Optimisation of a Stereoconvergent Process Catalysed by Whole Yeast Cells

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"No amount of experimentation can ever prove me right; a single experiment can prove me wrong."

- Albert Einstein

Summary

Stereochemistry, defined as being the study of the spatial behaviour of atoms in molecules and complexes, has become increasingly important in the pharmaceutical sector. In particular, the existence and pharmacological effects of pharmaceutical enantiomers has given rise to significant research efforts, aiming not only to study the biological effects of enantiopure products but also establishing methods of obtaining chiral molecules in their enantiopure form.

One of these methods, involving the use of enantioselective hydrolytic enzymes, has received considerable attention. Utilising the catalytic potential of epoxide hydrolase, an enzyme found in a wide variety of living organisms, highly reactive racemic epoxides may be obtained in their enantiopure form together with their corresponding *vicinal* diols. These two enantiopure products may themselves be biologically active or, in turn, serve as precursors during the synthesis of high value enantiopure pharmaceuticals.

The project described in this thesis had four main objectives. Firstly, to optimise the previously established enantioselectivity exhibited by whole cells of *Rhodotorula glutinis* towards terminal epoxides using styrene oxide as a model substrate. Successful completion of this goal illustrated that a pH of 7.2, optimal temperature of 15 °C and an initial substrate concentration of 50 mM yielded the most promising reaction. Further studies also showed that this reaction may be run as a salt free process, reducing costs and following a more environmentally friendly approach.

One of the major challenges during the hydrolysis of epoxides is the fact that these substrates tend to be highly insoluble in water. The enzyme, on the other hand, is far more active in its native aqueous medium. For this reason the second objective was to investigate not only the effects of two commonly used water miscible organic solvents (DMSO & DMF), but also the possibility of utilising solubility enhancing cyclodextrins for this reaction. It was found that hydroxypropyl-β-cyclodextrin (HPB) had a far greater solubilsation potential than the two solvents investigated. In addition, HPB was found to have the least negative effects on the reaction and was therefore shown to be a viable alternative to the use of solubility enhancing organic solvents.

The third goal of this project was to investigate the possibility of scaling the reaction up to a bench scale bioreactor, thereby not only investigating the factors that influence such a scale-up, but also to investigate the economic viability of using whole cells in a bioreactor to catalyse the enantioselective hydrolysis of a terminal epoxide. Initial reaction rate, enzymatic stability, reaction enantioselectivity and optimal cell/buffer ratios were all investigated and the optimal conditions reported. Recycling the whole yeast cells by means of micro-filtration was found to be ineffective.

Finally, as downstream processing of the products of a bioreaction contribute significantly to the costs involved, the use of a selective liquid-liquid extraction step was investigated, not only to separate the products from the reaction media, but also to simultaneously separate the residual epoxide and produced diol from one another. It was established that solvents with low log P values were best suited for the simultaneous extraction of both the epoxide and diol from the reaction medium. Solvents with high log P values, however, were useful for the selective extraction of the residual epoxide from the reaction medium.

Keywords: Epoxide hydrolase, enantioselective resolution, optimisation, co-solvents, temperature, bioreactor, liquid-liquid extraction, terminal epoxides, micro-filtration, whole yeast cells, cyclodextrins.

Opsomming

Stereochemie, wat as die studie van die ruimtelike gedrag van atome in molekules en komplekse gedefineer word, het toenemend belangrik vir die farmaseutiese sektor geraak. In besonder het farmakologiese effekte van farmaseutiese enantiomere tot betekenisvolle navorsing gelei. Die doel hiermee is nie slegs om die biologiese effekte van enantiosuiwer produkte te bestudeer nie, maar ook om metodes te vestig om chirale molekules in hul enantiosuiwer vorm te bekom.

Een van hierdie metodes, wat enantioselektiewe hidrolitiese ensieme gebruik, het heelwat aandag ontvang. Deur die katalitiese potensiaal te gebruik van epoksiedhidrolase, 'n ensiem wat in 'n verskeidenheid lewende organismes voorkom, kan hoogs reaktiewe rasemiese epoksiede en hul ooreenstemmende *visinale* diole in enantiosuiwer vorm bekom word. Hierdie twee enantiosuiwer produkte kan self biologies aktief wees of as voorgangers gedurende die sintese van waardevolle enantiosuiwer farmasetiese produkte dien.

Die projek wat in hierdie tesis beskryf word, het vier hoofdoelwitte. Eerstens, om die voorheen bepaalde enantioselektiwiteit van heel selle van *Rhodotorula glutinis* teenoor terminale epoksiede te optimiseer deur van stireenoksied as modelsubstraat gebruik te maak. Suksesvolle bereiking van hierdie doelwit het getoon dat 'n pH van 7.2, 'n optimale temperatuur van 15 °C en 'n aanvanklike substraatkonsentrasie van 50 mM die mees belowende resultate lewer. Verdere studies het ook getoon dat hierdie reaksie in 'n soutvrye omgewing kan plaasvind wat die kostes van die reaksie verminder en dit meer omgewingsvriendelik maak.

Een van die grootste uitdagings gedurende die hidrolise van epoksiede is die feit dat hierdie substrate redelik onoplosbaar in water is. Die ensiem, aan die ander kant, is baie meer aktief in 'n waterige omgewing. Om hierdie rede was die tweede doelwit om eerstens die effekte van twee algemene wateroplosbare organiese oplosmiddels (DMSO en DMF) te ondersoek en om tweedens die moontlike gebruik van siklodekstriene vir verbetering van oplosbaarheid te ondersoek. Dit is gevind dat hidroksipropiel-β-siklodekstrien (HPB) 'n heelwat hoër potensiaal as solibiliseerder as die twee organiese oplosmiddels het. Verder is gevind dat HPB die minste negatiewe effekte op die reaksie het en dus as 'n lewensvatbare alternatief tot organiese oplosmiddels vir verbetering van die substraat se oplosbaarheid gebruik kan word.

Die derde doelwit van hierdie projek was om die moontlikheid te ondersoek om die reaksie na 'n bankskaal bioreaktor op te skaal en hierdeur nie slegs die faktore wat so 'n opskaling beïnvloed nie, maar ook die ekonomiese lewensvatbaarheid van die gebruik van heel selle in 'n bioreaktor vir die enantioselektiewe hidrolise van 'n terminale epoksied te ondersoek. Aanvanklike reaksietempo, ensiemstabiliteit, die enantioselektiwiteit van die reaksie en die optimale sel/buffer-verhouding is ondersoek en die optimale kondisies gerapporteer. Hergebruik van die heel gisselle na mikrofiltrasie was onsuksesvol.

Aangesien stroomaf verwerking van die produkte van 'n bioreaksie heelwat tot die koste bydra, is die gebruik van vloeistof-vloeistofekstraksie laastens ondersoek. Die doel hiermee was nie slegs om die produkte uit die reaksiemedium te verwyder nie, maar ook om terselfdertyd die oorblywende epoksied van die geproduseerde diol te skei. Dit is gevind dat organiese oplosmiddels met lae log P-waardes die beste geskik is om die epoksied sowel as die diol gelyktydig uit die reaskiemengsel te verwyder. Oplosmiddels met hoë log P-waardes was die beste geskik om die epoksied selektief uit die reaksiemengsel te verwyder.

Sleutelwoorde: Epoksiedhidrolase, enantioselektiewe resolusie, optimisering, hulpoplosmiddels, temperatuur, bioreaktor, vloeistof-vloeistofekstraksie, terminale epoksiede, mikrofiltrasie, heel gisselle, siklodekstriene.

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CHAPTER 1

General Introduction

ABSTRACT

Chirality and enantiopure products have a significant influence on the pharmaceutical industry. In this Chapter the effects are discussed with specific reference to the pharmaceutical, pharmacological, economic and regulatory implications involved. The uses and sources of enantiopure epoxides and *vicinal* diols are summarized while the application of the epoxide hydrolase enzyme as a biocatalytic route to the aforementioned products is discussed in more detail. A summary of the *racemic* epoxides which have previously been successfully resolved using epoxide hydrolase from microbial origin is presented. As almost all bioprocesses need to be optimised to some extent after the initial screening, the approaches previously used to achieve such optimisation are discussed and specific examples summarised.

1. CHIRALITY AND ENANTIOPURE PRODUCTS IN THE PHARMACEUTICAL INDUSTRY

1.1 Definitions

Chirality is defined by Collet *et al.* [1] as the geometric property that is responsible for the non-identity of an object with its mirror image. A chiral object may exist in two enantiomorphic forms (enantiomers), which are mirror images of one another. Such forms lack inverse symmetry elements, that is, a centre, a plane and an improper axis of symmetry. Objects that possess one or more of these inverse symmetry elements are superimposable on their mirror images and are said to be achiral.

Compounds that exist in two forms that are non-superimposable mirror images show optical activity meaning that they rotate the plane of polarised light in opposite directions. This property is shown not only by an asymmetric carbon molecule (i.e. one with four different substituents as shown in Figure 1.1), but also by other atoms such as sulphur, phosphorus and some metal atoms. Compounds differing only in their capacity to rotate plane-polarised light in opposite directions are known as enantiomers [2] while a 50:50 mixture of the enantiomers of a specific compound is known as a racemate [3]. Such a mixture would not rotate plane-polarised light, it can therefore be said to be optically inactive.

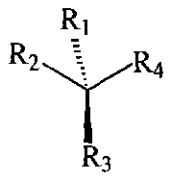

Figure 1.1 An asymmetric carbon atom where $R_1 \neq R_2 \neq R_3 \neq R_4$

Although more than one convention exists for naming the individual enantiomers, the Cahn-Ingold-Prelog system is currently recommended. In this method the ligands around the chiral centre are given a priority according to the IUPAC sequence rules. The molecule is then positioned with the ligand with the lowest priority away from the viewer. If the sequence of the remaining three ligands is arranged so that the highest to the lowest priority is in a clockwise manner, the molecule is assigned the R or *rectus*; the counter clockwise sequencing is given the S or *sinister* designation [3].

1.2 The Pharmaceutical and Pharmacological Implications of Chirality

The chiral nature of living systems has obvious implications on biologically active compounds interacting with them. On a molecular level, chirality is an inherent property of the "building blocks of life", such as amino acids and sugars, and therefore, of peptides, proteins and polysaccharides. As a result, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry and dissimilar activities can be observed when comparing the activities of two different enantiomers [4].

Drug action is the result of pharmacological and pharmacokinetic processes. Various examples exist where the enantiomers of drugs show differences in their bioavailability, distribution, and metabolism and excretion behaviour and where stereochemical parameters have a fundamental significance in their action. One example is that of the β-blocker propranolol, commonly used for the treatment of hypertension. The less active (R)-enantiomer is far more susceptible to first-pass metabolism than the 100 fold more active (S)-enantiomer [4] potentially allowing for a reduction in the total administered dose.

The Food and Drug Administration (FDA) has divided the enantiomers of chiral drugs into three distinct groups i.e. [5]:

- Both enantiomers have similar desirable effects that could be identical, or could differ in the magnitude of effects, e.g.
    - Both enantiomers of dobutamine are positive inotropes [6];
    - Both enantiomers of warfarin and phenprocoumon are anti-coagulants [7];
    - The enantiomers of bupivicaine both produce local anesthesia, and it is therefore desirable to have both enantiomers present [8].
- One enantiomer is pharmacologically active and the other is inactive, e.g.
    - The enantiomers of the quinolones and the β-lactam antibiotics are all antibacterial substances in which one enantiomer is pharmacologically active and the other is inactive [9].
    - The (S)-enantiomer of ibuprofen, an anti-inflammatory agent has been found to be active while the (R)-enantiomer is inactive. *In vivo*, an isomerase enzyme converts the (R)-enantiomer to the active (S)-form [10].
- Each enantiomer has a completely different activity, e.g.
    - (+)-sotalol is a type 3 antiarrhytmic while (-)-sotalol is a β-blocker [11].
    - (+)-ketamine is a hypnotic, while (-)-ketamine is responsible for undesired side-effects [12].

1.3 The Economic and Regulatory Implications of Chirality

Economic interests are obvious and essential driving forces in the development of new substances and technological improvements. A survey by Caner *et al.* [13] involving the worldwide and FDA approved pharmaceuticals during the period of 1983 to 2002 shows a rapid decrease in the amount of racemates marketed worldwide (from 37% down to 6% of the total amount of pharmaceuticals approved). During the same period an increase from 26% to 55% was shown for pharmaceuticals being marketed as single enantiomers. Interestingly their survey showed that an overwhelming majority of the pharmaceuticals marketed as single enantiomers contained multiple chiral centres (84%) compared to those containing only one chiral centre (16%). Today, chiral drugs continue to be a significant force in the global pharmaceutical market, unfortunately more recent statistics are not available.

Another consequence is that of the so-called chiral switch. Chiral switches are pharmaceuticals that were previously marketed as racemates or as mixtures of diastereomers but have since been developed as single enantiomers [14]. Even though the FDA does not consider a chiral switch to be a new chemical entity (as it has essentially been approved previously) [15], chiral switches allow companies to market their pharmaceuticals with lower total doses of the active ingredient, enhanced therapeutic windows, reduction of the variability between patients and more precise estimations of the dose-response relationships [13].

Recognition of potential pharmacological activity differences of pharmaceutical enantiomers has led to increased attention by regulatory authorities. In the United States, the FDA released a policy statement for the development of new stereoisomeric drugs [8] and during 1994 the Drugs Directorate of the Health Protection Branch (HPB) (in Canada) set out guidelines to sponsors of new drug submissions on specific areas to be addressed during the development of chiral drugs. The European Union (EU) Committee on Proprietary Medicinal Products (CPMP) released its final guidelines on the investigation of chiral substances in December 1993 [5]. In contrast to this, the South African Medicines Control council has set specific guidelines, but no official regulation has been endorsed as of yet [16].

2. ENANTIOPURE EPOXIDES AND DIOLS

2.1 Enantiopure epoxides

Epoxides (also referred to as oxiranes) are compounds that contain highly reactive cyclic ether groups consisting of a three-membered-ring which is approximately an equilateral triangle. Chemically, epoxides are formed by alkene peroxidation (also know as the Prilezhaev reaction) [3], by converting alkenes to halohydrins and then to epoxides [3] or by the Johnson-Corey-Chaykovsky reaction (the conversion of a carbonyl to an epoxide by a method of methylene transfer) [17]. *In vivo* epoxides are produced by cytochrome P450 oxidation reactions, for example the metabolism of xenobiotics such as aromatics and alkenes [59]. The structural strain that exists because of the equilateral ring, grants epoxides their unique chemical reactivity [3] which allows for the use of epoxides in many synthetic routes. For this reason they are widely accepted as invaluable synthons and precursors during organic synthesis. Figure 1.2 illustrates some of the possibilities when reacting epoxides with nucleophiles, acids, bases and reducing and oxidising agents.

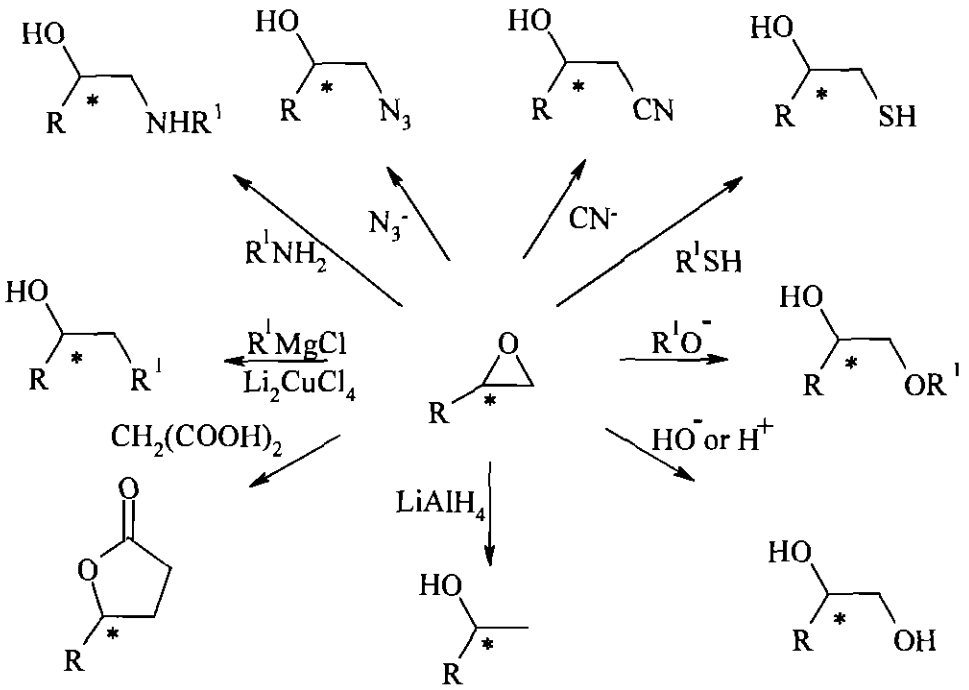

Figure 1.2 Reaction of epoxides with nucleophiles, acids, bases and reducing and oxidising agents [18] (Chiral centres are denoted by *).

As shown in Figure 1.2 epoxides may also contain a chiral centre and, should this be the case, would exist in two enantiomeric forms. An enantiopure epoxide may in turn impart its chirality or stereochemical properties to a product, yielding for example single enantiomer or

enantiopure pharmaceuticals. Some examples of the classes of enantiopure pharmaceuticals that have previously been synthesized using enantiopure epoxides are shown in Table 1.1, illustrating their importance and usefulness to the pharmaceutical industry.

Table 1.1 Examples of single enantiomer pharmaceuticals that have previously been synthesized using enantiopure epoxides or *vicinal* diols as precursors

Pharmaceutical class	**Examples**	**Indication**	**Ref.**
β-blockers	(S)-Propranolol	Cardiac arrythmias,	[19]
	(S)-Atenolol	cardioprotection after	[20]
	(R)-Nifénalol	myocardial infarction and	[21]
	(S)-Timolol	hypertension	[22]
Antimicrobials	Chloramphenmicol	Bacterial infection	[23]
	(-)-*cis*-Fosfomycin		[24]
	α-(-)-bisabolol (or Levomenol)		[25]
Chemotherapeutics	(2R,3S)-Paclitaxel	Cancers of the ovaries, breasts, lungs, neck and head amongst others	[26]
Anti-retrovirals	Indinavir	HIV infection	[27]
	Saquinavir	(Human immunodeficiency	[28]
	Ritonavir	virus)	[29]
Anti-depressants	(S)-Fluoxetine	Clinical depression, obsessive-compulsive disorder and bulimia nervosa amongst others.	[30]
Calcium channel blockers	(2R,3S)-Diltiazem	Hypertension, angina pectoris and arrhythmia	[31]

2.2 Sources of enantiopure epoxides

The various chemical and biological routes that can be utilised to access enantiopure epoxides have been well documented (other than obtaining them directly from the chiral pool). A short summary of these is shown in Tables 1.2 and 1.3 for chemical and biological routes respectively, with specific reference to the substrates and catalysts used during the syntheses.

Table 1.2 Chemical routes to enantiopure epoxides

Reaction	Substrates	Catalysts	Ref.
Asymmetric epoxidation	Olefins, sulfides, Unsaturated α,β-ketones Allylic alcohols	Chiral organic peracids, oxaziridines, borates, poly-L-Leucine, transistion metals with chiral ligands and chiral metalloporphyrins	[31], [32], [33], [34], [35], [36], [37], [38], [39], [40], [41], [42]
Epoxidation of enantiopure precursors	Halohydrins such as (S)-2-chloroalkanoic acids	Lithium aluminium hydride and potassium hydroxide	[43]

Table 1.3 Biological routes to enantiopure epoxides

Reaction	Substrates	Catalysts	Ref.
Epoxidation	Olefins	Mono-oxygenases	[20], [44], [45], [46], [47], [48]
Kinetic resolution of racemic epoxides	Functionalised and unfunctionalised epoxides	Lipases, Epoxide Hydrolases	See Table 1.6

2.3 Enantiopure *vicinal* diols

Vicinal diols, also sometimes referred to as *vicinal* glycols, are chemical compounds containing two adjacent (or *vicinal*) hydroxyl groups [3]. Chemically, *vicinal* diols may be formed by the hydroxylation of alkenes or the acid catalysed hydration of epoxides [3]. As with epoxides, enantiopure *vicinal* diols may also impart their chirality to a product, making them useful as pharmaceutical precursors. In addition, epoxides and diols can be stereospecifically interconverted and can therefore be seen as being synthetic equivalents [49]. Table 1.1 gives some examples of enantiopure pharmaceuticals that have been produced using enantiopure *vicinal* diols as precursors.

Enantiopure diols may also be biologically active themselves rather than being used as precursors during synthesis. Examples of biologically active 1,2 diols include (S)-

Guaifenesin, an expectorant used to provide relief during acute respiratory tract infections, and the centrally acting muscle relaxants (S)-Mephenesin and (S)-Chlorphenesin [50].

2.4 Sources of enantiopure *vicinal* diols

As with epoxides various methods have been developed to produce enantiopure *vicinal* diols as illustrated by the examples in Table 1.4 and Table 1.5 for chemical and biological routes respectively.

Table 1.4 Chemical routes to enantiopure *vicinal* diols

Reaction	Substrates	Catalysts	Ref.
Dihydroxylation	Olefins	Osmium and Sharpless chiral ligands	[51], [52]
Kinetic resolution	Terminal epoxides	Jacobsen-metalloporphyrins	[53]
Asymmetric ring opening of epoxides	*Meso*-epoxides	Jacobsen-metalloporphyrins, anilines	[54], [55]

Table 1.5 Biological routes to enantiopure *vicinal* diols

Reaction	Substrates	Catalysts	Ref.
Dihydroxylation	Olefins	Dioxygenase	[56], [57]
Kinetic resolution	Esters, epoxides	Lipase, epoxide hydrolase	[58]

3. EPOXIDE HYDROLASES

3.1 Background

Epoxide hydrolases (EC 3.3.2.3) are versatile catalysts that catalyse the hydrolytic kinetic resolution (HKR) of various different epoxides yielding their corresponding *vicinal* diols (glycols) as products. *In vivo,* this process detoxifies the otherwise harmful and chemically reactive epoxides (Figure 1.3) by hydrolysing the reactive epoxides to form inactive, water-soluble transdihydrodiol metabolites [59,60].

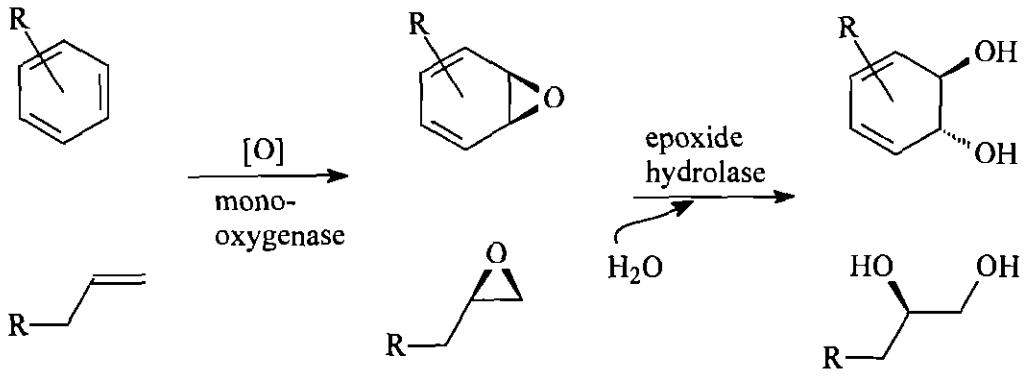

Figure 1.3 Metabolism of aromatics and alkenes mainly found in eukaryotic cells.

Enzymatic catalysis often proceeds stereoselectively, and in these cases, it is possible to use the enzyme *in vitro* to catalyse the enantioselective HKR of epoxides, yielding an enantiopure epoxide and its *vicinal* diol as products. These can in turn be used for various synthetic applications both in the fine chemicals as well as the pharmaceutical industry. For this reason a multitude of authors have committed their research to finding new sources of this enzyme, elucidating the mechanistic action and optimising these reactions in order to yield the most commercially viable reactions possible.

3.2 Mechanistic Aspects

EHs belong to the superfamily of α/β-hydrolase fold enzymes. This family is characterised by a catalytic triad consisting of a catalytic nucleophile and a charge relay system, formed by a histidine residue and an acidic residue [61]. The crystal structure of Aspergillus niger EH [62] (Figure 1.4), expressed in Escherichia coli, is available online at http://www.biochem.ucl.ac.uk/bsm/enzymes.

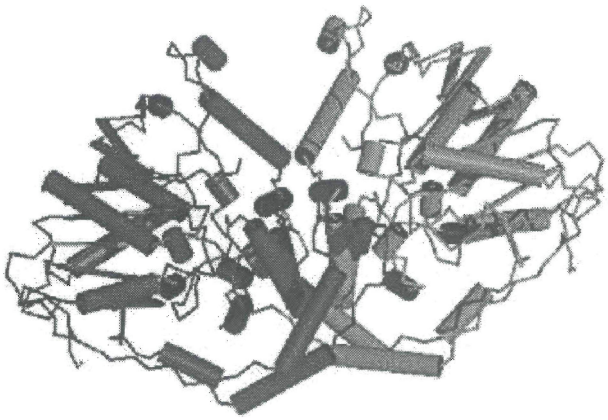

Figure 1.4 X-ray crystallography structure of *Aspergillus niger* EH showing the α and β chains

The mechanism of this enzyme involves a carboxylate residue, aspartate, which performs a nucleophilic attack on either of the carbon atoms in the epoxide ring. This leads to the formation of a glycol-monoester intermediate. Simultaneously a proton from an adjacent tyrosine residue is transferred. In a second step; the ester bond is hydrolysed by a hydroxyl ion which is provided by water (with the aid of histidine), leading to the liberation of the *vicinal*-diol (Figure 1.5).

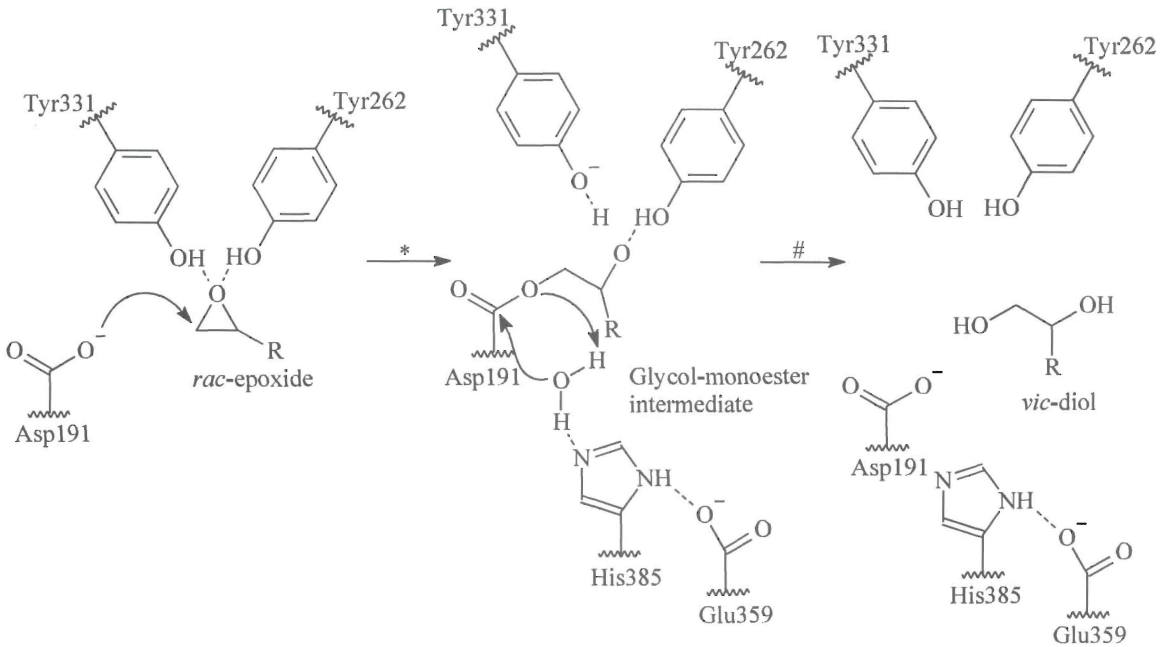

Figure 1.5 Proposed mechanism for the hydrolysis of a monosubstituted terminal epoxide by EH from *Rhodotorula glutinis* (*: Nucleophilic attack, #: Hydrolysis, Tyr: Tyrosine, Asp: Aspartate, His: Histidine, Glu: Glutamine). Adapted from [60,61,63].

3.3 Enantioselective Hydrolysis of Epoxides

In contrast to the majority of kinetic resolutions, where the absolute configuration of the stereogenic centres are not involved in the reaction, the HKR of epoxides may take place via four different pathways (Figure 1.6).

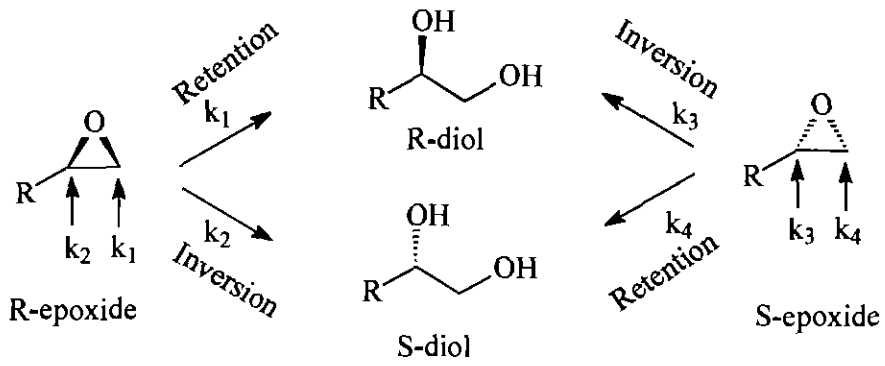

Figure 1.6 Enzymatic hydrolysis of epoxides proceeding with retention or inversion of configuration [60].

Nucleophilic attack of the hydroxide ion on the less-hindered primary carbon of the R epoxide (k_1) leads to the formation of the R-diol and therefore the reaction takes place with retention of the original configuration. Attack at the stereogenic centre (k_2) leads to the formation of the S-diol and the configuration is therefore inverted. Similarly attack of the S-epoxide at the primary carbon leads to retention (k_4) and attack at the stereogenic centre (k_3) leads to inversion [64].

When evaluating biocatalytic processes there are two important factors to consider namely the regioselectivity and the enantiomeric ratio (E). The regioselectivity is defined as being the ratio of retention versus inversion (k_1/k_2 and k_4/k_3), while E is expressed as the ratio of the reaction rate of the enantiomers (k_1+k_2/k_3+k_4). Instead of determining the rate constants (k_1-k_4), the initial reaction rates of the enantiomers can be mathematically linked to the conversion (c) of the reaction and the percentage enantiomeric excesses (%e.e.) or optical purities of both the substrate (%e.e.$_S$) and the product (%e.e.$_P$) [60].

To determine these values a few simple equations are used:

$$\%e.e. = \left(\frac{[R]-[S]}{[R]+[S]}\right)\times 100 \tag{1.1}$$

where [R] and [S] are the respective concentrations (mol/dm^3) of the two enantiomers and %*e.e.* is the enantiomeric excess expressed in terms of %.

$$c = \left(\frac{S_0 - S}{S_0}\right)\times 100 \tag{1.2}$$

Were c is the conversion of the substrate (%) and S_0 and S are the initial substrate concentration and the substrate concentration after hydrolysis respectively (mol/dm^3). Values from the aforementioned equations can then be used to determine the enantiomeric ratio of a reaction. The dependence of the selectivity and the conversion of the reaction for the substrate is:

$$E = \frac{\ln[(1-c)(1-e.e._S)]}{\ln[(1-c)(1+e.e._S)]} \tag{1.3}$$

Where E is the enantiomeric ratio of the reaction, c is the conversion (%) of the substrate and $e.e._S$ is the enantiomeric excess of the substrate.

And for the product:

$$E = \frac{\ln[1-c(1+e.e._P)]}{\ln[1-c(1-e.e._P)]} \tag{1.4}$$

Where E is the enantiomeric ration of the reaction, c is the conversion (%) of the substrate and $e.e._P$ is the enantiomeric excess of the product.

These two equations however do not yield reliable results at very low or very high conversions. In these cases the following equation can be used which only uses the relative values of enantiomeric excess in contrast to the absolute value of conversion [60].

$$E = \frac{\ln\left[\frac{e.e._P(1-e.e._S)}{(e.e._P+e.e._S)}\right]}{\ln\left[\frac{e.e._P(1+e.e._S)}{(e.e._P+e.e._S)}\right]} \qquad (1.5)$$

These calculated E values are however only valid if the regioselectivity of the enzyme is absolute; in other words, if attack only takes place at one of the two carbon atoms and mixed pathways do not occur. Since verification of this requires the use of sophisticated techniques, it is very common that authors only report %*e.e.* and *c* values [64].

3.4 Structural classes of epoxides and their hydrolysis

Various authors have reported the successful enantioselective hydrolysis of epoxides with EH originating from mammals, micro-organisms, such as bacteria, fungi and yeasts, insects and plants. These epoxides can be divided into four different structural classes (Types 1-4, Figure 1.7) [64].

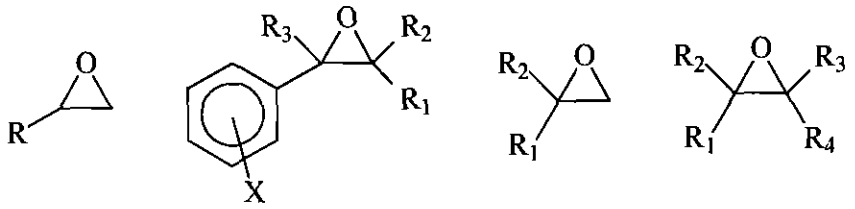

Figure 1.7 Four structural classes: monosubstituted epoxides (Type 1), styrene oxide-type epoxides (Type 2), 2,2-disubstituted epoxides (Type 3), 2,3-disubstituted and trisubstituted epoxides (Type 4)

Table 1.6 provides a summary of the epoxides successfully hydrolysed by microbial EH over the past few decades, illustrating the vast amount of substrates as well as the enantiopreference of the enzymes and its microbial sources.

Table 1.6 Epoxides successfully hydrolysed by microbial EH (BEH: Bacterial epoxide hydrolase, FEH: Fungal epoxide hydrolase, YEH: Yeast epoxide hydrolase).

Enzymatic hydrolysis of monosubstituted epoxides (type 1)						
R				*Enantio-preference*	*Enzyme Source*	*References*
n-C_3H_7; *n*-C_4H_9; *t*-C_4H_9; *n*-C_5H_{11}; *n*-C_6H_{13}; *n*-C_8H_{17}; *n*-$C_{10}H_{21}$				R	BEH	[65,66,67,68]
CH_2Cl; $C(CH_3)_2O(CO)C(CH_3)_3$; CH_2OPh				S	BEH	[69,70,71]
n-C_4H_9; *n*-C_6H_{13}; *t*-C_4H_9; 2-, 3- or 4-pyridyl; $CH_2CH(OEt)_2$; CH_2Cl; CH_2OH; CH_2OPh; CH_2O(*o*-, *m*-, *p*-CH_3Ph); CH_2OCH_2Ph; CH_2O-3-naphthyl				R	FEH	[66,68,72,73, 74,75,76,77, 78,79]
5,5-Dimethyl-1,3-diox-2-yl; CH_2O(*p*-NO_2Ph)				S	FEH	[80,81,82]
$CH{=}CH_2$; $(CH_2)_2{-}CH{=}CH_2$; $(CH_2)_4{-}CH{=}CH_2$; CH_2OCH_2Ph; CH_3; C_2H_5; *n*-C_3H_7; *n*-C_4H_9; *n*-C_5H_{11}; *n*-C_6H_{13}; *n*-C_8H_{17}; *n*-$C_{10}H_{21}$				R	YEH	[68,83,84,85 86]
CH_2OH; CH_2Cl; CH_2OCH_2Ph				S	YEH	[86]
Enzymatic hydrolysis of styrene oxide-type epoxides (type 2)						
R1	R2	R3	X			
H	H	H	*p*-CH_3, *o*-Cl, *m*-Cl	R	BEH	[71]
H	H	H	H	S	BEH	[87]
H	H	CH_3	H	R	BEH	[70]
H	H	H	H	S or R	FEH	[68,73,79,88]
H	H	CH_3	H	S or R	FEH	[73,88,89]
Indene oxide, dihydronaphthalene oxide				2S	FEH	[90,88,89]
H	CH_3	H	H	2R/2S	FEH	[73,88,89]
H	H	CH_3	*p*-H, *p*-F, *p*-Cl, *p*-Br, *p*-iBu, *p*-CN, *p*-CH_3, *o*-CH_3, *m*-CH3, *o*-Cl, *m*-Cl	S or R	FEH	[91,92,93]
CH_3	H	H	H	R or S	FEH	[73,88,89]
H	H	H	*p*-F, *p*-Cl, *p*-Br, *p*-CH_3, *p*-NO_2, *p*-CN, *m*-NO_2, *o*-NO_2	S/nd	FEH, YEH	[91,93,94,95, 96]

R1	R2	R3	X	*Enantio-preference*	*Enzyme Source*	*References*
H	H	H	*o*-CH_3, *o*-Cl	nd	YEH	[91]
H	CH_3	H	H	2S	YEH	[86,97]
H	H	H	H	R	BEH, YEH	[98 ,68,71,86, 91,97]
H	H	CH_2Cl	*2-,4*-F	R	FEH	[99]
Indene oxide, 1-(2,3-dihydrobenzol[b]furyl-4-yl)-1,2-oxirane				R	BEH, YEH, FEH	[100]
CH_3, CH_2CH_3, $CH_2CH_2CH_3$, $CH_2(CH_2)_2CH_3$, $CH_2(CH_2)_4CH_3$	H	H	H	1R,2S	mEH	[101]

Enzymatic hydrolysis of 2,2-disubstituted epoxides (type 3)

R_1	R_2			
CH_3	*n*-C_6H_{13}	nd	BEH	[65]
C_2H_5	*n*-C_5H_3	S or R	BEH	[67,69]
CH_3	$(CH_2)_2Ph$	S	BEH	[69]
CH_3	CH_2Ph	S	BEH	[69]
CH_3	*n*-C_4H_9, *n*-C_5H_{11}, *n*-C_7H_{15}, *n*-C_9H_{19}	S or R	BEH	[67,69]
CH_3	$(CH_2)_4$-Br	S	BEH	[69]
CH_3	$(CH_2)_mC\equiv C(CH_2)_nCH_3$, $(CH_2)_mCH=CH(CH_2)_nCH_3$, (m = 1, 2, 5, m + n = 5)	R or S	BEH	[102]
CH_3	$(CH_2)_3CH=CH_2$	S	BEH	[69]
CH_3	$(CH_2)_nOSiR_3$, $(CH_2)_nOCH_2CH=CH_2$, $(CH_2)_nCH_2CH_2CH_3$, $(CH_2)_nOCH_2Ph$ (n= 1, 2), $(CH_2)_2O(CH_2)_2OCH_3$, CH_2CN, $CH_2CH(OEt)_2$, CH_2N_3	R or S	BEH	[103]
CH_3	*n*-C_5H_{11}	R or S	FEH	[72]
CH_3	*n*-C_5H_{11}	R	YEH	[83]
CH_3	*n*-C_3H_7, *n*-C_5H_{11}	R or S	YEH	[73]

Enzymatic hydrolysis of 2,3-disubstituted epoxides (type 4)

R_1	R_2	R_3	R_4	*Enantio-preference*	*Enzyme Source*	*References*
H	*n*-C_4H_9, *n*-C_8H_{17}	H	CH_3, C_2H_5, n-C_8H_{17}, n-C10H21	2S	mEH	[104,105]
H	*n*-C_4H_9	H	$(CH_2)_{10}OH$	2S	mEH	[104]
H	*n*-C_8H_{17}	H	$(CH_2)_7CO_2H$	2S	mEH	[104]
H	$(CH_2)_2OH$, $(CH_2)_2OCH_3$	H	*n*-C_5H_{11}	2S	mEH	[105]
H	CH_3, C_2H_5, *n*-C_3H_7	H	*n*-C_4H_9, *n*-C_5H_{11}	R or S	mEH/ sEH	[105,106]
H	CH_2CH_2OH, $CH_2CH_2OCH_3$	H	*n*-C_5H_{11}	R or S	sEH	[106]
CH_3	H	H	*n*-C_4H_9, *n*-C_5H_{11}, *n*-C_6H_{13}	1S	BEH	[69]
H	C_2H_5	H	n-C_4H_9	2S	BEH	[69]
C_2H_5	H	H	n-C_3H_7	1S	BEH	[69]
H	CH_3	H	n-C_4H_9	2S	BEH	[107]
H	CH_2Cl, CH_2CH_2Cl	*n*-C_4H_9	H	R,R	BEH	[108]
H	CH_3	H	n-C_3H_7, n-C_5H_{11}	R or S	FEH	[72,73]
CH_3	H	H	n-C_3H_7, n-C_5H_{11}	2R/2S	FEH	[72,73]
H	CH_3, C_2H_5	CH_3	H	S	YEH	[86]
H	CH_3, C_2H_5	H	CH_3	S	YEH	[86]
Cyclohexene oxide, cyclopentene oxide				nd	FEH, YEH	[68,86,97]

Enzymatic hydrolysis of trisubstituted epoxides (type 4)						
R_1	R_2	R_3	R_4	*Enantio-preference*	*Enzyme Source*	*References*
H	$(CH_2)_2C(OAc)(CH_3)CH=CH_2$	CH_3	CH_3	1S	BEH	[109]
$n\text{-}C_4H_9$, $n\text{-}C_5H_{11}$, $n\text{-}C_6H_{13}$	H	CH_3	CH_3	S or R	BEH	[110]
H	Ph	CH_3	CH_3	nd	FEH	[88]
H	$(CH_2)_2C(CH_3)CHCH_2Cl$	CH3	CH3	nd	BEH	[111]
Epoxyaurapten				nd	BEH	[111]
1,2-Limonene oxide				S	BEH, YEH	[86,112]
1-Methyl-cyclohexene oxide				2S	BEH	[113,114]

4. THE OPTIMISATION AND UPSCALING OF BIOCATALYTIC REACTIONS

During the last decade a multitude of biocatalyst have been discovered that catalyse the synthesis of economically important fine chemicals. Almost all bioprocesses, however, need to be optimised to some extent after the initial screening in order to yield maximum productivity, stability and selectivity with minimal waste. Various strategies have been employed to reach these and other goals including substrate induction [115], optimisation of growth medium composition [115], addition of co-solvents [116,117] and variation in the reaction conditions such as pH and temperature [118,119].

Previously the enantioselective biocatalysis of styrene oxide and three of its nitro derivatives was established [96]. These reactions, however, presented a multitude of problems. Although a 98 % pure epoxide could be obtained in most cases after relatively short reaction times, selectivity was not absolute. Chemical hydrolysis of styrene oxide decreased the yield of pure product that could be obtained by competing with the enzymatic hydrolysis. Even though it was shown that the substrates did not have to be in solution for catalysed hydrolysis to take place, the insolubility of the substrates in aqueous buffer was thought to limit the productivity of the reaction, as well as the potential of the reaction to be scaled up to a reactor other than a large stirred batch reactor. For these reasons the reactions were optimised considering various factors namely pH, temperature, initial substrate concentration and

whether a cell free extract would have advantages above whole cells [120]. It was found that temperatures below 15 °C significantly increased both the selectivity of the reaction as well as the stability of the enzyme even though activity was decreased. Initial substrate concentrations between 50 mM and 70 mM were found to yield the highest initial reaction rates, even though these values far exceed the maximum solubility of the substrates in aqueous buffer.

4.1 Substrate modification and substrate concentration

The ability of an enzyme to recognize the chirality of a substrate depends mainly upon the substrates steric shape. Consequently, through variation in the substrate structure, the selectivity of the enzyme can be increased by creating a better fit with the enzyme. The easiest method of achieving this is by either adding or removing protective groups of different sizes and/or polarities [60]. The electronic effects of the substrate also play a role. For example, in the case of EHs, the enzymatic mechanism involves a nucleophilic attack at one of two carbon atoms (Figure 1.8). The electronic effects of a group substituted to the epoxide ring will influence the preferential site of attack and therefore may promote or antagonise the enzymatic reaction.

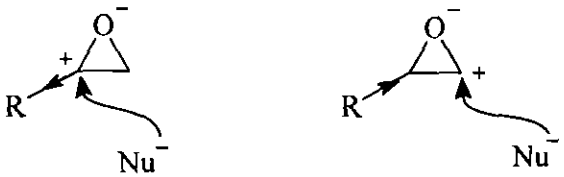

Figure 1.8 Nucleophilic attack of an epoxide ring at the stereochemical centre (A) and at the primary carbon atom (B). Attack is promoted at each point if R = electron withdrawing (A) and R = electron donating (B) respectively.

The initial concentration of substrate can play a major role as well. Both substrate and product inhibition have previously been observed at certain concentrations. Obviously increasing the initial substrate concentration will lead to a larger amount of product formed during the reaction, possibly leading to product inhibition. This problem is usually remedied by constantly removing the product from the reaction mixture. Another possible outcome of increasing the initial substrate concentration is an increase in reaction rate as well as selectivity [120]. This phenomenon remains relatively unexplained in most cases since the reaction rates continue to increase far above the solubility of the generally insoluble epoxides,

and are therefore performed in biphasic systems. It has been proposed that this is sometimes due to an activation of the enzyme in the presence of the product [125].

4.2 Temperature variation

Generally it is believed that enzymes, as with other catalysts, exhibit their highest selectivity at low temperatures [60]. It has however been proposed that the selectivity of an enzyme is dependant on the so-called 'racemic temperature' (T_{rac}) [121]. From the Gibb's-equation it can be seen that only the entropy-term ($\Delta\Delta S^{\neq}$), and not the enthalpy ($\Delta\Delta H^{\neq}$), is influenced by the temperature.

$$\Delta\Delta G^{\neq} = \Delta\Delta H^{\neq} - T \bullet \Delta\Delta S^{\neq} \qquad (1.6)$$

Where G is the Gibbs energy term, H is the enthalpy-term (joule), T is the temperature (kelvin) and S is the entropy-term (joule per kelvin).

$$\text{If } \Delta\Delta G^{\neq} = 0 \text{ then } T = T_{rac} = \frac{\Delta\Delta H^{\neq}}{\Delta\Delta S^{\neq}} \qquad (1.7)$$

Where T_{rac} is the *racemic* temperature of the reaction (kelvin).

The selectivity of an enzymatic reaction therefore depends on the temperature as follows:

- At temperatures less than T_{rac} the contribution of entropy becomes very small and the stereochemical outcome of the reaction is dependant upon the activation enthalpy difference ($\Delta\Delta H^{\neq}$). As a consequence the optical purity of the product will decrease with increasing temperature.
- At temperatures greater than T_{rac} the reaction is controlled mainly by the activation entropy difference ($\Delta\Delta S^{\neq}$), therefore the optical purity of the product will increase with increasing temperature.

4.3 Variation of pH

Reactions catalysed by hydrolases are usually preformed in aqueous buffer systems with pH values considered as being the optimal for that specific enzyme. Due to the fact that the conformation of an enzyme is dependant upon its ionisation state, it is reasonable to assume that the pH of the buffered solution and the type of buffer may influence the selectivity of the

enzyme [60]. Hydrolytic enzymes have however been shown to be active in a broad pH-activity range and variations are therefore acceptable in cases where a reasonable activity can be maintained.

4.4 Immobilisation

Immobilisation of an enzyme or enzyme containing microbial cell has commonly been attempted in order to increase the stability of the enzyme. This can be accomplished either by covalent bonding, for example the immobilisation of peroxidase onto silica microparticles [122], by entrapment of the cells/enzymes, for example the immobilisation of whole cells of *Rhodosporidium toruloides* within calcium alginate beads [123], or by cross-linking the cells/enzymes through the formation of a linkage between the individual enzymes/cells [124]. Even though immobilisation has been employed very successfully in the past with EH enzymes or whole cells containing EH, the benefits achieved should always be weighed against the additional costs and labour necessary to implement the investigated technology.

4.5 Medium engineering

4.5.1 Salt free processes

The use of plain water instead of an aqueous buffer for HKR is a relatively unexplored field since it is commonly accepted that any major changes in pH would adversely affect the reaction. Although excluding the buffer probably would not increase the selectivity nor conversion rate of the enzyme, it does have the advantage of simplifying the process and therefore, lowering the production cost. Monfort *et al.* [125] previously showed that there was no significant difference during the hydrolyses of 1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane in plain water as opposed to 100 mM phosphate buffer with *Aspergillus niger* EH.

4.5.2 Water miscible organic solvents

The effects of several water miscible organic solvents have previously been investigated with the aim of increasing the solubility of hydrophobic substrates for kinetic resolution. These include dimethyl sulfoxide (DMSO), dimethylformamide (DMF), 1,4-dioxane, acetonitrile (MeCN), ethanol (EtOH) and tetrahydrofuran (THF). After several investigations DMSO and DMF have generally been found to be the most biocompatible. Table 1.7 provides a summary of some of these reactions.

Table 1.7 Summary of reactions where substrate solubility was increased through the use of water miscible organic solvents.

Substrate	Enzyme	Co-solvents tested	References
2-methoxyphenol	Peroxidase	DMSO	[122]
1,2-epoxyoctane	Epoxide hydrolase	EtOH, MeCN	[126]
N-benzoyl-L-arginine ethyl ester, N-acetyl-L-tyrosine ethyl ester, Hippuryl-L-phenylalanine, Olive oil	Pancreatic trypsin, Chymotrypsin, Carboxypeptidase A, Pancreatic lipase	EtOH, MeCN, 1,4-dioxane, DMSO	[127]
1-(2,3-dihydrobenzo[b]furan-4-yl)-1,2-oxirane	Epoxide hyrdolase	DMSO, DMF	[100]
para-Nitrostyrene oxide	Epoxide hydrolase	DMSO, DMF, Acetone, MeCN MeOH, EtOH, 2-propanol	[21], [128],[129]
1,2-epoxyhexane	Epoxide hydrolase		[130]

Some of these co-solvents have shown stability enhancing effects [122], but in most cases lead to decreases in both activity and stability [128], especially at high concentrations. It is believed that they strip water from the enzymes, leading to the unfolding of the enzyme with exposure of the inner hydrophobic residues. They may also alter the protein structure by direct interactions with protein solvation sites, either by hydrophobic or hydrogen bonding [117].

4.5.3 Water immiscible organic solvents

The use of water immiscible solvents, also referred to as non-conventional aqueous biocatalysis [131], results in a biphasic system (consisting of an apolar organic phase and an aqueous phase). In this case the stability of the enzyme in the organic phase is the main challenge [131]. Table 1.8 gives some examples of the reactions previously investigated.

Table 1.8 Summary of reactions where substrate solubility was increased through the use of water immiscible organic solvents.

Substrate	Enzyme	Co-solvents tested	Ref.
N-benzoyl-L-arginine ethyl ester, N-acetyl-L-tyrosine ethyl ester, Hippuryl-L-phenylalanine, Olive oil	Pancreatic trypsin, Chymotrypsin, Carboxypeptidase A, Pancreatic lipase	EtOAc, toluene	[127]
1-(2,3-dihydrobenzo[b]furan-4-yl)-1,2-oxirane	Epoxide hydrolase	Cyclohexane, Toluene, 1,1,2-Trichloro-trifluoroethane, MTBE, Methyl isobutyl ketone, n-Butanol	[100]
1,2-epoxyhexane	Epoxide hydrolase	Dodecane, decane, hexane, Octane, Cyclohexane, dichloromethane, diethylether, ethylacetate	[130]

5. CONCLUSION

Obtaining biologically active compounds in their enantiopure form is of great importance to modern society, a statement which is especially true for the pharmaceutical industry. Amongst the methods utilised to obtain enantiopure products, the kinetic resolution of racemates using enantioselective catalysts in the form of naturally occurring enzymes seems to have significant potential. This effective, economical and environmentally friendly method may be tailored and optimised to suit specific production needs and will undoubtedly play a significant future role in the industrial production of enantiopure products.

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CHAPTER 2

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.

1. INTRODUCTION

Terminal epoxides (for example styrene oxide) are important chiral synthons [1] and have enjoyed the attention of biotechnologists and biochemists worldwide [2,3,4]. 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 (*e.e.*) of >98% and 15% of the formed diol with an *e.e.* 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 *e.e.* of 98% and a 39% yield of the formed diol with an *e.e.* of 99% can be achieved after 40 hours. Even though the reaction time of the biocatalytic route is more appealing (3 hours) 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 *e.e.* 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 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

Rhodotorula 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 HP-5 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 t_R min and (R) 12.3 t_R min. 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 litre 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 hours) the cells were harvested at room temperature (24 °C) by centrifugation (3 500 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 (3 500 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 hours 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 (3 500 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. 10 mL of each suspension was transferred into 50 mL glass bottles and incubated at 15 °C for 5 minutes (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 (Figure 2.1). 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.

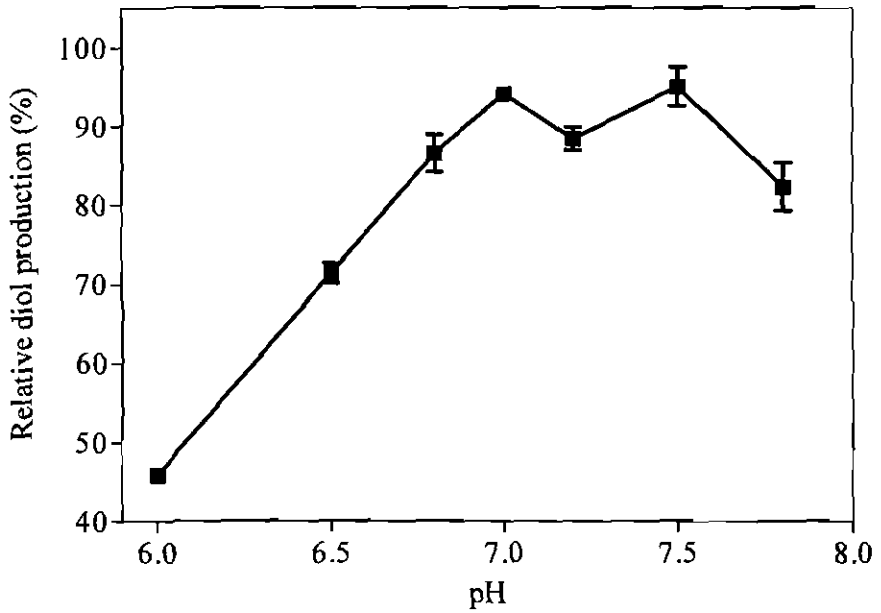

Figure 2.1 Formation of 1-phenyl-1,2-ethane diol after biohydrolysis (30 minutes, 30 °C) of SO with *R. glutinis* (▲) at various pH values. Relative diol production is defined as the concentration diol obtained (mM · min^{-1} · g^{-1}) relative to the maximum concentration obtained.

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 Figure 2.2. Again the whole cells demonstrated optimal activity over a broad range of temperatures (30 – 50 °C).

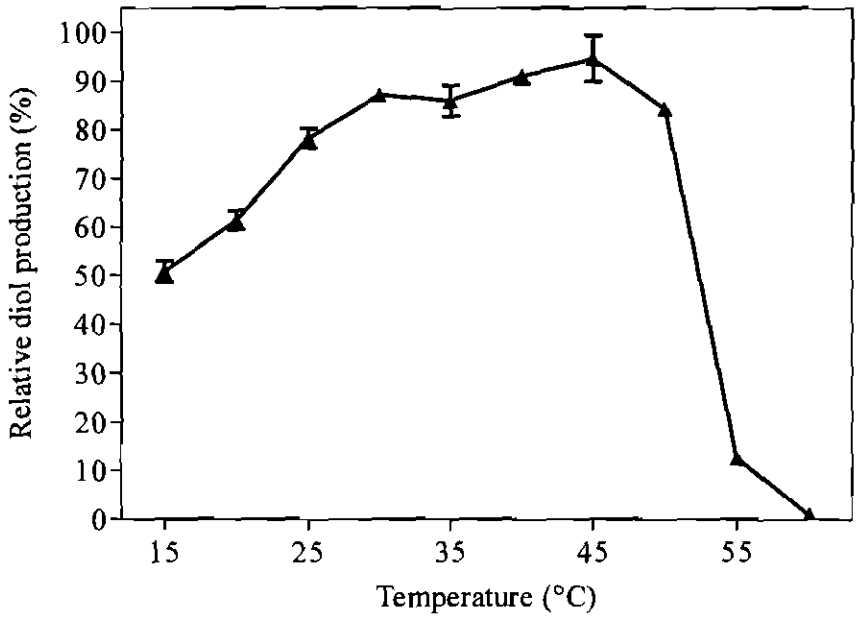

Figure 2.2 Formation of 1-phenyl-1,2-ethane diol after biohydrolysis of SO with *R. glutinis* at various temperatures. Relative diol production is defined as the product obtained ($mM \cdot min^{-1} \cdot g^{-1}$) relative to the maximum amount of product obtained.

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 *Aspergillus 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 hour of incubation. When investigating the selectivity, however, it became clear that increased temperatures have an adverse effect on the selectivity of the reaction (Figures 2.3 & 2.4).

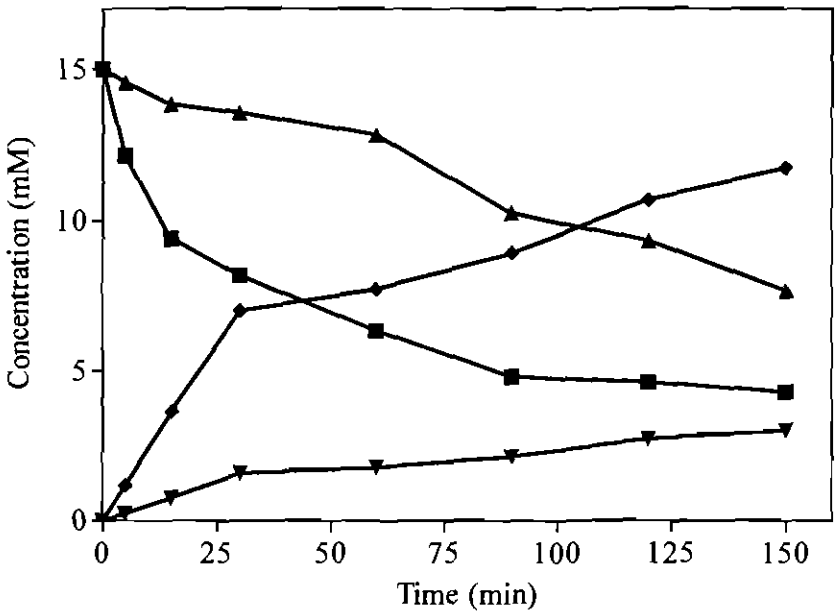

Figure 2.3 Hydrolysis of racemic SO (30 mM, pH 7.5) at 40 °C with *R. glutinis*. (▲-(S)-epoxide, ■-(R)-epoxide, ♦-(R)-diol, ▼-(S)-diol).

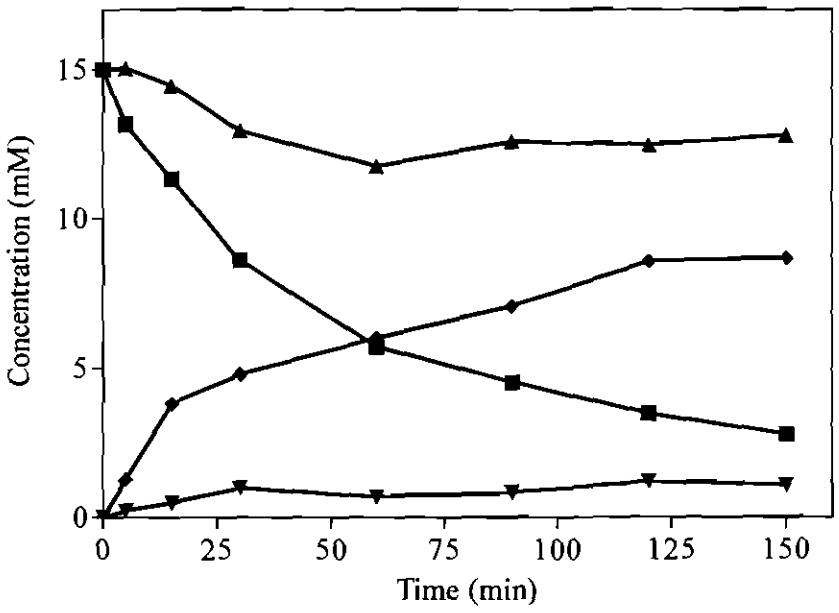

Figure 2.4 Hydrolysis of racemic SO (30 mM, pH 7.5) at 15 °C with *R. glutinis*. (▲-(S)-epoxide, ■-(R)-epoxide, ♦-(R)-diol, ▼-(S)-diol).

At 15 °C (Figure 2.4) a substrate enantiomeric excess ($e.e._s$) of 64% was reached after 150 minutes compared to 28% reached at 40 °C (Figure 2.3). 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.

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-hour reactions are given in Table 2.1. Enantiomeric ratios (E) were not calculated since the regioselectivity of this enzymatic reaction has not been determined.

Table 2.1 Hydrolysis of SO (30 mM) with *Rhodotorula glutinis* (UOFS Y-0653) at various reaction temperatures after 2 hours ($e.e._s$: % enantiomeric excess of the substrate, $e.e._p$: % enantiomeric excess of product, c: conversion of substrate, $yield_s$: % yield of (S)-SO, $yield_p$: % yield (R)-1-phenyl-1,2-ethane diol.)

Temperature (°C)	$e.e._s$ (%)	$e.e._p$ (%)	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

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, Figure 2.2), while the increase in selectivity led to higher substrate yields. Since $e.e._s$ 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 the experimental section that for each experiment chemical hydrolysis was determined and subtracted from the total hydrolysis to obtain the biocatalysed hydrolysis. The chemical hydrolysis (after 2 hours - 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 minutes). Previously epoxide hydrolase from *Aspergillus 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

$$\frac{d[S]}{dt} = -k[S][E] \qquad (2.1)$$

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]* >> *[S]* (500 µl cells vs. 20 mM SO). Therefore Equation 2.I can be rewritten as a first order equation.

$$\frac{d[S]}{dt} = -k'[S] \qquad (2.2)$$

Solving Equation 2.2 it follows that

$$\ln\frac{[S]}{[S_o]} = -k't \tag{2.3}$$

where $[S_o]$ is the initial substrate concentration (mol · dm^{-3}), $[S]$ the substrate concentration (mol · dm^{-3}) after time t (120 minutes) 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

$$\ln\frac{[E]}{[E_o]} = -k''t' \tag{2.4}$$

where $[E]$ is the remaining active enzyme concentration (i.e. enzyme activity) after heat treatment, $[E_o]$ 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 Equation 2.4 for each temperature of heat treatment. An example is shown in Figure 2.5 (20 °C).

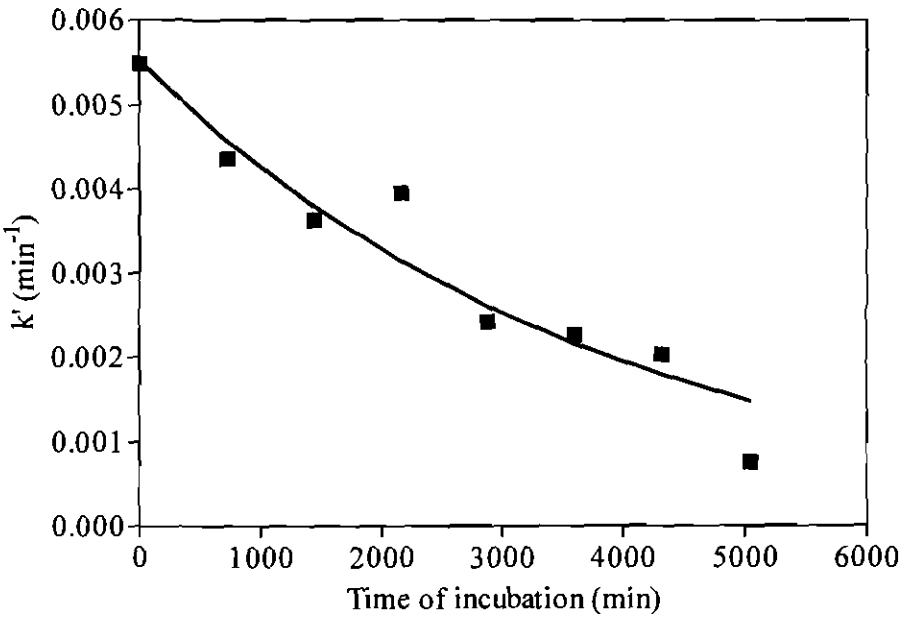

Figure 2.5 Residual enzymatic activity *(k[E])* after heat treatment at 20 °C.

$t_{1/2}$ (half life), which is defined as the time needed to decrease the initial enzyme activity by 50%, can be obtained by solving Equation 2.4 for $[E] = 0.5\ [E_o]$.

$$t_{1/2} = \frac{0.693}{k''} \tag{2.5}$$

The k'' and $t_{1/2}$ values for each heat treatment are presented in Table 2.2. Standard deviations were determined with solvstat [16].

Table 2.2 Rate constants and half-life values obtained at different temperatures.

Temperature (°C)	T (K)	$10^3/T$ (K^{-1})	k'' x 10^{-4} (min^{-1})	$t_{1/2}$ (hours)
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

Since (Arrhenius' law)

$$\ln k'' = \ln A - \frac{E_a}{RT} \tag{2.6}$$

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 $J \cdot mol^{-1} \cdot K^{-1}$) and T the absolute temperature (K), plotting $\ln(k'')$ as a function of $1/T$ a straight line is obtained (Figure 2.6) with the slope

$$Slope = -E_a/R \tag{2.7}$$

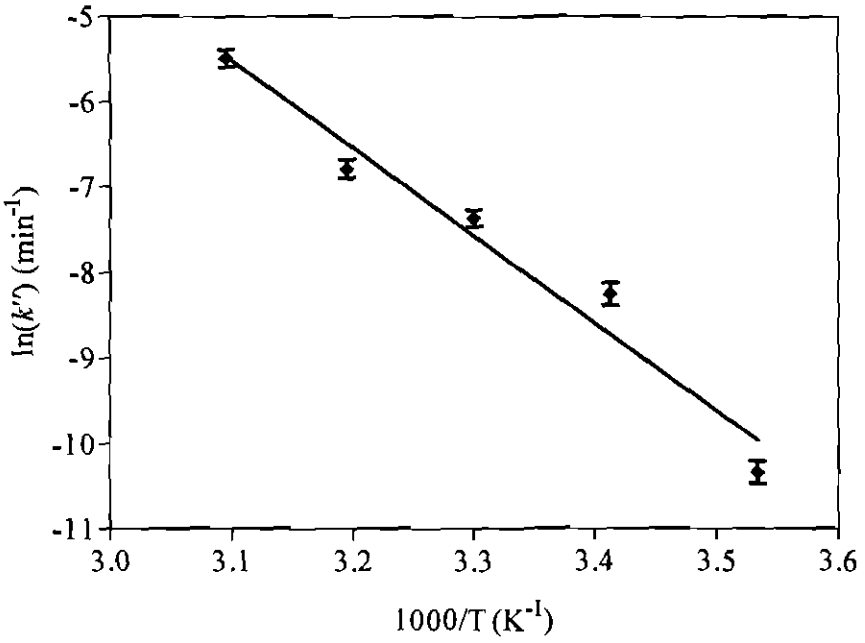

Figure 2.6 Arrhenius plot of ln(k') vs. $1/T$. Standard deviations are illustrated by error bars.

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 *Aspergillus 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 hours.

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 Figure 2.7.

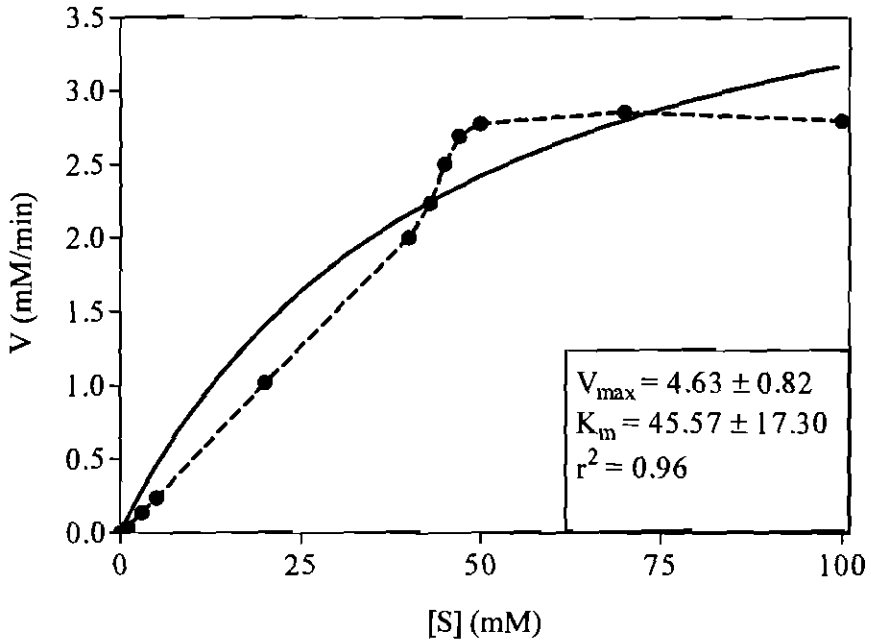

Figure 2.7 Hydrolysis of substrate over time (v) at various initial SO concentrations (S) during hydrolysis by *R. glutinis* (dashed line) and a theoretical Michaelis-Menten curve fit (solid line).

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 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 equation (Equation 2.8) was used (Figure 2.7).

$$v = \frac{v_{max} S}{K_m + S} \tag{2.8}$$

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.

3.4 Salt free process

Monfort *et al.* [18] recently described the use of crude recombinant epoxide hydrolase from *Aspergillus 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 *Rhodotorula glutinis*, two reactions were run parallel, one with phosphate buffer as reaction medium and the other demineralised water (Figure 2.8).

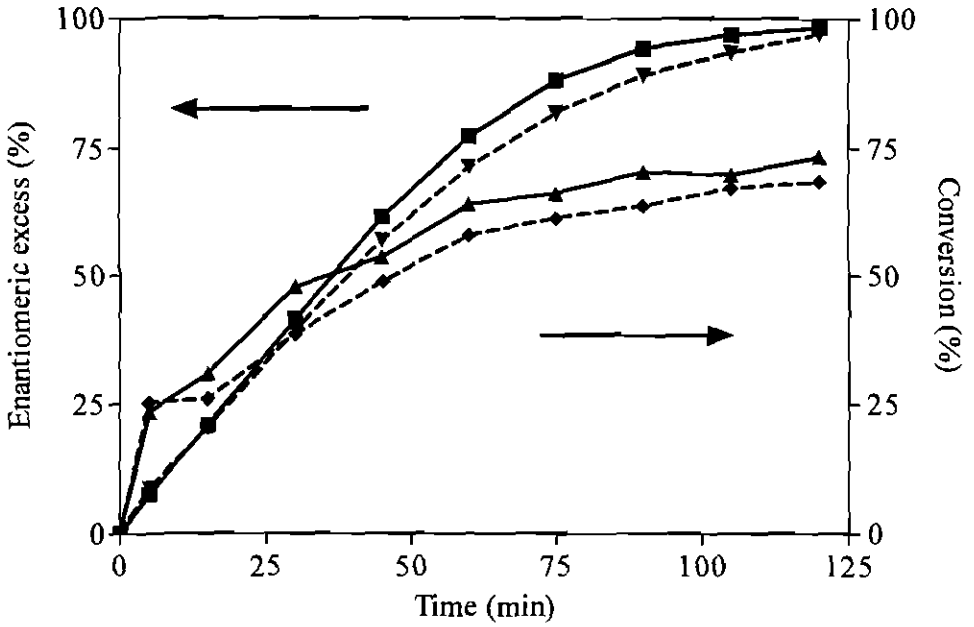

Figure 2.8 Kinetic resolution of SO (20 mM initial concentration) with *Rhodotorula glutinis* (whole cells, 15 °C, pH 7.0) using 50 mM phosphate buffer (solid line) or demineralised water (dashed line) as reaction media. Enantiomeric excess values are represented by ■ and ▼, while conversion ratios are represented by ▲ and ◆ respectively.

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 °C - 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% *e.e.*. 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% $e.e._s$, depending on the final process requirements.

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CHAPTER 3

Hydroxypropyl-β-cyclodextrin induced complexation for the biocatalytic resolution of a poorly soluble epoxide.

*Enzyme and Microbial Technology 2007, **40**, 228-235*

ABSTRACT

The use of a cyclodextrin induced inclusion complex can be very useful during the biohydrolysis of poorly soluble substrates. By using hydroxypropyl-β-cyclodextrin (HPB), the water solubility of styrene oxide (SO) was increased 7 fold (from 2.12 g/L to 15.11 g/L at 15 °C) (20% w/v) and at 10% (w/v) HPB SO was almost 3 times (9.71 g/L at 15 °C) more soluble than in 10% dimethylsulfoxide (DMSO) (w/v) (3.80 g/L at 15 °C). Increasing concentrations of HPB did not influence the enzymatic stability of epoxide hydrolase (EH) from *R. glutinis* contrary to the organic co-solvents which negatively influenced it in the order DMSO > dimethylformamide (DMF). HPB does, however, negatively influence the EH activity of the biocatalyst *Rhodotorula glutinis* UOFS Y-0653. When compared to the inhibition resulting from the addition of organic co-solvents, the inhibition was in the order DMF > HPB > DMSO. Inactivation constants of, 4.54 ± 0.32, 3.29 ± 0.28 and 1.81 ± 0.13 (weight ratio^{-1} w/v) were determined for DMF, HPB and DMSO respectively. Neither DMSO nor DMF influenced the selectivity of the reaction while HPB decreased it slightly. Thermal studies yielded inactivation energy (E_a) values of 84.0 kJ/mol, 82.3 kJ/mol and 86.0 kJ/mol for DMSO, DMF and HPB respectively.

1. INTRODUCTION

Enzymes from various microbial sources have previously been shown to enantioselectively catalyse the hydrolysis of a wide range of epoxides as discussed in a variety of reviews [1,2,3]. Many substrates however, suitable for biocatalysis, are poorly soluble in water, for example *para*-nitro styrene oxide [4], 2-methyl-2-pentyl oxirane [5] and 1,2-epoxyoctane [6], which can have a detrimental effect on the reaction rate. Since the activity of most enzymes is higher in their natural aqueous media than in organic media [7,8] and the effectiveness of a biotechnological process depends on the close contact between the catalyst and the substrate [9], various strategies have been developed to overcome the problem of poor solubility. Concomitantly, increasing the substrate concentration is also a important factor in the industrial implementation of a process. Strategies previously investigated include the use of pure organic solvents (monophasic organic solution) [10], biphasic reaction media (consisting of an aqueous phase and a water insoluble organic phase) [11,12],water soluble organic co-solvents (monophasic aqueous solution) [5,11,12], supercritical fluids and liquid gasses [13], detergents [5] and ionic liquids [14,15].

Variation of the reaction medium by the addition of water-miscible organic co-solvents such as methanol, *tert*-butanol, acetone, dioxane, acetonitrile, dimethylformamide (DMF) and dimethylsulfoxide (DMSO) is a method commonly used to improve the activity, selectivity and stability of enzymatic reactions [4,16,17,18]. These co-solvents have the potential to increase activity by increasing the dissolution of poorly soluble substrates [18], but can inhibit the enzyme by changing its conformation through denaturation [19]. In addition, high co-solvent concentrations lead to the deactivation of the enzyme by stripping away the essential bound water from the surface of the enzyme [16]. In previous studies DMSO was found to be most biocompatible (least inhibitory) above DMF, acetone and acetonitrile with epoxide hydrolase (EH) preparations from *Aspergillus niger* [20], while the relative activity of lipase from *Streptomyces rimosus* was increased more than 3-fold with 2.5% (v/v) 1,4-dioxane while being stable in up to 50% (v/v) of the same co-solvent [21]. These examples illustrate that the effect of co-solvents have to be investigated for each enzymatic reaction to establish the most compatible solvent.

Another method to improve activity and selectivity when dealing with poorly soluble substrates is molecular complexation, which has become an interesting topic in recent years. Molecular complexation can be useful for improving selectivity, separation and solubilisation of various molecules. Cyclodextrins (CDs), a family of cyclic oligosaccharides composed of

α-1,4-linked α-D-glucopyranose subunits [22], are valuable molecular complexation agents, especially in the pharmaceutical industry. One research group has presented numerous examples where they used CDs for biotechnological applications, including the esterification of oleic acid to butyl oleate [23], the hydrolysis of troilein [24] and the enantioselective hydrolysis of (R,S)-ketoprofen ethyl ester [25] by lipase from *Candida rugosa*. In all of the aforementioned examples an increased conversion yield attributed to an enhancement of accessibility of the enzyme to the substrate was illustrated. CDs also have the ability to inhibit enzymes. Monteiro *et al.* [26] described the hydrolysis of diloxanide furoate, an antiparasitary agent, by lipase from *Candida cilindracea* in the presence of various cyclodextrins. It was found that CDs solubilise and stabilise the drug, while inhibiting the lipase enzyme.

Previously it was established that increasing the initial substrate concentration of styrene oxide (SO), during hydrolysis catalysed by *Rhodotorula glutinis* [27], from 20 mM to 50 mM, led to a significant increase in the initial reaction rate (1.02 mM/min to 2.78 mM/min). No further increase was observed above 50 mM [28]. The aim of this study, therefore, was to improve the substrate solubility with a minimum effect on enzyme activity, stability and selectivity. To achieve this, the effect of hydroxypropyl-β-cyclodextrin (HPB) was compared to the effect of two well known miscible organic solvents, namely DMSO and DMF. HPB was selected for this investigation due to the positive results obtained in a previous study by Kim *et al.* [25]. To avoid confusion and simplify comparison of these two groups, which possess appreciably different solubilisation mechanisms, both the cyclodextrin and organic co-solvents will be referred to as solubility enhancing additives or just additives.

2. MATERIALS AND METHODS

2.1 General

Rhodotorula glutinis (UOFS Y-0653) was obtained from the yeast culture collection of the University of the Free State (South Africa). Reactions were analysed by chiral gas chromatography (GC, Thermo Finnigan Focus gas chromatograph equipped with FID and a Thermo Electron AS3000 auto sampler) on a 25 m Chrompack Chirasil-dex CB fused silica cyclodextrin capillary column 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: styrene oxide, 90 °C, t_R (R) 15.35 min. and t_R (S) 16.85 min., 1-phenyl-2-ethanediol, 150 °C, t_R (S) 16.78 min. and t_R (R) 17.53 min. 1-Pentanol was used as an internal standard during GC analysis (at 90

°C: t_R 4.48 min., at 150 °C: t_R 1.25 min.). Spiking samples with a small amount of enantiopure substrate or product were used to identify the absolute configuration of the enantiomers.

2.2 Cultivation and preparation of whole yeast cells

Yeasts were grown in 1 L shake-flask cultures 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 a vitamin solution (0.2% v/v) in a Labcon® rotary platform shaking incubator at 27 °C (180 rpm). The vitamin solution was chosen due to availability and contained (mg/L): biotin 20.0, calcium pantothenate 200.0, folic acid 0.2, inositol 1000.0, niacin 40.0, *p*-amino benzoic acid 20.0, pyridoxine HCl 40.0, riboflavin 20.0 and thiamine HCl 40.0. At late growth phase the cells were harvested by centrifugation (3 500 g, 5 min) and washed with phosphate buffer (KH_2PO_4/K_2HPO_4, 50 mM, pH 7.2). The washed cells were resuspended (25% w/v) in phosphate buffer containing 10% (v/v) glycerol (pH 7.2), frozen and stored in 50 ml centrifuge tubes (20 ml per tube) below −18 °C. No noteworthy decrease in activity was observed after 3 months of storage.

2.3 Solubility of SO

In 1.5 ml micro-centrifuge tubes (polypropylene) phosphate buffer (50 mM, pH 7.2) and various concentrations (% w/v) of the relevant additive were added to a final volume of 1 ml. After adding an access of SO (200 µl), the micro-centrifuge tubes were incubated (15 °C or 25 °C) while continuously being shaken (200 rpm) in a temperature regulated shaking water bath. After 24 hours, 250 µl samples were taken from the aqueous phase, extracted with ethyl acetate (250 µl), dried over anhydrous sodium sulphate and analysed by chiral GC.

2.4 Effect of additives on pH

To 25 ml glass bottles (with screw caps and rubber septa) containing phosphate buffer (50 mM, pH 7.5) the relevant additive was added at various concentrations (% w/v) to a final volume of 10 ml. The solutions were shaken for 30 minutes (200 rpm) and the final pH values measured using a Metrohm 744 pH meter.

2.5 Enzymatic stability

Phosphate buffer solutions (50 mM, pH 7.2) containing various concentrations of the relevant additive were prepared. Suspended frozen cells were thawed (20 ml), centrifuged (3 500 g, 5 min) and the supernatant discarded. After resuspending the cells in additive containing

phosphate buffer solutions to a total volume of 20 ml, the suspensions were incubated at 15 °C for 30 minutes. Subsequently the additive containing buffer was removed by centrifugation (3 500 g, 5 min). After washing the cells twice with additive free phosphate buffer, they were resuspended in phosphate buffer (50 mM, pH 7.2). The remaining enzymatic activity was determined by pre-incubating the suspensions for 5 minutes (allowing temperature equilibration) followed by the addition of SO to a final concentration of 20 mM. During incubation at 15 °C, while continuously being shaken (200 rpm), 500 µl samples were drawn at various time intervals and analysed by chiral GC

2.6 Activity and Selectivity

Phosphate buffer solutions (50 mM, pH 7.2) containing various concentrations of the relevant additive were prepared. Suspended frozen cells were thawed (20 ml), centrifuged (3 500 g, 5 min) and the supernatant discarded. After resuspension (in additive containing buffer to a total volume of 20 ml) the enzymatic activity was assayed as described in Section 2.5.

2.7 Thermal stability

Phosphate buffer solutions (50 mM, pH 7.2) containing 10% (w/v) of the relevant additive were prepared. The thawed, washed and resuspended frozen cells (see Section 2.5) were aliquoted (500 µl) into 1.5 ml micro-centrifuge tubes. These micro-centrifuge tubes were incubated at various temperatures in a shaking water bath (200 rpm). At specific time intervals micro-centrifuge tubes were removed and shock frozen in liquid N_2 to ensure accurate measurements. After all the micro-centrifuge tubes had been removed, they were thawed and the remaining enzymatic activity assayed as discussed before (Section 2.5). The only exception was that the samples were incubated for 2 hours before extraction.

3. RESULTS AND DISCUSSION

3.1 Solubility of SO

Increasing the low water solubility of SO, determined to be 2.7 g/L (22.5 mM/mL) in phosphate buffer (50 mM, pH 7.2) at room temperature (25 °C) and 2.2 g/L (18.6 mM/mL) at 15 °C, could result in an increased reaction rate by increasing the dissolution rate of SO. Furthermore, increased solubility increases the reaction's potential use in a membrane bioreactor, for example during the filtration of a reaction medium after bioconversion [29]. For the removal of the reaction medium, the products ought to be soluble to avoid product retention. Since the optimal reaction temperature for this reaction is 15 °C [28], the solubility

of SO with various concentrations of the additives was determined at this temperature. The average results (experiments were done in triplicate) are shown in Figure 3.1.

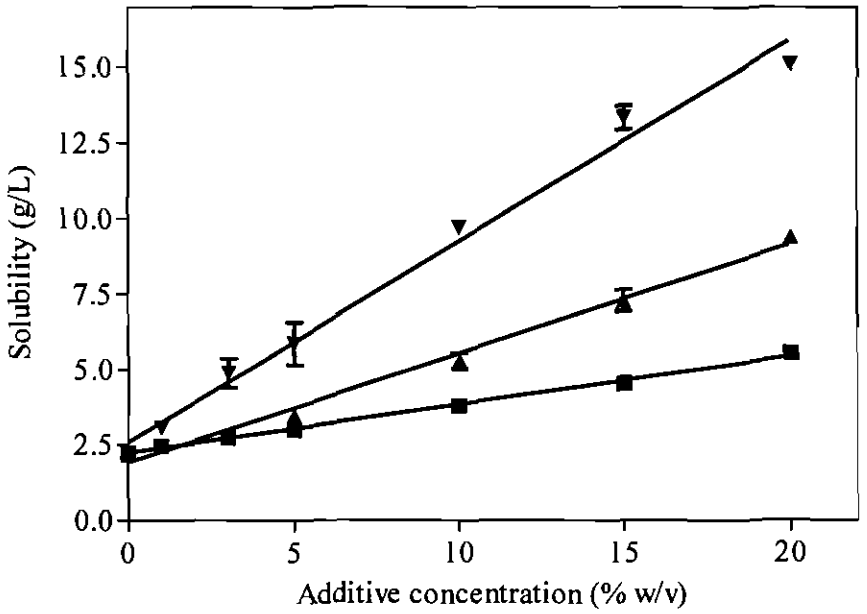

Figure 3.1 Solubility enhancing effect of three additives, DMSO (■, $R^2 = 0.98$), DMF (▲, $R^2 = 0.98$) and HPB (▼, $R^2 = 0.98$), on SO in phosphate buffer (50 mM, pH 7.2) at 15 °C.

For all the concentrations investigated the solubilisation effect was in the order: DMSO < DMF < HPB. A linear increase in solubility with increasing additive concentration was observed for all three additives. When repeating these experiments at 25 °C, the same trends were observed. These results correspond with data presented by Nellaiah *et al.* [20], who reported the same order for DMSO and DMF while investigating the solubilisation of *para*-nitrostyrene oxide. While both DMSO and DMF improved the solubility of SO, the most significant increase in solubilisation was observed for HPB through the formation of an inclusion complex. At 10% HPB (w/v), SO was almost twice as soluble (10.5 g/L) than in 10% DMF (5.3 g/L) and 2.8 times more than in 10% DMSO (3.8 g/L). At 20% HPB, 18.8 g/L SO (157 mM) was solubilised at 15 °C which clearly makes HPB very useful for the solubilisation of poorly soluble substrates. Interestingly, even though cyclodextrins are chiral and have been shown to have the ability to selectively solubilise single enantiomers [30,31], chiral analysis of the solubilised SO illustrated that selective complexation did not occur in this case.

3.2 Effect of additives on pH

An important variable is the influence of additives on the pH of the reaction medium [32]. Various authors have shown the effect of pH on enzyme catalysed reactions [e.g. 5,26,33,34], showing the need to investigate the effect of additive addition. Previously EH from *R. glutinis* was found to have optimal activity between pH 6.8 and 7.8 [28]. Concentrations ranging between 5 and 50% (w/v) were investigated for the three additives DMSO, DMF and HPB, the results of which are shown in Figure 3.2. In spite of the fact that the regular definition of pH cannot be applied directly in this study since the activity coefficient of the protons in solutions containing organic solvents may not be the same as their coefficients in solvent free water [32], good reproducibility was obtained (in triplicate).

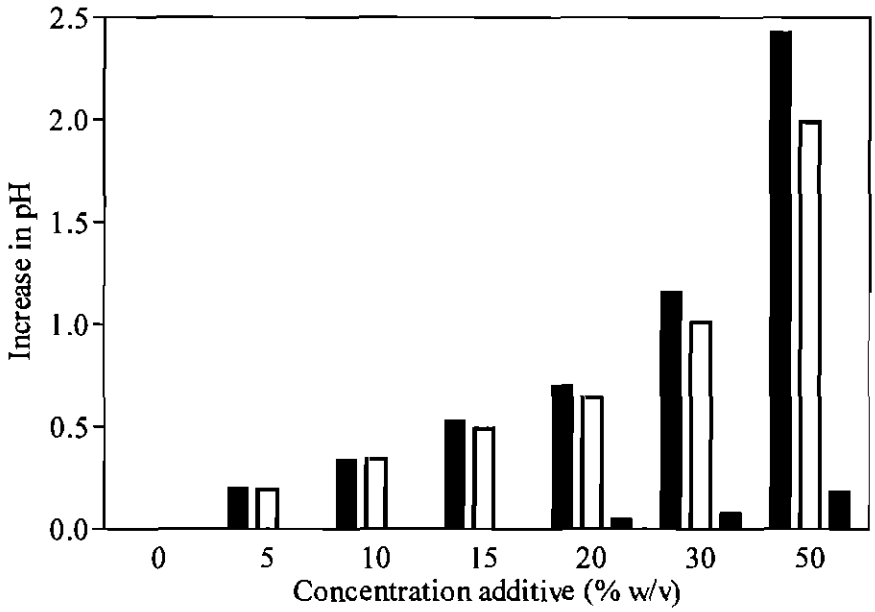

Figure 3.2 Effect of additives on the pH of a 50 mM phosphate buffer with an initial pH of 7.5. DMSO (filled bar), DMF (unfilled bar), HPB (banded bar).

The presence of DMSO and DMF resulted in an exponential increase in pH of the phosphate buffer solution (50 mM, initial pH 7.5) with increasing additive concentration ranging from 0.2 at 5% to 2.4 at 50% (for DMSO). This change can be explained by the fact that most co-solvents have acidic or basic properties which they confer to the aqueous solution. Co-solvents containing oxygen or nitrogen in their structure generally confer basic properties to the solution [32], as dipolar aprotic solvents such as DMSO and DMF do not act as hydrogen bond donors but rather as electron pair donors [35]. While DMSO and DMF seem to have the same influence on pH below 15% additive concentration, the pH in the presence of DMSO increased more significant than in the presence of DMF above 15%. These results compare

well to those reported by Shubhada and Sundaram [32]. The increased pH in the presence of DMSO and DMF can be explained in terms of either the pK_a of the solvent or the normalised solvent polarity parameter (E_T^N), which gives a measure of the availability of lone electron pairs within a molecule. According to this measure, a higher E_T^N indicates a more alkaline solvent. Since the E_T^N of DMSO and DMF is 0.444 and 0.386 respectively [35], DMSO is the stronger base, which correlates with the observed higher pH obtained in the presence of DMSO.

In contrast to DMSO and DMF, HPB had no significant effect (< 3%) on the pH of the solution. The data shows that when using basic or acidic co-solvents (i.e. DMSO and DMF) the final pH of the reaction medium has to be monitored. Alternatively a buffer such as Tris may be used to diminish the effects of co-solvent addition. On the other hand, due to the negligible effect HPB had on pH, no pH monitoring or control is required when using HPB as an additive, giving rise to yet another advantage of using HPB as a co-solvent.

3.3 Enzymatic stability

To determine which of the additives would deliver the most beneficial reaction; their effect on the stability of the enzyme was firstly investigated. To this end whole *R. glutinis* cells were incubated for 30 minutes (15 °C) in phosphate buffer (50 mM, pH 7.2) containing various amounts of the additives and the effect of this incubation period established by assaying the remaining enzymatic activity. Figure 3.3 illustrates the effect of various additive concentrations upon the initial reaction rate obtained after incubation (R_a) relative to the initial reaction rate achieved when no additive was used during incubation (R_0).

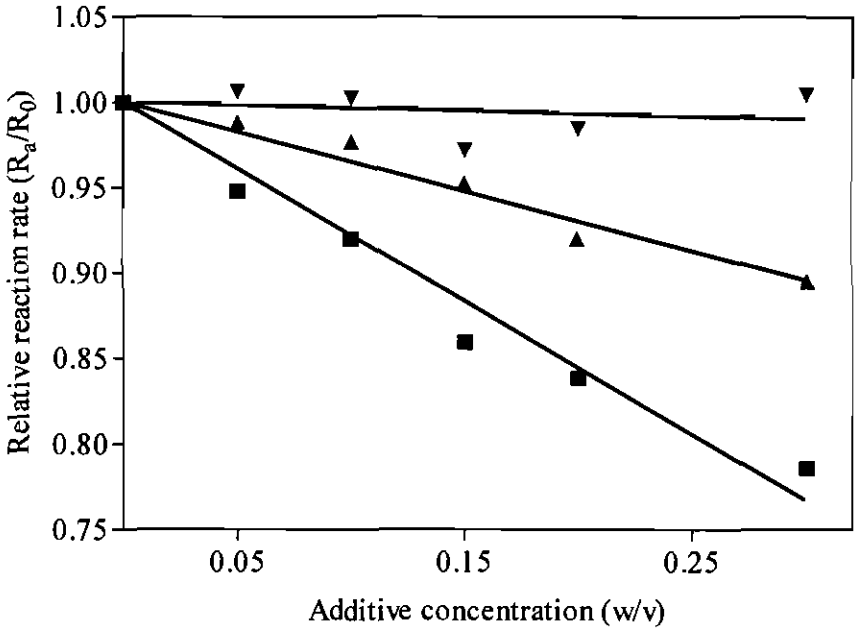

Figure 3.3 Effect of on the initial relative reaction rate of styrene oxide during hydrolysis with *R. glutinis* after 30 minutes of incubation (15 °C) with various concentrations of each of the three additives, DMSO (■), DMF (▲) and HPB (▼). R_0 = 0.27 mM/min.

The deactivation effect of the additives was in the order DMSO > DMF > HPB. Increasing concentrations of DMSO and DMF led to a linear deactivation of the enzyme. In contrast to this, increasing HPB concentrations had a negligible effect on enzymatic activity. For this reason HPB would be the additive of choice when maintained activity and enzyme reusability are the most important considerations for a specific reaction.

3.4 Activity and selectivity

The variation in the initial reaction rate as a result of increasing concentrations of each additive was subsequently established. As mentioned previously, increased substrate solubility can lead either to increased yields and substrate conversion rates due to an increased amount of substrate available to the enzyme, or to a decrease in reaction rates due to changes in the protein structure of the enzyme and disruption of the non-covalent forces [18]. To ensure that only the effects of the additives on the enzyme were to be investigated, an initial substrate concentration of 20 mM was used. In other words, at all additive concentrations, the substrate was completely solubilised. For comparative purposes the reaction rate (mM/min) achieved with each concentration of the different additives (R_a) can be reported relative to the rate achieved without any additives (R_0). The results are shown in Figure 3.4.

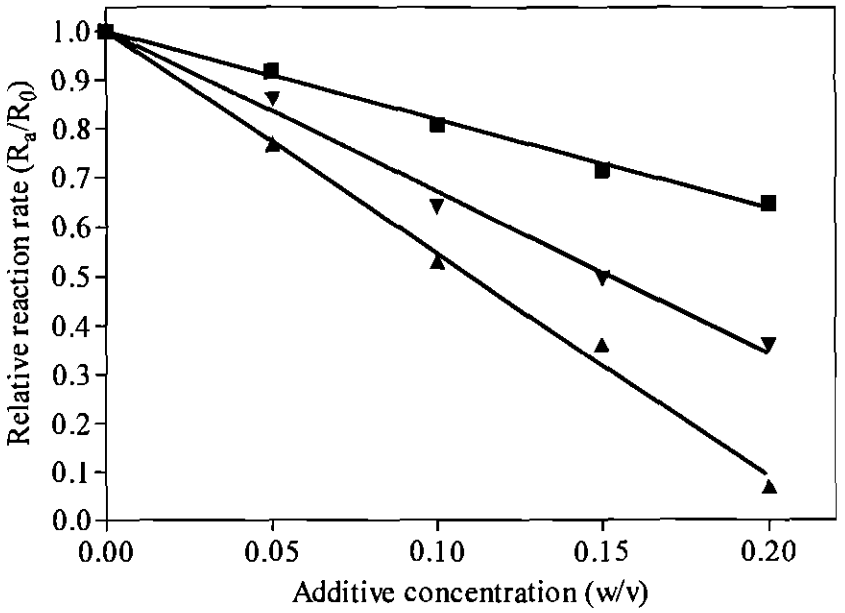

Figure 3.4 Effect of the three additives DMSO (■), DMF (▲) and HPB (▼) on the initial reaction rate of styrene oxide during hydrolysis with *R. glutinis*. R_0 = 0.29 mM/min

For each additive the decrease in initial reaction rate was directly proportional to the additive concentration. This inhibitory effect followed the order DMSO < HPB < DMF. However, when considering that this inhibitory effect is directly proportional to the additive concentration and that HPB has a far superior solubilisation effect, the picture changes. This can be illustrated by presenting the relative reaction rate as a function of the amount of additive required to attain a specific solubility of SO (Figure 3.5).

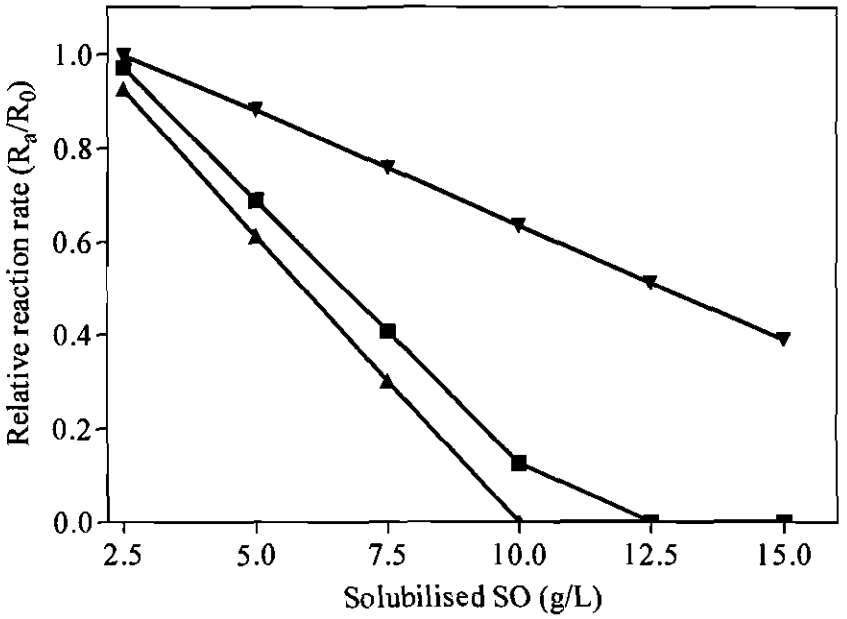

Figure 3.5 Effect of the three additives DMSO (■), DMF (▲) and HPB (▼) on the initial reaction rate of styrene oxide in the presence of the concentration additive necessary to solubilise a specified amount of SO.

For instance, if one assumes a 5 g/l concentration of SO is required for a feasible bio process which is double the normal solubility of SO at 15 °C (2.2 g/l), the addition of 17.2% (w/v) DMSO, 8.5% (w/v) DMF and 3.6% HPB would be required (Figure 3.1). According to Figure 3.4 this converts to a relative reaction rate for the enzyme of 69%, 61% and 88% in the presence of DMSO, DMF and HPB respectively. This clearly shows that at a specific substrate solubilisation, the use of HPB, due to its superior solubilisation ability, will yield the highest enzyme reaction rate.

According to Nellaiah *et al.* [20] the inhibition of an enzyme by an additive can be quantified by calculating either the additive concentration leading to a 50% decrease in initial reaction rate (half-inhibitory concentration), also known as the threshold concentration (C_{50}) [18], or by calculating the inactivation constant from the slope of the straight lines. These values are reported in Table 3.1. The half-inhibitory concentration of DMSO was determined through extrapolation of the obtained straight line. The correlation (R^2) of the straight line used for the calculations was above 0.99 for all three additives, i.e. DMSO, DMF and HPB.

Table 3.1 Half-inhibitory concentrations (C_{50}) and inactivation constants of the hydrolysis of styrene oxide with *R. glutinis* with three additives.

Additive	Half-inhibitory (threshold) concentration % (w/v)	Inactivation constant (v/w)
DMSO	28	1.81 ± 0.13
DMF	11	4.54 ± 0.32
HPB	15	3.29 ± 0.28

The inactivation constant and C_{50} values obtained for DMSO are in the same range as those established by Nellaiah *et al.* who reported values of 1.58 volume ratio^{-1} and 32% (v/v) respectively when investigating the hydrolysis of *para*-nitrostyrene oxide by an EH preparation from *A. niger*. DMF, however, was inhibitorier towards *R. glutinis* EH within whole cells when compared to *A. niger* EH. An inactivation constant and C_{50} value of 2.44 volume ratio^{-1} and 21% (v/v) respectively were reported [20]. Another similarity to this report is that the solvent with the greater solubilisation potential (DMF), was more inhibitory. According to the results obtained for both DMSO and DMF, the influence of the solvent on activity is inversely proportional to its influence on solubility, or in other words, increased solubility comes at the cost of decreased activity. The superior biocompatibility of DMSO above DMF for different enzymatic reactions, including the hydrolysis of epoxides by EH from whole cells of *R. glutinis* [36], has previously been shown by Morisseau *et al.* [4], Nellaiah *et al.* [20], and Azevedo *et al.* [17].

HPB, however, had a lower inactivation constant than DMF, and yet resulted in a substantial increase in the solubility of SO. While the results suggest that DMSO has the greatest biocompatibility for this reaction, it should be noted that, being an organic solvent and strong denaturant [15], both DMSO and DMF probably deactivate the enzyme through denaturation. It is likely that HPB on the other hand decreases the enzymatic activity by restricting the access of the enzyme to the substrate as a result of decreased permeation of the substrate across the cell membrane, stereochemically hindering the reaction at the active site of the enzyme or by stabilising the usually highly reactive substrate as is the case with diloxanide furoate [26]. Montero *et al.* [26] described stabilisation of the aforementioned substrate against enzymatic and alkaline hydrolysis through the addition of β-cyclodextrins. Previously (Figure 3.3) it was shown that HPB had a negligible effect on the stability of the enzyme.

This illustrates that the observed decrease in enzymatic activity in the presence of HPB is not due to denaturation of the enzyme, but rather due to the reversible inclusion complex formed with the substrate.

Increasing additive concentrations did not markedly affect the selectivity of the enzyme. Even though the time to reach a specific conversion increased significantly (activity), higher additive concentrations did not increase or decrease the enantiomeric excess of the substrate ($e.e._s$) that could be achieved at a specific substrate conversion. This was the case for all additives investigated. When comparing the effect of different additives on $e.e._s$, DMSO and DMF yielded similar results over the entire additive concentration range (5 – 20%). The use of HPB, however, did yield a slightly less selective reaction over the entire concentration range. It is suspected that this may be due to the nature of the cyclodextrin inclusion complex which may restrict access to the faster reacting (R) enantiomer more than the slow reacting (S) enantiomer. As can be seen from Figure 3.6 if the concentrations of additive necessary to solubilise 5 g/L SO are compared to one another, a less selective reaction is achieved with HPB than with any of the other additives.

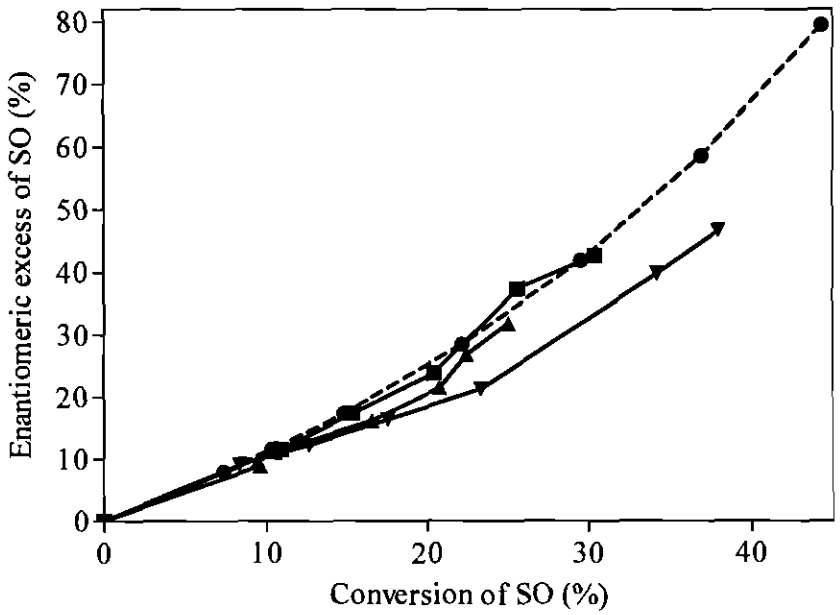

Figure 3.6 Enantiomeric excesses (%) of SO obtained vs. the conversion (%) of SO in the presence of the concentration DMSO (■), DMF (▲) or HPB (▼) (w/v) necessary to solubilise 5 g/L SO. The reaction without additive is represented by the dashed line (•).

It is possible that this negative effect of HPB could be overcome by selecting a different CD or a derivate thereof and this will be investigated in the future.

3.5 Thermal stability

The thermal stability of enzymes depends on their specific amino acid sequence, but in general high temperatures induce irreversible deactivation of enzymes. EH from *R. glutinis* is not stable at high temperatures [28], exhibiting a deactivation energy of 85.2 kJ/mol compared to the much higher value of 304 kJ/mol exhibited by *Rhizomucor meihei* [37].

To establish the effect of the three additives on the thermal stability of the enzyme, the cell suspension was incubated (without substrate) at various temperatures for different times in the presence of 10% (w/v) of each additive. Ln plots of the remaining activity as a function of incubation time yielded linear trends indicating first order deactivation kinetics. From the slopes of these lines the inactivation constants (k) in min^{-1} for each of the additives at various temperatures was obtained. The results showed an exponential increase in the inactivation constant above 40 °C illustrating the heat sensitivity of the specific enzyme. An often used measure of temperature induced inactivation is the half life ($t_{1/2}$) of the enzyme, which is defined as the time needed to decrease the initial enzyme activity by 50%. The half lives in the absence and presence of additives were calculated from the respective inactivation constants using Equation 3.1 and are illustrated in Figure 3.7.

$$t_{1/2} = \frac{0.693}{k} \tag{3.1}$$

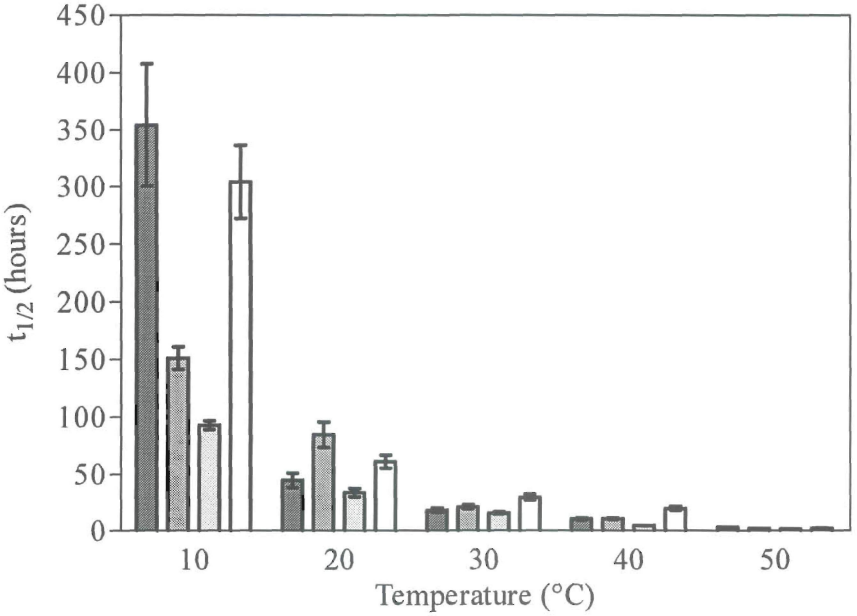

Figure 3.7 Half-lives obtained with no additive (■), 10% (w/v) DMSO (▩), DMF (▨) and HPB (□) at various temperatures.

According to Figure 3.7 the additives have a higher impact on thermal stability at lower temperatures. This can be explained by the increased contribution of temperature induced denaturation at higher temperatures, masking the possible detrimental effect of the additives. However, irrespective of temperature, DMF had the most detrimental influence on enzyme inactivation (74% reduction in $t_{1/2}$ at 10 °C and 60% at 50 °C compared to the $t_{1/2}$ obtained without any additives) followed by DMSO (57% reduction at 10 °C and 41% at 50 °C). The presence of HPB on the other hand had very little effect on the rate of deactivation compared to a reaction not containing any additive (14% reduction at 10 °C and 37% at 50 °C). At high temperatures (50 °C), the established half-lives are all in the same range, illustrating that at this temperature enzymatic deactivation occurs mainly as a result of temperature and not due to the additive used. While it was found that at 15 °C DMSO had a greater negative effect (Figure 3.3) upon the enzymatic stability of EH than DMF (within whole cells), with increasing temperatures, the presence of DMF has a greater negative effect upon enzymatic stability than in the presence of DMSO. Using the Arrhenius relationship (Equation 3.2), E_a, the activation energy of deactivation or thermal inactivation energy (J/mol) for the reaction can be established.

$$\ln k = \ln A - \frac{E_a}{RT} \tag{3.2}$$

where A is a constant, E_a the activation energy of deactivation or thermal inactivation energy (J/mol), R the universal gas constant (8.314 J $mol^{-1} \cdot K^{-1}$) and T the absolute temperature (K) was calculated in the presence of 10% (w/v) of each additive. These energies, as well as the correlation coefficients of the obtained straight lines used to calculate these values, are shown in Figure 3.8.

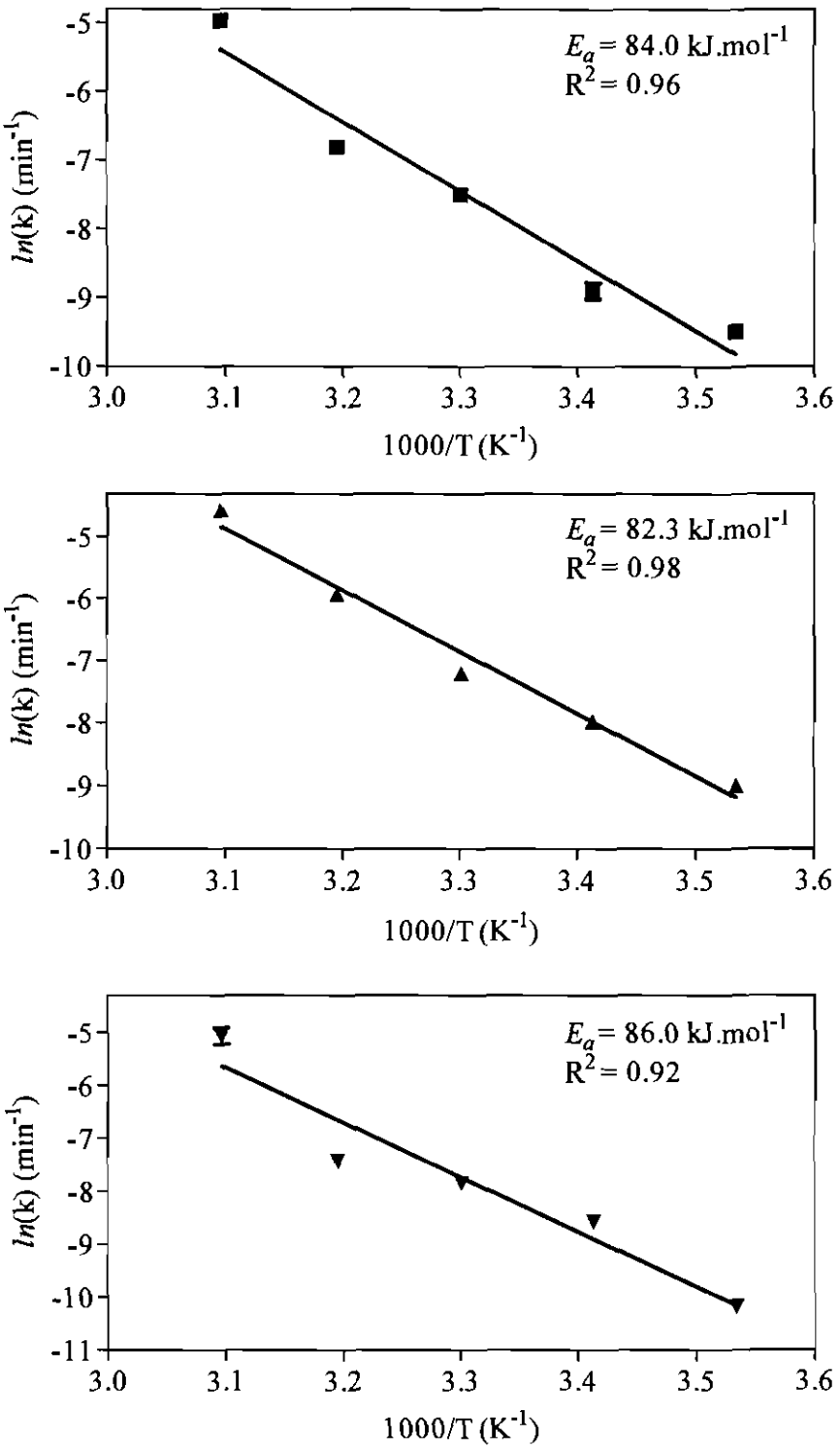

Figure 3.8 Arrhenius plot of ln(k) vs. $1/T$ in the presence of 10% (w/v) DMSO (■), DMF (▲) and HPB (▼).

The established E_a values illustrate that HPB had the least effect on the thermal stability of the enzyme. The obtained value of 86.0 kJ/mol is in the same order of magnitude as the value obtained without the use of an additive (85.2 kJ/mol) [28]. Although the difference in E_a with

and without HPB is too small to conclude significantly increased enzyme stability by HPB, it is clear that HPB does not contribute to the destabilisation of the enzyme at elevated temperatures. The values obtained for DMSO and DMF were 84.0 kJ/mol and 82.3 kJ/mol respectively, once again illustrating the deactivation order DMF > DMSO > HPB ≈ no additive.

4. CONCLUSION

It was shown that cyclodextrins, but specifically HPB, are useful tools to increase the solubility of poorly soluble substrates during hydrolysis reactions catalyzed by enzymes. In terms of the influence on activity the inhibition increased in the order DMSO < HPB < DMF. While the addition of HPB resulted in a higher decrease in activity and selectivity than observed in the presence of equal quantities of DMSO, much less HPB is required to attain a specific solubility. While this fact could deal with the point that HPB is more expensive than DMF and DMSO, it also implies that for a specific solubility, HPB had the least inhibitory effect on the enzymatic reaction rate. Furthermore it was also shown that no pH monitoring and adjustment is required when using HPB, resulting in a simplification of the production process.

Both enzymatic stability and enzymatic thermal stability were least affected by the addition of HPB compared to DMSO and DMF. This may be especially useful for the development of continuous reactors were the use of membranes necessitates completely soluble substrates and were the reuse of the catalyst (whole cells or purified enzyme) could become a process requirement. Finally, it should be stated that cyclodextrins are environmentally friendly and non-toxic compounds, unlike DMSO and DMF, which could be an additional benefit when selecting a biocatalysed reaction for a pharmaceutical application.

In an attempt to further elucidate the decrease in $e.e._s$ in the presence of HPB further work could include determining substrate-enzyme complexation energies using pure SO enantiomers in the absence and presence of HPB. An alternative approach would be to compare various CDs and their derivatives to one another. The aim of this would not only be to establish the best CD for this reaction but also to clarify the effect of CD structure upon the activity, selectivity and stability of the enzyme.

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CHAPTER 4

Bench-scale production of an enantiopure terminal epoxide in a stirred batch reactor

ABSTRACT

Various factors influencing the scale-up of a biocatalytic process were investigated, using the previously optimised enantioselective hydrolysis of styrene oxide (SO) by epoxide hydrolase (EH) as a model reaction. Scale-up studies were conducted in a temperature regulated, stirred batch reaction vessel with a total volume of 400 mL. A 10 fold increase in impeller speed was shown to increase the initial reaction rate by 156%, however, a simultaneous decrease in the selectivity of the reaction was observed. An impeller speed of 400 rpm was established to be optimal. Incremental increases in impeller speed, and therefore increased shear, had a negligible effect on the stability of the enzyme within the whole yeast cells over a 4 hour period. Increases in the amount of biocatalyst in the reaction vessel (g/L) increased the observed initial reaction rates but decreased the overall productivity of the reaction (based on the productivity number of the reaction). A cell/buffer ratio of 1:4 (20% w/w) was found to be optimal in terms of the productivity of the reaction. Repetitive batch operation incorporating micro-filtration was investigated but was found to be ineffective.

1. INTRODUCTION

Owning to the high reactivity of epoxides, they are important synthons for fine organic synthesis [1], a property emphasised by their ability to act as precursors to a variety of compounds [2]. Another factor, however, makes these compounds even more interesting. The fact that they can be enantioselectively hydrolysed by epoxide hydrolase (EH, EC 3.3.2.3), an enzyme found in a variety of living organisms [3], may allow for the production of enantiomerically pure epoxides and, in turn, high value single enantiomer pharmaceuticals such as (R)-Nifenalol [4]. Because of potential interest in resolutions catalysed by EH, various research groups have investigated the possible application of EH to a variety of substrates. This topic has been discussed in several reviews, a good example of which was published by Archelas & Furstoss [1].

The aforementioned interest has also led to various attempts to scale-up these enantioselective reactions, usually with the intention of developing an economically viable process by optimising the production rate, removing inhibitory products and minimizing waste. Batch reactors [5], repetitive batch reactors [6], fed-batch [7], continuous stir tank reactors [5,8], continuous flow through reactors [9,10], membrane reactors [11] and various others have all been investigated as possible methods to produce not only enantiopure epoxides, but also various other products. In most cases, however, stirred batch reactors or repetitive batch reactors have been most successful. In the case of terpenes for example, a recent review states that 18% of all papers published on this subject involved the use of stirred tank reactors, compared to only 3% involving membrane reactors [12].

Several factors have the ability to influence the productivity of enzymes within reactors. The use of a biphasic system within a stirred batch reactor for example (enantioselective hydrolyses of glycidyl phenyl ether), increases the average productivity 2.6 fold when compared to a single aqueous phase [13]. Shear stress for instance, which might be caused by increased agitation, may in turn cause interfacial inactivation of epoxide hydrolase within a stirred reactor [14]. In addition to the abovementioned factors, one other (often neglected) consideration has to be included, i.e. the separation of the biocatalyst and the product after conclusion of the reaction. This step is of importance as a result of potential interferences with downstream processing, contamination of the product or the need to recycle the biocatalyst [15]. The aforementioned concern has led to, amongst others, the application of methods such as micro-filtration [8] and ultra-filtration [16] of the reaction mixture retaining the biocatalyst while the product is located in the filtrate. The main obstacle for use of these

methods, however, is the fouling of the membrane which has been discussed by Meindersma *et al.* [15] and Zydney & Ho [17].

In this study the enantioselective hydrolysis of SO by a strain of *Rhodotorula glutinis* UOFS (Y-0653) with EH activity [18] was scaled up and the factors influencing the reaction were investigated. In addition, the previously established solubilisation potential of hydroxypropyl-β-cyclodextrin (HPB) [19] was applied to increase the solubility of the poorly soluble substrate. A stirred batch reactor was selected as these systems provide a homogeneous environment and a high degree of freedom for varying mixing and mass transfer rates [20]. The effects of the impeller speed and the concentration biocatalyst (g/L) were investigated in a batch reactor with a total volume of 400 mL. Furthermore the potential of biocatalyst recycling, a factor greatly contributing to the overall cost of a process, was investigated by running the reactor in a repetitive batch configuration. Finally, micro-filtration was employed in an attempt to separate the biocatalyst from the products.

2. MATERIALS AND METHODS

2.1 General

Rhodotorula glutinis (UOFS Y-0653) was obtained from the yeast culture collection of the University of the Free State. Reactions were monitored and analysed by chiral gas chromatography (GC). Analyses were conducted using H_2 as carrier gas on a Thermo Finnigan Focus GC equipped with a flame ionisation detector (FID) and a Thermo Electron AS3000 auto-sampler. A 25 m Chrompack Chirasil-DEX CB (Varian Inc.) fused silica cyclodextrin capillary column (ID: 0.25mm, Df: 0.25 μm) was used for enantiomer separation. Racemic styrene oxide and (R)-1-phenyl-1,2-ethane diol were obtained from Sigma-Aldrich, while (S)-styrene oxide was obtained from Fluka. Isocratic chiral GC analysis of the products after biohydrolysis were done as follows: SO; 90 °C, t_R (R) 15.35 min and t_R (S) 16.85 min, 1-phenyl-2-ethanediol; 150 °C, t_R (S) 16.78 min and t_R (R) 17.53 min. 1-Pentanol was used as an internal standard during GC analysis (90 °C: t_R 4.48 min, 150 °C: t_R 1.25 min). Spiking samples with a small amount of enantiopure substrate or product identified the absolute configuration of the enantiomers. 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

Dehydrated culture media was obtained from Biolab (Biolab Inc., Budapest) and was used throughout this study. Yeasts were grown in 1 L shake-flasks containing 200 ml yeast growth media (0.5% yeast extract, 2.0% malt extract, 0.5% peptone w/v) supplemented with 1.5% (w/v) glucose and 0.2% of a previously used vitamin solution [22]. The inoculated shake flasks were incubated in a temperature regulated Labcon® rotary platform incubator at 27 °C and agitated at 180 rpm. At late growth phase the cells were harvested by centrifugation (3 500 g, 5 minutes) and washed with phosphate buffer (50 mM, pH 7.2). The washed cells were resuspended in phosphate buffer (25% w/w) containing 10% (v/v) glycerol (pH 7.2), decanted into 50 mL centrifuge tubes with screw caps (20 mL per tube), frozen and stored below –18 °C. No significant loss of activity was detected after 2 months of storage after which time cells were discarded.

2.3 Batch reactor

It was previously shown by Yeates *et al.* [22] that temperature affects the EH activity of the yeasts. Therefore, a bench top, cylindrical batch reactor (400 ml total volume) fitted with a water mantle for temperature regulation, an interior Teflon® coating (to eliminate the possibility of inhibition by metal ions) and a dual impeller stirring rod was used for all experiments. The reactor was connected to a temperature regulating water circulator and a variable speed laboratory stirrer. The solid base of the reactor could be removed and replaced with a micro-filtration unit which was used for all relevant studies. A graphic representation of the reactor is given in Figure 4.1.

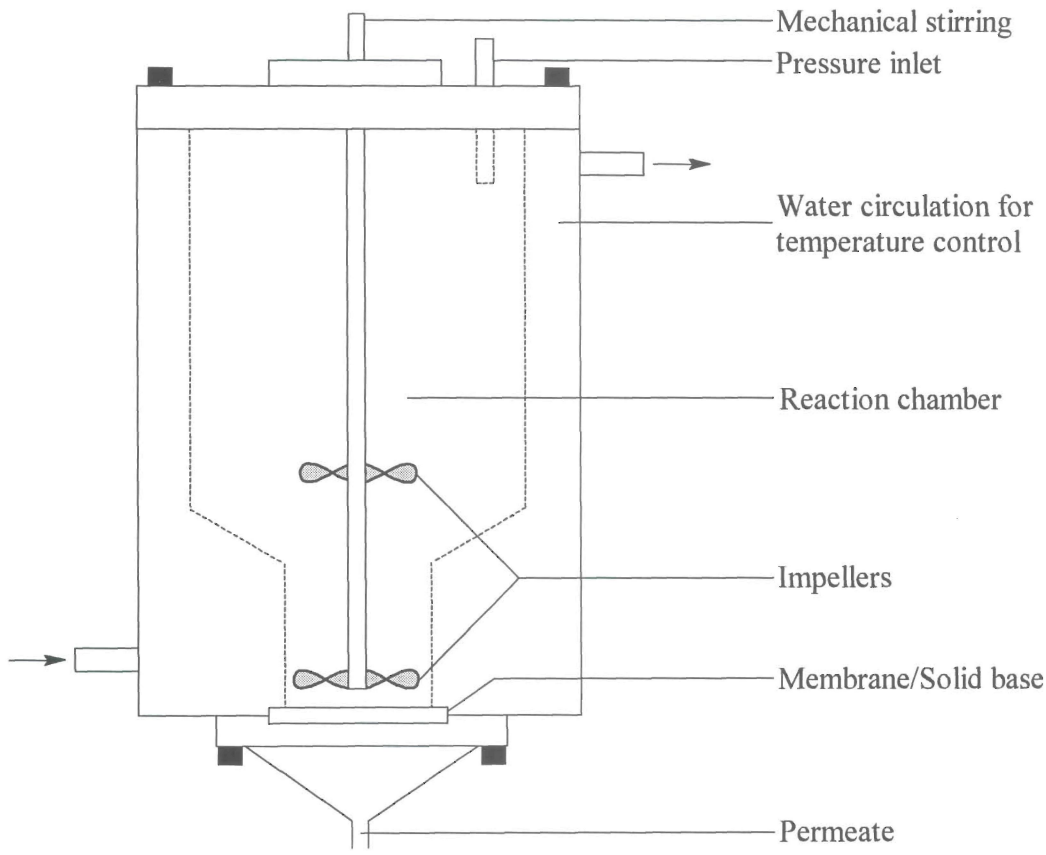

Figure 4.1 Schematic representation of the utilised stirred batch reactor.

2.3.1 The effect of impeller speed upon activity and selectivity

Frozen cells were thawed and centrifuged (3 500 g, 5 minutes). The glycerol containing buffer was replaced with 50 mM phosphate buffer (pH 7.2) containing 5% HPB. After allowing 5 minutes incubation in a water bath to allow for temperature equilibration at 15 °C, SO was added to a final concentration of 100 mM (240 µl in 20 ml suspension) and shaken vigorously to allow for maximum solubilisation. The suspension was transferred to the reactor and continuously stirred at various impeller speeds with a 50 Hz IKA Labortechnik RW 20.n laboratory stirrer. At various time intervals samples were drawn with a Hamilton® Gastight™ syringe (500 µl). The samples were immediately frozen through submersion in liquid nitrogen to prevent further hydrolysis. After all the samples had been drawn they were thawed and the residual epoxide and formed diol extracted with ethyl acetate (250 µl). Following these steps the ethyl acetate was dried over anhydrous sodium sulphate and analysed by chiral GC.

2.3.2 The effect of impeller speed upon enzymatic stability

Frozen cells were thawed, centrifuged (3 500 g, 5 minutes), washed and resuspended as before. The suspension was transferred to the reactor and stirred at various impeller speeds for 4 hours (15 °C). Hereafter SO was added to a final concentration of 100 mM and the reaction monitored over time by drawing samples as before.

2.3.3 Variation of the cell/buffer ratio

Yeast cells were cultivated as before. During the harvesting of the cells, however, they were suspended in phosphate buffer (50 mM, pH 7.2) containing 10% glycerol in different cell/buffer ratios before being frozen. Thawed cells of each ratio were added to the reactor, together with 48 µL SO, to a final volume of 20 mL (20 mM). The reaction was monitored over time by drawing samples as before.

2.3.4 Repetitive batch operation

Yeast cells were cultivated as before. After thawing, the cells were centrifuged (3500 g, 5 minutes) washed with phosphate buffer (50 mM, pH 7.2) and resuspended in phosphate buffer containing 5% (w/v) HPB. The cell/buffer suspension (1:4 or 20%) was added to the reactor together with racemic SO (2.4 mL) to a final volume of 200 mL (100 mM). The reaction mixture was continuously stirred at an impeller speed of 400 rpm (15 °C). The reaction was monitored over time by drawing and analysing samples as before. Upon complete hydrolysis (>98% enantiomeric excess) of the (R)-enantiomer the entire volume was removed from the reactor and the remaining (S)-enantiomer removed by washing the cells using centrifugation as before. Following this the cells were resuspended and introduced back into the reactor together with racemic SO (100 mM). The reaction was monitored by drawing and analysing samples as before.

2.3.5 Micro-filtration after hydrolysis

Thawed cell suspensions (200 mL) were washed and resuspended as described in Section 2.3.4 before being transferred into the batch reactor together with SO (100 mM). After 10 hours of stirring at an impeller speed of 400 rpm the solid base of the reactor was replaced with a micro-filtration unit containing a hydrophilic Nylon membrane. Various positive nitrogen pressures were applied to the reactor (20, 40, 60, 80 and 100 kPa). Four 47 mm supported Nylon membranes (Osmonics) were evaluated for the micro-filtration of the reaction medium with pore sizes of 0.22, 0.45, 0.8 and 1.2 µm. Spherical glass beads (2 mm diameter) were evaluated as a filter aid to decrease fouling of the membrane by yeast cells.

3. RESULTS AND DISCUSSION

3.1 The effect of impeller speed on activity and selectivity

As with all bioreactors, the turbulent stresses within the reactor may affect the observed activity of an enzyme. This may affect the operation, productivity as well as profitability of these reactors [21]. To determine the effect of increased agitation upon this reaction, initial reaction rates were determined at incrementally increasing impeller speeds within the reactor. Initial substrate concentrations were set at 100 mM as this concentration has previously been shown to yield a maximum rate of hydrolysis (50 mM – 100 mM) without exhibiting substrate or product inhibition [22]. The initial reaction rates (V) were determined from the slope of the initial decrease in substrate concentration. These values, relative to the achieved maximum initial reaction rate (V_{max}), are presented in Figure 4.2.

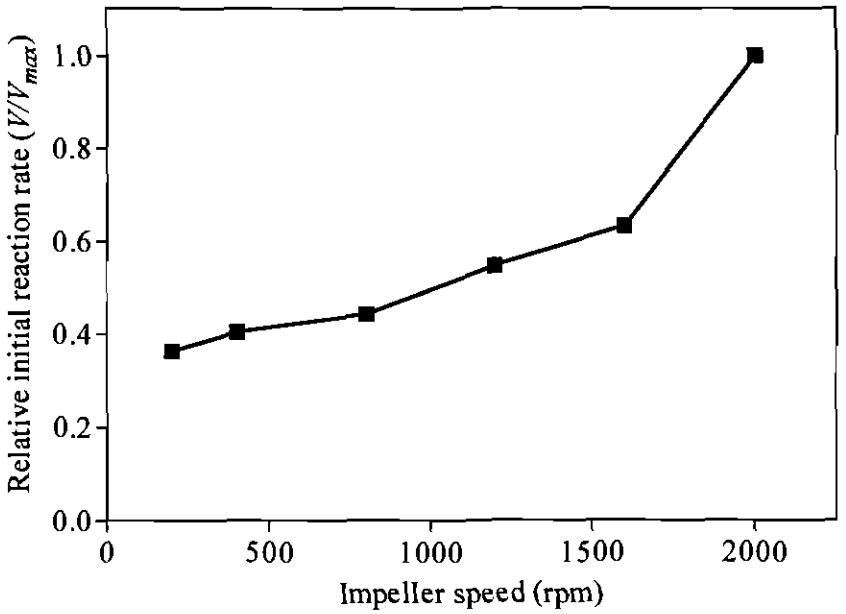

Figure 4.2 Reaction rates at various impeller speeds relative to the maximum reaction rate achieved (0.82 mM/min).

Increased impeller speeds led to a directly proportional increase in the observed initial reaction rates up to 1 600 rpm. Above this value a sharp increase in the initial reaction rate was observed. The reaction rate at 2000 rpm after 30 minutes (0.82 mM/min), for example, is 156% higher than the rate at 200 rpm (0.32 mM/min). This is probably due to an increased contact between the substrate and cells as a result of increased agitation as well as due to increased aeration. It should further be considered that, at the commencement of the reaction, 50% of the substrate is not in solution and, therefore, the sharp increase in reaction rate above 1 600 rpm could also be attributed to the emulsification of the substrate increasing the surface

area available for solubilisation and hence hydrolysis. From these results it seems that the highest impeller speed attainable would lead to the most advantageous reaction. It is, however, necessary to further investigate the effects of increased impeller speeds within a reactor. Previously it was established that increased temperatures increased activity but severely decreased the selectivity of the reaction [22]. Once again an increased reaction rate as a result of increased agitation was shown to negatively influence the selectivity of the reaction (Figure 4.3).

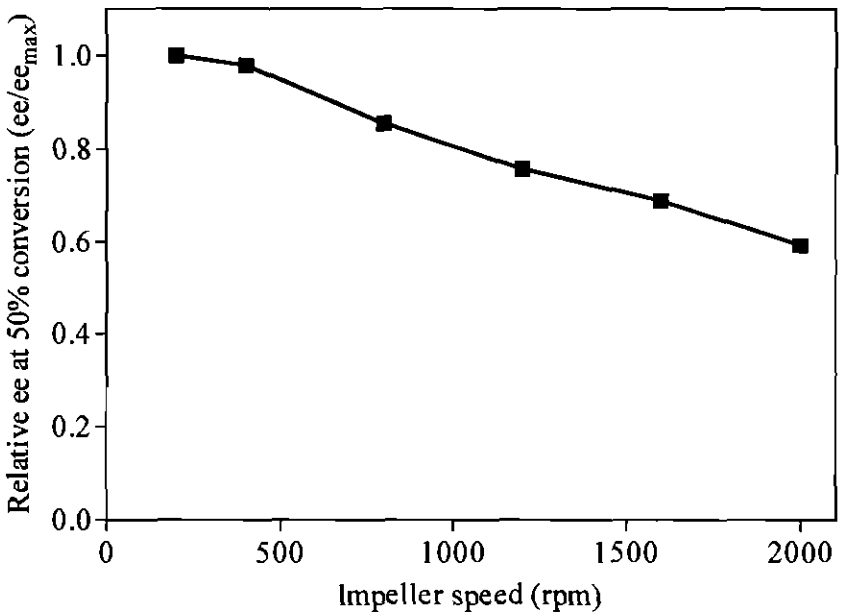

Figure 4.3 Enantiomeric excesses at 50% substrate conversion achieved using various impeller speeds relative to the maximum enantiomeric excess (*e.e.*) achieved.

Above 400 rpm the selectivity was found to be inversely proportional to the impeller speed, while the selectivity exhibited at 400 rpm was very similar to that at 200 rpm. As was the case with increased temperatures, the increase in the overall reaction rate could mainly be attributed to an increase in the reaction rate of the slow reacting S-enantiomer while the reaction rate of the fast reacting R-enantiomer remains largely unaffected (Figure 4.4). As a result, higher impeller speeds yielded lower *e.e.* values at the same degree of conversion and, therefore, a less selective reaction. Interestingly, at high impeller speeds such as 2000 rpm, almost no enantioselectivity was exhibited by the enzyme during the first 30 minutes of the reaction.

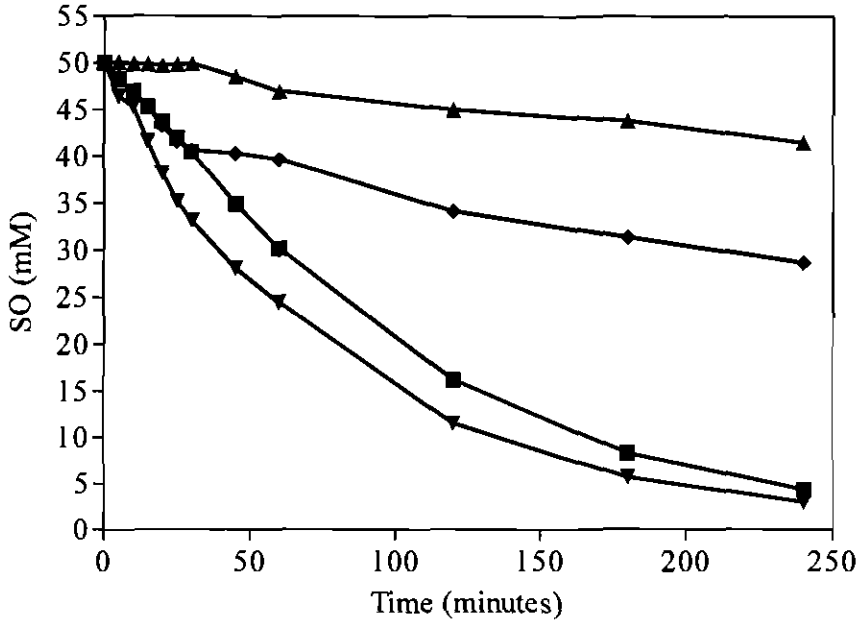

Figure 4.4 Hydrolysis of SO in a stirred tank reactor at an impeller speed of 200 rpm (R-SO: ▼, S-SO: ▲) vs. the hydrolysis at 2000 rpm (R-SO: ■ , S-SO: ◆)

Considering the effects of variation in the impeller speed within the reactor, 400 rpm was selected as the optimal impeller speed, yielding a faster initial reaction rate than at 200 rpm while maintaining high enantioselectivity.

3.2 The effect of impeller speed upon stability

Impeller speed may also affect the stability of the enzyme as a result of increased shear exerted upon the whole cells, possibly disrupting the cell walls and diminishing their previously established protective effect [22] against the environment. Kaya *et al.* [23] and Mukataka *et al.* [24] previously illustrated that the amount of shear and aeration experienced by an enzyme could potentially have negative effects upon the stability of the enzyme. To investigate the effect upon whole yeast cells they were incubated within the reaction at various impeller speeds before starting the reaction. After 4 hours of incubation a decrease in residual activity at high impeller speeds was observed, even though the effect was minimal (Figure 4.5). These results again illustrate the mechanical strength and protection offered by the whole yeast cells. Even after 4 hours at 2000 rpm inspection of the remaining cells under a light microscope showed minimal disruption of the cells and cell walls.

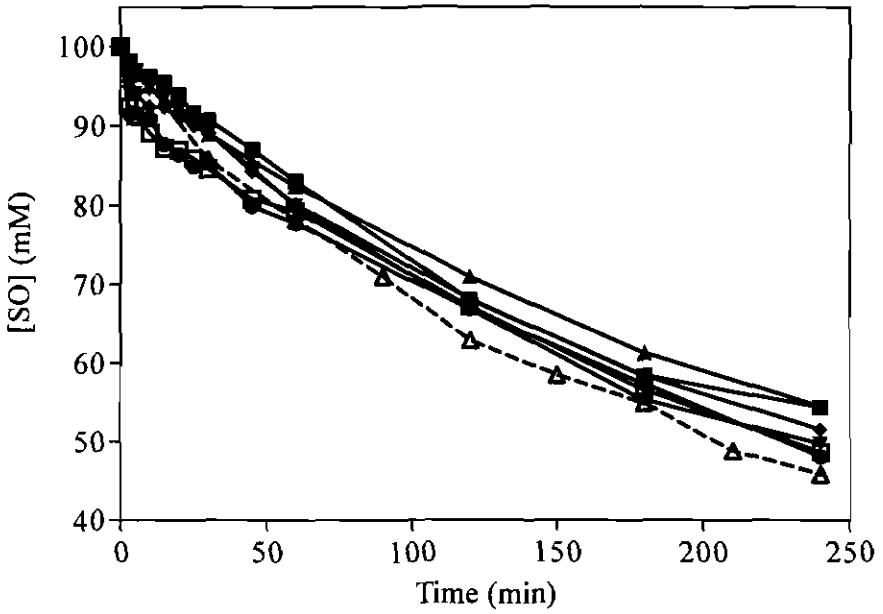

Figure 4.5 Hydrolysis of SO with whole cells from *R. glutinis* at an impeller speed of 400 rpm after four hour incubation periods at various impeller speeds (0 rpm:△ , 200 rpm:■, 400 rpm:▲, 800 rpm:▼, 1200 rpm:◆, 1600 rpm:●, 2000 rpm:□).

3.3 The effect of the cell/buffer ratio upon the reaction

Increasing the amount of cells, and therefore catalyst, available within the reaction may increase the reaction rate and possibly even the selectivity of the reaction [13]. An increased amount of catalyst, however, may not be the most effective approach. Monfort *et al.* [25] previously illustrated that 1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane can be enantioselectively hydrolysed (*e.e.*>99%) even when the concentration of catalyst is decreased by 75%, albeit at the expense of reaction time (increased by 500%). This increase may, however, be considered as being acceptable since the addition of more catalyst contributes significantly to the cost of an enzymatic reaction. In addition, increased catalyst concentration may also adversely affect the reaction as a result of increased viscosity (especially when utilising whole cells) as previously illustrated by Gong *et al.* [13].

To measure the productivity of a catalyst within a reaction, the productivity number (*PN*), may be calculated (Equation 4.1) [26].

$$PN = \frac{n_{prod}}{m_{dry}} \times t \qquad (4.1)$$

where n_{prod} is the amount of product formed (mol), m_{dry} is the amount of dry cell mass (g) and t is the time of the transformation(s). A high PN illustrates an efficient process that provides a high output per volume unit and time. The *PN* values obtained by using a range of cell/buffer ratio's as well as the initial reaction rates achieved are reported in Figure 4.6.

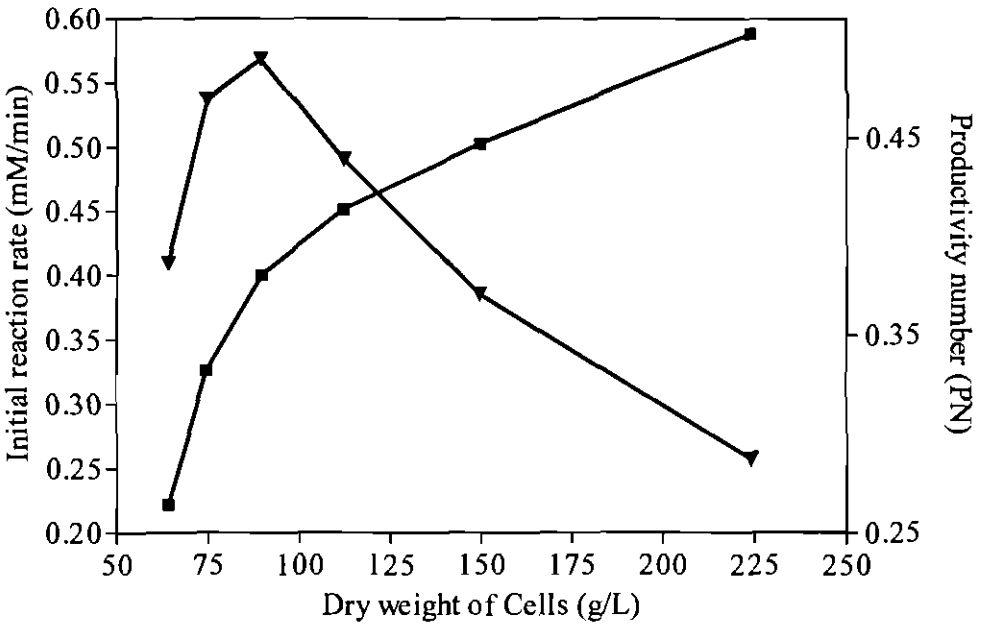

Figure 4.6 Initial reaction rates achieved with increasing amounts of cells (■) and the productivity number achieved with each cell/buffer ratio investigated (▼).

As expected a constant increase in the initial reaction rate was achieved with an increasing amount of cells available in the reaction. A much shorter reaction time would be required with an increased amount of cells to reach a product with 98% purity (as a result of the increased reaction rate), however, when the PN of the different amounts of cells is calculated, the highest investigated amount of catalyst looks less attractive. A compromise should therefore be reached between the required reaction time and the actual productivity of the catalyst. From Figure 4.6 it is evident that at 100 mM of SO a cell/buffer relationship of 1:4 or 20% (w/v) is most effective (89.5 g/L dry cell weight). The selectivity of the reaction was not significantly affected by the increase in the amount of cells (results not shown).

3.4 Repetitive batch operation

The possibility of re-using an enzyme within a reactor for catalysis was, amongst others, exemplified by Ishihara *et al.* [27]. They illustrated that, during the conversion of 1-acetoxy-2-alkanones to enantiopure 1-acetoxy-2-alkanols, a bakers' yeast cell free extract can be used repetitively (5 batches) with only a minimal decrease in activity (14% decrease).

Unfortunately, in this case, *R. glutinis* did not show the same capability. After the hydrolysis of 100 mM of epoxide in the stirred reactor (10 hours) the cells were removed, washed and re-used, the results are shown in Figure 4.7.

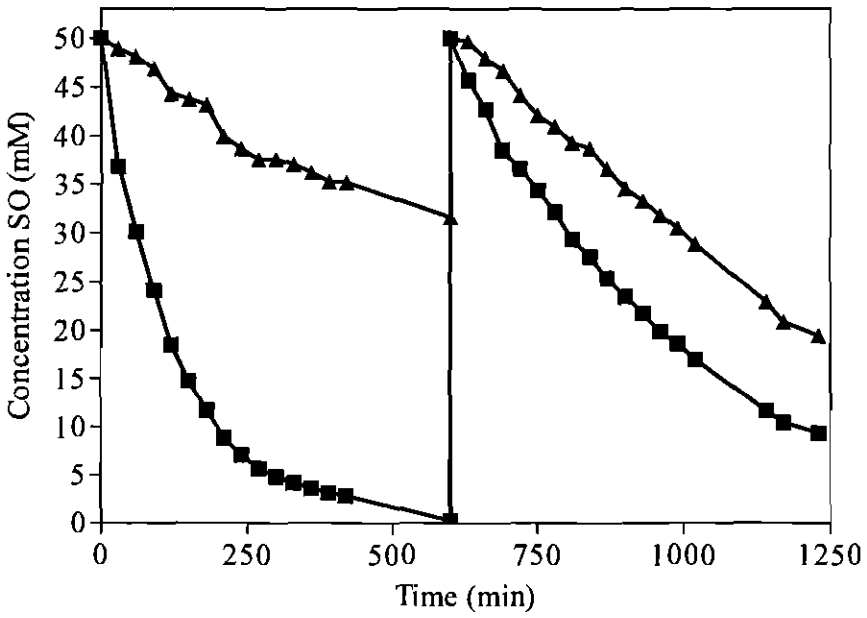

Figure 4.7 Hydrolysis of SO over time during repetitive batch operation (R-SO: ■, S-SO: ▲) in a stirred batch reactor.

The selectivity of the reaction decreased severely, yielding a product with an *e.e.* of only 35% after 630 minutes. This decrease in selectivity may be as a result of the washing procedure (centrifugation) or degeneration (and loss of selectivity) of the enzyme during the previous 10 hour reaction. The initial reaction rate achieved during the second batch (0.2 mM/min) was slightly higher than the final reaction rate of the first batch (0.11 mM/min), probably due to increased availability of substrate, but decreased over time to 0.11 mM/min. Considering these results, repetitive batch operation cannot be considered as a viable option to increase the productivity of this reaction without stabilisation of the enzyme. A potential solution to the observed decrease in activity is stabilisation through immobilization, an example of which was recently demonstrated for epoxide hydrolase from *Aspergillus niger* [28]. Immobilization of a recombinant enzyme allowed a repetitive batch reactor to be operated for seven cycles during the enantioselective hydrolysis of *para*-chlorostyrene oxide.

3.5 Micro-filtration after hydrolysis

The HPB in the reaction medium increases the solubility of SO to 50 mM at 15 °C, after complete hydrolysis of the fast reacting S-enantiomer, therefore, the theoretical maximum of

50 mM of the slow reacting R-enantiomer is in complete solution. This simplifies the separation of the reaction media and the products, involving the separation of a liquid and a solid, rather than an emulsion (two liquid phases) and a solid. A process such as micro-filtration [15] can now be applied to recycle the biocatalyst, followed by either liquid-liquid (Chapter 5) or solid phase extraction. Dead-end filtration of the reaction medium was, however, attempted with limited success. Under all the conditions investigated an almost immediate fouling of the utilised micro-filtration membrane was observed. An example is shown in Figure 4.8. Rapid fouling of the membrane was observed within the first 30 minutes of filtration after which the filtration rate stabilised at 0.3 ml/min. This fouling is probably mainly due to cake formation of the yeast cells, forming a second, less permeable membrane, but may also be due to particles, cells or biomolecules fouling the membrane pore structure [17]. These results correlate well with those reported by Krieg *et al.* [9] who illustrated an initial decrease in flow as a result of yeast cell fouling while investigating enantioselective hydrolysis in a continuous flow through membrane bioreactor.

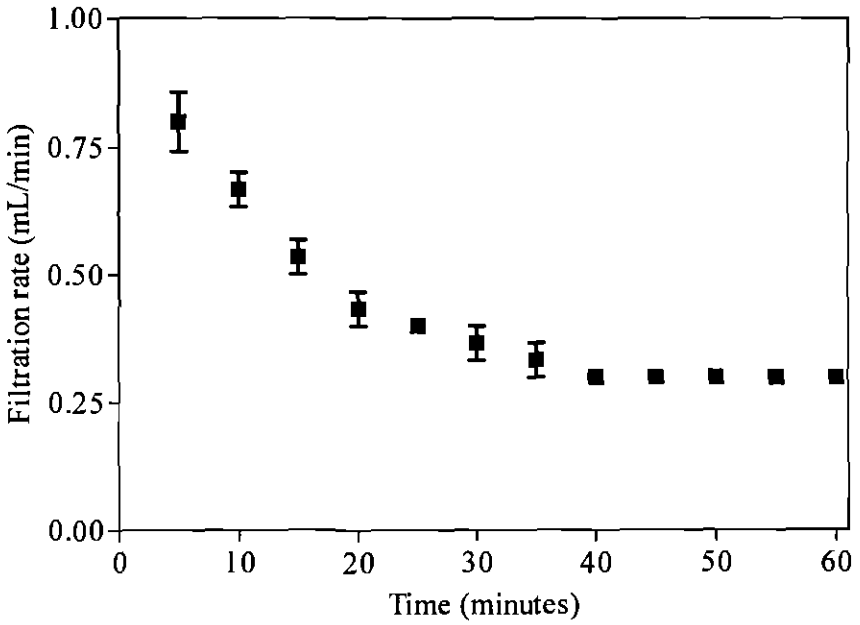

Figure 4.8 Fouling of a nylon membrane with a 0.8 μm pore size during dead-end filtration at a constant pressure of 80 kPa. Standard deviations are indicated by error bars.

Neither increased pressures nor the use of small spherical glass beads as a filtration aid could resolve this problem. Even though a filtration rate (0.5 ml/min) could be achieved and maintained using the glass beads (not shown), filtration of the entire reaction medium (200 ml) before the hydrolysis of all of the substrate could not be achieved. The fouling properties of whole yeast cells were previously illustrated by Le Roux *et al.* [29] during an investigation

into the application of chitosan as an antifouling agent. A further example, on a larger scale, was illustrated by Meindersma *et al.* [15] whereby aminopeptidase from *Pseudomonas putida* is used for the production of an enantiopure amino acid. Fouling of the filtration membrane again led to investigation into the use of filtration aids. The causes of, as well as techniques to decrease fouling experienced during microfiltration, have previously been discussed by Wakeman and Williams [30]. However, considering the fact that repetitive use of the yeast cells was not deemed as being efficient (Section 3.4), dead-end micro-filtration was not investigated further. In future experiments ultrafiltration, tangential flow or cross-flow filtration might be considered to solve the fouling phenomenon experienced.

4. CONCLUSIONS

This work illustrates the successful upscaling of a micro-reaction to a 100 mM batch reactor. Even though cells could not effectively be re-used, 1.54 g of (S)-styrene oxide (*e.e.*>98%) could be produced within 10 hours per 400 ml batch of cell suspension (9.2 $g \cdot L^{-1} \cdot day^{-1}$). This space time yield may be considered as being very low when compared to 2 850 $g \cdot L^{-1} \cdot day^{-1}$ as reported by Monfort *et al.* [25] for a similar reaction. It should, however, be considered that the use of whole cells as catalyst significantly decreases the costs involved in a biocatalytic reaction when compared to the use of purified enzymes as used in many other examples. Low impeller speeds led to slower reaction rates but concomitantly to a more selective reaction. A compromise between activity and selectivity was established to be 400 rpm while the optimal productivity was achieved with a cell/buffer ratio of 1:4 (20%). Although separation of the products and biocatalyst following the reaction could not be established by micro-filtration, future work may include stabilisation of the enzyme, allowing for its re-use, followed by different processes designed to overcome fouling.

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CHAPTER 5

Solvent facilitated selective extraction of terminal epoxides and their corresponding *vicinal* diols from an aqueous medium

ABSTRACT

Four *racemic* terminal epoxides were selected based on the fact that they had previously been shown to be candidates for hydrolytic kinetic resolution catalysed by epoxide hydrolase. Both the terminal epoxides and their corresponding *vicinal* diols were extracted from an aqueous medium (separately and from mixtures) to determine the suitability of different solvents for their extractive separation. Nine water immiscible organic solvents were selected for these extractions (based on their log P values). It was found that the epoxides were preferentially extracted from the aqueous phase irrespective of the solvent used. The best selectivity's were obtained with cyclohexane yielding selectivities of 100%, 95%, 69% and 32% for 1,2-epoxybutane, 1,2-epoxyoctane, styrene oxide and 1,2-epoxydodecane respectively. While there seems to be an increase in selectivity with increasing hydrophobicity of the solvent, no conclusive correlation could be established between the physico-chemical properties of the organic solvents, the substrates being extracted and the selectivity of the extraction. Generally, however, the properties of the diol dictated the outcome of the extraction. A low log P, and therefore, high water solubility of the diol led to efficient extraction of the epoxide from the mixture without removing the diol.

1. INTRODUCTION

One of the challenges facing the implementation of a bioprocess, is the downstream processing of the products of such a process, in other words, the purification of the products for further use. Several publications have, for example, considered the use of epoxide hydrolase (EH) enzymes to obtain either enantiopure epoxides, their corresponding diols or both [1,2]. During these reactions the EH enzyme selectively lowers the hydrolysis activation energy for one of the enantiomers, yielding, in most cases, an enantio-enriched mixture of the substrate and product. During the hydrolysis of styrene oxide (SO) catalysed by *Rhodotorula glutinis* [3] for example, racemic SO is converted to a mixture of (S)-SO and (R)-1-phenyl-1,2-ethanediol. Upon completion of the reaction, the residual substrate and formed product have to be separated and purified to be of significant use for example, in the pharmaceutical industry. This downstream processing contributes to the costs of the process. Cascaval *et al.* states that the separation cost of a biosynthesised product can be 20 - 60%, and in some cases up to 90% of the total manufacturing cost [4], which is one of the driving forces for the development of new extraction techniques. Only a few examples exist where researchers have been able to circumvent the separation altogether. Pedragosa-Moreau *et al.* illustrated the hydrolysis of SO by a combination of *Aspergillus niger* and *Beauveria sulferescens* [5]. The two enzymes have two different mechanisms yielding only one enantio-enriched product with a 100% yield. Another example is the hydrolysis of *meta*-chlorostyrene oxide catalyzed by *Solanum tuberosum* EH which yields only the corresponding (R)-diol after complete substrate conversion [6].

In some cases the *in situ* selective removal of the product is necessitated by factors such as product inhibition of the enzyme [7,8]. In other cases the removal may lead to an increase in the enantioselectivity and conversion rate of the reaction [9]. However, when none of these factors significantly influence the reaction, it is adequate to remove the biocatalyst and products by methods such as centrifugation [10] or filtration [11,12] (when enzyme recycling is important) after which the aforementioned products can be extracted from the supernatant. During this extraction the differences between the physical and chemical properties of the residual substrate and formed product, which are considerable in the case of terminal epoxides and their corresponding *vicinal* diols, allow the application of separation techniques such as gravitational column chromatography [11] and flash column chromatography [13]. Alternatively, the residual substrate and formed product may be extracted by organic solvents. Solvent extraction is defined as a method of separation based on the transfer of a solute from one solvent into another solvent (which is not, or only slightly, miscible with the first) when

the two solvents are brought into contact. The solute, being soluble in both phases will then partition in a definite proportion, which may be altered by changing specific parameters such as pH, solvents and ion pair formation [14]. By adjusting these chemical parameters, the residual substrate or formed product may in some cases, be extracted from the aqueous phase (selectively and completely) thus eliminating the need for further separation steps. After the preparative scale hydrolysis and non-selective extraction of the racemic epoxide 1-{2',3'-dihydrobenzo[b]furan-4'-yl}-1,2-oxirane by ethyl acetate for example, the product was dissolved in a mixture of heptane and ethyl acetate and repeatedly extracted with $Na_2B_4O_7$ in water. The resulting organic phase contained only epoxide while the aqueous phase contained only diol [15]. Another example is presented by Genzel *et al.* [16] who, after the hydrolysis of each of three investigated epoxides, extracted the residual epoxides with $CHCl_3$ and subsequently extracted the remaining diol with ethyl acetate.

The aim of this study was to explore the possible application of selective solvent extraction for the enantioselective hydrolysis of terminal epoxides catalysed by epoxide hydrolase from various origins. To this end the selective extraction of four terminal epoxides and their corresponding *vicinal* diols from an aqueous phase was investigated. Nine water immiscible solvents were selected and each of the epoxides and diols were extracted (both separately and from mixtures) and the resulting organic phases analysed. Finally, a correlation was sought between the physico-chemical properties of the solvents and solutes and their extraction potential.

2. MATERIALS AND METHODS

2.1 General

All epoxides and their corresponding diols as well as the organic solvents were purchased from Aldrich. The selected solvents were all of analytical grade and were used without further purification. Deionised water, obtained from a Milli-Q system (Millipore, Millford, MA), was used for all experiments.

2.2 Solvent extraction of substrates

An amount of epoxide, diol or a mixture of the two was added to deionised water to a final concentration of 10 mM and final volume of 1 ml in 2 ml micro-centrifuge tubes. One mL of the relevant organic solvent (ethyl acetate, 1-buatnol, dichloromethane, dichloroethane, di-iso-propylether, chloroform, toluene, carbon tetrachloride or cyclohexane) was added and the micro-centrifuge tube incubated at 15 °C (minimizing chemical hydrolysis of the epoxide) in

a shaking water bath (200 rpm) for 1 hour. Hereafter, a sample of the organic phase was taken, either from above or below (where organic solvents with higher densities than water were used) the aqueous phase and the amount of epoxide and diol determined by GC. All experiments were done in triplicate.

2.3 GC analysis

Because of the similar maximum absorbencies exhibited by the epoxides and their diols, a reversed phase polar column was used for gas chromatography (GC) analysis to evaluate concentrations. Calibrations were completed with each individual organic solvent containing either epoxide or diol. Analysis of all substrates was conducted on a Thermo-Finnigan Focus gas chromatograph equipped with FID and a Thermo-Electron AS1000 auto sampler. Both epoxides and diols were analysed with an Econocap capillary column (30 m, 0.32 mm). The following retention times (R_t) and temperatures (Temp) using H_2 as carrier gas were obtained for the 8 different substrates (Figure 5.1). **1**; Temp: 40 °C, R_t: 3.20 min, **2**; Temp: 110 °C, R_t: 1.70 min, **3**; Temp: 110 °C, R_t: 3.18 min, **4**; Temp: 160 °C, R_t: 4.29 min, **5**; Temp: 90 °C, R_t: 3.21 min, **6**; Temp: 150 °C, R_t: 1.55 min, 7; Temp: 140 °C, R_t: 2.90 min, **8**; Temp: 180 °C, R_t: 4.20 min.

1-4 **5-8**

1, 5 R = C_2H_5
2, 6 R = C_6H_5
3, 7 R = C_6H_{13}
4, 8 R = C_8H_{17}

Figure 5.1 Structures of the terminal epoxides (**1-4**) and *vicinal* diols investigated (**5-8**).

3. RESULTS AND DISCUSSION

To investigate the possibility of selectively extracting either the residual substrate (epoxide) or formed product (diol) after biocatalysed kinetic resolutions, four terminal epoxides were selected. All of the aforementioned epoxides (Figure 5.1) were previously enantioselectively hydrolysed by epoxide hydrolase enzymes from several sources [3,17,18,19], leading to the formation of enantiomerically enriched *vicinal* diols.

The extraction of a substrate from an aqueous phase is determined by the physico-chemical properties of both the substrate and the extracting solvent. While various methods can be employed to quantify and relate these properties, we used the log P value to compare and evaluate solvent and solute properties. Log P is the log of the distribution coefficient of the relevant compound between water and the immiscible organic solvent octanol when in neutral form. [20]. For comparative purposes the log P for each of the epoxides and their corresponding diols were calculated using appropriate software (ACD/Log P DB version 4.54, Advanced Chemical Development Inc., Toronto, Canada) and are reported in Table 5.1.

Table 5.1 Log P values for each of the terminal epoxides and *vicinal* diols investigated.

No.	Terminal epoxide	Log P	No.	*Vicinal* diol	Log P
1	1,2-Epoxybutane	0.66 ± 0.24	**5**	1,2-Butanediol	-0.81 ± 0.22
2	Styrene oxide	1.61 ± 0.28	**6**	1-Phenyl-1,2-ethane diol	0.04 ± 0.31
3	1,2-Epoxyoctane	2.78 ± 0.24	**7**	1,2-Octanediol	1.32 ± 0.22
4	1,2-Epoxydodecane	3.84 ± 0.24	**8**	1,2-Dodecanediol	2.38 ± 0.22

From the equation of log P (Equation 5.1),

$$log\,P = log\left(\frac{[X]_{Octanol}}{[X]_{Aqueous}}\right) \tag{5.1}$$

where [X] is the concentration (mol/dm^3) of the compound X in the octanol and water phases, it follows that a compound with a log P of 0 is as soluble in water as it is in octanol. A negative log P value denotes that the compound is more soluble in water than in octanol, while a positive value indicates that the compound is more soluble in octanol than in water. According to Table 1 compound **5** is more soluble in water than in octanol, while **3, 4** and **8** are almost insoluble. All the epoxides investigated are poorly soluble in water in the order **4** > **3** > **2** > **1**.

Each of the epoxides and diols under investigation were added to an aqueous phase, thereby simulating the conditions achieved after a biocatalytic reaction. Racemic substrates were used since the factors governing their extraction from aqueous phase are identical to those of the pure enantiomers. Hereafter they were extracted with each of the nine immiscible organic solvents (Table 5.2) for which log P values were also calculated.

Table 5.2 Log P, water solubility and electrical dipole moment values for the nine selected immiscible organic solvents. Log P values were calculated as before while all other values were obtained from literature [21].

Solvent	Log P	Solubility in Water (% m/v)	Electrical dipole moment (D)
Ethyl acetate	0.71 ± 0.20	8.08	1.78
1-Butanol	0.88 ± 0.18	7.40	1.77
Dichloromethane	1.19 ± 0.24	1.73	1.60
Dichloroethane	1.41 ± 0.23	0.50	1.80
Di-iso-propylether	1.68 ± 0.22	1.20	1.13
Chloroform	1.76 ± 0.30	0.80	1.04
Toluene	2.68 ± 0.17	0.06	0.37
Carbon tetrachloride	2.83 ± 0.41	0.07	0.00
Cyclohexane	3.39 ± 0.16	0.01	0.00

The solvents were chosen to cover a wide range in terms of log P, i.e. from 0.71 for ethyl acetate to 3.39 for cyclohexane. It is clear from Table 5.2 that the log P is closely related (inversely) to the physically determined dipole moments confirming that the log P gives a good indication of the polarity of the solvents and hence of the epoxides and diols.

Weijers previously illustrated the kinetic resolution of **1** using *Rhodotorula glutinis* [17]. An enantiomeric excess of the residual substrate ($e.e._s$) of >98% (yield: 16%) and an enantiomeric excess of the product **5** ($e.e._p$) of 25% was achieved. Following the completion of the hydrolysis reaction, **1** was extracted from the aqueous phase with ethyl acetate while saturation of the aqueous phase with NaCl facilitated the extraction of **5**.

To investigate the selective extraction of the epoxide, 10 mM of both **1** and **5** were added to an aqueous phase and extracted with each of the nine solvents. It was found that the separate extractions of the substrates from the aqueous phase yielded the same results as the extraction of the epoxide/diol mixture and hence only the combined extraction results were reported. The fraction of the solute that is extracted in a single step is given by Equation 5.2 [14].

$$\theta = \frac{C_O V_O}{C_O V_O + C_W V_W} \tag{5.2}$$

C_O and C_W represent the concentrations (mol/dm^3) in the organic and aqueous phase respectively, and V_O and V_W represent the phase volumes (dm^3). Since V_O *and* V_W are both equal to 1, Equation 5.2 simplifies to Equation 5.3.

$$\theta = \frac{C_O}{C_O + C_W} \tag{5.3}$$

The obtained fractions of the epoxide (θ_E) and diol (θ_D) are presented in Figure 2.

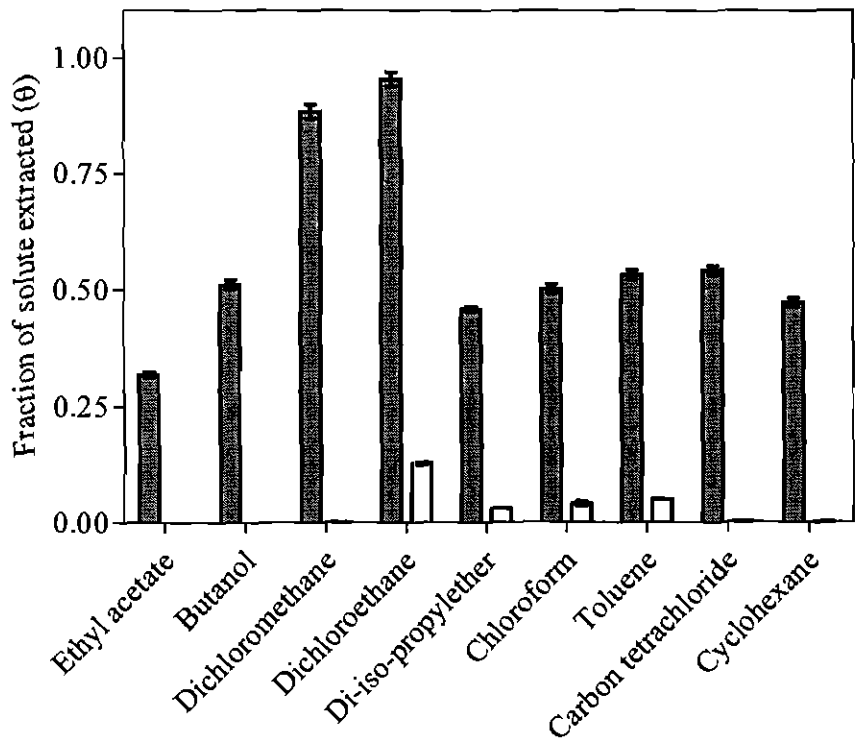

Figure 5.2 The fraction of **1** (filled bar) and **5** (unfilled bar) extracted by each of nine organic solvents from water.

Extraction of **1** and **5** showed that all the solvents selectively extracted the epoxide from the aqueous phase (Figure 5.2). There were five organic solvents (ethyl acetate, butanol, DCM, CCl_4 and cyclohexane) that selectively extracted the epoxide without extracting any diol. None of the aforementioned solvents did, however, extract 100% of the epoxide. The selectivity can be improved using two possible approaches, i.e. either by increasing the phase ratio or by using multiple extraction phases [14]. Either of these approaches, when applied after completion of resolution, would lead to an organic phase containing enantiopure epoxide

and an aqueous phase containing enantioenriched/enantiopure diol. Interestingly, none of the solvents had the ability to extract a significant amount of **5** and, therefore, extraction of this substrate would require additional steps. While initially there seems to be an increase in epoxide extraction with increasing log P of the solvent, the extraction suddenly drops at log P ≥ 1.68. It would thus seem that the solvent must have some polarity to be able to remove **1** effectively. Considering that **1** has a log P of 0.66 and hence is slightly polar itself, it does make sense that a slightly polar solvent would yield higher extraction efficiency than the non-polar cyclohexane for example. The low extraction of the diol is understandable considering its log P of -0.81, suggesting a highly polar diol which prefers to remain in the aqueous phase.

The kinetic resolution of **2** has also been enantioselectively catalysed by *R. glutinis* ($e.e._s$: >98%, yield: 30%) [22]. In this case both **2** and **6** were simultaneously extracted from the aqueous phase with ethyl acetate. During the current study most solvents were more effective removing **2** than **1**, with a decrease in efficiency in the solvent log P range between 0.88 and 1.68 (Figure 5.3).

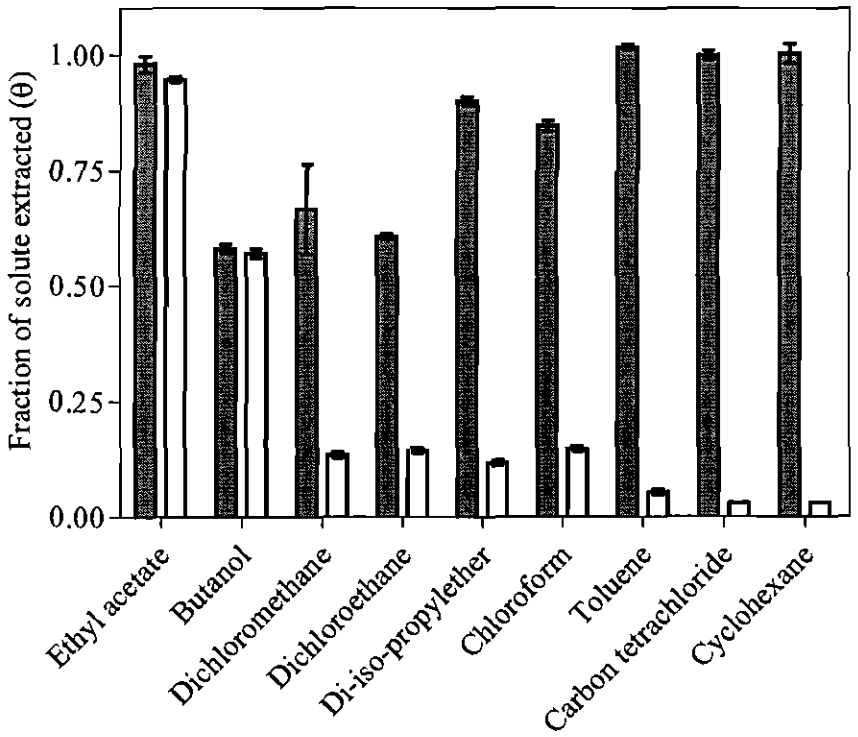

Figure 5.3 The fraction of **2** (filled bar) and **6** (unfilled bar) extracted by each of nine organic solvents from water.

Substrate **6** (log P = 0.04) is not as soluble in water as was the case with the previously investigated diol (log P = -0.81). This resulted in an increased extraction of the diol and hence a subsequent decrease in the selectivity of the extraction solvents. However, solvents with high log P values still preferentially extracted the epoxide mainly due to the decreased extraction of the diol. A definite trend was observed during these extractions. As the log P of the solvents increased, so the selectivity of the extraction increased. Both CCl_4 and cyclohexane selectively extracted the epoxide yielding a product with 95% purity. Simultaneous extraction of nearly 100% of both the epoxide and diol was observed with ethyl acetate.

Selective extraction of **3** and **7** was far less successful than the previous substrates investigated (Figure 5.4). Botes *et al.* [18] previously demonstrated that a strain of *Rhodosporidium araucariae* selectively catalyses the hydrolysis of **3** with an enantiomeric ratio (E) of >200 ($e.e._s$: >98%, yield: 49%, $e.e._p$: 87%, yield: 48%). Once again ethyl acetate was used to extract both **3** and **7** simultaneously from the aqueous phase.

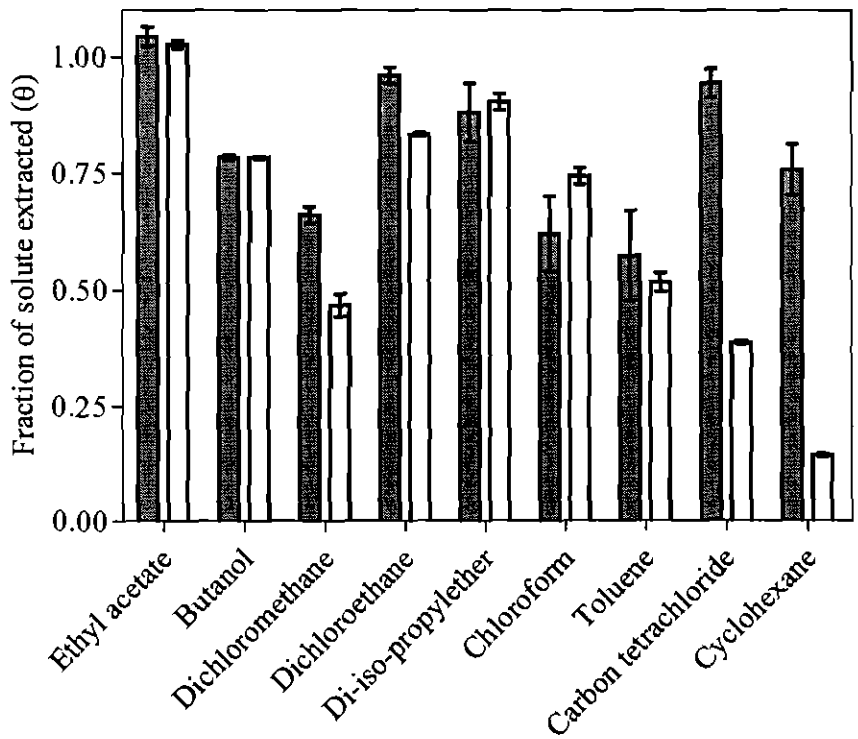

Figure 5.4 The fraction of **3** (filled bar) and **7** (unfilled bar) extracted by each of nine organic solvents from water.

The poor selectivity of the extraction can once again be attributed to the higher log P of the diol. It is interesting to note, however, that while the increase in log P of the diol (0.04 to 1.32) resulted in an increase in the fraction extracted, this tendency does not hold for the epoxide where no significant increase in the extraction had occurred in spite of a similar increase in log P (1.61 to 2.78). According to the trends observed during the previous two extractions, a solvent with a log P higher than that of cyclohexane may be more suitable. Cyclohexane, being the most successful selective extraction agent, yielded a product of 68% purity. Again ethyl acetate would be the solvent of choice for complete extraction of both substrate and product.

Selective extraction of **4** and **8** was not very successful (Figure 5.5). Botes *et al.* previously illustrated the enantioselective hydrolysis of **4** by *Rhodotorula aurantiaca* (E = 12). Substrates **4** and **8** were simultaneously extracted from an aqeous phase with ethyl acetate.

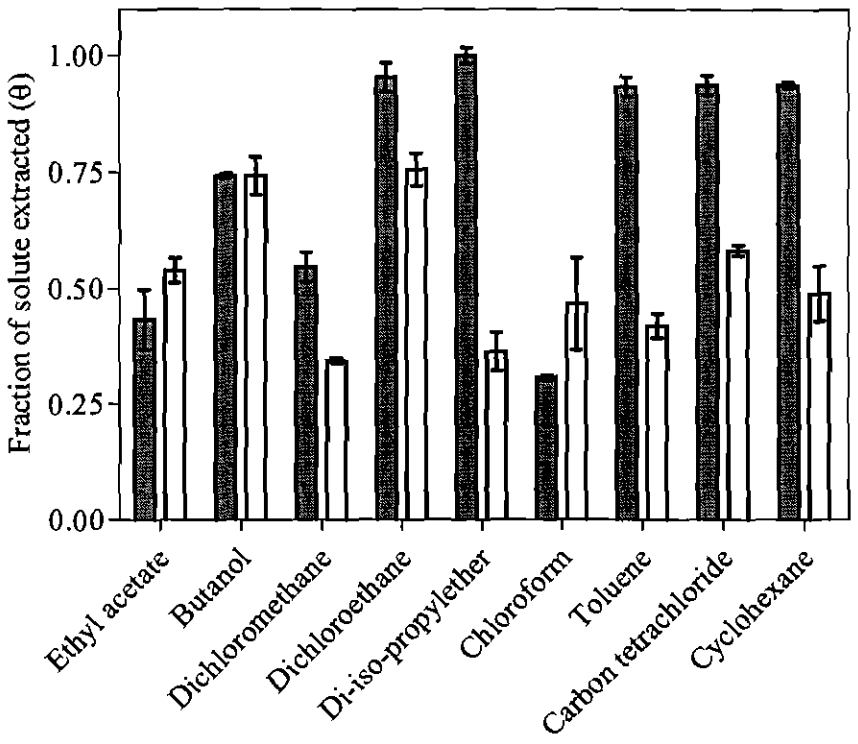

Figure 5.5 The fraction of **4** (filled bar) and **8** (unfilled bar) extracted by each of nine organic solvents from water.

Due to the high log P value of epoxide **4** the polar solvents ethyl acetate, 1-butanol and dichloromethane were not very effective in extracting the epoxide from the aqueous phase.

The higher log P value of diol **8** made selective extraction of the epoxide less viable. A certain amount of success was however achieved with solvents that themselves have high log P values. Again, other solvents with even higher log P values could be more successful. Multiple extraction phases or an increased phase ratio with solvents such as ethyl acetate or butanol would facilitate the extraction of all the epoxide and diol simultaneously.

To evaluate the success of each solvent to selectively extract the epoxide to the organic phase without extracting the diol, the latter may be considered as being a contaminant to the organic phase. The selectivity of the solvent can then be calculated using Equation 5.4,

$$Selectivity\ (\%) = \left(\theta_E - \theta_D \Big/ \theta_E + \theta_D \right) \times 100 \qquad (5.4)$$

where θ_E and θ_D refer to the fractions of the epoxide and diol extracted, respectively. The results obtained when performing this calculation are shown in Figure 5.6.

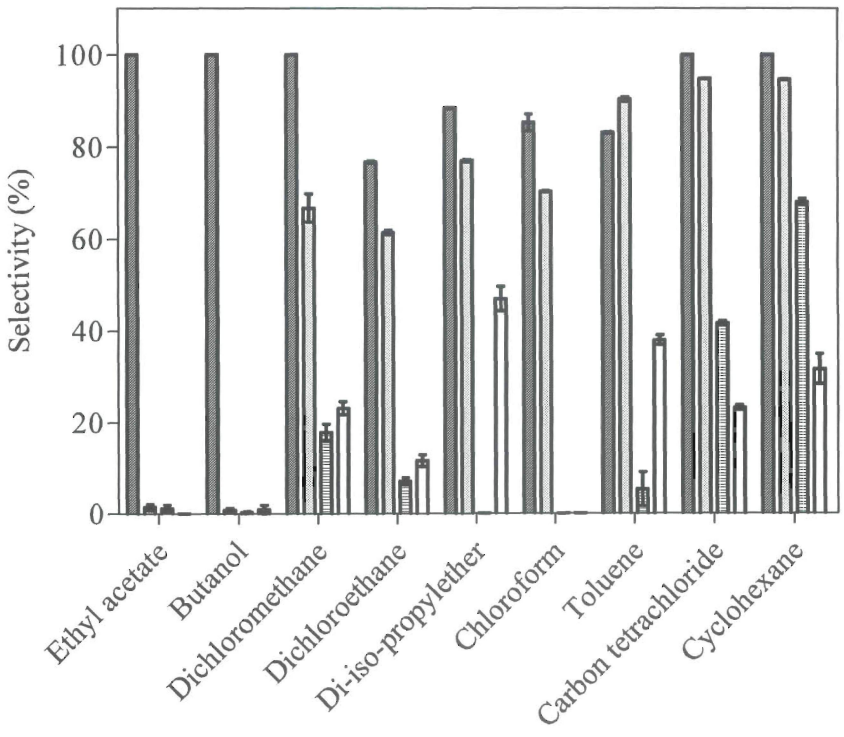

Figure 5.6 Extraction selectivity (%) exhibited by various organic solvents for the extraction of **1** (■), **2** (▨), **3** (▤) and **4** (□) from an aqueous phase.

Low selectivity occurs where θ_E and θ_D values are both high or both low. A high selectivity is usually the result of high θ_E values and low θ_D values. From Figure 5.6 it is clear that epoxides with low log P values such as **1** and **2** were most selectively extracted. It is interesting to note that the difference in log P values between the epoxides **1** - **4** and their corresponding diol **5** – **8** remains constant in the order of 1.47 (with the exception of **2** and **6** where the difference is 1.57, probably due to the effect of the phenyl group). This means that the difference in the log P value of the epoxide and its corresponding diol is less important than the absolute log P value of the substrates. In that sense it seems that an increase in the log P value of the substrate results in a decrease in the selectivity of the extraction. In terms of the solvents the highest selectivity was achieved with solvents that have high log P values such as CCl_4 and cyclohexane. The two aforementioned solvents also resulted in the best extraction for epoxides **3** and **4**, which however remained lower than the extraction attained for epoxide **1** and **2**. This can be explained by the fact that the log P values of the corresponding diols (**7** and **8**) are also higher, in other words, they are less soluble in water and have a tendency to be extracted by the organic solvent.

4. CONCLUSION

This work illustrated the possible use of selective solvent extraction to obtain the products of previously reported biological reactions. No direct correlation between the physico-chemical properties of the solvents and their selective extraction potential could however be found. Nevertheless, certain general trends were observed. In cases were both substrate and product are to be extracted simultaneously from an aqueous medium, solvents with low log P values, such as ethyl acetate or butanol, were found to be best suited. This allows for the extraction of the *vicinal* diols, which are generally far more water soluble than their corresponding epoxides. When the epoxide needs to be removed selectively, however, a solvent with a high log P is best suited. This methodology allowed for the extraction and concurrent purification of two of the four epoxides with >95% purity. Solvents with higher log P values than those investigated could possibly allow for the separation of epoxides and diols such as **4** and **8**. Finally, this work illustrates the importance of selecting the appropriate solvent when using solvent extraction with subsequent GC analysis for example, as a method to evaluate biological reactions conducted in aqueous media. Using an extracting solvent with a high log P value may yield erroneous results (unless precautions are taken) when evaluating the concentrations of both the substrate and the product.

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CHAPTER 6

Summary and concluding remarks

ABSTRACT

In this chapter the main findings of the work described in this thesis, which was aimed at investigating various factors that influence a biocatalytic reaction for the production of an enantiomerically pure compound, are summarised and the future prospects highlighted. The specific challenges and achievements experienced are revised while specific reference is made to the publications that have resulted from this study.

1. GENERAL

The previously established enantioselective activity of a strain of *Rhodotorula glutinis* (UOFS Y-0653) towards terminal epoxides [1] was used as a model reaction during all investigations. The factors investigated include the optimisation of the reaction (Chapter 2), the use of co-solvents and solubility enhancing additives (Chapter 3), the enantiopure production within a stirred batch reactor (Chapter 4) and solvent facilitated downstream selective product extraction (Chapter 5). It is therefore clear that various aspects of a full process stream for the production of a biocatalyzed resolved racemic terminal epoxide were elucidated in this thesis, i.e. from the actual biocatalytic resolution, the optimization of the reaction environment, the possible upscaling of the reaction and finally the separation of the epoxide and the formed *vicinal* diol.

2. OPTIMISATION OF A BIOCATALYTIC REACTION

In Chapter 2, it was shown that the pH as well as the reaction temperature influence the relative activity exhibited by the enzyme. High temperatures (30 °C - 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, however, 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 significantly longer reaction time was required to reach 98% *e.e.*. Furthermore, low temperatures were shown to markedly increase enzymatic stability. Considering the aforementioned temperature effects, a temperature of 15 °C was selected as the optimal temperature for this reaction. In an attempt to minimize the total costs involved in this reaction and simplify the scale-up thereof, it was shown that this process is not negatively influenced when exchanging the phosphate buffer reaction medium with unbuffered deionised 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% $e.e._s$, depending on the final process requirements. The results of this study were published in Enzyme and Microbial Technology in 2007 [2]

3. THE USE OF CO-SOLVENTS AND SOLUBILITY ENHANCING ADDITIVES

Having attained an optimized reaction, it was the purpose in Chapter 3 to improve the solubility of the substrate to further improve on the reaction efficiency. It was shown that cyclodextrins in general, but specifically hydroxypropyl-β-cyclodextrin (HPB), are useful compounds to increase the solubility of poorly soluble substrates during hydrolysis reactions catalyzed by enzymes. In terms of the influence on activity, the inhibition increased in the order dimethylsulfoxide (DMSO) < HPB < dimethylformamide (DMF). While the addition of HPB resulted in a higher decrease in activity and selectivity than observed in the presence of equal quantities of DMSO, much less HPB is required to attain a specific solubility. While this is beneficial since HPB is more expensive than DMF and DMSO, it also implies that for a specific solubility, HPB had the least inhibitory effect on the enzymatic reaction rate. Furthermore it was also shown that no pH monitoring and adjustment is required when using HPB, resulting in a simplification of the production process. Both enzymatic stability and enzymatic thermal stability were least affected by the addition of HPB compared to DMSO and DMF. This may be especially useful for the development of continuous reactors were the use of membranes necessitates completely soluble substrates and were the reuse of the catalyst (whole cells or purified enzyme) could become a process requirement. Finally, it should be mentioned that cyclodextrins are environmentally friendly and non-toxic compounds, unlike DMSO and DMF, which could be an additional benefit when selecting a biocatalysed reaction for a pharmaceutical application. The results of this study were published in Enzyme and Microbial Technology in 2007 [3]

4. THE USE OF A BATCH REACTOR FOR ENANTIOMER PRODUCTION

With an optimized reaction, the next step was to investigate the whether this reaction is suited for upscaling. This was illustrated in Chapter 4 where the micro-reaction used previously was scaled up to a 100 mM batch reactor. The achieved space time yield of 9.2 $g \cdot L^{-1} \cdot day^{-1}$ may be considered as being low when compared to 2 850 $g \cdot L^{-1} \cdot day^{-1}$ as reported by Monfort *et al.* [4] for a similar reaction. It should, however, be considered that the use of whole cells as catalyst significantly decreases the costs involved in a biocatalytic reaction when compared to the use of purified enzymes as used in many other examples. Low impeller speeds led to

slower reaction rates but concomitantly to a more selective reaction. A compromise between activity and selectivity was obtained at 400 rpm while the optimal productivity was achieved with a cell/buffer ratio of 1:4 (20%). Separation of the products and biocatalyst following the reaction could not be established by micro-filtration as a result of severe fouling caused by the whole yeast cells. Even though cells could not effectively be re-used, 1.54 g of (S)-styrene oxide (*e.e.*>98%) could be produced within 10 hours per 400 ml batch of cell suspension.

5. SOLVENT FACILITATED SELECTIVE PRODUCT EXTRACTION

In the last step, i.e. after the completion of the reaction, it is important to separate the residual epoxide and formed *vicinal* diol in order to obtain the purified compound required. The possible use of selective solvent extraction to obtain the products of previously reported biological reactions was investigated in Chapter 5. No direct correlation between the physico-chemical properties of the solvents and their selective extraction potential could be found. Nevertheless, certain general trends were observed. In cases were both substrate and product are to be extracted simultaneously from an aqueous medium, solvents with low logP values, such as ethyl acetate or butanol, were best suited. This allows for the extraction of the *vicinal* diols, which are generally far more water soluble than their corresponding epoxides. When, on the other hand, the epoxide has to be removed selectively, a solvent with a high logP is best suited. This methodology allowed for the extraction and concurrent purification of two of the four epoxides with >95% purity. Solvents with higher logP values than those investigated could possibly allow for the separation of epoxides and diols such as 1,2-epoxydodecane and 1,2-dodecanediol. Finally, this work illustrated the importance of selecting the appropriate solvent when using solvent extraction with subsequent GC analysis for example, as a method to evaluate biological reactions conducted in aqueous media. Using an extracting solvent with a high logP value may yield erroneous results (unless precautions are taken) when evaluating the concentrations of both the substrate and the product.

6. SUMMARY

During this study various factors which influence the development of a biocatalytic process were investigated, from the initial optimisation of the process, its scale-up to a bench-scale process through to the downstream processing of the products. Two international scientific publications followed from this work, the first of which illustrates the optimisation of a biocatalytic process and explains the effect of environmental considerations such as temperature, pH and substrate concentration [2] while the second illustrates the use of cyclodextrins, rather than organic solvents, to increase the aqueous solubility of the generally

insoluble substrates [3]. The scale-up studies and studies on the downstream processing of the residual substrate and products currently remain unpublished.

This work may, in future, be used as a foundation for studies into the optimisation of other biocatalytic processes with the goal of establishing certain trends within families of biocatalysts or groups of substrates. Most markedly, however, it may lead to the development of environmentally friendly processes using only easily renewable whole yeast cells as biocatalyst. With pure water as reaction medium, minimum addition of energy to regulate low temperatures and the additional of environmentally benign cyclodextrins, even the most insoluble substrates may undergo enantioselective hydrolysis, thereby allowing access to their enantiopure forms. Even though the recovery and re-use of the biocatalyst was not as successful as was initially hoped, the fact that this process may be scaled to a bench-scale reaction opens avenues for further investigations into the stabilisation and recovery of the biocatalyst with the final goal of setting up an economically feasible and environmentally friendly industrial process. Various approaches may be followed to separate the residual substrate and produced product after the completion of a biocatalytic process. One of these, known as solvent facilitated selective extraction, was shown to be effective in certain cases depending on the properties of the substrate, product and extraction solvent. The fact that substrate and product may be easily separated in a single step based on differences in the chemical properties was, however, clearly shown and opens the door to further studies into the application of other technologies to achieve this goal and to develop the final step in an industrial process.

7. FUTURE PROSPECTS AND CHALLENGES

- Although various optimal conditions have been established during this work for the enantioselective hydrolysis of styrene oxide by *R. Glutinis*, the question of whether these conditions would also be optimal for most other reactions catalysed by whole yeast cells remains unanswered. Using the established conditions various other reactions should be investigated to determine whether the effects observed during this study are applicable only to *R. Glutinis* or to a far greater range of organisms that exhibit epoxide hydrolase activity.

- To further elucidate the decrease in the enantiomeric excess of the substrate in the presence of HPB, further work should include determining substrate-enzyme complexation energies using pure styrene oxide enantiomers in the

absence and presence of HPB. An alternative approach would be to compare various cyclodextrins (CDs) and their derivatives to one another. The aim of this would not only be to establish the best CD for this reaction but also to clarify the effect of CD structure upon the activity, selectivity and stability of the enzyme.

- Within the reactor two main obstacles were observed during scale-up. Firstly the enzyme, although very stable to immediate changes in its environment, did not maintain its selectivity for a period long enough to allow for re-use. In addition the process of micro-filtration was unsuccessful as a result of fouling and the time necessary to filter the reactor contents. Future work may, therefore, include stabilisation or immobilisation of the enzyme, allowing for its re-use, followed by different processes designed to overcome fouling and decrease the time required to separate the cells and reaction media.

- A study involving a far greater number of solvents and substrates might potentially result in a better correlation between the properties of the substrates to be extracted and the solvent used when using solvent facilitated selective extraction. Future work may include this as well as comparing the effectiveness and costs of this method to preparative chromatographic methods of purification such as flash chromatography, solid phase extraction, size exclusion chromatography and ion exchange chromatography.

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