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Journal of Molecular Catalysis B: Enzymatic 13 (2001) 37–47

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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The enantioselective catalytic hydrolysis of racemic 1,2-epoxyoctane in a batch and a continuous process

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Received 20 December 1999; received in revised form 5 June 2000; accepted 12 July 2000

Abstract

In this study, the effect of the two co-solvents, acetonitrile (MeCN) and ethanol (EtOH), on the solubility of 1,2-epoxyoctane (epoxide), and on the reactivity of the catalytic hydrolysis to 1,2-octanediol (diol) by *Rhodosporidium toruloides* was investigated for final application in a flow-through bioreactor. The solubility of epoxide increased exponentially with the addition of both EtOH and MeCN. However, this increased solubility was at the cost of the reactivity of the enzyme, which showed a decrease with increasing co-solvent concentration, most predominant for MeCN. At 20% EtOH the solubility increased from about 6 to 10 mM, while the initial reaction rate has approximately halved, however, without loss in selectivity. When increasing the epoxide concentration (from 2 to 100 mM) at 20% EtOH, there is an initial linear increase in the initial production rate of the diol (V_{diol}) which reaches a plateau at 40 mM. There is a dip in V_{diol} at around 9 mM, indicating the possible effect of the one- and two-phase system. In the flow-through bioreactor, a %ee of 35 and 46% was achieved for the (S)-epoxide and (R)-diol, respectively.

Keywords: Enantioselective catalysis; Epoxide hydrolysis; Bioreactor; Solubility; Membrane

1. Introduction

Epoxides are valuable intermediates in the synthesis of many bioactive compounds [1]. Since several of the bioactive compounds are chiral and since the biological activity of the enantiomers of these chiral compounds can differ vastly, it is imperative to obtain these enantiomers in optically pure form. For this purpose, optically pure reagents are needed, and therefore, the synthesis of enantiopure epoxides has become a very active area of research.

Although numerous biologically catalyzed enantioselective epoxidations are available [2–4], for many epoxides only the racemate is obtainable, requiring some form of resolution [5]. This has resulted in the continuous research to find better enantioselective catalytic routes. Recent work has shown the highly enantioselective hydrolysis of 1,2-epoxyoctane (epoxide) to 1,2-octanediol (diol) by *Rhodosporidium toruloides* as illustrated in Fig. 1 [6].

With this catalyst, high enantioselectivities ($E >$ 100) and initial reaction rates (>300 nmol min^{-1} $mg^{-1}_{dry\ weight}$) were obtained [7]. An added advantage is that the enzyme does not have to be isolated for activity, nor do the cells have to be active. The obvious benefit and suitability of this reaction is thus,

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1381-1177/01/$ – see front matter
PII: S1381-1177(00)00229-0

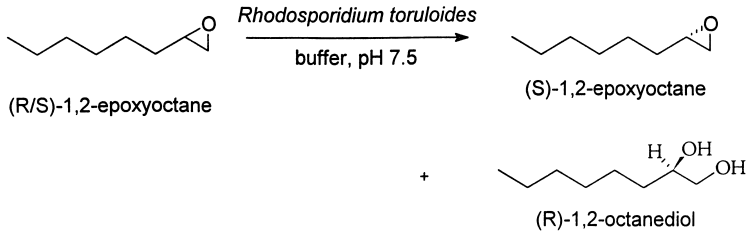

Fig. 1. Hydrolytic resolution of racemic 1,2-epoxyoctane by resting yeast cells.

apparent and the question of commercial viability, i.e. upscalability comes to mind.

Although various methods are available for upscaling, one option could be a bioreactor with a membrane for containing the yeast cells. There are different ways of utilizing a membrane in a reactor. If the substrate migrates across a membrane, reacts and migrates back across the same membrane, then the mass transport is diffusive. If the substrate moves across the membrane, reacts and is removed by a different route then the transport is convective. The membrane can also be used either to retain or to immobilize the biocatalyst. There are many variations on these four different setups, for example where an apoenzyme is immobilized on a membrane to facilitate the recognition and transport of only one of the enantiomers [8]. Another example of a convective system would entail a fixed bed process in a flow-through bioreactor (similar to a chromatographic columns [9]), as was used in this study.

The low solubility of many organic substrates in the aqueous environment of the biocatalyst is a known problem not only for enantioselective catalysis [10], but also for drug development [11]. This is the case with 1,2-epoxyoctane, which has a much lower solubility than the 20 mM epoxide concentration (or higher) used in the small-volume batch studies conducted by Botes et al. [12,13]. However, due to the proximity of the substrate and enzyme in such a small batch process, the difference in reactivity of solubilized versus emulsified epoxide is not critical. It is, however, not certain what influence such a two-phase system, i.e. an emulsion, might have on a flow-through bioreactor. The considerations include, for example, whether the emulsion would pass through the membrane, how the reaction rate is influenced by a one- or two-phase system and whether co-solvents could improve the solubility and thus, mass transfer of the epoxide into the yeast cells.

To obtain a better understanding of the influence of solubility and emulsions on the biocatalyst activity and functionality of a bioreactor, the influence of two co-solvents, acetonitrile (MeCN) and ethanol (EtOH), on the catalytic hydrolysis of 1,2-epoxyoctane was investigated. This study first established the effect of the co-solvents on the GC analysis, then evaluated their influence on the enzyme activity in batch studies, and finally tested the optimized reaction (in terms of solubility and reactivity) in a flow-through bioreactor.

2. Experimental

2.1. Materials

R. toruloides was obtained form the Yeast Culture Collection of the University of the Orange Free State (South Africa). The growth medium was purchased from Biolab (South Africa). Glycerol and EDTA was obtained from Merck (South Africa) and Saarchem (South Africa), respectively. Buffers were obtained form Merck (South Africa). All reagents were of analytical grade and used without further purification. Single distilled, deionized water (pH 7.0) was used. 1,2-epoxyoctane and 1,2-octanediol was obtained from Fluka and Aldrich, respectively. The bioreactor was made by the Department of Instrumentmaking (Potchefstroom University, South Africa).

3. Methods

3.1. Analysis

For the monitoring and quantification of the reactions, a GC equipped with FID was used. Chiral

separation was attained with a fused silica cyclodextrin column (β-DEX 120, 30 m × 0.25 mm, 0.25 μm film). Due to its higher sensitivity, H_2 instead of N_2 was used as carrier gas. The epoxide and diol were analysed at 58°C and 130°C, respectively. The respective retention times were Rt (58°C) = 21.0 and 21.3 min for (R)- and (S)-1,2-epoxyoctane, respectively, and Rt (130°C) = 10.3 and 10.6 min for (S)- and (R)-1,2-octanediol, respectively. The absolute configurations of the epoxide and diol had been established previously [14]. Quantification was done from calibration curves.

3.2. Preparation of frozen yeast cells

R. toruloides was grown at 30°C in 1 l shake flask cultures containing 200 ml YM medium supplemented with 1% glucose (w/v). At late growth phase (48–72 h) the cells were harvested by centrifugation (5000 g, 10 min, 4°C), washed with phosphate buffer (50 mM, pH 7.5), centrifuged and frozen at −18°C in phosphate buffer containing glycerol (10%) and EDTA (1 mM) in micro-centrifuge tubes (0.5 ml of cells per tube).

3.3. Effect of co-solvents on quantification and solubility

Irrespective of whether analysis was done for the epoxide/diol calibration, the epoxide solubility studies, or for following reaction progress, the sampling was always done in the same manner. Samples were accurately removed (Hamilton syringes) from the mixture into micro-centrifuge tubes. Samples where then extracted with an equal volume of ethyl acetate (EtOAc). EtOAc fractions were dried over Na_2SO_4 prior to GC analysis.

To determine the solubility of epoxide in the co-solvents, 0.5 ml epoxide was added to 5.0 ml phosphate buffer containing the specified amount of co-solvent. Enough epoxide was present to ensure a two-layer system. The mixture was stirred at 30°C for 24 h in glass bottles with screw tops fitted with septa. Stirring was discontinued 1 h prior to analysis to ensure that only solubilised and not emulsified epoxide is extracted from the phosphate aqueous phase. Samples were subsequently extracted with EtOAc, dried and analyzed on GC. Using the specific calibration curve for each co-solvent concentration, the solubility of epoxide was established.

3.4. Effect of co-solvents on reaction rate and selectivity

For the batch reactions, the cells were defrosted (approximately 1 ml of cells), washed with phosphate buffer (50 mM, pH 7.5), and resuspended in 4 ml of buffer (containing different co-solvent concentrations) in glass bottles with screw tops fitted with septa. Epoxide was added to a final concentration of 20 mM. The mixtures were stirred at 30°C in a time course experiment. Samples were taken at regular intervals and analyzed. The reaction rate was determined form the initial slope of the diol production over time (nmol $mg^{-1}_{\text{wet cells}}$ min^{-1}).

3.5. Effect of substrate on reaction rate and selectivity at 20% EtOH

Similar to the experiments of the solvent effect, these experiments were also conducted at 30°C. The only difference was that the co-solvent concentration was kept constant and the epoxide concentration was varied form 2 to 100 mM.

3.6. Flow-through bioreactor

For the reactor experiments, a flow-through reactor as illustrated in Fig. 2 was used. The feed has a

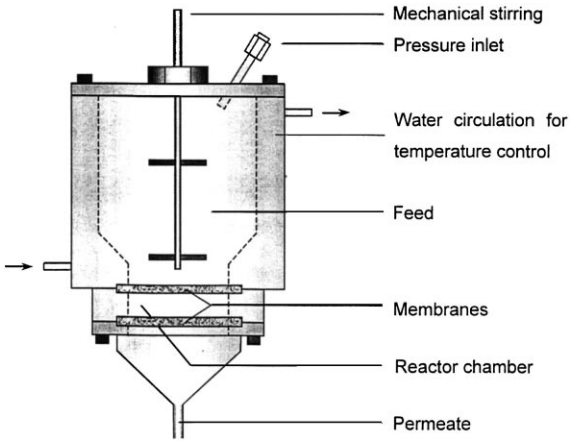

Fig. 2. A schematic setup of the flow-through bioreactor.

300 ml bulk feed capacity. The reactor itself has a diameter of 47 mm and a height of 10 mm, with a membrane on either side of the reactor. On the feed side of the reactor a 1 mm pore radius copper membrane was used while the permeate side consisted of a 0.45 μm pore diameter nylon membrane (47 mm diameter from Osmonics) supported on a 10 μm sintered metal membrane. The membrane on the feed side ensured the stirring in the feed bulk would not effect the reactor chamber itself, while the membrane on the permeate side was chosen to ensure that the cells were not washed from the reactor during permeation.

The bulk feed was stirred at 1000 rpm to ensure emulsification of the epoxide into the buffer. The temperature of the bulk feed was maintained at 30°C by a temperature controlled mantle surrounding the feed connected to a circulating water bath. 1.0 g of wet cells that had been thawed and washed with phosphate buffer were placed inside the reactor chamber and screwed into position. The feed phase consisted of a 20% EtOH phosphate buffer solution containing 20 mM of epoxide. Compressed air was used to apply a pressure (driving force) on the feed phase. The pressure was controlled by a regulator to ensure a constant flow rate of 0.3 ml min^{-1} through the reactor. Samples were taken from the permeate at regular intervals and extracted with EtOAc prior to GC analysis.

4. Results and discussion

4.1. Effect of co-solvents on quantification and solubility

It was assumed that the presence of co-solvents would influence the extraction of epoxide and/or diol by ethyl acetate. Therefore, the effect of the co-solvents on the calibration of the GC was first established. Three co-solvent concentrations were investigated: 0, 10 and 20% for MeCN and 0, 15 and 30% for EtOH. For the calibration, an epoxide and diol stock solution (20 mM each) was freshly prepared in a phosphate buffer containing the specified amounts of co-solvent prior to analysis. From the stock solution a dilution series (2, 6, 10, 16 and 20 mM) was extracted with EtOAc and injected. The surface area of the peaks was then plotted against the concentration range injected. In Fig. 3, an example is presented of two calibration curves of diol containing 15 and 30% EtOH, respectively.

After obtaining the calibration curves for all the different MeCN and EtOH co-solvent concentrations for both the epoxide and the diol, the slope of these calibration curves (straight line fitted on the calibration data points) was calculated. These calculated slopes of the calibration curves were then plotted as a function of the co-solvent concentration both for EtOH (Fig. 4) and for MeCN (Fig. 5). The values of the *Y*-axis for

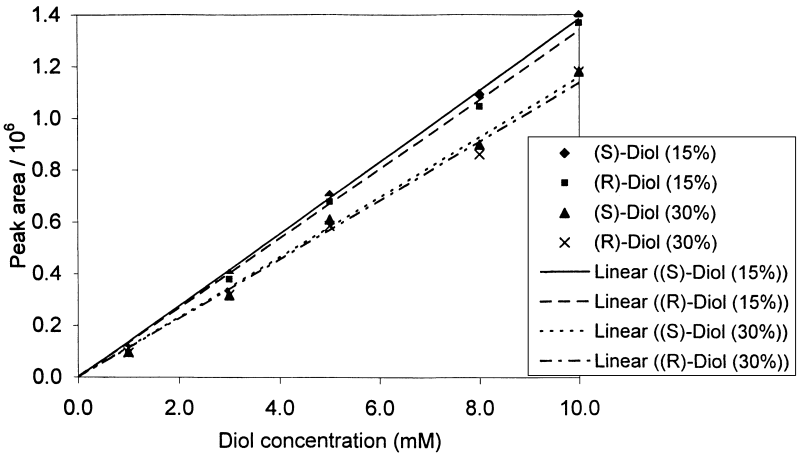

Fig. 3. GC calibration curve of diol extracted from a 15 and 30% EtOH buffer.

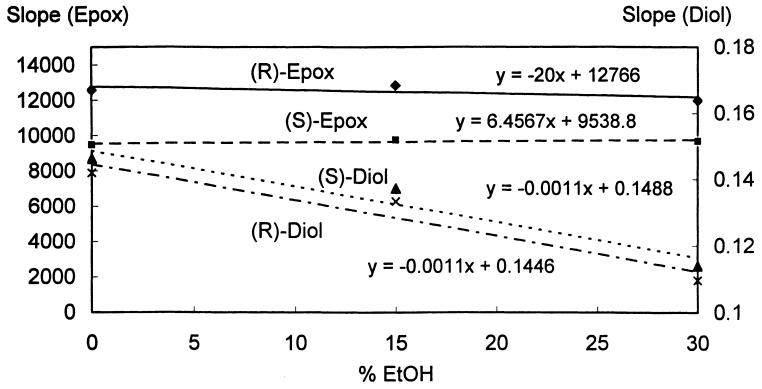

Fig. 4. The influence of EtOH as co-solvent on the slope of the (peak height or area as a function of concentration) calibration curve of epoxide and diol.

both graphs are arbitrary units based on the surface area (diol) or height (epoxide) of the GC-peaks as a function of concentration.

The difference in the slopes for the (R)- and (S)-epoxide and diol observed in Figs. 4 and 5 results from the GC yielding different slopes for the two enantiomers. This means that the two peaks were detected for a racemic mixture for each calibration point, upon integration, yielded different areas, in spite of the epoxide and diol being racemic mixtures.

The results clearly show the influence of the co-solvents and their concentration on the extraction of epoxide and diol from an aqueous phase using EtOAc. EtOH and MeCN have similar effects on the extraction of the diol and the epoxide, with very low, if any, effect on the extraction of the epoxide, while causing a decrease in the extractability of the diol. Although the addition of the relatively polar co-solvents slightly decreases the polarity of the aqueous phase, this effect is not enough to keep the relatively hydrophobic epoxide in the aqueous phase. However, the decreased aqueous polarity seems to be enough to increase the solubility of the polar diol (more than the epoxide), resulting in a decreasing concentration of extracted diol. This explains the decrease in the slopes observed for the diol in the presence of both EtOH and MeCN.

For the solubility experiments, the calibration equations obtained from Figs. 4 and 5 were used for calculating the absolute concentrations of the

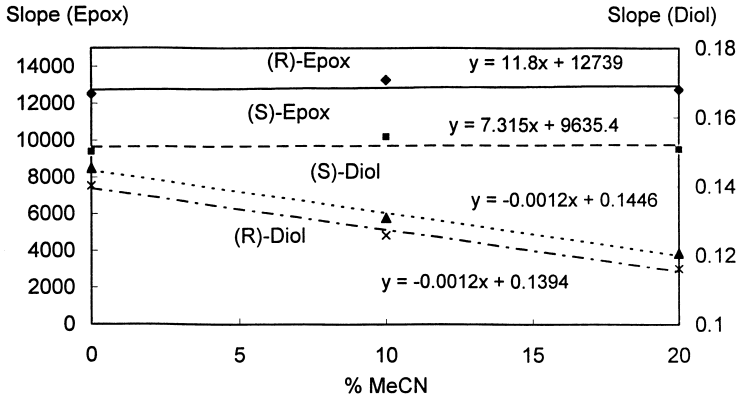

Fig. 5. The influence of MeCN as co-solvent on the slope of the (peak height or area as a function of concentration) calibration curve of epoxide and diol.

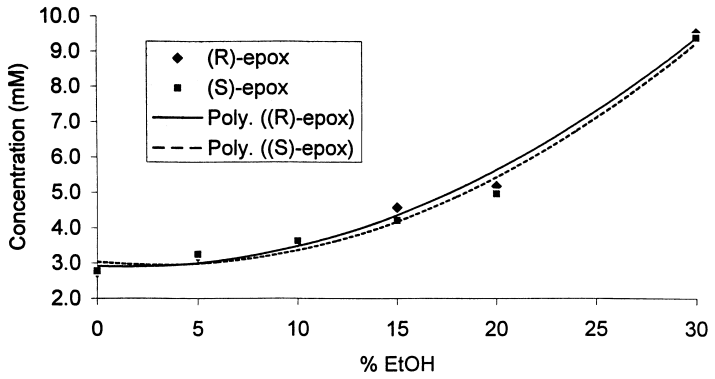

Fig. 6. The influence of EtOH as co-solvent on the solubility of epoxide (Poly.: polynomial function used to fit the data).

respective epoxide and diol extracted. When comparing the solubility graphs for EtOH and MeCN (Figs. 6 and 7), it is remarkable that both co-solvents, for all practical purposes, have the same effect on the solubility of the epoxide. Both show an exponential increase in solubility with increasing co-solvent concentration. This also means that at low concentrations, the co-solvents have little influence on the solubility of epoxide. This is specifically the case for the effect of MeCN.

Considering the initial objective, i.e. to increase the solubility of epoxide for application in a bioreactor, it would be essential to use as high a co-solvent concentration as possible. It is thus, imperative to investigate the effect of the two co-solvent concentrations on the activity of the cells.

4.2. Effect of co-solvents on reaction rate and selectivity

Two phenomena are noticeable when comparing the breakdown of epoxide in the absence and presence of co-solvent as illustrated in Fig. 8, where the breakdown of epoxide in the absence (a) and presence (b) of co-solvent (at various concentrations) is illustrated.

With the addition of co-solvent, the rate of (R)-epoxide breakdown decreases (25 min instead of 10 min). Secondly, in the absence of any co-solvent, both enantiomers are broken down and enantioselectivity is only a function of the difference in the rate of breakdown as previously shown by Botes et al. [12], who found similar results except at very high epoxide concentrations (>20 mM).

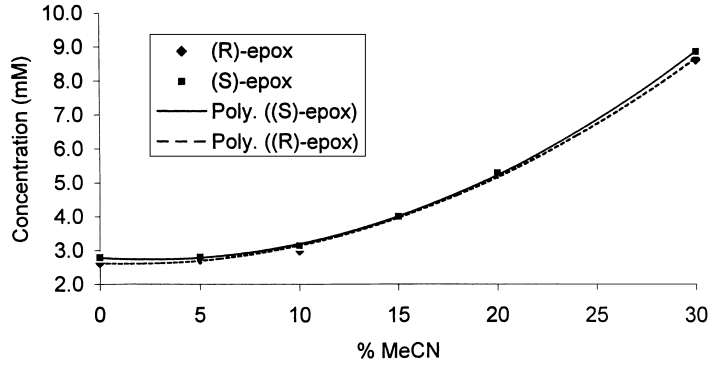

Fig. 7. The influence of MECN as co-solvent on the solubility of epoxide (Poly.: polynomial function used to fit the data).

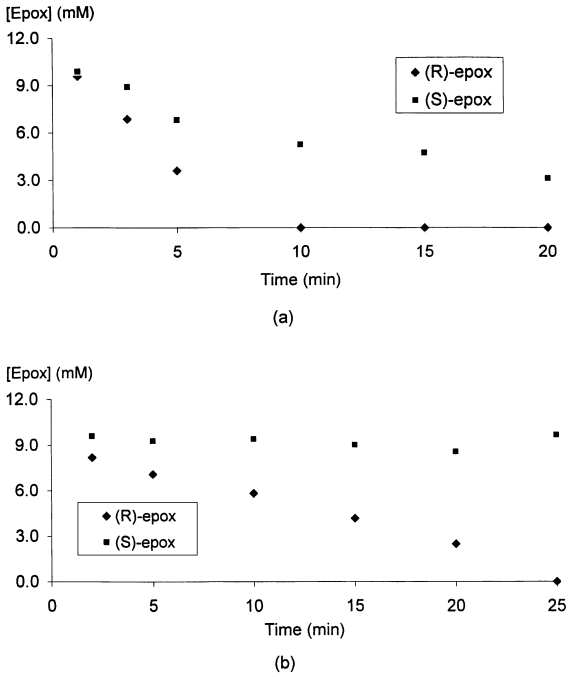

Fig. 8. Influence of co-solvent on the breakdown of (R)- and (S)-epoxide by *R. toruloides* at a total (epoxide) = 20 mM ((a) 0% EtOH and (b) 20% EtOH).

However, in the presence of co-solvent, it seems that the hydrolysis of (S)-epoxide is totally inhibited, irrespective of the co-solvent concentration, as shown in Fig. 8(b). This seems to indicate that the presence of co-solvent results in a decrease in the breakdown rate of (R)-epoxide, while completely inhibiting the breakdown of (S)-epoxide. This means that the presence of co-solvents not only decreases the reaction rate but also increases the enantioselectivity.

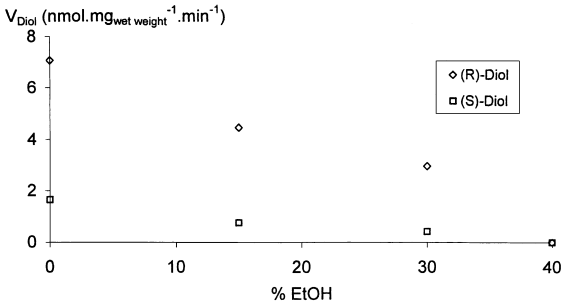

Fig. 10. Initial reaction rates (V_{diol}) as a function of the EtOH concentration.

When comparing the breakdown of epoxide and the formation of diol a similar trend was observed. Instead of presenting the raw data of the production of diol as a function of time, the reaction rates for diol synthesis where calculated and are presented as a function of co-solvent concentration (for MeCN in Fig. 9 and for EtOH in Fig. 10).

It is clear that irrespective of whether MeCN or EtOH was used, an increase in co-solvent concentration resulted in a decrease in the reaction rate for both enantiomers, which confirms the results obtained from the epoxide breakdown. However, while the reaction rate decreases for both enantiomers, the ratio of the

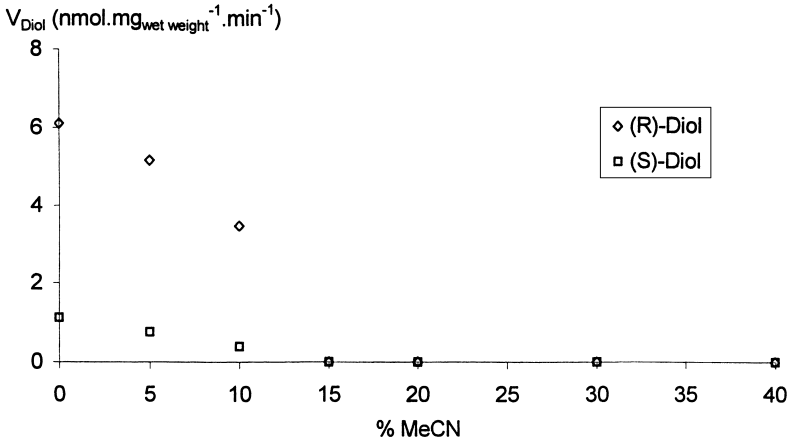

Fig. 9. Initial reaction rates (V_{diol}) as a function of the MeCN concentration.

reaction rate for the two diols increases (from five to eight for MeCN and from five to seven for EtOH), implying an increase in enantioselectivity with increasing co-solvent concentration. One could deduce that the increase in the selectivity of the production of diol is the result of the increase in the selectivity of the epoxide breakdown. However, this would be incorrect, since (S)-epoxide breakdown is completely inhibited in the presence of co-solvent, irrespective of its concentration, while the selectivity in the production of the diol is dependent on the co-solvent concentration.

Although the preference for the (R)-epoxide, i.e. the enantioselectivity is increased, the regioselectivity is not absolute [6]. This means that the (R)-epoxide can be attacked at either one of the epoxide carbon atoms. If the attack is from the least hindered carbon atom, then the (R)-diol is formed (retention of configuration), but if the attack occurs at the more hindered carbon atom, then inversion of configuration occurs, resulting in the (S)-diol being formed.

From these results, it seems that irrespective of whether one is interested in enantiopure epoxide or diol, the presence of co-solvent would be beneficial as it increases selectivity in both cases. The co-solvent concentration on the other hand would be determined by the product of interest. For enantiopure epoxide production, a minimum of co-solvent would ensure that no (S)-epoxide is broken down while minimising the inhibition of the (R)-epoxide breakdown. For enantiopure diol, a compromise has to be found between the decrease in reaction rate and the increase in enantioselectivity with increasing co-solvent concentration. For the useful operation of a reactor on the other hand, the increase in epoxide solubility as a function of co-solvent concentration becomes important.

When comparing the effect of co-solvent concentration on the increase in epoxide solubility and decrease in the reaction rate, the following observations seem significant. First, MeCN has a much stronger inhibitory effect on diol production, while simultaneously having less effect on epoxide solubility, than EtOH. At 15% MeCN, all enzyme activity has been destroyed. When comparing this with the solubility data (Fig. 7), it is clear that at 15% MeCN, no significant increase in solubility is observable. This implies that MeCN is not practical as a co-solvent, since no benefit is obtained in terms of epoxide solubility in the co-solvent concentration range where the enzyme is still active.

However, in the presence of EtOH, enzyme activity is only lost above an EtOH concentration of 30%. Comparing this with the solubility data (Fig. 6), where a clear increase in solubility is observed above 10%, implies that EtOH could successfully be used in a flow-through bioreactor, in the concentration range of 10–30%, as it would:

- Increase the epoxide solubility.
- Increase the enantioselectivity both of the epoxide breakdown and the diol production.
- Only slightly reduce the rate of the enantioselective hydrolysis, which becomes less important anyway in a continuous membrane system, where upscaling becomes easy.

4.3. Effect of substrate concentration on reaction rate and selectivity at 20% EtOH

It was mentioned that one of the reasons for using a co-solvent would be the increased solubility, and therefore concentration of substrate, in the bioreactor. However, what would the benefit be for increasing the epoxide concentration? To establish and quantify this substrate concentration effect, the influence of substrate (epoxide) concentration on reaction rate was investigated in a 20% EtOH buffer system (the optimum co-solvent concentration determined previously). Since the solubility of epoxide at 20% EtOH is known (approximately 9 mM), the study would also indicate if and what the effect of an aqueous-organic interface would be on the reaction rate in terms of the production of the diol. The initial diol production rate for both enantiomers as a function of epoxide concentration is illustrated in Fig. 11.

Two clear regions of change in reaction rate with increasing substrate concentration can be distinguished in Fig. 11, namely the region below 40 mM, which seems to follow first order kinetics, and the region above 40 mM, following zero-order kinetics. Initially, there is a linear gradual increase with increasing epoxide concentration, reaching its plateau at an epoxide concentration of 40 mM. This plateau region gives an indication of the V_{max} (maximum reaction rate) for the hydrolysis (approximately $3.5\,\text{nmol}\,\text{mg}^{-1}_{\text{wet weight}}\,\text{min}^{-1}$ for (R)-diol).

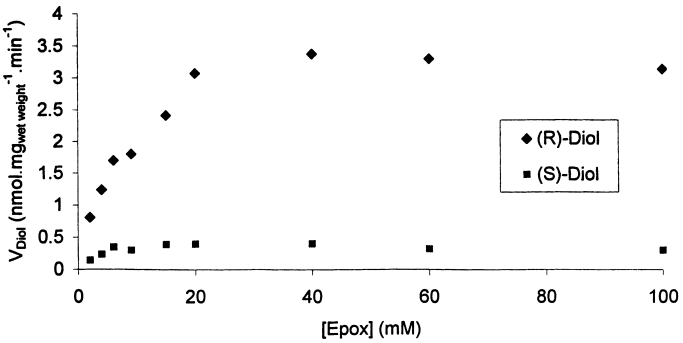

Fig. 11. Initial reaction rates (V_{diol}) as a function of epoxide concentration at 20% EtOH.

As the epoxide concentration is further increased, the reaction rate starts to decrease for both the (R)- and the (S)-diol, suggesting that inhibition occurs above 40 mM. However, in terms of enantioselectivity the ratio of the reaction rates for the two enantiomers gradually increases from five until it finally reaches 10 at 60 and 100 mM. This selectivity can also be expressed in terms of the enantiomeric excess (E), which was derived as follows [15]. The enantiomeric conversion (ξ) can be expressed in terms of the enantiomeric excess of the substrate (ee_s) and the product (ee_p)

$$\xi = \frac{ee_s}{ee_s + ee_p} \tag{6.1}$$

and since

$$ee_s = \left(\frac{\xi}{1-\xi}\right) ee_p \tag{6.2}$$

the enantiomeric ratio (E) can be expressed in terms of the ee_p and ξ

$$E = \frac{\ln\left[(1-\xi)\left(1-(\xi/(1-\xi))\,ee_p\right)\right]}{\ln\left[(1-\xi)\left(1+(\xi/(1-\xi))\,ee_p\right)\right]} \tag{6.3}$$

When calculating E using Eq. (6.3), then a gradual increase from approximately $E \cong 60$ to 140 was observed in the concentration range of 2–20 mM epoxide. This implies that E improves as the substrate concentration increases. This confirms what had been deduced before in terms of the ratio of the reaction rates. Above 20 mM, E remains similar to E obtained at 20 mM.

There is one more point to be considered in terms of Fig. 11. It was stated that initially the reaction rate for both enantiomers gradually increased with increasing substrate concentration. However, in the region of an epoxide concentration between 6 and 9 mM, the linear reaction rate increase is disturbed, which might have been highly coincidental was it not for the solubility maximum of epoxide lying in this range. This means that initially (below 9 mM), the system consists only of the yeast cells in a homogeneous buffer solution containing dissolved epoxide. Above 9 mM, the homogeneous solution has turned into an emulsion, i.e. the two-phase system containing the cells in the aqueous phase, and the hydrophobic epoxide phase.

Similar to the region of 2–6 mM, a linear increase in reaction rate as a function of epoxide concentration is again observed over the concentration range of 9–20 mM. However, the slope in the two-phase system is smaller than the slope observed for the one-phase system. Both the kink between 6 and 9 mM, and the difference in the slope before and after epoxide saturation has been reached, clearly shows the influence of solubilization on the reaction rate, and hence the enzyme kinetics. A possible explanation for the decrease in the slope above solubilization could be as follows. Above the solubility limit, an increase in the epoxide concentration will not necessarily mean a proportionally increased access of epoxide to the enzyme, but rather a proportional increase in the surface area of the interface as the emulsion droplets increase in size and number.

4.4. Flow-through bioreactor

One flow-through bioreactor experiment was conducted, where a 20% EtOH was added as co-solvent, to

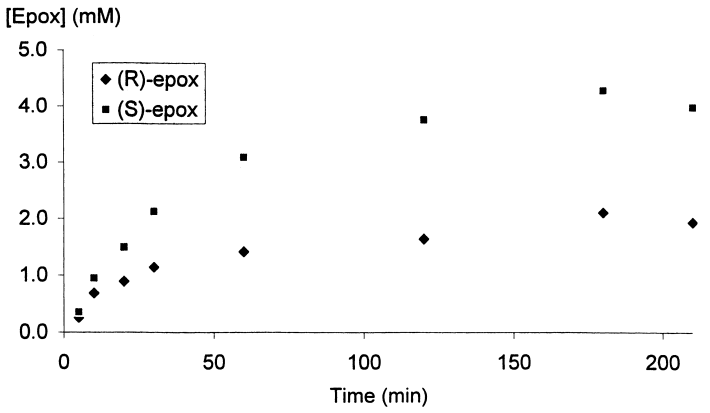

Fig. 12. Epoxide production as function of time in a flow-trough bioreactor.

improve the solubility and thus, the amount of epoxide in contact with the cells in the reactor. The amount of epoxide and diol permeating from the reactor is illustrated in Figs. 12 and 13, respectively. The %ee for the (S)-epoxide and the (R)-diol were 34.8 and 45.8%, respectively. Although these values are much lower than the %ee obtained in batch processes [6,7], it should be noted that these results were obtained form only a single reactor experiment.

In a batch reactor, as used above, the epoxide concentration decreases as it is converted to diol. However, in a reactor, there is continuously a new supply of epoxide and the epoxide concentration is maintained at the initial levels. It would thus, be expected that the kinetics would behave different from that of the batch reactions. One example is that optimum selectivity was only attained after 50 min of permeation. It is also interesting that initially both the epoxide and diol concentrations were very low. One possible explanation would be that the epoxide is absorbed by the yeast cells prior to conversion and only reaches the permeate once the cells have been saturated, i.e. a quasi-stationary state had been reached.

In terms of enzyme stability, the experiment has shown that no decrease in activity was observed for the duration of the experiment (more than 3 h). It is interesting to note that when adding up the total amount of epoxide and diol in the permeate once a quasi-stationary state had been reached, the total concentration is 9 mM. This is approximately the maximum solubility of epoxide at 20% EtOH as illustrated before confirming that only the solubilized epoxide passes through the bioreactor.

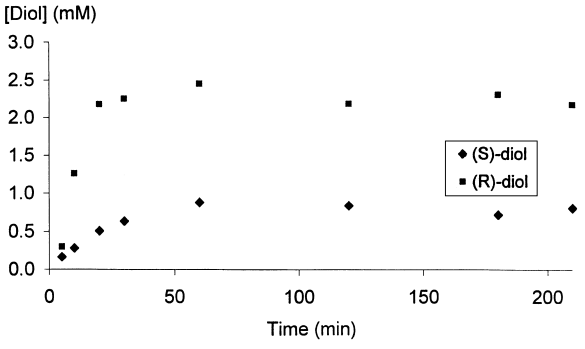

Fig. 13. Diol production as function of time in a flow-trough bioreactor.

5. Conclusion

In this study, two co-solvents (EtOH and MeCN) were investigated in terms of their influence on the solubility of epoxide as well as the influence they have on the catalysed hydrolysis of epoxide to diol. It was shown that the solubility of epoxide increases exponentially as a function of co-solvent concentration. Further, it was illustrated that both co-solvents decrease both the rate of diol production and the enantioselectivity of the hydrolysis. However, when

MeCN is present the reaction rate decreases faster than any increased benefit that would be derived from the increased solubility of epoxide. Thus, MeCN is not suitable as a co-solvent for a continuous type catalytic process.

From the study on the effect of epoxide concentration on the reaction rate, two observations were made. First, the reaction rate initially increases following first order kinetics until about 40 mM, and subsequently it follows zero order kinetics. Secondly, it was shown that a one-phase system versus a two-phase environment had an influence on the kinetics of the enzyme-catalysed hydrolysis. In a two-phase system, increasing the epoxide concentration has less effect on the rate of diol production than in a one-phase environment.

Finally, although the flow-through bioreactor yielded (S)-epoxide and (R)-diol with a %ee of only 34.8 and 45.8%, respectively, this experiment has clearly illustrated the feasibility of the enzyme catalysed reaction in a continuous type reaction.

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