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Optimisation of the biocatalytic resolution of styrene oxide by whole cells of *Rhodotorula glutinis*

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Abstract

Various studies have described the optimisation of the hydrolysis of epoxides through epoxide hydrolase, however, far fewer have investigated the specific application of whole cells containing the enzyme. For this reason the enantioselective biocatalytic hydrolysis of styrene oxide by *Rhodotorula glutinis* UOFS Y-0653 through the use of whole cells was explored. It was found that a pH of 7.2, temperature of 45 °C and an initial substrate concentration of 50 mM led to maximum enzymatic activity. The whole cells were resistant to a changing environment. High temperatures were found to increase enzymatic activity but decrease enantioselectivity. At low temperatures (15 °C) enantioselectivity was significantly increased leading to an increase in both enantiopure substrate yield and the enantiomeric excess of both the substrate and product. No substrate inhibition was observed at initial substrate concentrations as high as 100 mM. The low deactivation energy (85.2 kJ/mol) obtained for this hydrolysis reaction suggests thermal instability of the enzyme. No significant effect on the reaction was observed when using unbuffered water instead of phosphate buffer as reaction medium.

Keywords: Yeast epoxide hydrolase; Enantioselectivity; Styrene oxide; Optimisation; Whole cells

1. Introduction

Terminal epoxides (for example styrene oxide) are important chiral synthons [1] and have enjoyed the attention of biotechnologists and biochemists worldwide [2–4]. Consequently numerous enzymatic catalysts have been identified with the ability to catalyse the enantioselective hydrolysis of these epoxides to their corresponding *vicinal* diols. Through the use of the epoxide hydrolase (EH) enzyme, enantioselective hydrolysis of epoxides is catalysed via the *trans* antiperiplanar addition of water to the epoxide ring, resulting in the formation of the corresponding *vicinal* diol [5]. Although biocatalytic routes have previously been reported that yield both the (*R*)- and (*S*)-enantiomers of styrene oxide (SO) [i.e. 6], these routes are not always optimal. In fact, although many different micro-organisms exhibit enantioselective activity towards a variety of chiral substrates, these reactions are sometimes not feasible as an alternative to existing chemical catalysts. An example of such a biocatalytic route, illustrating the moderate enantioselective EH activity of *Rhodotorula glutinis* (UOFS Y-0653) towards SO, was previously demonstrated [7]. The reaction yielded 19% SO with an enantiomeric excess (ee) of >98% and 15% of the formed diol with an ee of 39%. A chemical approach to this reaction was illustrated by Kureshy et al. [8] using a dimeric homochiral Co(III) Schiff base complex as chemical catalyst. They established that a 46% yield of SO with an ee of 98% and a 39% yield of the formed diol with an ee of 99% can be achieved after 40 h. Even though the reaction time of the biocatalytic route is more appealing (3 h) the yield of enantiopure substrate and product is far less.

Many examples of enantioselective biocatalytic enzymes can be found in literature, for example lipase [9], EH [6,10,11] and alcohol dehydrogenase [12]. One approach used to improve these reactions is to optimise them with respect to reaction conditions. Preferably such a reaction would achieve a high ee for both substrate and product while maintaining high yields of the residual epoxide and the formed diol in the shortest time possible. By varying certain parameters such as pH, temperature, co-solvent and substrate concentration these reactions can be tuned to yield

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optimal activity and enantioselectivity. An increase of 40% in the initial relative activity of the enzyme, for example, was demonstrated by Tang et al. [10] after a 25 °C increase in reaction temperature. Cleij et al. [13] reported another interesting phenomenon using a two-liquid-phase reaction with a soluble EH extract from *Aspergillus niger* LCP 521 for the enantioselective resolution of *para*-bromo-α-methyl styrene oxide. In this biphasic system the selectivity of the reaction increases 13-fold when compared to a regular batch reaction in aqueous phase.

During the present study the EH activity exhibited by whole cells of *R. glutinis* [7] was optimised, through the use of SO as substrate, in an attempt to obtain conditions that would deliver a higher yield of enantiopure substrate and product within a reasonable reaction time. A further objective was to elucidate the behaviour of the whole cells at various reaction conditions.

2. Materials and methods

2.1. General

R. glutinis (UOFS Y-0653) was obtained from the yeast culture collection of the University of the Free State. In all the experiments samples were collected using the sacrificial technique of extraction, simultaneously halting the reaction and extracting the remaining substrate and formed product. These samples were analysed by gas chromatography (GC, Hewlett-Packard 6890 Plus equipped with FID) on a HP4^{TM} non-polar column (non-chiral analysis) and a β-Dex^{TM} 120 (Supelco) fused silica cyclodextrin capillary column (chiral analysis) using H_2 as carrier gas. Racemic SO and (*R*)-1-phenyl-1,2-ethane diol were obtained from Aldrich, while (*S*)-SO was obtained from Fluka. Chiral GC analysis of the isolated products after biohydrolysis were done as follows: SO, 90 °C, t_R(*R*) 9.9 min and t_R(*S*) 10.3 min, 1-phenyl-2-ethanediol, 150 °C, (*S*) 11.8 min t_R and (*R*) 12.3 min t_R. Spiking samples with a small amount of enantiopure substrate or product identified the absolute configuration of the individual enantiomer peaks. The phosphate buffers used were prepared by mixing different volumes of 1 M KH_2PO_4 and K_2HPO_4 stock solutions into 1 L of deionised water to obtain the required pH values at a 50 mM concentration. If necessary, final pH values were set with NaOH and HCl.

2.2. Cultivation and preparation of whole yeast cells

Yeasts were grown in 1 L shake-flask cultures, within a Labcon® temperature regulated rotary bed incubator (27 °C, 180 rpm), containing 200 mL YM media (0.5% yeast extract, 2.0% malt extract, 0.5% peptone w/v) supplemented with 1.5% (w/v) glucose and vitamins (0.2%, v/v). The vitamin solution consisted of 0.02% biotin, 2.00% calcium pantothenate, 0.002% folic acid, 10% inosital, 0.4% niacin, 0.2% *p*-aminobenzoic acid, 0.4% pyridoxine hydrochloride, 0.2% riboflavin and 0.4% thiamine HCl (w/v) dissolved in double distilled water. At late growth phase (72 h) the cells were harvested at room temperature (24 °C) by centrifugation (3500 × *g*, 5 min, maximum acceleration) and washed with phosphate buffer (50 mM, pH 7.5). The washed cells were resuspended (25%, w/v) in phosphate buffer containing 20% (v/v) glycerol, aliquoted into either 1.5 mL micro-centrifuge tubes (500 μL per tube) or 50 mL centrifuge tubes (20 mL per tube) and frozen at −20 °C.

2.3. Chemical hydrolysis

For every experimental condition where the biocatalytic activity was determined another set of experiments was done in the absence of biocatalyst, thus determining the non-selective chemical hydrolysis under the specified conditions. Subsequently these values resulting from chemical hydrolysis were subtracted from the total hydrolysis observed in the presence of biocatalyst. The results presented in this paper are thus limited to the actual biocatalytic activity resulting from the presence of the biocatalyst only.

2.4. Effect of pH upon the activity

Frozen cell suspensions (500 μL) were thawed, centrifuged (3500 × *g*, 5 min, 24 °C) and washed with the appropriate phosphate buffers before being resuspended (500 μL in 1.5 mL micro-centrifuge tubes). SO (10 μL of a 1 M DMSO stock solution) was added to give a final concentration of 20 mM. The reaction mixtures were incubated at 30 °C for 30 min while continuously being shaken in a shaking water bath (200 rpm). Extraction with 250 μL of ethyl acetate terminated the reactions. The ethyl acetate fraction was analysed by chiral GC.

2.5. Effect of temperature upon the activity, selectivity and stability

2.5.1. Effect upon activity and selectivity

Frozen cell suspensions (pH 7.5) were thawed (500 μL in 1.5 mL micro-centrifuge tubes) after which SO (15 μL of a 1 M DMSO stock solution) was added to give a final concentration of 30 mM. This initial substrate concentration was used to prevent the complete chemical hydrolysis of the substrate at high temperatures within the investigated reaction time. The reaction mixtures were incubated at various temperatures with continuous shaking (200 rpm). At different time intervals one micro-centrifuge tube was removed and the residual epoxide and formed diol extracted with 250 μL ethyl acetate. The ethyl acetate fractions were analysed by chiral GC.

2.5.2. Effect upon the stability

Frozen cells were thawed (500 μL in 1.5 mL micro-centrifuge tubes) and incubated at various temperatures with continuous shaking (200 rpm). At specific time intervals a micro-centrifuge tube was removed and shock frozen in liquid N_2. To determine the remaining enzymatic activity all the frozen micro-centrifuge tubes were thawed and the activity assayed at 15 °C by adding SO to a final concentration of 20 mM. After 2 h the residual epoxide and formed diol were extracted as before and analysed by chiral GC.

2.6. Effect of substrate concentration upon the activity

Frozen cells (pH 7.5) were thawed (500 μL in 1.5 mL micro-centrifuge tubes) after which a specified amount of SO (1 M DMSO stock solution) was added to give a range of initial concentrations. The reaction mixtures were incubated at 45 °C and extracted with ethyl acetate (250 μL) at various time intervals while continuously being shaken (200 rpm). The ethyl acetate fractions were analysed by non-chiral GC.

2.7. Salt free process

Frozen cells (20 mL) were thawed and centrifuged (3500 × *g*, 5 min, 24 °C), the supernatant discarded and replaced with either phosphate buffer (50 mM, pH 7.0) or demineralised water (pH 7.0) before being resuspended. Ten milliliters of each suspension was transferred into 50 mL glass bottles and incubated at 15 °C for 5 min (allowing temperature equilibration) before the addition of SO to a final concentration of 20 mM. At different time intervals 500 μL samples were drawn, extracted with ethyl acetate (250 μL) and analysed by chiral GC.

3. Results and discussion

3.1. Effect of pH upon activity

To determine the optimal pH for the EH enzyme, *R. glutinis* was used to catalyse the hydrolysis of SO. The optimal pH for the hydrolysis reaction was determined by analysing the amount of formed diol by GC analysis (Fig. 1). According to the control study (phosphate buffer not containing cells) variation of the pH between 6.0 and 7.8 had a negligible effect on the chemical stability of the substrate.

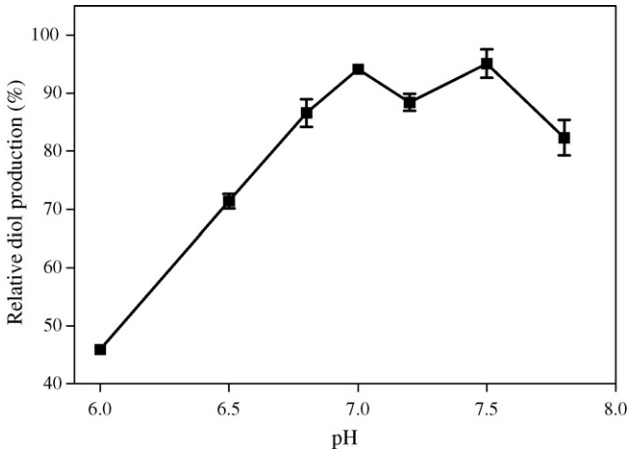

Fig. 1. Formation of 1-phenyl-1,2-ethane diol after biohydrolysis (30 min, 30 °C) of SO with *R. glutinis* (■) at various pH values. Relative diol production is defined as the concentration diol obtained (mM min^{-1} g^{-1}) relative to the maximum concentration obtained.

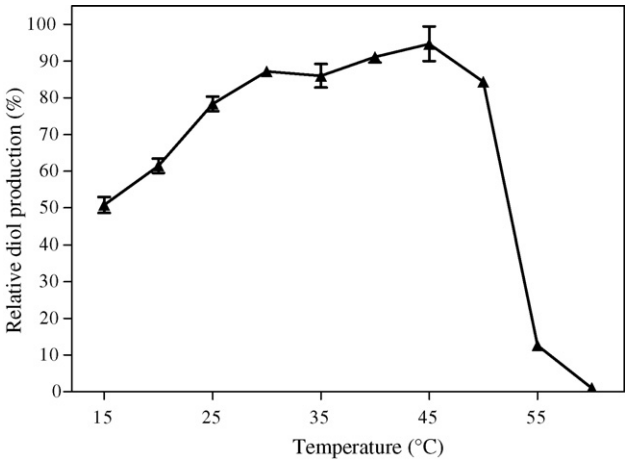

Fig. 2. Formation of 1-phenyl-1,2-ethane diol after biohydrolysis of SO with *R. glutinis* at various temperatures. Relative diol production is defined as the product obtained (mM min^{-1} g^{-1}) relative to the maximum amount of product obtained.

The enzyme exhibited high activity across a broad range of pH values (6.8–7.8). The ability of the whole cells to efficiently catalyse the reaction over a wide pH range is probably due to the physical protection offered by the cell membrane against the immediate environment. A decrease in activity was observed at both pH < 6.0 as well as pH > 7.8, leading to the conclusion that a pH value of between 7.0 and 7.5 would be most advantageous for this reaction. This correlates well with previous work preformed using resting yeast cells at a pH value of 7.5 [3,14]. Furthermore, the obtained results correlate well with the optimal pH range obtained by Botes with EH enzyme from *Rhodosporidium toruloides* CBS 0349 [15].

3.2. Effect of temperature upon the activity, selectivity and stability

3.2.1. Effect upon activity and selectivity

The optimal temperature for the hydrolysis reaction was determined. The results are shown in Fig. 2. Again the whole cells demonstrated optimal activity over a broad range of temperatures (30–50 °C).

The whole cells exhibited decreased relative activity at temperatures below 30 °C and higher than 50 °C (deactivation of enzyme). Nellaiah et al. [11] previously reported that an enzyme preparation from *A. niger* showed an increase in initial reaction rate up to 35 °C while losing nearly 60% of its original activity at 40 °C after 1 h of incubation. When investigating the selectivity, however, it became clear that increased temperatures have an adverse effect on the selectivity of the reaction (Figs. 3 and 4).

At 15 °C (Fig. 4) a substrate enantiomeric excess (ee_s) of 64% was reached after 150 min compared to 28% reached at 40 °C (Fig. 3). Interestingly 4.3 mM of the fast reacting (*R*)-enantiomer was still present at 40 °C, while only 2.8 mM was present at 15 °C. This illustrates that the lower temperature increased the rate at which the (*R*)-enantiomer was hydrolysed while slowing the hydrolysis of the (*S*)-enantiomer, thereby, enhancing selectivity and confirming the difference in activation energy for the reaction of the two enantiomers with the enzyme.

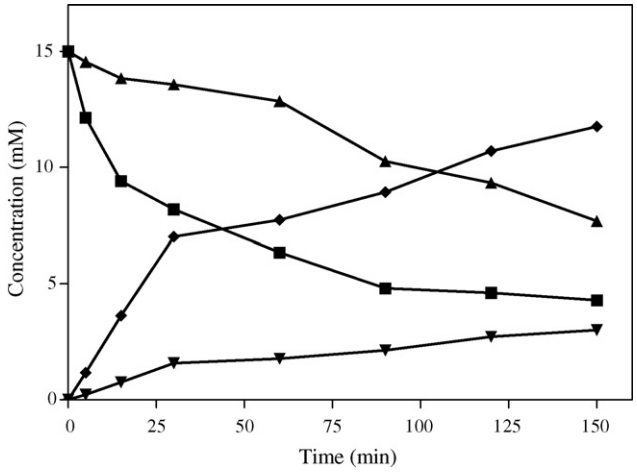

Fig. 3. Hydrolysis of racemic SO (30 mM, pH 7.5) at 40 °C with *R. glutinis*. (▲-(*S*)-epoxide, ■ (*R*)-epoxide, ♦ (*R*)-diol, ▼ (*S*)-diol).

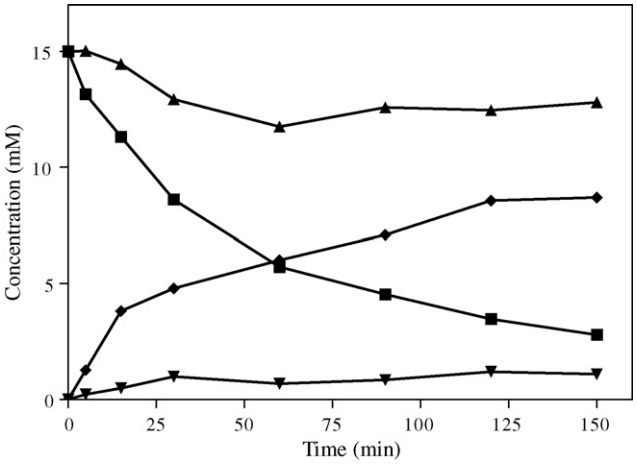

Fig. 4. Hydrolysis of racemic SO (30 mM, pH 7.5) at 15 °C with *R. glutinis*. (▲ (*S*)-epoxide, ■ (*R*)-epoxide, ♦ (*R*)-diol, ▼ (*S*)-diol).

Table 1
Hydrolysis of SO (30 mM) with *Rhodotorula glutinis* (UOFS Y-0653) at various reaction temperatures after 2 h

Temperature (°C)	ee_s (%)	ee_p (%)	c (%)	$Yield_s$ (%)	$Yield_p$ (%)
50	23	37	41	36	27
40	34	43	54	31	36
30	47	58	45	41	35
20	34	69	43	38	24
15	56	67	47	42	29
10	22	77	19	49	17
5	19	78	16	50	16

ee_s: % enantiomeric excess of the substrate, ee_p: % enantiomeric excess of product, c: conversion of substrate, $yield_s$: % yield of (*S*)-SO, $yield_p$: % yield (*R*)-1-phenyl-1,2-ethane diol.

To determine the optimal temperature for the highest enantioselectivity, time-course reactions were performed at various temperatures with an initial epoxide concentration of 30 mM. The results of the biocatalysed hydrolysis (compensated for chemical hydrolysis) after 2-h reactions are given in Table 1. Enantiomeric ratios (*E*) were not calculated since the regioselectivity of this enzymatic reaction has not been determined.

The results demonstrated the most optimal reaction at temperatures of 15 °C and below, illustrating an enantioselectivity enhancing effect at lower temperatures. Decreases in activity at lower temperatures led to decreasing conversion values (correlating with the increased activity at higher temperatures, Fig. 2), while the increase in selectivity led to higher substrate yields. Since ee_s was found to be greatest at 15 °C while maintaining a high $yield_s$ and c, this temperature was selected as being optimal for this reaction. For all the temperatures investigated the selective hydrolysis of the epoxide (*R*)-enantiomer and the selective formation of the (*R*)-diol was observed.

It was stated in Section 2 that for each experiment chemical hydrolysis was determined and subtracted from the total hydrolysis to obtain the biocatalysed hydrolysis. The chemical hydrolysis (after 2 h—for the same experiment as described above) expressed in terms of the epoxide converted to diol as a function of temperature was 47% (50 °C), 23% (40 °C), 16% (30 °C), 7.2% (20 °C) and 0.0% (15 °C). As expected the chemical hydrolysis decreased with decreasing temperature. The nearly exponential increase in the uncatalysed reaction rate, as a function of temperature, is as was expected for purely chemical reactions. Since no chemical hydrolysis was observed at reaction temperatures of 15 °C and below the enzymatic reaction no longer has to compete with the chemical reaction, increasing the potential of a highly selective reaction.

3.2.2. *Effect upon the stability*

Generally, low temperatures increase enzymatic stability, increasing potential use for slow reacting substrates, higher initial substrate concentrations and repetitive use. To examine this, cells were incubated at various temperatures and times before determining the enzymatic activity at 15 °C (120 min). Previously epoxide hydrolase from *A. niger* has been shown to follow initial linear deactivation with increasing temperatures [11] and first order deactivation kinetics were supposed. Similarly for this reaction it can be assumed that

$$\frac{d[S]}{dt} = -k[S][E] \quad (1)$$

where $d[S]/dt$ is the change in substrate concentration over time, and $[S]$ and $[E]$ are the substrate and enzyme concentrations, respectively (mol dm^{-3}). Further experiments (discussed in Section 3.3) illustrated that increasing $[S]$ led to an increase in reaction rate and therefore it was assumed that $[E] \gg [S]$ (500 μL cells versus 20 mM SO). Therefore Eq. (1) can be rewritten as a first order equation:

$$\frac{d[S]}{dt} = -k'[S] \quad (2)$$

Solving Eq. (2) it follows that

$$\ln\frac{[S]}{[S_0]} = -k't \quad (3)$$

where $[S_0]$ is the initial substrate concentration (mol dm^{-3}), $[S]$ the substrate concentration (mol dm^{-3}) after time t (120 min) at 15 °C and k' the rate constant (min^{-1}) containing the initial rate constant k and the enzyme concentration $[E]$ (mol dm^{-3}). When assuming that the decrease in enzyme activity during heat treatment results solely from a decrease in the active enzyme concentration it follows that during heat treatment:

$$\ln\frac{[E]}{[E_0]} = -k''t' \quad (4)$$

where $[E]$ is the remaining active enzyme concentration (i.e. enzyme activity) after heat treatment, $[E_0]$ the initial active enzyme concentration (mol dm^{-3}), k'' the rate constant for thermal decomposition (min^{-1}) and t' the time of heat treatment (min). Since $k' = k[E]$, by plotting k' as a function of the incubation time t', k'' can be obtained using Eq. (4) for each temperature of heat treatment. An example is shown in Fig. 5 (20 °C).

$t_{1/2}$ (half life), which is defined as the time needed to decrease the initial enzyme activity by 50%, can be obtained by solving Eq. (4) for $[E] = 0.5[E_0]$:

$$t_{1/2} = \frac{0.693}{k''} \quad (5)$$

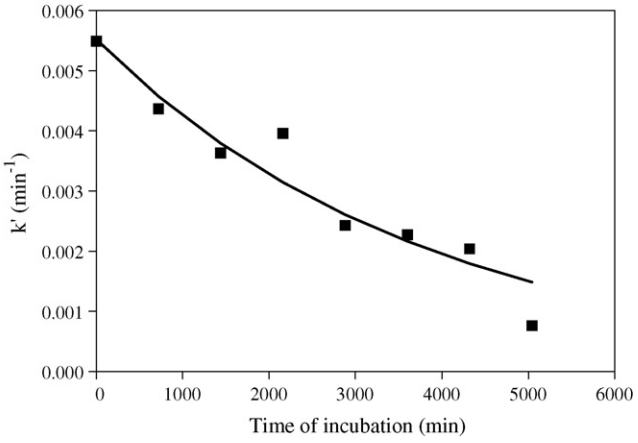

Fig. 5. Residual enzymatic activity ($k[E]$) after heat treatment at 20 °C.

Table 2
Rate constants and half-life values obtained at different temperatures

Temperature (°C)	T (K)	$10^3/T$ (K^{-1})	$k'' \times 10^{-4}$ (min^{-1})	$t_{1/2}$ (h)
10	283	3.53	0.33 ± 0.05	354.52
20	293	3.41	2.60 ± 0.37	44.41
30	303	3.30	6.34 ± 0.65	18.20
40	313	3.20	11.28 ± 1.28	10.24
50	323	3.10	41.36 ± 4.36	2.79

The k'' and $t_{1/2}$ values for each heat treatment are presented in Table 2. Standard deviations were determined with solvstat [16].

Since (Arrhenius' law):

$$\ln k'' = \ln A - \frac{E_a}{RT} \quad (6)$$

where A is a constant, E_a the activation energy of deactivation or thermal inactivation energy ($J\,mol^{-1}$), R the universal gas constant ($8.314\,J\,mol^{-1}\,K^{-1}$) and T the absolute temperature (K), plotting $\ln(k'')$ as a function of $1/T$ a straight line is obtained (Fig. 6) with the slope:

$$\text{Slope} = \frac{-E_a}{R} \quad (7)$$

Accordingly, the E_a for this reaction is 85.2 kJ/mol. This value is low when compared to other enzymes such as *Rhizomucor meihei* lipase (304 kJ/mol) [17] and *A. niger* epoxide hydrolase (177 kJ/mol) [11]. The obtained values for E_a and $t_{1/2}$ (at various temperatures) indicate a relatively low thermal stability, which further affirms the advantage of using low temperatures. At 10 °C there is very little decrease in activity with the enzyme retaining 72% of its original activity after 180 h.

3.3. Effect of substrate concentration upon the activity

To determine the substrate concentration that would give the highest initial reaction rate, hydrolysis was preformed using whole cells with increasing amounts of initial substrate concentration. The decrease in the concentration of epoxide was measured over time by GC analysis. By plotting the slope of the initial reduction in epoxide concentration (V) against the substrate concentration (S), the initial concentration leading to the highest initial reaction rate could be determined. The results are shown in Fig. 7.

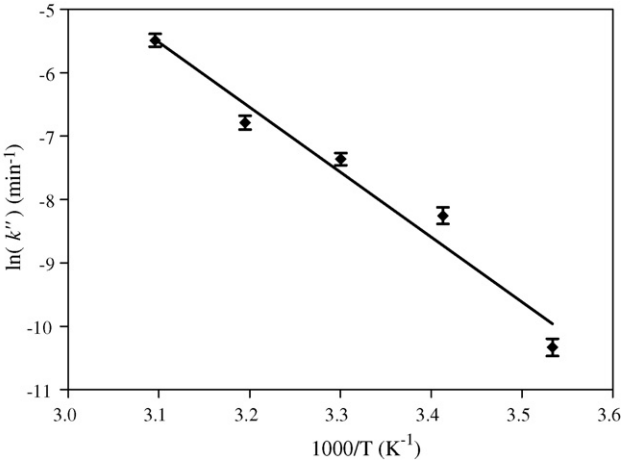

Fig. 6. Arrhenius plot of $\ln(k'')$ vs. $1/T$. Standard deviations are illustrated by error bars.

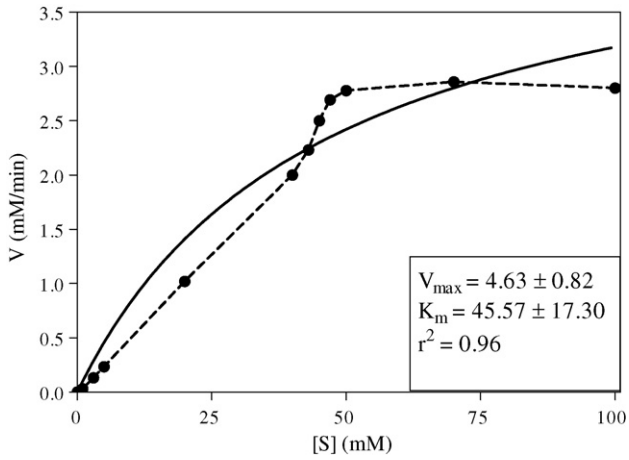

Fig. 7. Hydrolysis of substrate over time (V) at various initial SO concentrations (S) during hydrolysis by *R. glutinis* (dashed line) and a theoretical Michaelis–Menten curve fit (solid line).

It was found that an increase in the initial substrate concentration lead to an increase in the initial reaction rate. However, at substrate concentrations higher than 50 mM the initial reaction rate was no longer dependant upon an increase in the substrate concentration. The highest initial reaction rates (V_m) and the corresponding maximum substrate concentrations (S_m) leading to these rates were 2.78 mM min^{-1} and 50 mM, respectively. At S_m it can be assumed that either all the active enzyme-binding sites are saturated and therefore more epoxide molecules would have no effect or the poor solubility of SO in the aqueous phosphate buffer becomes rate limiting (±24 mM with 2% (v/v) DMSO). The S_m is however much higher than the soluble concentration, leading to the conclusion that the reaction can take place at the interface between the aqueous phase and the unsolubilised substrate. To describe the relationship between the initial substrate concentration and the initial reaction rate the known Michaelis–Menten Eq. (8) was used (Fig. 7):

$$V = \frac{V_{max} S}{K_m + S} \quad (8)$$

Using solvstat [16], a maximum initial velocity (V_{max}) of 4.63 ± 0.82 and a Michaelis constant (K_m) of 45.57 ± 17.30 were estimated. The obtained data did not follow Michaelis–Menten kinetics accurately as can be seen from the large standard deviations. For this reason it was concluded that further studies would be necessary to elucidate the kinetics of this whole cell reaction.

The results that were obtained during this study are slightly different to those reported by Nellaiah et al. for *A. niger* [11]. The sharp increase in initial reaction rate during hydrolysis decreases at concentrations higher than 20 mM. This effect is attributed to the poor solubility of the substrate since no substrate inhibition is present.

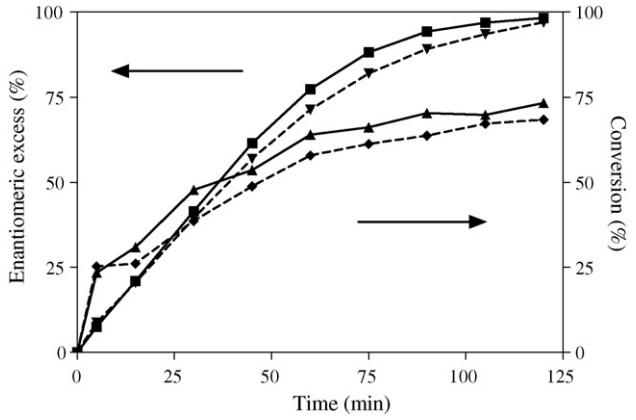

Fig. 8. Kinetic resolution of SO (20 mM initial concentration) with *Rhodotorula glutinis* (whole cells, 15 °C, pH 7.0) using 50 mM phosphate buffer (solid line) or demineralised water (dashed line) as reaction media. Enantiomeric excess values are represented by ■ and ▼, while conversion ratios are represented by ▲ and ♦, respectively.

3.4. Salt free process

Monfort et al. [18] recently described the use of crude recombinant epoxide hydrolase from *A. Niger* origin in unbuffered water, a so-called "salt free" process. This allows simplification and cost reduction of the process. Their results demonstrate an insignificant difference when using a phosphate buffer or unbuffered water as reaction medium. To determine whether the same is true for the hydrolysis of SO with whole cells of *R. glutinis*, two reactions were run parallel, one with phosphate buffer as reaction medium and the other demineralised water (Fig. 8).

The obtained results illustrate the same negligible difference between using either of the two reaction media, indicating that the phosphate buffer could be replaced with unbuffered water. From this it can be assumed that the reaction does not lead to any significant changes in pH since the negative effects of pH variation have been illustrated. Except for the previously mentioned lower production costs or the costs of downstream processing, the use of demineralised water could have advantages when used in scaled up reactors, which depend upon membranes for their functionality.

4. Conclusion

Enantioselective hydrolysis of terminal epoxides by yeast EH is influenced by various factors such as pH, temperature, co-solvent and initial substrate concentration. During this work it was shown that the pH as well as the reaction temperature could influence the relative activity exhibited by the enzyme. High temperatures (30–50 °C), substrate concentrations between 40 and 60 mM and neutral pH values (6.5–7.5) led to increased enzymatic activity. It was concluded that, for optimal enzymatic activity, this reaction had to be operated at 45 °C and at a pH of 7.2.

The benefit of higher reaction rates at increased temperatures was overshadowed by the observation that an increase in temperature was responsible for a severe decrease in enantioselectivity. Lower temperatures (15 °C) on the other hand increased the hydrolysis rate of the fast reacting (*R*)-enantiomer and significantly reduced the hydrolysis rate of the slow reacting (*S*)-enantiomer. At even lower temperatures (i.e. 10 °C) selectivity was increased even further but reaction rate decreased such that a longer reaction time was required to reach 98% ee. Furthermore, low temperatures were shown to markedly increase enzymatic stability.

This process can be simplified by exchanging the phosphate buffer reaction medium with unbuffered water as this does not lead to a significant decrease in the activity or selectivity of the reaction.

While it would be ideal to apply those conditions that yield highest activity to complete a reaction within the shortest time, in practice, as shown in this study, it is often not possible. This means that some compromise has to be found between the different parameters such as pH, temperature, substrate concentration and solubility, ratio of catalyst to substrate (as these parameters influence reaction rates), enantioselectivity, activity and reaction time to reach 98% ee_s, depending on the final process requirements.

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