B)  $[E]_0 = 0.16 \mu\text{M}$ . Initial racemic styrene oxide concentration in the organic phase was 20 mM in both cases. Symbols indicate experimental data. Lines show model simulations of both the organic (full lines) and aqueous (broken lines) phase concentrations.

reaction, resulting in low aqueous phase concentrations of both enantiomers. The simulations show that the aqueous phase concentration of the (R)-enantiomer is below  $K_m^R$  (Fig. 3B) and therefore the (S)-enantiomer is also converted, thus reducing the effective enantioselectivity of the conversion. In the biphasic system it is important, therefore, to maintain sufficiently high concentrations of the (R)-enantiomer in the aqueous phase to inhibit the conversion of the (S)-enantiomer.

A resolution was also tested at an initial epoxide concentration of 126 mM (Fig. 4). The (R) and (S)-enantiomer concentrations in the organic phase decreased to 29 mM and 53 mM, respectively, corresponding to an e.e. of 28%, after which the reaction was terminated. After 4,000 min the concentrations of the two enantiomers and therefore also the e.e. started to become constant, deviating from the simulated curves calculated with  $k_d = 5.6 \times 10^{-6} \text{ s}^{-1}$ . This indicates that enzyme inactivation occurred at a faster rate than expected. The data was fitted to obtain a larger first-order inactivation rate constant,  $k_d = 7.1 \times 10^{-6} \text{ s}^{-1}$ .

In all three experiments fitted values for  $k_d$  were higher than the value measured in the Lewis cell when no reaction takes place. Epoxides are very reactive compounds and



**Figure 4.** Resolution of styrene oxide in the Lewis cell. The initial racemic styrene oxide concentration in the organic phase was 126 mM. Initial enzyme concentration in the aqueous phase was  $0.054 \mu\text{M}$ . Symbols indicate experimental data (R =  $\square$ , S =  $\triangle$ , e.e. =  $\times$ ). Simulation lines are shown for two different values of first order inactivation rate constant:  $k_d = 5.6 \times 10^{-6} \text{ s}^{-1}$  (dotted lines)  $k_d = 7.1 \times 10^{-6} \text{ s}^{-1}$  (full lines).

toxic effects of epoxides on mono-oxygenases, used in alkene epoxidation, have been reported (Habets-Crutzen and de Bont, 1985; Prichanont et al., 1998; Stanley et al., 1992), although the specific mechanism for inactivation is still unknown. The higher enzyme inactivation rates found under reaction conditions for the resolutions may be caused by substrate toxicity to epoxide hydrolase and it is possible that this effect depends on the epoxide concentration. This is supported by the observation that when pure styrene oxide is used as the second phase, epoxide hydrolase is inactivated quickly and conversion stops before a reasonable enantiomeric excess is obtained (results not shown). Further investigation into toxic effects of both the epoxide and diol are currently in progress. With fitted values of  $k_d$ , there is good agreement between experimental data and model simulations.

### Maximizing the Yield of Pure Enantiomer

As discussed, a high enough concentration of the (R)-enantiomer must be present in the aqueous phase to inhibit the conversion of the (S)-enantiomer. The concentration of both enantiomers in the aqueous phase is dependent on the relative rates of mass transfer, enzymatic reaction, and enzyme inactivation. Given that for a kinetic resolution the maximum attainable yield of pure enantiomer is 50%, the yield of (S) at time  $t$ , defined as:

$$Y_S = \frac{S_t}{S_0 + R_0}, \quad (15)$$

has to be maximized for highest process profitability. Process times should also be as short as possible to maximize space-time yield and to limit the proportion of epoxide converted by non-enantioselective chemical hydrolysis. This latter effect is most important when the rates of enzymatic hydrolysis and spontaneous hydrolysis are comparable, which at this operating pH occurs when enzyme concentra-



tions are in the range of a few nanomolar. Low initial enzyme concentrations and enzyme inactivation also result in longer process times (for example, Fig. 4), in which a higher proportion of substrate is converted by spontaneous hydrolysis.

Based on the framework provided by Ramelmeier and Blanch (1989) for analyzing reaction regimes in two-liquid-phase systems, we derive an equation to determine the maximum enzyme concentration that should be used under certain mixing conditions, for which mass transfer limitations are sufficiently small, to achieve a good resolution in the shortest possible process time. We assume that mass transfer and reaction are processes in series. This assumption is justified since for all resolutions carried out in the Lewis cell, the Thiele modulus,  $\phi$ , which gives information on the ratio of the kinetic rate of reaction relative to the rate of mass transfer, is much less than 0.3. The Thiele modulus is defined as  $\phi = \sqrt{k'[E]D/k_a^2}$  (Westerterp et al., 1993) where  $k'$  is an apparent enzymatic reaction rate constant defined as:

$$k' = \frac{k_{cat}^S}{S_a + \left( \frac{R_a}{K_m^R} + \frac{D_S}{K_i^{D_S}} + \frac{D_R}{K_i^{D_R}} + 1 \right) K_m^S} \quad (16)$$

Assuming a pseudo-steady state with  $dS_a/dt = 0$ , and substituting  $J_S$  from Eq. (3) and  $S_i$  from Eq. (1) in the mass balance over the aqueous phase (Eq. (10)),

$$k_a \left( \frac{S}{m} - S_a \right) \frac{a}{(1 - \Phi)} = -r_S \quad (17)$$

By rearrangement and using the reaction rate constant  $k'$  defined in Eq. (16) we obtain:

$$\frac{\left( \frac{S}{m} - S_a \right)}{\left( \frac{S}{m} \right)} = \frac{(S_i - S_a)}{S_i} = \frac{(k'[E] + k_c) S_a}{k_a \left( \frac{a}{1 - \Phi} \right)} \frac{1}{S/m} \quad (18)$$

This equation describes the dependency of the aqueous phase substrate concentration gradient over the film for mass transfer on the operating parameters which determine the rates of mass transfer and reaction. If there are no mass transfer limitations then the aqueous phase will be in equilibrium with the organic phase and  $(S_i - S_a)/S_i = 0$ . We assume that mass transfer limitations are sufficiently small when,

$$\frac{S_i - S_a}{S_i} < 0.1. \quad (19)$$

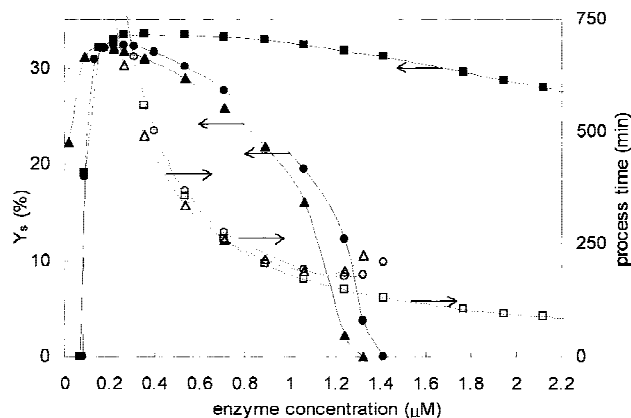
If the operating parameters  $k_a$ ,  $a$  and  $\Phi$ , are fixed, then the enzyme concentration  $[E]$  for which mass transfer limitations will cause only a small reduction in the yield, can be calculated from this equation. Due to enzyme inactivation the active enzyme concentration decreases during the course of the reaction. It follows that the predicted maximum enzyme concentration, obtainable from Eq. (18), is a "lower limit value" so that the actual enzyme concentration which

may be used will be slightly higher. It is clear from Eq. (18) that when enantioselectivity is almost absolute, in the case that  $k_{cat}^S \rightarrow 0$ , or  $K_m^S$  is very high, so that  $k'$  is small, mass transfer limitations have a limited effect on decreasing the yield of pure (S)-enantiomer.

## Resolution of Styrene Oxide in a Batch Emulsion Reactor

Using the mathematical model, simulations were made to study the effect of varying enzyme concentration and specific interfacial area on  $Y_S$  at 95% e.e. and on the process time required to reach 95% e.e. (Fig. 5). The influence of the value of  $k_d$  was also studied (for  $a = 300 \text{ m}^{-1}$ ). As expected, process times decrease with increasing enzyme concentration. When the mass transfer rate is low ( $a = 300 \text{ m}^{-1}$ ) the yield of (S) decreases rapidly when the enzyme concentration is increased. At an enzyme concentration of  $1.4 \text{ } \mu\text{M}$ , the predicted yield is close to zero. If no inactivation were to take place, a lower range of enzyme concentrations could be used to carry out a resolution since sufficient active enzyme would initially be present to reach the desired conversion. When  $k_d$  has the value  $9.5 \times 10^{-6} \text{ s}^{-1}$  the lowest enzyme concentration which catalyzes conversion to 95% e.e. is  $0.1 \text{ } \mu\text{M}$ . With a much higher mass transfer rate ( $a = 1,000 \text{ m}^{-1}$ ), however, mass transfer limitations become important only for very high enzyme concentrations and low process times can be achieved with a relatively low decrease in  $Y_S$ . The simulations thus predict that the maximum enzyme concentration which can be used to obtain a good resolution is higher with a higher interfacial contact area.

Based on these predictions, and to test the validity of Eq. (18), we tested the effect of a higher mass transfer rate with

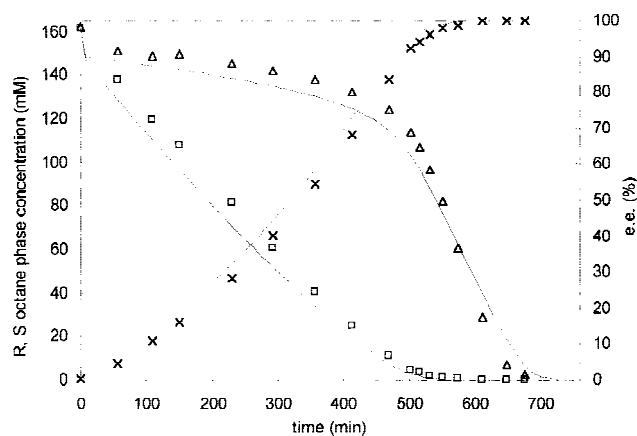


**Figure 5.** Effect of process parameters on resolution in an emulsion system. Simulations of the effect of enzyme concentration ( $[E]$ ) and specific interfacial area ( $a$ ) on the yield of pure (S)-enantiomer ( $Y_S$ ) (closed symbols) at 95% e.e. and on the process time required to reach 95% e.e. (open symbols):  $a = 1,000 \text{ m}^{-1}$  ( $\blacksquare$ ,  $\square$ ) for  $k_d = 9.5 \times 10^{-6} \text{ s}^{-1}$ . For  $a = 300 \text{ m}^{-1}$ , two cases are shown:  $k_d = 9.5 \times 10^{-6} \text{ s}^{-1}$  ( $\bullet$ ,  $\circ$ ) and  $k_d = 0$  ( $\blacktriangle$ ,  $\triangle$ ); For all the simulations  $k_a = 2.3 \times 10^{-5} \text{ ms}^{-1}$ ;  $S_0 = R_0 = 162 \text{ mM}$ ;  $\Phi = 0.2$ ; total volume = 150 ml.

respect to operation in the Lewis cell by working with an emulsion where a larger interfacial area can be achieved. In Eq. (18)  $k'$  is a function of substrate and product concentrations, which vary during conversion. A simplification can be made by considering the relative magnitudes of substrate and product concentrations with respect to the Michaelis-Menten parameters (Table I).  $D_S$  and  $D_R$  are on the same order of magnitude as the respective  $K_i$  values and will not influence  $k'$  to a great extent. Under good resolution conditions, when conversion of (S) is still low,  $S_a \gg K_m^S$  and when  $R_a \ll K_m^R$ ,  $k' = k_{cat}^S/S_a$ , and since  $k_c \ll k'$ ,  $k_c$  can be eliminated so that Eqs. (18) and (19) simplify to,

$$\frac{k_{cat}^S[E]}{k_a \left( \frac{a}{1-\Phi} \right) S_i} \frac{1}{S_i} < 0.1. \quad (20)$$

In the emulsion system tested, with  $\Phi = 0.2$  and stirring at 200 rpm, the visually observed droplet diameters of approximately 3–5  $\mu\text{m}$  corresponded to a specific interfacial area of 250–400  $\text{m}^{-1}$ , but for calculation the specific interfacial area was fixed at 300  $\text{m}^{-1}$ . The initial epoxide concentration in the organic phase was 324 mM, and using the criterion from Eq. (20) (with  $S_i = 3.7$  mM), the maximum enzyme concentration which should be used to minimize the effect of mass transfer limitations is 0.30  $\mu\text{M}$ . The uncertainty in the droplet diameter means that this calculated enzyme concentration could vary between 0.26 and 0.41  $\mu\text{M}$ . The resolution was performed in such an emulsion system with an initial enzyme concentration  $[E]_0 = 0.40$   $\mu\text{M}$  (Fig. 6). Using this higher enzyme concentration, a good resolution could still be obtained since the active enzyme concentration decreased during the course of the reaction due to inactivation. From the experimental data, the enzyme first-order inactivation rate constant was fitted to

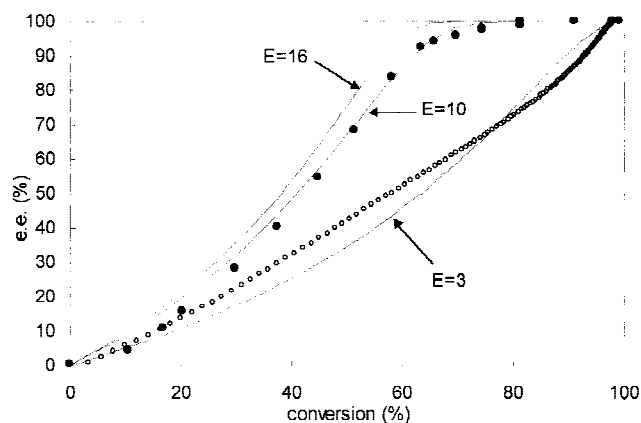


**Figure 6.** Resolution of styrene oxide in the batch emulsion reactor. The resolution was carried out in a total volume of 150 ml with  $\Phi = 0.2$  stirring at 200 rpm, for which  $a = 300$   $\text{m}^{-1}$ . Styrene oxide was fed to the organic phase at 324 mM. The initial enzyme concentration was 0.40  $\mu\text{M}$ . Symbols indicate experimental data (R =  $\square$ , S =  $\triangle$ , e.e. =  $\times$ ). Lines show simulation with a fitted first order inactivation constant  $k_d = 9.5 \times 10^{-6}$   $\text{s}^{-1}$ . At 96% e.e. the yield of (S)-enantiomer was 30%.

$k_d = 9.5 \times 10^{-6}$   $\text{s}^{-1}$ . The average active enzyme concentration over the course of the reaction, when the initial enzyme concentration is 0.40  $\mu\text{M}$ , can be calculated as 0.34  $\mu\text{M}$  using the approximation,  $\langle [E] \rangle / [E]_0 = 1/0.6k_d t_{95\%} + 1$  (Wolff et al., 1999).

For the conditions in this experiment, the model predicts a yield,  $Y_S$ , of 31.1% and a process time of 575 min (Fig. 5, for  $k_d = 0$ ,  $a = 300$   $\text{m}^{-1}$  and  $[E] = 0.34$   $\mu\text{M}$ ). There is good agreement between experimental data and model predictions and the uncertainty in specific interfacial area is of small importance since simulations show that  $Y_S$ , at 95% e.e., is relatively constant at 30% for enzyme concentrations ranging from 0.26 to 0.41  $\mu\text{M}$ , even for  $a = 250$   $\text{m}^{-1}$ . After 530 min, the e.e. reaches 96% with an analytical yield of (S) of 29.7% (Figure 6). The conversion curve obtained (Fig. 7) was fitted to the equation derived by Chen et al. (1982) which relates e.e., conversion and E, to obtain an effective E value of 10. This is an improvement over the conversion in the Lewis cell of Figure 3A, for which the effective E value was 3, but it is still lower than the intrinsic E value for the conversion of this substrate, of 16 (Fig. 7), suggesting that some mass transfer limitations still occurred under the chosen conditions. This may be true at the beginning of the reaction, when transfer of substrate from the organic phase starts and enzyme activity is highest, and when most of the (R)-enantiomer has reacted, so that (R)-enantiomer aqueous phase concentrations are not high enough to inhibit conversion of the (S)-enantiomer. Furthermore, the intrinsic E value of 16 was determined with the kinetic parameters and does not take into account the effect of spontaneous hydrolysis. The measured E value of a resolution of styrene oxide in a single aqueous phase system at pH 9, including spontaneous hydrolysis, was 13.8 (Lutje Spelberg et al., 1998).

A higher enzyme inactivation rate than that found for operation with a flat interface was expected due to the large increase in interfacial contact area. Studies on the inactivation of enzymes in liquid/liquid systems (Ghatorae et al.,



**Figure 7.** Comparison of resolution enantioselectivity in the Lewis cell and emulsion reactors. (O) simulation of the resolution carried out in the Lewis cell (Fig. 3A); (●) experimental points of the resolution carried out in the emulsion reactor (Fig. 6). Fitted E values and corresponding curves are: E = 10 and E = 3 as shown. A theoretical curve for the intrinsic E value (16) is also shown.

1994) suggest that the amount of enzyme inactivated is proportional to the interfacial contact area. From the inactivation data (Fig. 2), it is clear that the presence of the interface caused part of the enzymatic inactivation. Since interfacial areas must be large enough so that mass transfer is not limiting, investigations are currently under way to determine exactly how inactivation rate and specific interfacial area are related in our system.

Product inhibition becomes a limitation in this system only when diol concentrations exceed 1 M. This is because of the high diol inhibition constants relative to the affinity constants for both enantiomers of the substrate. To remove the diol formed during hydrolysis, ultrafiltration of the emulsion in a module external to the batch reactor could be carried out, recycling the organic phase and part of the aqueous phase with the enzyme back to the reactor. The applicability of this option has not been studied further here, but has recently been demonstrated for in situ removal of an inhibitory product (Cauwenberg et al., 1999).

## CONCLUSIONS

We have shown that the kinetic resolution of racemic epoxides by an enantioselective epoxide hydrolase can be carried out in an n-octane/aqueous two-phase system to overcome the problem of low epoxide aqueous solubility. A kinetic model was developed which could describe the bioconversion sufficiently accurately. Simulations showed that good resolution and high yields of enantiomerically pure epoxide can be obtained when mass transfer is not limiting. Based on the model, an equation for predicting the highest enzyme concentration which may be used to obtain a good resolution under defined mass transfer conditions and in a short process time was derived. This could be confirmed by a resolution of racemic styrene oxide at high concentration in a batch emulsion reactor, carried out under the predicted operating conditions. Agreement between predicted and observed yield of pure enantiomer was good.

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## NOMENCLATURE

a	Specific interfacial area (interfacial area/total liquid volume), (1/m)
A	Interfacial contact area (m <sup>2</sup> )
D	Diffusion coefficient (m <sup>2</sup> /s)
D <sub>S</sub> , D <sub>R</sub>	(S) and (R) diol enantiomers, aqueous phase concentrations (mol/m <sup>3</sup> )
[E]	Enzyme concentration (mol/m <sup>3</sup> )
⟨[E]⟩	Average enzyme concentration during a resolution (mol/m <sup>3</sup> )
E	Enantiomeric ratio ( $k_{cat}^R/K_m^R/k_{cat}^S/K_m^S$ )
J	Substrate flux (mol/m <sup>2</sup> · s)
k <sub>a</sub>	Mass transfer coefficient (m/s)
k'	Apparent reaction rate constant (m <sup>3</sup> /mol s)
K <sub>m</sub> <sup>S</sup> , K <sub>m</sub> <sup>R</sup>	Michaelis-Menten parameters (mol/m <sup>3</sup> )
k <sub>cat</sub> <sup>S</sup> , k <sub>cat</sub> <sup>R</sup>	Michaelis-Menten parameters (1/s)
K <sub>i</sub> <sup>SD</sup> , K <sub>i</sub> <sup>RD</sup>	Product inhibition constants for (S) and (R) diol (mol/m <sup>3</sup> )

k <sub>c</sub>	Spontaneous hydrolysis kinetic constant (1/s)
k <sub>d</sub>	First-order enzyme inactivation constant (1/s)
m	Epoxide partition coefficient (organic/aqueous)
S, R	(S) and (R) epoxide enantiomers, organic phase concentrations (mol/m <sup>3</sup> )
r <sub>S</sub> , r <sub>R</sub>	Rate of conversion of (S) and (R) enantiomers (mol/m <sup>3</sup> · s)
S <sub>a</sub> , R <sub>a</sub>	(S) and (R) epoxide enantiomers, aqueous phase concentrations (mol/m <sup>3</sup> )
S <sub>p</sub> , R <sub>i</sub>	(S) and (R) epoxide enantiomers, concentrations at the interface (aqueous side) in equilibrium with organic phase concentrations (mol/m <sup>3</sup> )
Y <sub>S</sub>	Yield of (S)-enantiomer

### Greek Symbols

ϕ	Thiele modulus
Φ	Volume fraction of organic phase (organic phase volume/total volume)

### Subscripts

0	at time = 0
t	at time = t

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