OPTIMISATION OF CONDITIONS FOR THE RESOLUTION OF 1,2-EPOXYOCTANE IN A BIOREACTOR

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B. Pharm

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Summary

Due to recent legislation requiring the determining of the pharmacokinetic effect of both enantiomers separately, of any new racemic drug before commercialisation, much research is done to improve and optimise methods for obtaining chirally pure compounds important for the pharmaceutical industry, for example epoxide precursors.

To date most experiments regarding the biocatalytic chiral separation of 1,2-epoxyoctane has been done in batch processes. The aim of this study was to optimise the enantioselective hydrolysis of 1,2-epoxyoctane by *Rhodosporidium toruloides* in both a batch and continuous process. The batch process was optimised in terms of stir speed, biomass (cell) concentration and reaction time, while the flow-through reactor (continuous process) was optimised with regards to the flow rate as a function of the pressure and the amount of chitosan and biomass in the reactor.

Initial inconsistencies of epoxide concentrations in preliminary studies were found to be due to adsorption by reaction and sampling vessels, and the lower than expected solubility of 1,2-epoxyoctane (3.85 mM instead of 6 mM as reported by previous investigators).

The results from the batch process suggest that as the reaction time increases, the % ee_{epox} increases initially, but decreases after 40 minutes. Optimum yield in terms of % ee_{epox} were obtained at medium stir speed (400 rpm) and biomass (cell) concentration (13 %). Below these values the % ee_{epox} increases with an increase in stir speed and/or biomass concentration. Above these values however, the increased stir speed and/or biomass concentration causes abrasion between cells, which negatively affects the % ee_{epox}. The % ee_{diol} reached a steady state after 10 minutes, and the effect of the different operating conditions on % ee_{diol} was negligible.

In the flow-through reactor chitosan was used as a spacer material (antifouling agent) to help decrease the fouling due to biomass deposition. The use of chitosan as a spacer ensured higher and stabilised flow rates for extended periods of time. In initial studies 0.5 g chitosan increased the flow rate by 34 % with a resistance removal of 25 %. For 1 g chitosan these values were 130 % flow increase and 57 % resistance removal. The flow rate was optimised in relation to the chitosan amount, biomass (cell) amount and pressure. The maximum flow rate was obtained at a pressure of 40 kPa, using the minimum amount of cells (0.4 g) and a maximum amount of chitosan (1.6 g).

Keywords: Biocatalysis, 1,2-epoxyoctane, process optimisation, chitosan.

Opsomming

As gevolg van nuwe wetgewing wat ingestel is en wat bepaal dat die farmakokinetiese effek van beide enantiomere afsonderlik bepaal moet word voordat nuwe rasemiese geneesmiddels gekommersialiseer kan word, word baie navorsing gedoen om metodes vir die verkryging van opties suiwer chirale verbindings, vanaf bv. epoksied voorlopers te verbeter en te optimiseer. Sulke metodes vind belangrike toepassing in die farmaseutiese industrie.

Tot op hede is die meeste eksperimente rakende die biokatalitiese chirale skeiding van 1,2-epoksie-oktaan as lotprosesse gedoen. Die doel van hierdie studie was om die enantioselektiewe hidrolise van 1,2-epoksie-oktaan deur *Rhodosporidium toruloides* in beide lot- en kontinue prosesse te optimiseer. Die lotproses is geoptimiseer in terme van roerspoed, biomassa- (sel) konsentrasie en reaksietyd, terwyl die deurvloeireaktor (kontinue proses) geoptimiseer is met betrekking tot die vloeitempo as 'n funksie van die druk en die hoeveelheid kitosaan en biomassa in die reaktor.

Daar is gevind dat aanvanklike variasies in epoksiedkonsentrasies in voorlopige studies veroorsaak is deur adsorpsie aan die houers, asook die laer as verwagte oplosbaarheid van 1,2-epoksie-oktaan (3.85 mM in stede van 6 mM soos gerapporteer deur vorige navorsers).

Die resultate van die lotproses suggereer dat 'n toename in die reaksietyd die % ee_{epoks} aanvanklik laat styg, maar dat 'n daling na 40 minute plaasvind. Medium roerspoed (400 rpm) en biomassa- (sel) konsentrasie (13 %) het 'n optimum opbrengs in terme van die % ee_{epoks} gelewer. Onder hierdie waardes het 'n toename in roerspoed en/of biomassakonsentrasie tot hoër % ee_{epoks} gelei. Bo hierdie waardes het 'n toename in roerspoed en/of biomassa-konsentrasie wrywing tussen die selle veroorsaak wat die % ee_{epox} negatief beïnvloed. Na 10 minute het die % ee_{diol} 'n bestendige toestand bereik en die effek van die verskillende operasionele kondisies op % ee_{diol} is onbeduidend.

In die deurvloeireaktor is kitosaan as spasieerder (teenverstoppingsmiddel) gebruik om sodoende die verstopping, toegeskryf aan biomassaneerslag te verminder. Die gebruik van kitosaan as spasieerder het hoër en stabiele vloeitempo's vir langer tydsperiodes verseker. In aanvanklike studies met 0.5 g kitosaan het die vloeitempo met 34 % toegeneem en weerstandsverwydering van 25 % is verkry. Die waardes verkry met 1 g kitosaan was 130 % toename in vloei en 57 % weerstandsverwydering onderskeidelik. Die vloeitempo is geoptimiseer relatief tot die hoeveelheid kitosaan, hoeveelheid biomassa (selle) en druk. Die

maksimum vloeitempo is verkry by 'n druk van 40 kPa deur gebruik te maak van die minimum hoeveelheid selle (0.4 g) en 'n maksimum hoeveelheid kitosaan (1.6 g).

Sleutelwoorde: Biokatalise, 1,2-epoksie-oktaan, prosesoptimisering, kitosaan.

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CHAPTER 1

Introduction

1. LITERATURE

Enantiomers are molecules that as non-superimposable mirror images of one another are almost identical, except that one physical (rotation of polarised light) and one chemical (reaction with optically active compound) property differs. If the two enantiomers are in a 50:50 ratio, it is called a racemic mixture. Due to their similarity, enantiomers are very difficult to separate and thus it is very common to find drugs that are sold as racemic mixtures. Enantioselectivity, however, plays an important role in the pharmacodynamics and kinetics of drugs. This means that at least half of a racemic drug could be inactive, or even cause side effects [1].

One of the approaches that can be used for the separation of enantiomers is by enzymatic resolution [2, 3]. Enzymes, being chiral, in many instances react almost exclusively with one of the two enantiomers. The two resulting compounds, i.e. the enantiomerically pure product that is formed and the remaining enantiomer, can hence be separated easily. In this study the yeast, *Rhodosporidium toruloides,* and the terminal epoxide 1,2-epoxyoctane, was used as enzymatic catalyst and racemic substrate, respectively.

Epoxides are useful as intermediates for various organic syntheses [4, 5]. They are highly reactive, because of their strained three membered ring structure and electronic polarisation and react easily, particularly with nucleophiles. Enantiopure epoxides can thus be used as intermediates for a range of reactions, including the synthesis of compounds that are used in pharmaceutics, food products and agrochemicals [6, 7]. Comprehensive research efforts have been directed towards the synthesis of optically pure epoxides. Enzymatic resolution (biological synthesis) can be used to convert readily available and inexpensive racemic mixtures of epoxides into enantiopure epoxides.

Some of the operating conditions (which include temperature, pH, substrate concentration and ions) for the specific yeast and substrate combination in a batch process have been optimised previously [8]. Further optimisation was therefore deemed necessary in terms of stir rate and cell (biomass) concentration. Most of the research done to date has been performed in a batch reactor setup. It was decided to also try and optimise a flow-through reactor (continuous process) that have been used previously [8, 9], in terms of flow rate and the enantiomeric excess of the substrate and product.

The current (flow-through) reactor design is basically a two-step process reactor, which means that the reaction is done in the reactor, and the product and remaining enantiomer, have to be

separated in a second, subsequent step. A membrane was hence used to retain the cells in the reaction chamber, while allowing the permeation of the unreacted substrate as well as the converted product.

The occurrence of fouling is a major disadvantage for all membrane processes [10]. Fouling reduces productivity, shortens the membrane life and impairs the capabilities of the membrane. Usually spacers or antifouling aids are used to help reduce the effects of fouling. The spacers most commonly used are Kieselguhr (or diatomaceous earth) and perlite, but both have problems and limitations in terms of being health hazards and having problems with waste disposal [11, 12]. In this study the possible use of chitosan as a spacer was investigated. Chitosan is inert, chemically stable, non-toxic for humans and the environment, and inexpensive.

2. AIM AND OBJECTIVES

The aim of this study was to optimise the operating conditions for the stereoselective hydrolysis of 1,2-epoxyoctane by *Rhodosporidium toruloides*.

The first objective was to optimise the reaction conditions in a batch reactor process. This was done by investigating the influence of cell concentration and stir speed on the enantiomeric excess of both the substrate and the product. The second objective was to optimise the flow rate in a flow-through reactor. It was decided to test the antifouling capabilities of chitosan in this reactor. Attempts were made to optimise and stabilise the flow rate by using chitosan as a spacer. The effect that the higher and stabilised flow rate might have on the reaction, was also investigated.

3. OUTLINE OF THIS STUDY

In Chapter 2, a literature review is presented to compare studies that have been done previously, and also to show what still needs to be done in this specific field of study. Four main areas of interest are presented in the literature study. Firstly, the importance of racemates and enantiomers in general is discussed. Secondly, the existing methods for enantiomer separation, and in particular the separation and possible uses of epoxides are examined. Thirdly, hydrolase

enzymes are investigated according to their sources in nature. Lastly, a classification of the available reactors and their various advantages and drawbacks is given.

During initial studies, continual inconsistencies in terms of epoxide concentration were observed. In Chapter 3 experimental work is presented aimed at giving possible explanations for these inconsistencies. The various possible causes that are reported on include extraction method efficiency, chemical degradation, evaporation and adsorption to reaction and sampling containers. In a second section, an in depth review is given on previously found information regarding optimum conditions for the reaction done with *Rhodosporidium toruloides* and 1,2-epoxyoctane. As a further contribution to the optimisation of this reaction, the effect of stir rate and cell concentration on the enantiomeric excess of the substrate and product is presented.

In Chapter 4 results on the further optimisation of a continuous membrane reactor process are presented. A flow-through reactor was used, with the reaction chamber containing cells connected to the bottom of the feed tank. The effect of fouling by the biomass (on the membrane) on flow rate was investigated and is discussed in this chapter. The efficiency of chitosan as a spacer (antifouling agent) to help optimise (i.e. control) and stabilise the flow rate in the reactor is shown.

In Chapter 5 a discussion and evaluation of the research done, and how the different systems (batch and continuous) compare to each other, is presented. A summary, some recommendations and conclusions are included for further references.

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CHAPTER 2

Literature review

This project was aimed at the optimisation of conditions for the chiral resolution of 1,2-epoxyoctane in both batch and flow through reactors. In this chapter there is looked at enantiomers, and the available separation methods of racemates. More specifically, epoxides and epoxide hydrolase enzymes are examined. To try and simplify all the various types of reactors that exist, a classification is made of the types most commonly used.

1. ENANTIOMERS AND THE RESOLUTION OF RACEMATES

Enantiomers are molecules that are non-superimposable mirror images that are identical, in terms of their physical properties, except that the direction of rotation of polarised light differs, and in their chemical properties, except that they react differently to optically active reagents [1].

Enantioselectivity plays a role in the pharmacokinetics of drugs, which includes adsorption, distribution, metabolism and excretion, as well as the pharmacodynamics of drugs, i.e. the interaction of the drug with the receptor [1]. It is, however, quite common for mixtures of enantiomers (if in a 50:50 ratio, then these mixtures are called racemates) to be marketed as a single drug, even though at least half of the mixture not only may be inactive for the desired biological activity, but may be responsible for various side effects [1 – 3].
The following possibilities exist:

- The enantiomers can be equipotent.
- The enantiomers can have the same type of activity, but with different potencies.
- One enantiomer can have no effect at all. In this case the enantiomer with the desired effect is called the eutomer, while the other enantiomer is called the distomer.
- Some enantiomers even cause adverse or opposing effects, for example picenadol.
- It is also possible that one enantiomer has a secondary function, for example propoxyphene.
- Finally, the two enantiomers can have different therapeutic activities.

In general, about 25 % of all drugs are sold as racemic mixtures. The unwanted isomers are not removed, since it is often quite expensive to separate the enantiomeric impurities. There are two ways of eliminating the unwanted enantiomeric substances. This can be done by synthesising pure chiral products, or by separating the impurities from racemic mixtures.

The synthesis of enantiomerically pure compounds, especially pharmaceuticals in which biological activity resides in only one of the enantiomers, has become important research areas

in organic chemistry [4]. It is important to try and find better, or more economically viable ways, to either separate the racemates, or to synthesise pure chiral products.

There are various ways to achieve the resolution of racemates.

1.1. MECHANICAL SEPARATION

This process usually necessitates a recrystallisation process, followed by a mechanical separation. Thus only compounds in which the enantiomers crystallise separately can be separated in this way. It is also time-consuming and is usually not feasible for use on large scale [5, 6].

1.2. CHROMATOGRAPHIC RESOLUTION

Resolution can be achieved with liquid or gas chromatography. This method is based on the use of chiral packing material or chiral mobile phase, which causes different retention times for the different enantiomers [5, 6].

1.3. ENZYMATIC RESOLUTION

Enzymes have been found to be chiral and extremely selective in their reaction with enantiomers. Frequently they react almost exclusively with only one of the enantiomers. The two different compounds (the remaining enantiomer and the formed product) can be separated more easily [5, 6].

1.4. FORMING DIASTEREOMERS

When a racemate reacts with an optically pure compound a mixture of diastereomers is formed. These have different physical and chemical properties and can be separated more easily [5, 6].

1.5. SYNTHETIC CHIRAL MEMBRANES

Membranes have been used as selective permeative barriers. The chiral membranes preferentially let through only one of the enantiomers. This results in enrichment of one of the enantiomers. Figure 2-1 illustrates the basic principle on which this type of membrane works.

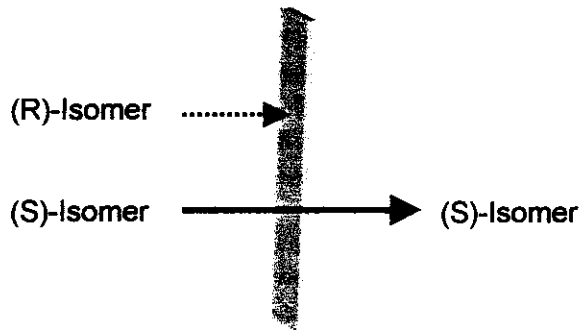

Figure 2-1. Enantioselective membranes that preferentially transport one of the two isomers of a racemic mixture [7].

2. EPOXIDES

The epoxide functional group is a very useful intermediate in organic synthesis. Due to their electronic polarisation and the strain of the three membered ring structure, epoxides are highly reactive molecules, which react readily with a number of reagents, in particular nucleophiles.

Figure 2-2 and Figure 2-3 show some of the different types of reactions that epoxides can undergo. Enantiopure epoxides are versatile chiral building blocks in organic synthesis and can be used as key intermediates in the synthesis of optically pure bioactive compounds like pharmaceuticals and agrochemicals. Examples of these are leukotriene, GABOB (γ-amino-β-hydroxybutyric acid) and products with biological activity. Lately even some steroids, antibiotic compounds, β-blockers and even HIV protease inhibitors have been produced from optically pure epoxides. Therefore extensive research efforts have been directed towards the chemical synthesis of optically active epoxides. Various chemical and biological production methods have been reviewed [4, 8 – 14].

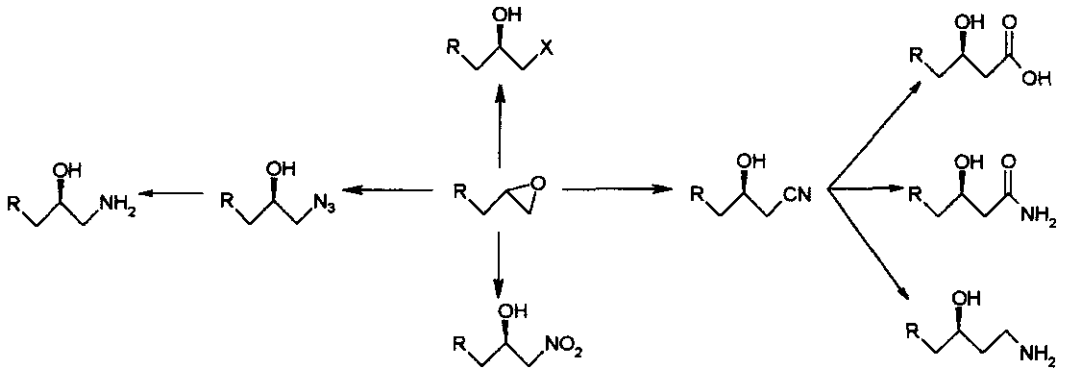

Figure 2-2. Chiral selective transformation from terminal epoxides [13].

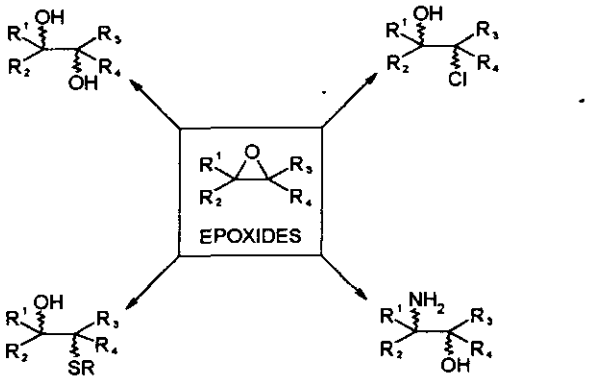

Figure 2-3. Ring opening reactions facilitated by nucleophilic attack of halides, carbon, nitrogen, oxygen, sulphur or amines on epoxides [8, 13].

There are two main methods of synthesising chiral epoxides, although the second is actually a separation of racemates. Thus, chiral epoxides can be attained by either synthesising the epoxide by using a chemical (chiral) substance, or by using biological materials (for example enzymes) to separate the specific enantiomer from a racemic mixture.

2.1. CHEMICAL SYNTHESIS OF CHIRAL EPOXIDES

These chemical methods include amongst others the epoxidation reaction catalysed by metals (Sharpless method), where transition metals are used as catalysts. Chemical synthetic methods, especially when using the Sharpless reagent, result, in the majority of cases, in epoxides having enantiomeric excesses greater than 90 % [5, 6, 8].

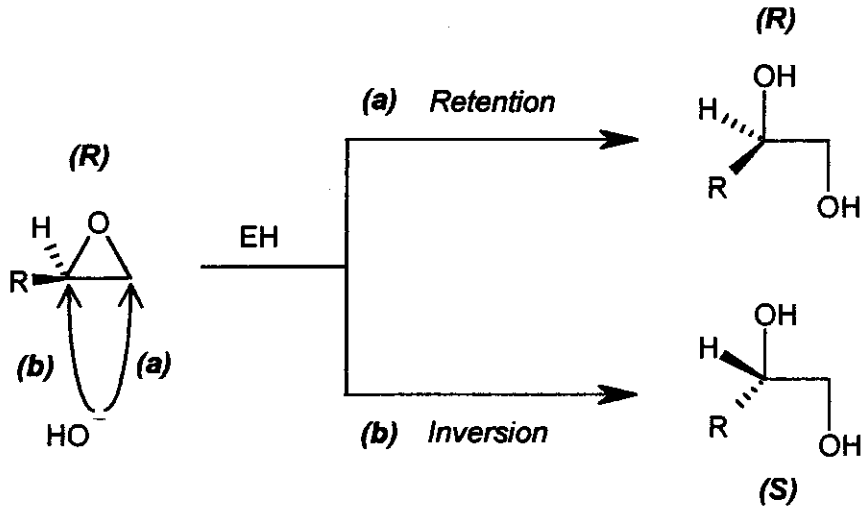

Figure 2-4. Retention (a) and inversion (b) of configuration during the biocatalytic hydrolysis of terminal epoxides [16, 17].

However, the use of EH enzymes for the production of enantiopure epoxides has a few disadvantages that have to be taken into account. These include a potentially low substrate specificity, necessity to work in water, product inhibition and maximum yield of 50 % of the required epoxide. If, however, the target of the transformations is the transformed diol, a number of approaches have been adopted in order to optimise yields [8, 18].

Hydrolytic kinetic resolution of racemic epoxides offers a convenient route to obtain single enantiomer synthons for enantiopure fine chemicals. Since both the remaining epoxide and the diol product (employed as cyclic sulphates or sulphites) are useful as reactive intermediates, high product recovery can be achieved. Terminal epoxides are arguably the most important subclass of epoxides, serving as building blocks for organic chemistry [9, 19]. Asymmetric hydrolytic catalysts, able to resolve these inexpensive racemates into optically active epoxides and vicinal diols, have thus become an important focus of research [15, 19].

The productivity of the routes available are limited, however, by the low aqueous solubility of epoxides, which is typically a few grams per litre, the loss of epoxide due to chemical hydrolysis, and enzyme inhibition at high diol concentrations [12]. Attempts are constantly being made to try to improve the solubility of epoxides and to develop reactors where product inhibition can be minimised.

EHs are almost ubiquitous in nature and can be found in mammals, plants, insects and microorganisms. Mammalian EH (mEH) have been more extensively studied until very recently, but the last few years more microbial EHs have been discovered and researched [15, 20].

3.1. MAMMALIAN LIVER MICROSOMAL EPOXIDE HYDROLASES (mEH)

Mammalian liver microsomal epoxide hydrolases have been shown to display exceptionally high enantioselectivity towards a wide range of substrates. They also exhibit much greater enantioselectivity than microbial EHs in the asymmetric hydrolysis of *meso*-epoxides to chiral diols. However, their limited availability has prevented scientists from viewing them as a potential asymmetric catalyst for the production of chiral epoxides and diols, in the same manner, for example, that lipases/esterases have been used in organic synthesis [18]. A discouraging fact is that their use for preparative scale biotransformations is still limited despite the fact that some of the genes encoding these proteins have been cloned [17].

3.2. BACTERIAL EPOXIDE HYDROLASES

It was discovered that EHs from microbial origin could be used satisfactorily [15, 20]. It is apparent that epoxide hydrolases from bacterial sources are far more widespread than was thought five years ago. In general, these microbial enzymes do not exhibit such a broad substrate range as mEH, although, as studies continue, more microbial EH enzymes are discovered, extending the range of substrates, which can be hydrolysed with acceptable enantioselectivity [18].

Three of the strains most commonly used are from the genera *Rhodococcus, Mycobacterium* and *Nocardia.* Many of these organisms contain EHs that are highly enantioselective. Research has only just begun to examine the substrate ranges of these enzymes in terms of their enantioselectivity, with high enantioselectivities only occurring in a fairly limited number of cases where the substrate has a strict substitution pattern. It is also significant that most of the enzymes show the same sense of enantioselectivity with regard to the substrates tested, even going as far as showing similar switches in enantioselectivity when comparing mono- and disubstituted epoxides. These similarities probably indicate that the enzymes are related in evolutionary terms [15, 18].

3.3. FUNGAL EPOXIDE HYDROLASES

One of the earliest reported uses of a fungal epoxide hydrolase for the synthesis of optically enriched compounds were in 1972 [18]. To date some of the strains used most often include *Aspergillus niger, Beauveria sulfurescens* and *Diplodia gossipina* [15, 18, 21].

Like their bacterial counterparts, EHs from fungal sources are probably more widely distributed than was thought five years ago. The enantioselectivity of various fungal epoxide hydrolases obviously varies with substrate structure, but also between strains, such that enantiocomplementary pairs are often available [18].

On the basis of the available data, the stereoselectivity of microbial epoxide hydrolases can be estimated to a fair extent, which facilitates the practical use of these recently discovered enzymes for the production of enantiopure epoxides and vicinal diols. However, more detailed studies have to be done to elucidate reaction mechanisms, thus verifying the stereospecifity of the enzymes [17].

3.4. YEAST EPOXIDE HYDROLASES

A few of the yeasts most commonly used, are *Rhodotorula glutinis, Saccharomyces cerevisiae* and *Trichosporon mucoides. Rhodotorula glutinis* displays epoxide hydrolase activity with an exceptional substrate range and due to superb enantioselectivity in most cases, represents an important achievement in this field [15, 18].

Rhodosporidium and *Trichosporon* preferentially hydrolyse *(R)*-1,2-epoxides with retention of configuration. The epoxide hydrolases of these yeast strains are membrane-associated. This means that, after all soluble enzymes are extracted, the total enzyme activity is retained in the cell debris, and that no epoxide hydrolase activity is found in the supernatant [19].

Yeasts (specifically red yeasts) are especially selective for monosubstituted epoxides, and more specifically *Rhodotorula araucarae, Rhodosporidium toruloides* and *Rhodotorula glutinis.* On the contrary, fungal epoxide hydrolases show high enantioselectivities for more functionalised epoxides. Regardless of the enzyme source, the enantiopreference for the tested (R)-configured oxirane was predominant and only a few exceptions were reported. The mechanism of hydrolysis by yeast EH is given in Figure 2-5 [16, 17].

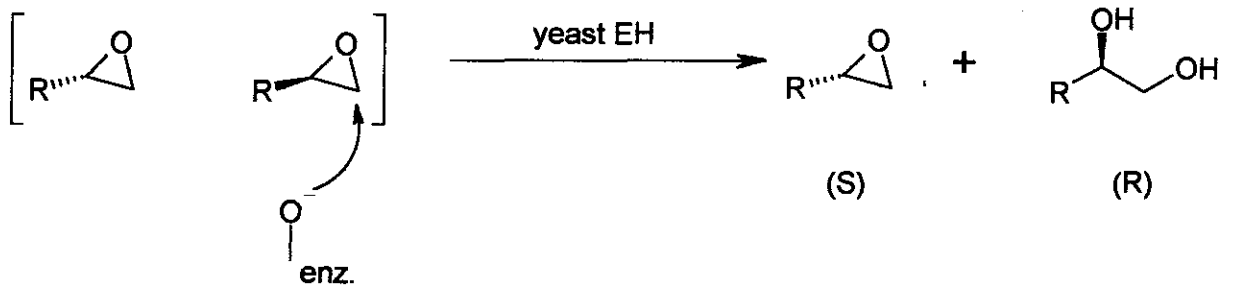

Figure 2-5. General mechanism of hydrolysis by yeast epoxide hydrolase enzymes [15].

3.5. IMMOBILISED IMPRINTED EPOXIDE HYDROLASES

In attempts to influence the enantioselectivity of immobilised EH, derivatised EH are imprinted with different molecules (imprinters) prior to cross-linking. This procedure is called "immobilised protein imprinting".

Immobilised protein imprinting enables scientists to modify the enantioselectivity of an EH. Most remarkable of this process of imprinting the protein is that one can reverse the enantioselectivity of an enzyme from the same source and exactly the same strain. Usually, opposite enantiopreference towards a substrate is only obtained by using EHs from different bacteria [22].

4. BIOREACTORS: MAIN CATEGORIES & CONFIGURATIONS

4.1. INTRODUCTION

Biocatalytic membrane reactors combine selective mass transport with chemical reactions, while the selective removal of products from the reaction site increases the conversion of product-inhibited or thermodynamically unfavourable reactions. Membrane reactors using biological catalysts can be used in production, processing and treatment operations. Furthermore, the recent trend towards environmentally friendly technologies make these membrane reactors particularly attractive because they do not require additives, are able to function at moderate temperatures and pressures, and reduce the formation of by-products. The catalytic action of enzymes is extremely efficient and selective. Compared with chemical catalysts, enzymes demonstrate higher reaction rates, milder reaction conditions and greater

stereospecificity. Their potential applications have led to a series of developments in several technology sectors: (1) the induction of microorganisms to produce specific enzymes; (2) the development of techniques to purify enzymes; (3) the development of bioengineering techniques for enzyme immobilisation; and (4) the design of efficient productive processes [23].

Biocatalysts:

The biocatalysts used can either be free or immobilised. Furthermore, they can be used as whole cells, or the enzymes can be extracted from the cells. These different configurations have some advantages and disadvantages, which have to be taken into consideration when planning which reactor to use. Other factors that have to be taken into consideration, include the type of biocatalyst, the substrate used and type of membrane used.

Phases:

Reactors can be divided into two groups, according to the amount of phases. The phases usually refer to the type and number of different organic or inorganic liquids that are used in the reactors. Reactors can have only one phase (single phase) or they can have more than one phase (biphasic or multiphasic). The phases can either be mixed together, or separated by a membrane. These two different systems also have their advantages and disadvantages.

Membranes:

In this chapter membranes were used to classify the reactor types, although there are no rigid rules concerning classifications. The classifications are usually just made in an attempt to simplify the understanding of all the different types of reactors that can be used. For the purpose of this section, the membrane reactors were classified according to the way in which the membranes are used and the function they perform in the reactor. Accordingly, the two groups are:

(1) the one-step process (integrated reaction/separation processes) where the membrane takes part in the reaction as well as the separation process; and

(2) the two-step process (classical unit operation) where the membrane is only used for separation after the reaction has taken place [24].

4.2. ONE-STEP PROCESS

This process implies that the reaction and separation processes take place in, on or across the membrane (Figure 2-6).

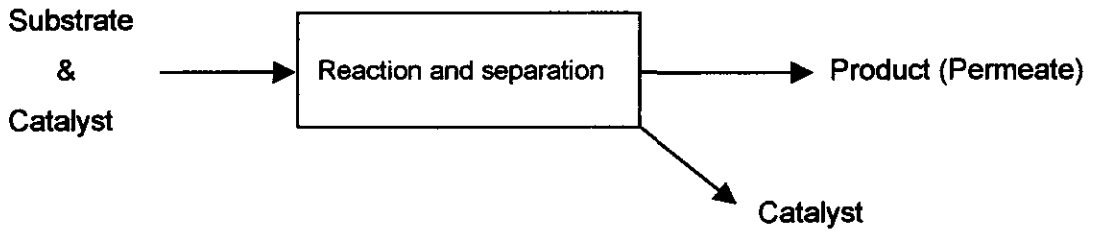

Figure 2-6. One-step process.

Under one-step processes two sub-groups were identified:

- Immobilisation
- Biphasic or multiphasic systems

Table 2-2 is a summary of the advantages and disadvantages of these two methods.

4.2.1. Immobilisation

Five aspects need to be considered when developing an immobilised enzyme system. These are: the source of the enzyme, the support membrane used, the immobilisation procedure, the regeneration procedure and the proposed reactor configuration [25].

The immobilisation of biocatalysts can lead to an activity change. This is not only caused by the immobilisation itself, but can also be caused by diffusion limitations in the immobilised biocatalyst system. The measured apparent activity of the immobilised system should be compared to the activity of the free-enzyme system [26].

The immobilisation methods most often used (Figure 2-7) are gelification by using carrier beads (adsorption) or covalent bonding (attachment) and microencapsulation (entrapment behind a barrier) [27].

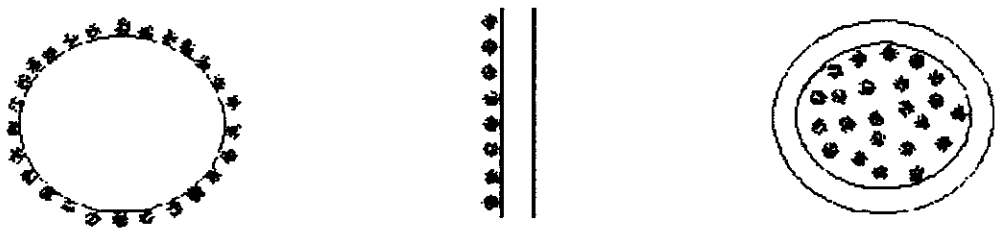

Figure 2-7. Different methods of cell-immobilisation [27].

4.2.1.1. Immobilisation by gelification

When an enzyme solution is flushed through an ultrafiltration membrane that rejects the enzyme molecules, the enzyme will accumulate on the membrane surface and deposit as a thin gel layer characterised by enzymatic catalytic activity. When the biocatalyst is immobilised on the surface, flushing the substrate solution along the enzymatic gel can cause the conversion of substrate to product in the retentate stream.

Enzyme attachment can take place by:

- Ionic binding to ion-exchanger supports.
  e.g. *cellulose*.
- Adsorption through van der Waals interactions to hydrophobic supports.
  e.g. *polypropylene* and *Teflon*.
- Covalent binding between the amino or carboxy groups of amino acids and the support membrane.
  Usually formed by active bridge molecules: CNBr and bi- or multifunctional reagents such as glutaraldehyde [23].

4.2.1.2. Microencapsulation

The microencapsulation process (Figure 2-8) involves the formation of a spherical polyanionic gel containing the cells, which is subsequently deposited on a polymeric membrane. The inner gel is then liquified, to allow its diffusion out of the capsule, leaving the membrane and the contained cells behind. Both the porosity of the membrane and the size of the microcapsule can be varied to accommodate many reactant-product systems.

The basic concept of encapsulating a cell suspension in a semipermeable polymer membrane and allowing the cells to multiply and excrete a desired product that remains within the microcapsule, has been extended to the production scale [27].

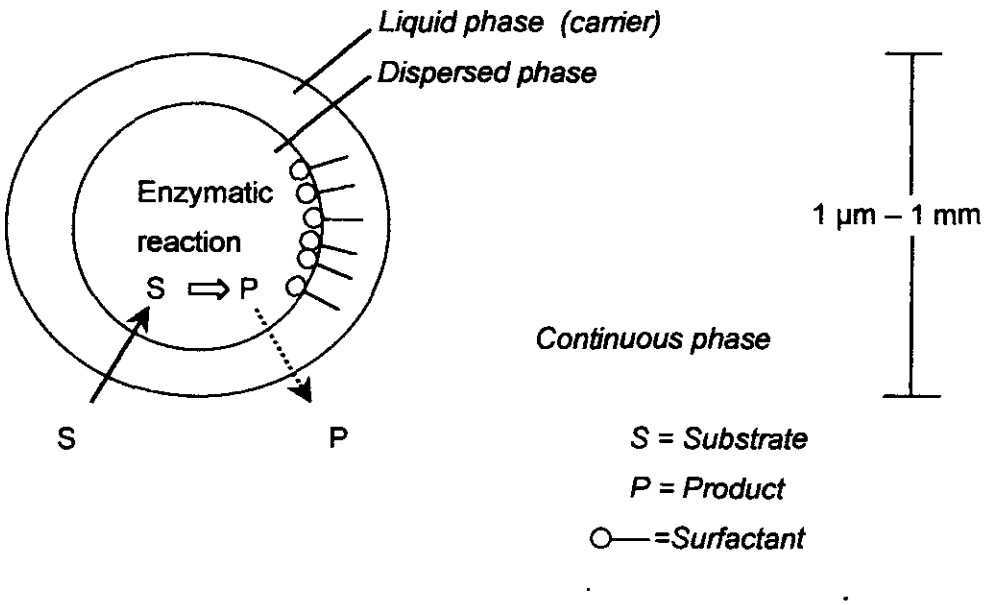

Figure 2-8. Schematic representation of microencapsulation [28].

4.2.1.3. Ultrafiltration (UF) membrane reactors

This technique is usually used when the substrate has a higher molecular mass than the product. It is important that the substrate and the product are both soluble in the same solvents. By choosing a membrane with the appropriate pore size (Figure 2-9), the substrate is transported to the enzyme immobilised in or on the membrane, but the substrate cannot enter through the membrane. The product, on the other hand, can freely pass through, where it can be recovered. It is important to compare the transport rate with the reaction rate, to ensure that the substrate reaches the enzyme, has enough time to be converted and that the product is transported to the other side of the membrane [23].

Ultrafiltration is a process usually used to retain macromolecules or colloids from solutions. The main field of application for ultrafiltration is in the dairy, food and textile industries. It is also used in metallurgy, pharmaceutical and water treatment applications. To retain emulsions and suspensions, microfiltration (MF) has to be used. Apart from its application in biotechnological processes for cell harvesting and/or in bioreactors, MF is also used for analytical purposes, sterilisation, water purification and clarification of beverages [29].

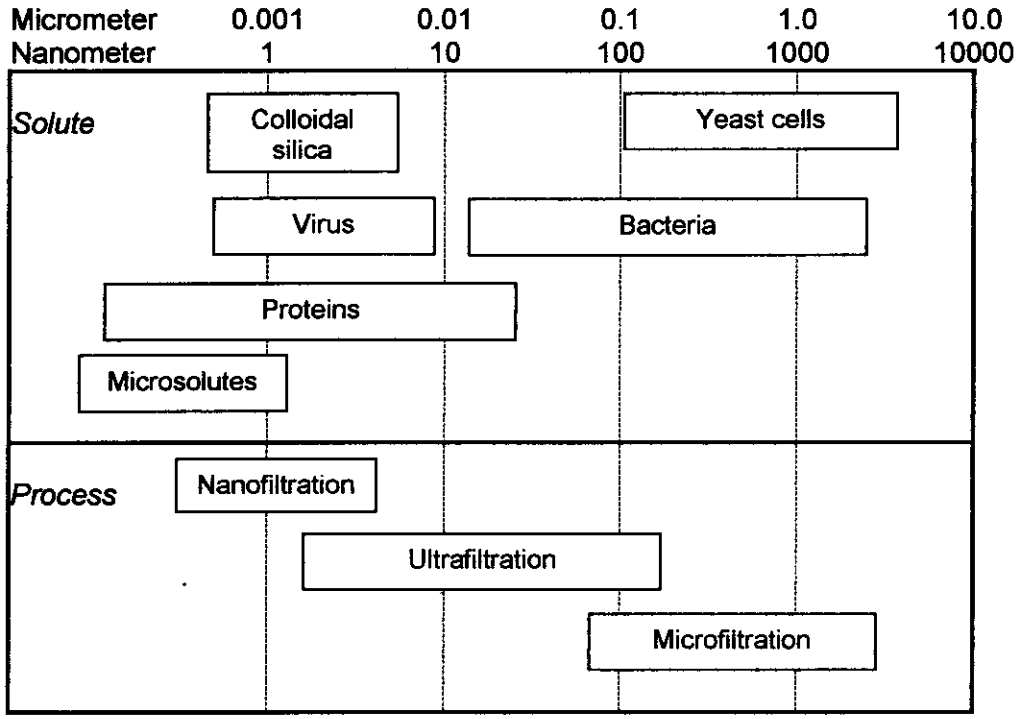

Figure 2-9. Range of various types of membranes [29]

A major problem and disadvantage of UF is, however, fouling. Fouling is when flux decline is not reversible by simply altering operating conditions. Fouling reduces productivity, shortens membrane life and it impairs fractionation capabilities of the membranes [30].

4.2.1.4. Reactors with immobilised enzymes

This type of reactor is used to prevent enzymes and cells from being deactivated by shear stress, by segregating the biocatalyst in the membrane module (either in the lumen or the shell side). The membranes then act as selective separation barriers, while providing structural support for the biocatalysts. The biocatalysts can either be loaded within the porous structure of the membrane, or on the surface of the membranes.

These systems are able to retain the cells in a low shear environment with a continuous supply of nutrients and co-factors and the simultaneous removal of metabolic products. In spite of the advantages of using membrane bioreactors with/without perfusion for culturing cells and producing large amounts of product, these systems do have some limitations. Due to the high cell densities, the transport of nutrients, including oxygen and products to and from the cells, can be limited, resulting in necrotic regions and the possible demise of the system [23, 27, 31].

In general, it is the mass transport resistance that primarily influences the performance of these reaction systems. In order for a reaction to function at its optimal performance, it should work in a reaction-limited regime rather than a diffusion-limited regime. The system is essentially controlled by kinetics and the mass-transfer limitation is negligible [23, 25].

A variation on this type of reactors is where the membranes have been "activated", by giving them catalytic function, while still retaining their permeative selective characteristics.

- Advantages
  - High enzyme effectiveness and bioreactor productivity is possible.
  - With convective transport of substrate through membranes, the residence time that the substrate is in contact with the enzyme can be controlled.
  - The product can be removed efficiently.

This process is especially beneficial for large molecular-weight substrates, fast reactions (to minimise unwanted secondary reactions) and for multiple series/parallel reactions such as with co-factors [27].

4.2.2. Biphasic or multiphasic bioreactors

This type of bioreactor consists of two (or more) liquid phases [10, 31, 32]. The phases are separated by a membrane, and are dispersed by mixing. There are different configurations that can be used. The liquid phases can either be aqueous/aqueous or aqueous/organic or organic/organic. The most commonly used system is the aqueous/organic system. Different designs have been used to try and increase the bioreactor inventory of reactant and to reduce potential reactant/product inhibition.

In some cases biphasic (organic and aqueous) membrane reactors can be used in conjunction with UF membranes. Biphasic reactors can be used if the substrate and the product have different solubilities. One example of this is when the reaction used is with an ester and its hydrolysed products. Biphasic reactors use an enzyme-loaded membrane, which is then located between two immiscible liquid phases. Through diffusion the substrate is transported to the enzymes on the membrane surface. The reaction occurs on the membrane and the formed product then moves through the membrane. The product is extracted into the second phase, the aqueous phase, which is flushed along the other side of the membrane [23].

4.2.2.1. Multiphasic enzyme hollow fibre reactors

One of the systems frequently used is the multiphase enzyme hollow fibre reactor, also known as a tube-and-shell module (Figure 2-10) [23]. This type of reactor has been successfully employed for the efficient kinetic resolution of various racemic mixtures [10, 25 – 27, 33].

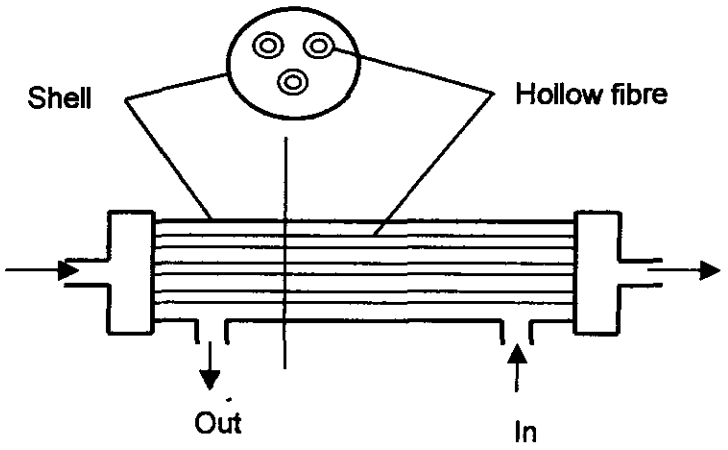

Figure 2-10. A hollow fibre membrane assembled in a tube-and-shell module.

In the standard design, hollow fibre membranes are potted together at each end and sealed in a housing (usually tubular in design) so as to separate the extracapillary space (ECS) from the lumen space (LS) [27].

Using high surface area to volume hollow fibre membranes containing enzymes to separate the aqueous and organic streams, the substrate is brought close to the catalyst, reducing diffusion limitations [27].

There are mainly two methods of operation: (see Table 2-1 for a comparison)

1. Substractive resolution
   The unreacted stereoisomer of the substrate is recovered in the organic stream.
2. Product recovery (resolution)
   This is where the optically pure product of the enzymatic reaction is recovered in the aqueous phase.

Table 2-1. A comparison of the two main methods of operation of multiphasic enzyme hollow fibre reactors [34, 35].

Product recovery (resolution)	**Substractive resolution**
Performance of multiphase bioreactor is significantly more sensitive to the operating conditions than in substractive resolution	A higher Thiele modulus reflects a lower internal diffusion rate relative to the enzymatic reaction rate which results in a lower effective enantiomerical selectivity with a resultant lowering in optical purity
Considerations for practical applications: • Greater surface area reactor with lower enzyme charge • Relative thin membrane with high enzyme charge	
Membrane thickness has an effect on product recovery Thin membranes to be used if high optic purity is desired	Membrane thickness has a minimal effect
Optically pure product from the enzymatic reaction is recovered in the aqueous phase	Unreacted substrate is recovered in the organic phase
Unlike substractive resolution, a high optical purity of the product can not be obtained for product recovery when the intrinsic enzyme stereoselectivity is too low	

4.2.2.2. Flat sheet reactors

Although these reactors have a lower ratio of surface area to volume than hollow fibre membranes, it has all the advantages thereof. It also overcomes all of the disadvantages of hollow fibre reactors. Flat sheet reactors can be used as spirally wound modules or they can be assembled in plate and frame modules (Figure 2-11) [27].

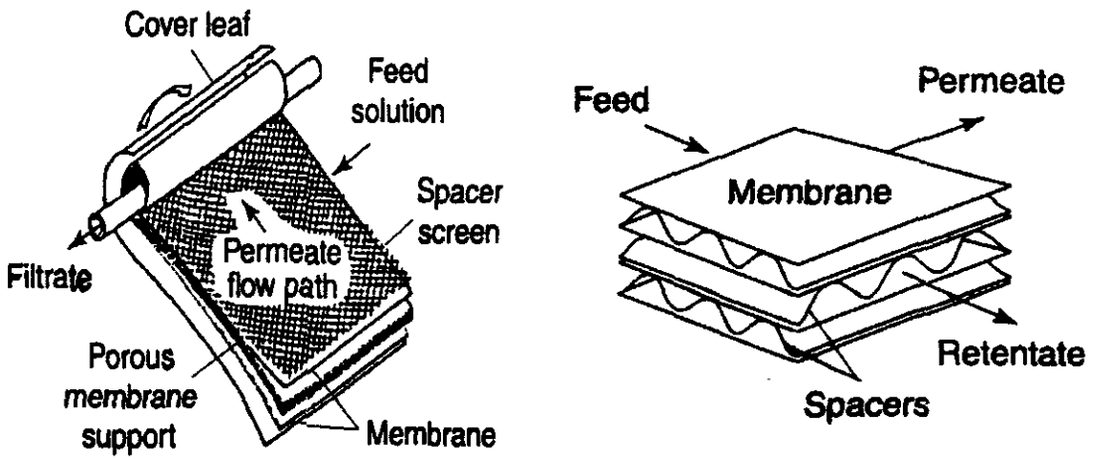

Figure 2-11. Flat-sheet membrane assembled in spiral wound (left) and plate and frame (right) modules [23].

4.2.2.3. Entrapped enzyme membrane reactors

This type of system is normally used when the substrate or product used is sparingly soluble. It can also be used if the product exerts a feedback inhibition on the enzyme reaction.

Table 2-2. Advantages and disadvantages of the two one-step processes

Process	Advantages	Disadvantages	*Ref* *
1. Immobilisation:			
Gelification	• Highly stable	• Steady state performance lowered if product inhibition occurs • Denaturation of enzyme with binding • Difficult to replenish enzymes • Only useful if initial denaturation step is negligible	[23]
Microencapsulation	• High concentration and purity of enzymes • Cells are protected	• Product recovery needs additional steps • Diffusive limitations can occur • Feedback inhibition on cell productivity • Difficult to fabricate capsules smaller than 200 μm.	[27, 28]
UF† membranes (between 2 UF membranes)	• Avoid inhibition and inactivation by product • Useful for producing pure enantiomers • Overcomes low water solubility • No polar products formed in organic phase • Can be used to separate enantiomers	• Can find traces of substrate in aqueous phase	[23, 33]
Reactors with immobilised enzymes	• Improved stability, productivity, purity and quality • Biocatalyst stabilised for longer gives steady and sustained output • Reduced waste • Easy downstream separation and lower recovery costs	• Varying fluid sheer stresses • Possible contamination • Unsteady culture environment • Uncontrollable conditions in packed bed • Mass transfer limitations <u>Limitations:</u> • More aseptic connections • Need more monitoring and control than batch processes	[23, 27]

* Ref: References
† UF: Ultrafiltation

Table 2-2 (continued).

Process	Advantages	Disadvantages	*Ref* *
2. Biphasic or Multiphasic Bioreactors:			

Multiphasic enzyme hollow fibre reactors	• High surface area to volume ratio
	• Can isolate cells from shear and contamination
	• Product easily retained and concentrated
	• Substrates and membranes are in direct contact
	• Lower bulk mass transfer limitations
	• High surface area contact between phases
	• Maintain phase separation during process
	• Can minimise toxicity of organic solvent towards enzyme activity
		• Downstream separation is difficult:
		  • Emulsion formation
		  • High intensity mixing
		• Fouling and clogging of filters
		• Difficult to access cell mass and maintaining a well-defined intrafibre spacing
		• Can get disrupted by cell growth or gas production
			[27]

Flat sheet reactors	• Access to cell space is possible • Can carefully control cell space between the two membranes		[27]
Entrapped enzyme membrane reactors	• Reduces diffusion limitations	• Poor distribution coefficients • Low diffusion coefficients • Phase separation • Product recovery problems	[27]

* Ref: References

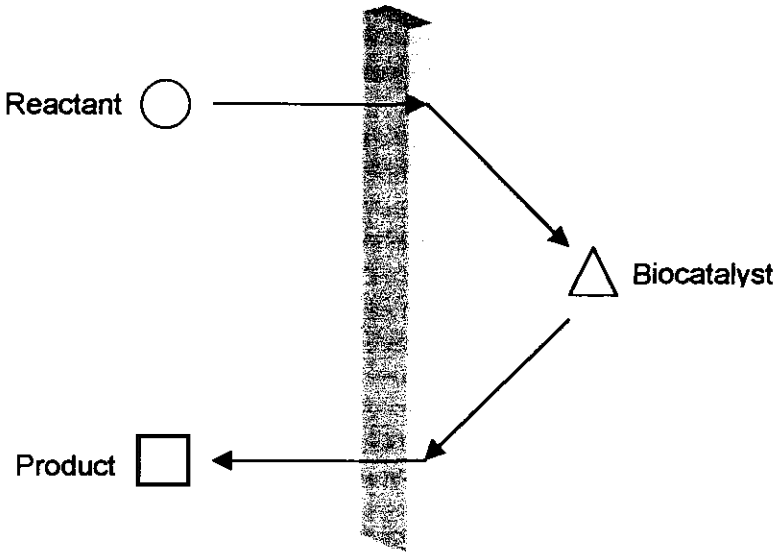

Figure 2-13. Operating principle of MBB [31].

Since the mass transfer in this system is controlled by diffusion in the aqueous phase, the organic phase hydrodynamics and membrane thickness does not significantly affect the rate of mass transfer.

Often silicone rubber is used as membrane material. However, some organic solvents can cause silicone rubber to swell. This can cause the formation of a two-phase system, which has the potential of forming emulsions and also of causing product loss. These disadvantages should be taken into consideration when selecting an organic solvent [31].

4.3.1.2. Extractive fermentation

This process can be used to lower the effect of end product inhibition, by using the water immiscible phase to remove fermentation products *in situ*. Product extraction into the solvent occurs by diffusion across the dense membrane under a concentration driving force. In addition, liquid feed of the growth substrate and the substrate are pumped directly into the bioreactor to increase the rate of delivery of these compounds to the aqueous phase. In this system the product accumulates in a pure solvent phase and thus product recovery problems associated with emulsion formation are avoided. It was shown that no phase breakthrough of either phase across the membrane was observed. Lowering the dilution rate led to higher biomass concentrations, however, the specific activity was significantly reduced [36].

4.3.1.3. External membrane module

There are several advantages of an external membrane module compared to the internal membrane reactors described in 4.2.1.1 and 4.2.1.2. The size of the module, and thus the membrane surface area, is not restricted, and the aqueous phase can be recirculated at a high Reynolds number inside the membrane tubing, thus generating a positive transmembrane pressure differential on the aqueous (non-wetting) phase [36].

4.3.2. Separation of product, enzyme and substrate by the membrane

4.3.2.1. In situ *product removal (ISPR)*

In this process, the product is selectively removed from the vicinity of the biocatalyst as soon as it is formed. This can also provide further benefits for the subsequent downstream processing. If the biocatalyst and product are not allowed to mix, it eliminates the problem of separating the catalyst and the product. ISPR-methods can also be used to increase the productivity/yield of a given biocatalytic reaction.

For an effective application, the separation technique must have a high capacity for the target molecule (on a mass basis) to reduce the quantities of adsorbents, complexing agents and solvents required. It should also exhibit the required selectivity for the target molecule. For separation techniques based on adsorption/complexation, there is a need for resins with increased capacity. If high capacity resins are used, less of it has to be added to adsorb the molecules [37].

4.3.2.2. Crossflow microfiltration (CFMF)

In CFMF the process fluid flows parallel to the membrane (Figure 2-14). The different models of CFMF operations have been reviewed [38]. Separation is achieved on the basis of the large size difference between the cells and the other components of the feed, rather than a small density difference, which centrifuges and settling ponds attempt to exploit.

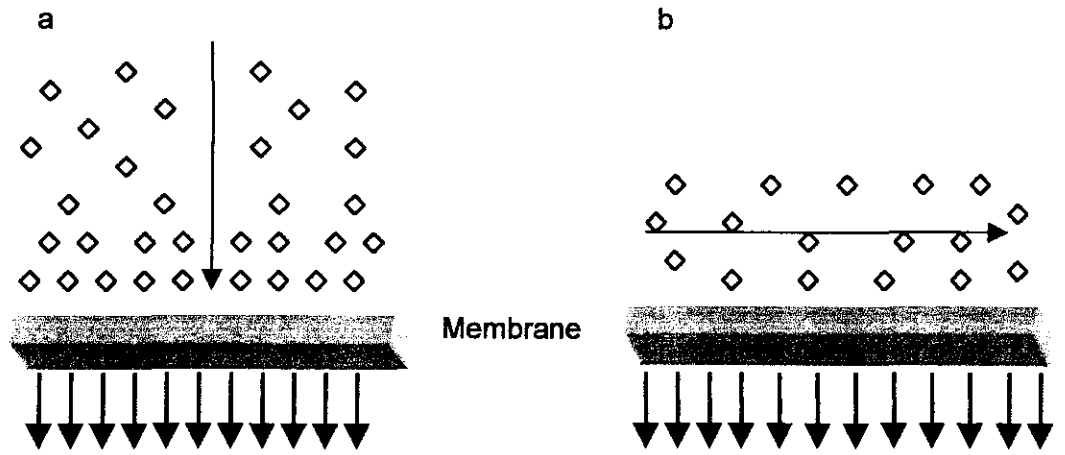

Figure 2-14. Principles of (a) dead-end filtration and (b) cross-flow filtration. In dead-end filtration the flow causes the build-up of the filter cake, which may prevent efficient operation. This is avoided in cross-flow filtration where the flow sweeps the membrane surface clean [39].

4.3.3. Recirculated enzymes

In this type of bioreactor, enzymes are circulated or re-used in the aqueous phase. This means that the enzymes can react with more than one substrate molecule. The role of the micro-porous membrane is to separate the bulk phases, while offering a high surface area of contact between the two phases [27].

4.3.4. Biphasic or multiphasic systems

4.3.4.1. Direct contact two-phase bioreactors

Although the direct contact two-phase bioreactor design offers many advantages there are still significant problems in its operation. Due to intimate contact of the two liquid phases, the high intensity of mixing, and the presence of many biological surfactants, the organic phase becomes heavily emulsified, making phase separation difficult [36].

4.3.4.2. Porous membrane bioreactors

In this design the two liquid phases are in contact via a membrane. The design offers the advantages of a direct-contact system, but avoids the key problem of emulsification. However, there can be operational difficulties in immobilising the liquid interface within the membrane pores, and in the presence of surface-active biological material, the positive transmembrane pressure applied to the non-wetting phase needs to be strictly controlled to prevent phase breakthrough. As an alternative to porous membranes a design employing dense membranes

for example, silicone rubber, has been described. Membrane mass transfer is well described by a solution-diffusion model, and in previous studies it was found that when the membrane/aqueous partition coefficient was high, membrane resistance to the mass transfer was insignificant [31, 36, 40].

4.3.4.3. Dense membranes

Because phase breakthrough does not occur with dense membranes, this design has all the advantages of its porous-membrane counterpart, but avoids the operational complexities introduced by the requirement for strict control of transmembrane pressure. However, as in porous membrane processes, positive pressurisation of the aqueous phase (non-wetting phase) is still needed to immobilise the liquid interface.

Provided sufficient membrane area is available to meet the biotransformation rate requirements, these membranes can help satisfy all the criteria of a good bioreactor design for a two-phase biocatalytic process [31, 36].

Table 2-3. Advantages and disadvantages of the four two-step processes.

Process	Advantages	Disadvantages	*Ref* *
1. Biocatalysts compartmentalised:			
Compartmentalised in reaction vessel	• Direct contact of membrane with both substrate and biocatalyst • Diffusional resistance is limited	• Concentration polarisation phenomena • Fouling of membranes • Need appropriate fluid-dynamic conditions and reactor design to control performance at steady state	[23, 31]
Extractive fermentation	• Removes inhibitory products as they are formed • Keeps reaction rate low • Potential to keep recovery costs low	• Energy intensive maintenance of vacuum if large amounts of carbon dioxide are formed • Need to sparge fermentor with pure oxygen if yeast cells are used • Accumulation of toxic by-products in fermentor • Large bleed stream is necessary	[41]
External membrane module	• Size of membrane and thus membrane surface area is not restricted • Aqueous phase can be recirculated at a high Reynolds number inside membrane tubing, generating a positive pressure differential in aqueous phase • Can achieve high overall mass-transfer coefficients • Simple way to immobilise the liquid interphase • Phase breakthrough cannot occur • Do not need to control the magnitude of the positive pressure differential		[36]

* Ref: References

Table 2-3 (continued)

Process	Advantages	Disadvantages	*Ref* *
2. Separation of product, enzyme and substrate:			
In situ product removal (ISPR)	• Removes product from vicinity of biocatalyst as soon as it is formed • Can overcome inhibitory/toxic effects • Shifts unfavourable reaction equilibria • Minimises product losses due to degradation or uncontrolled release • Reduces number of downstream processing steps		[37]
Crossflow microfiltration (CFMF)	• Tangial movement of fluid helps remove most of rejected material from membrane surface • Accumulation of cells at filter surface is minimalised • Resistance to flow rate increases at a lower rate than conventional filtration • No contamination of cell concentrate with flocculants or filter aids • Does not generate aerosols • Sterile operation is possible	• Moisture content of cell suspension is generally higher than that of a suspension produced by conventional filtration	[38]
3. Recirculated enzymes			
Transporting enzyme membrane bioreactors	• Stable interface • Easy to scale up • No emulsification problems • Enzymes re-used and can react with more than one substrate		[27]

* Ref: References

Table 2-3 (continued)

Process	Advantages	Disadvantages	*Ref* *
4. Biphasic or Multiphasic systems			
• Direct contact two-phase bioreactors	• No product recovery problems avoiding emulsification • No phase breakthrough across membrane • Low solubility can be overcome with 2nd organic phase	• Phase separation is difficult due to: □ Intimate contact between the two liquid phases □ High intensity of mixing □ Presence of many biological surfactants □ Heavy emulsification of organic phase • Not ideal for integration of the biocatalytic step into an organic synthesis	[36]
• Porous membrane bioreactors	• Provides a fixed aqueous/organic interface within the bioreactor • Allows direct phase contact • No phase mixing • Avoids emulsification • No separation procedure is required	• Relies on careful control of transmembrane pressure to avoid phase "breakthrough" and thus emulsification • Biological surface active agents are formed • "Breakthrough" pressure changes as reaction proceeds	[31]
• Dense membranes	• No need for strict control of transmembrane pressure • Problem associated with fouling and bulk-phase breakthrough will not occur • High interfacial contact area • No emulsion formation • No strict control of pressure needed • No biocatalyst immobilisation needed		[31, 36]

* Ref: References

5. SUMMARY

Enantiopure compounds and their synthesis remain two very important areas of research, since enantioselectivity plays a major role in the pharmacokinetics and the pharmacodynamics of drugs. Subsequently the separation of enantiomers is important to eliminate side effects and undesired activities of drugs.

Epoxides is a very useful group in the synthesis of enantiopure compounds. They are reactive and can undergo reactions with a number of nucleophiles. Enzymatic resolution methods can easily convert inexpensive and readily available racemic epoxides into enantiopure products.

About two thirds of biotransformations reported on non-natural compounds in the last twenty years used hydrolase enzymes. The EH enzymes used for these reactions have the following advantages [20] :

(1) They do not require any cofactors (e.g. NAD(P)H / NAD(P)) and only need water to react with the substrate.
(2) They are widely available from a number of sources.
(3) They remain catalytically active in non-aqueous media.
(4) They frequently show remarkable chemo-, regio- and stereoselectivity whilst accepting a wide range of substrates.

Most of the results of EH enzymes from yeasts that have been investigated the last few years have been encouraging. Excellent E-values, high reaction rates and high turnover frequencies (catalytic activity) were displayed for C-6 to C-8 1,2-epoxyalkanes by several strains belonging to the genera *Rhodotorula* and *Rhodosporidium* [19].

While reactors can be categorised into various different groups, these classifications can be very different, depending on the different entities in the reactor that are important to different researchers. There are three main parts of the reactors, which usually are of importance: (1) the membrane and its role in the reactor, (2) the biocatalyst and its state in the reactor and (3) the phases of the different components in the reactor.

Before choosing a bioreactor to work with, the advantages and disadvantages of all the reactors have to be looked at. It is also best to try and see which of the reactor types will be compatible with the system that will be used. There should be looked at the phases, biocatalyst and membranes that will be used in the setup.

A study done previously [19] with several yeast strains belonging to the genera *Rhodotorula* and *Rhodosporidium* has shown excellent E-values and high reaction rates for C-6 to C-8 1,2-epoxyalkanes. This, coupled with the advantages of using hydrolases enzymes, led to the decision to further investigate the hydrolysis reaction of 1,2-epoxyoctane (a C-8 1,2-epoxyalkane) to 1,2-octanediol. The biocatalyst used for this reaction is *Rhodosporidium toruloides*. A dead-end reactor (two-step process reactor) that has been used previously [3, 42] was chosen for the further optimisation of reaction conditions.

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CHAPTER 3

Optimisation of process variables in a batch bioreactor

1. INTRODUCTION

The use of single enantiomers and the manufacturing of enantiomerically pure compounds has become a growing area of development in the fine chemicals industry [1, 2]. The success of these companies depends mainly on their ability to synthesise novel chemical entities. Another important motivation is the optimisation of marketable drugs, agrochemicals, food products and cosmetics [1 – 3].

When a product is marketed as a single enantiomer, a high enantiomeric excess (ee, > 98 %) (high enantiomeric purity) is required. The % ee (Equation 3-1) is derived from the concentrations of the two enantiomers [1, 4]:

$$\% ee = \left| \frac{C_R - C_S}{C_R + C_S} \right| \times 100 \qquad (3\text{-}1)$$

where C_R and C_S are the individual concentrations (mM) of the (R)- and (S)-enantiomers, respectively. Enantiomeric excess is important for all catalysed syntheses. These include both chemical and biochemical catalysis, where chemical catalysis is usually done with transition metals, while in biochemical catalysis enzymes are used [5]. For the reaction done in this study a biochemical catalyst was used, and it should be remembered that the ee of the formed diol does not depend only on the enantioselectivity of the reaction, but also on its regioselectivity [4].

According to the scheme in Figure 3-1, only one product is formed. This would only be true if this reaction was both 100 % stereo- and regioselective. Stereoselectivity is the enzyme's preference for reacting with only one of the enantiomers of the racemic substrate [4]. Regioselectivity gives an indication of how selectively the enzyme attacks only one of the carbon atoms of the oxirane ring [6], and thus is an indication of whether the enzyme converts the (R)-enantiomer to only the (R)-diol, or both (R)- and (S)-diol [4].

The single most important factor however for the optimisation of processes is the enantioselective performance of the catalyst. The evaluation of this property has been greatly facilitated by the use of the enantiomeric ratio, E [3, 7].

$$E = \frac{\ln\left[\left(1-ee_s\right)/\left(1-\frac{ee_s}{ee_p}\right)\right]}{\ln\left[\left(1+ee_s\right)/\left(1-\frac{ee_s}{ee_p}\right)\right]} \qquad (3\text{-}2)$$

where ee_S and ee_P are the ee values of the substrate (epoxide) and the product (diol), respectively. By definition, E is an intrinsic property of the enzyme, consequently the E value cannot change unless the inherent values of the enzyme changes [1].

1.1. HYDROLYSIS OF 1,2-EPOXYOCTANE

The hydrolysis of 1,2-epoxyoctane (epoxide) to 1,2-octanediol (diol) by *Rhodosporidium toruloides* is a highly enantioselective reaction (Figure 3-1).

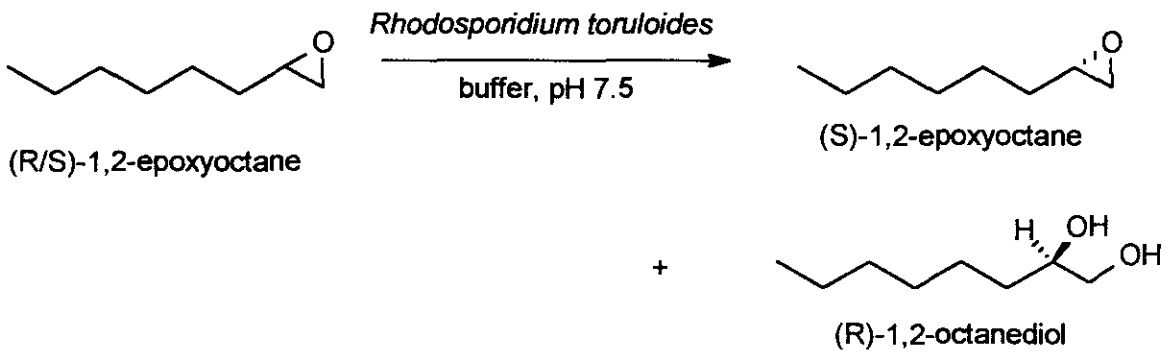

Figure 3-1. Hydrolysis of 1,2-epoxyoctane to 1,2-octanediol.

There are a few advantages of using this reaction, including a high enantioselectivity (E = 85-200), a high reaction rate (172 $\mu mol^{-1}.mg.protein^{-1}$) and an enhanced stereoselectivity (if a higher substrate concentration is used). Other advantages are that the enzyme responsible for the reaction need not be isolated and the yeast cells do not have to be active [8].

1.2. SOLUBILITY OF 1,2-EPOXYOCTANE

The only work done previously concerning the solubility of 1,2-epoxyoctane, was a study testing the effect of co-solvent concentrations on the solubility of 1,2-epoxyoctane in 50 mM phosphate buffer [8, 16]. A solubility of about 18 mM in 30 % ethanol (EtOH) and about 16 mM in 30 %

acetonitrile (CH_3CN) was obtained. Other researchers also used CH_3CN as a co-solvent, but only used solutions with concentrations of 10 mM [9] and 1 mM [10] epoxide. Another co-solvent that has also been used with 1,2-epoxyoctane is dimethyl sulphoxide (DMSO), and epoxide solutions with concentrations of 70 mM epoxide were used [11]. According to these results [16], the solubility of the epoxide in 50 mM phosphate buffer (i.e. 0 % co-solvent) was about 6 mM.

Previously batch processes have been done with epoxide concentrations ranging from 1 to 10 mM [12] or 20 mM [8, 13] up to 500 mM [14]. In the only example of a continuous system the epoxide concentration used was 20 mM [4, 8]. It is safe to assume that in these studies the epoxide, at concentrations higher than 6 mM, was present as emulsion droplets.

1.3. AIM AND OBJECTIVES

Due to repeated inconsistencies regarding the epoxide concentrations added and analysed, a thorough investigation was carried out to find possible causes explaining the loss of epoxide during experimentation. For this purpose the effect of sampling, evaporation, reaction and sampling containers and chemical degradation was investigated.

The most commonly used reactor processes for biocatalysis are batch processes [3]. For reactions done with the yeast, *R. toruloides*, the cell concentrations most often used were 20 % (w/v) suspensions [14 – 16], where the total epoxide concentration was obtained by adding the concentrations of both of the enantiomers [(R)- and (S)-epoxyoctane].

Research done previously [16, 17] optimised the batch process in terms of the influence that temperature, pH and ions have on the activity of the enzyme. It has been shown that E improves with higher substrate concentrations, but only for concentrations up to 20 mM and thereafter E stayed about the same [16]. As 20 mM is above the solubility of the epoxide, the stir speed in a batch process will probably play an important part in the efficiency and even the selectivity of the reaction. Another variable is the amount of cells that are in the reactor, which should also play an important role in the efficiency and thus indirectly also in the selectivity of the reaction. Up to now most batch processes have been done with a cell concentration of 20 % (w/v). It was consequently decided to further optimise the process, by examining the effect that the stir speed and cell concentrations in a batch reactor would have on the enantiomeric purity.

2. EXPERIMENTAL

2.1. MATERIALS

Rhodosporidium toruloides (UOFS Y-0471) was obtained from the Yeast Culture Collection of the Department of Microbial, Biochemical and Food Biotechnology of the University of the Free State (South Africa). The vitamin solution was also purchased from the same department. The chemicals for the growth medium were purchased from Biolab (South Africa). 1,2-Epoxyoctane and 1,2-octanediol were obtained from Aldrich (South Africa), while the glycerol and buffer materials (K_2HPO_4 and KH_2PO_4) were purchased from Saarchem (South Africa). Analytical grade ethyl acetate (EtOAc) was bought from Acros Organics. Deionised water (pH 7.0) (Millipore Milli-Q Plus® CPMQ004R1) was used throughout. All reagents were of analytical grade and were used without further purification. The details of the reaction and sampling vessels are presented in Table 3-1.

Table 3-1. Information regarding reaction and sampling vessels used.

	Supplier	Cat. No.	Lid	Septum
Large Epps	Merck	0030 120.086	-	
Small Epps	Merck	0030 121.023	-	
McCartneys	Merck	215/0054/20	Screw caps	Fitted rubber
Amber bottles	National scientific supply	B7921	Holed caps	Teflon-silicone 22 mm
2 ml glass vials	National scientific supply	C4000-1	Screw caps 5182-0717	Teflon/red rubber C4010-30

2.2. METHODS

2.2.1. Analysis

For the analysis and quantification of the reactions, a Thermo Finnigan Focus GC equipped with FID and AS 3000 autosampler, was used. The chiral separation (see Appendix 1) was achieved using a fused silica β-cyclodextrin column (CP Chirasil-DEX CB, 25 m x 0.25 mm, 0.25 µm film). H_2 was used as carrier gas, because it has a higher selectivity than N_2 or He. The epoxide and diol were analysed at 70 °C and 140 °C, respectively. The retention times were Rt (70 °C) = 21.5 min and 21.8 min, respectively, for the (R)- and (S)-1,2-epoxyoctane (see Appendix 2) and the retention times were Rt (140 °C) = 11.9 min and 12.3 min, respectively, for the (S)- and (R)-1,2-octanediol. The absolute configuration of the epoxide and diol had been established

previously [18]. Butanol was used as an internal standard and its retention times were Rt (70 °C) = 4.0 min and Rt (140 °C) = 1.65 min. Quantification was done using calibration curves.

2.2.2. Preparation of frozen yeast cells

Growth media consisting of 20 % (w/v) malt extract, 15 % (w/v) glucose, 5 % (w/v) peptone and yeast extract was used for the cultivation of the yeast cells. 2 % (v/v) of filter sterilised vitamin solution was added to the growth media. After inoculation, the growth media were cultivated at 27 °C for 4 days on a Labcon® rotary shaker (180 rpm). The cells were harvested by centrifugation (5000 g, 5 min, 4 °C). The cells were washed twice with phosphate buffer (50 mM, pH 7.5) and resuspended (50 % w/w) in phosphate buffer containing 20 % (v/v) glycerol. The cells were frozen in 50 ml centrifuge tubes and stored at –18 °C. Cells were thawed prior to each experiment.

2.3. PRELIMINARY STUDY

2.3.1. Extraction method efficiency

Before doing any bioconversions, initial experiments were done to see if the extraction method used in a previous study [16], was suitable for taking samples from the batch process reactor. Four different extraction (sampling) methods were investigated (Table 3-2). For the extraction of the samples, 1.5 ml Safe-Lock Eppendorf Tubes (large Epps) were used.

Table 3-2. Various sampling preparation and extraction methods

Run No.	Sampling vessel	Extraction method
1	McCartney (10 ml)	Full volume (10 ml)
2	McCartney (10 ml)	Sample (500 µl)
3	Large Epp (500 µl)	-
4	Large Epp (500 µl)	Full volume (500 µl)

In runs 1 and 2 a 10 ml solution of 5 mM epoxide in phosphate buffer was prepared in glass bottles with screw caps and fitted septa (McCartney). Run 1 was immediately extracted using a full volume extraction, i.e. adding 10 ml EtOAc to the McCartney. Run 2 was first stirred for 10 minutes at 30 °C, before a 500 µl sample was taken with a Hamilton syringe. For runs 3 and 4 the solutions were made in large Epps. Run 3 was with a 500 µl solution (5mM) of epoxide in EtOAc, i.e. no extraction was done. Run 4 was with a 500 µl solution of epoxide in phosphate buffer, to which 500 µl EtOAc was added (full volume extraction). All samples, consisting of phosphate buffer and 5 mM 1,2-epoxyoctane (epox), were extracted with an equal volume of

shaken for 3, 20 and 24 hours respectively. At these pre-determined time-intervals an Epp was removed and a full volume extraction was done by adding 500 µl EtOAc to the Epp. The EtOAc fractions were dried over Na_2SO_4 and analysed by GC. After extraction, the 24 hour used Epp was washed with EtOAc, by adding approximately 1 ml of EtOAc to the Epp and vortexing it for about 10 seconds. Washing was repeated 5 times. Subsequently 500 µl EtOAc was added to the Epp and it was placed in the shaking water bath and shaken for another 24 hours. After the second 24 hours, the Epp was removed and the EtOAc was dried over Na_2SO_4 and analysed by GC.

2.3.6. Efficiency of sampling method from 2 ml vials

This experiment was done to establish if epoxide was adsorbed by 2 ml screw top vials with holed caps and loose septa. A concentration of epoxide higher than the solubility was used, to ensure that the evaporation from the vials would have a minimal effect on sampling.

Table 3-3. Sampling from 2 ml vials

Run No.	Sampling method	Method of separation
1	Opened the bottle	Centrifuge for 3 min
2	Opened the bottle	Left standing for 10 min
3	Sampled through septa	Centrifuge for 3 min
4	Sampled through septa	Left standing for 10 min

Four solutions of 10 mM epoxide in phosphate buffer were made (Table 3-3). All vials were vortexed for 2 minutes and thereafter separation of the two phases (EtOAc and buffer) was achieved either by centrifugation (3 min) or by allowing the samples to stand for 10 minutes (gravitational separation). After separation the samples were dried over Na_2SO_4 in a second 2 ml glass vial before analysis by GC.

2.3.7. Solubility of 1,2-epoxyoctane

Since the optimum temperature for the hydrolysis reaction was previously found to be 30 °C [17], it was decided to determine the solubility at this temperature. An excess of epoxide (250 µl, i.e. 218 mM) was added to 7.5 ml of phosphate buffer in 20 ml amber bottles. Magnetic Teflon-coated stirring bars were placed in the bottles. The bottles were sealed with Parafilm® and stirred for 24 hours to ensure a state of equilibrium in the bottle. After 24 hours stirring was ceased and the samples were left for another half an hour at 30 °C to eliminate the possibility of sampling emulsion droplets of the epoxide present in the buffer. 500 µl samples were taken

through the septa with a Hamilton syringe, extracted with EtOAc in 2 ml glass vials, dried over Na_2SO_4 again in 2 ml glass vials, and analysed by GC. The runs were done in triplicate.

2.3.8. Adsorption of epoxide on 0.45 µm nylon membranes and chitosan

An epoxide concentration of 4 mM was used to determine the adsorption of epoxide to a membrane and to chitosan. The chitosan used was high molecular weight chitosan flakes (Aldrich), and the membrane used was a 0.45 µm pore diameter nylon membrane (47 mm diameter from Osmonics). For the experiments, one membrane was cut into smaller pieces, (smaller than about 10 mm^2) while about 0.6 g of chitosan was used. Control runs, in which no membrane nor chitosan was added, were done. The buffer, epoxide, magnetic stirring bars and membranes or chitosan were added to the amber bottles and capped. Parafilm® was used to seal the bottles, before they were placed in a water bath (30 °C) and stirred for 24 hours. After stirring, the bottles were left in the water bath for another half an hour to eliminate the possibility that the samples contain epoxide suspended in the buffer as emulsion droplets. Samples were taken through the septa with a Hamilton syringe and extracted with EtOAc in 2 ml glass vials and dried over Na_2SO_4 (in 2 ml glass vials) before analysis by GC. All the runs were done in triplicate.

2.4. OPTIMISATION OF STIR SPEED AND BIOMASS CONCENTRATION

A flow-through reactor that has been used previously [16], was slightly modified to enable batch processes to be performed in it. The lower part of the reactor was sealed of with a tight-fitting Teflon disk (Figure 3-2), transforming the feed tank into a batch type reactor. The reactor was connected to a water bath to maintain a temperature of 30 °C. Solutions of 20 ml of cells and phosphate buffer were used per batch experiment.

Since no pressure would be applied, the pressure inlet was capped to prevent evaporation. A Hamilton 1 ml Gas Tight-Teflon® tipped syringe was used for sampling. The original needle of the syringe was removed and a modified elongated needle was attached to permit sampling from the bottom of the reactor. Before sampling, the stirrer was switched off, the cap of the sampling valve was removed, the needle was put in through the valve and a sample was taken. The cap of the sampling valve was replaced immediately after a sample had been obtained. Samples of 600 to 700 µl were acquired and placed in 20 ml amber bottles. From this a 500 µl sample was immediately removed and extracted with an equal amount of EtOAc in a 2 ml glass vial. The extractions were then dried in 2 ml glass vials over Na_2SO_4 before analysis by GC.

Samples, taken at 0.5, 2.5, 5, 10, 15, 20, 30 and 40 minutes, were analysed for both 1,2-epoxyoctane and 1,2-octanediol.

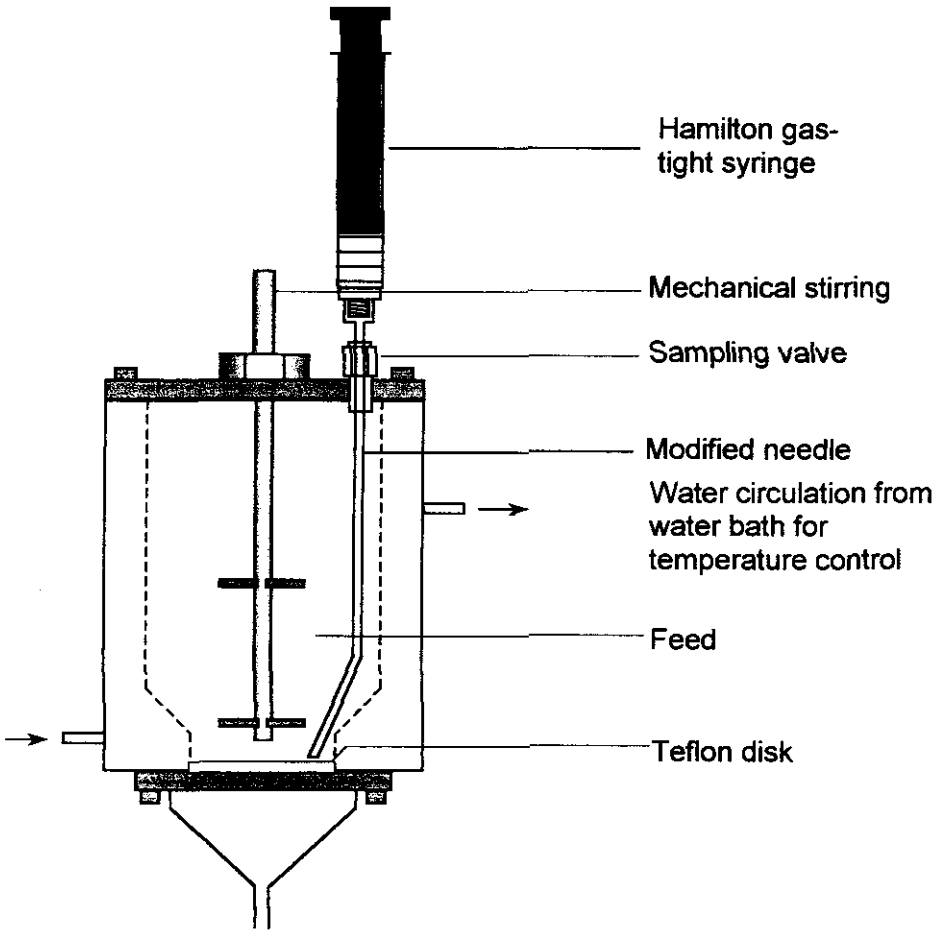

Figure 3-2. Batch reactor with sampling valve, 1 ml Hamilton Gas Tight-Teflon® tipped syringe and removable modified Luer Lock needle and fitting.

Four stir speeds (72, 240, 600 and 960 rpm) and four cell concentrations (3, 5, 10 and 20 % (w/v)) were used. The experimental conditions for each run are shown in Table 3-4.

Table 3-4. Experimental conditions for the two variables (stir speed and cell concentrations).

	Cell concentrations (%) (w/v)			
	3 %	5 %	10 %	20 %
Stir speed (rpm)	72	72	72	72
	240	240	240	240
	600	600	600	600
	960	960	960	960

3. RESULTS AND DISCUSSION

In an attempt to explain repeated epoxide loss during experimentation, the following experimental conditions were investigated prior to the further optimisation of the batch process: the effect of sampling, evaporation, reaction and sampling containers and chemical degradation.

3.1. PRELIMINARY STUDY

3.1.1. Extraction method efficiency

The total epoxide concentrations for the four runs in this set of experiments are presented in Table 3-5 (see Table 3-2 for the experimental conditions for each run).

Table 3-5. Different sampling methods to test extraction efficiency.

Run No.	Epox conc. (mM) †
1	6.00
2	3.70
3	5.07
4	6.36

† Total extracted epoxide concentration (initial epox concentration: 5 mM)

To ensure that no emulsion is formed, an epoxide concentration of less than the solubility (as obtained from [8, 16]) was used. According to the results, all the epoxide initially present is recovered when a full volume extraction is used, irrespective whether it is from a large Epp (run 1) or a McCartney (run 4). However, only 74 % of the epoxide was recovered when a sample was taken (run 2), instead of a full volume extraction. When no extraction is used (control: run 3) it can be seen (Table 3-5) that all the epoxide is recovered.

However, for runs 1 and 4, the extracted epoxide concentrations were higher than the initial concentration of 5 mM. This can be explained by the fact that some of the EtOAc dissolves in the phosphate buffer and thus the volume of EtOAc that remains and participates in the actual extraction, is less. If the extraction volume is less, the final concentration of epoxide analysed on the GC, will be higher. It can be seen that in the third run (where no buffer was used) the

concentration was 5 mM. This supports the fact that the EtOAc partly dissolves in the buffer as according to literature the solubility of EtOAc in water is 8.79 % (w/w) [19].

In an attempt to explain the low concentrations of run 2, two further variables were investigated: chemical breakdown and evaporation. Chemical breakdown might occur during the 10 minutes that the solution was stirred before the sample was taken. Since large Epps were used in most of the extractions (runs 2, 3 and 4), the possibility of evaporation/adsorption from these specific Epps was investigated.

3.1.2. Chemical degradation

GC analysis of the samples was done for 1,2-epoxyoctane and 1,2-octanediol (Table 3-6). While the epoxide concentrations decreased with time, no diol had formed. From the absence of any diol, it could be derived that chemical breakdown of the epoxide had not taken place. It was decided to next test the evaporation of epoxide from the Epps.

Table 3-6. Examining possible chemical degradation

Time (h)	Total epox conc.† (mM)	Total diol conc.‡ (mM)
0.5	5.95	0.00
1.0	4.01	0.00
2.0	2.81	0.00
3.0	2.31	0.00
3.5	2.60	0.00

† Total epoxide concentration from extraction (initial concentration: 6 mM)

‡ Total diol concentration from extraction

3.1.3. Evaporation

In Table 3-7 the weight changes of Epps containing epoxide and diol are presented. According to the results no significant evaporation from the large Epps occurred. This, together with the fact that chemical breakdown did not occur, can therefore still not explain the decrease in epoxide concentration for run 2 (section 3.1.1). It was hence decided to investigate whether a) the epoxide adsorbs to the Epps, or b) McCartneys are suitable for these experiments.

Table 3-7. Weight changes after 90 hours for epox and diol

	Mass before (g)	Mass after (g)	Decrease (%)	Average reduction (%)	Std. Dev. (%)
EPOX†	1.2065	1.2062	0.0249	0.0164	0.0120
	1.2600	1.2599	0.0079		
DIOL‡	1.5738	1.5738	0.0000	0.0119	0.0169
	1.6785	1.6781	0.0238		

† Epoxide amount: 200 µl

‡ Diol concentration: 1 M in phosphate buffer

3.1.4. Adsorption of epoxide in Epps, McCartneys and amber bottles

The first problem observed in this set of experiments was that the zero point's concentration was only 1.5 mM (Table 3-8) instead of 6 mM. This again confirms the results from run 2 (extraction done in section 3.1.1), since extraction was also used in these sets of experiments.

Table 3-8. Possible adsorption to Epps and the effectiveness of using McCartneys

		Avg. conc. (mM)	Std. Dev. (mM)	Error† (%)
Zero point		1.53	-	-
McCartney	"Control"	1.39	0.0265	1.91
20 ml Amber bottles	"Control"	1.45	0.0052	0.36
	+ Large Epp	1.24	0.0656	5.27
	+ Small Epp	0.96	0.0293	4.24

† Error (%) is calculated by: (Std. Dev / Avg. conc.) x 100

The slightly lower concentrations that were obtained with the McCartneys, compared to the 20 ml amber bottles, can be explained by the fact that the McCartneys have to be opened (solid caps) before a sample can be taken, during which evaporation can occur (boiling point of epoxide is 60 - 62 °C). The loose septa and holed caps of the amber bottles permit sampling, (using a Hamilton syringe) without opening the bottle.

It is clear that the small Epp, after only five hours, had adsorbed 54.9 % epoxide, while the large Epp had adsorbed only 18.9 % of the epoxide. According to the manufacturers the Epps are identical, however, it is remarkable to see the huge difference in adsorption of epoxide in the different size Epps.

Since the adsorption by the small Epps was so high, it was decided not to use them for any part of the extraction or sampling processes. Since the adsorption by the large Epps was lower, it was decided to test their adsorption over a longer period of time.

3.1.5. Adsorption of epoxide in large Epps

As a full volume extraction was done, the concentration of the zero point run was 20 mM (Table 3-9). However, over time the epoxide concentrations decreased drastically. No diol (product of chemical hydrolysis) was, however, found in the samples, confirming the results obtained previously that investigated possible chemical breakdown (section 3.1.2).

Table 3-9. Epoxide concentrations sampled

Time (h)	Total conc. † (mM)
0	20.0
3	8.2
20	2.5
24	2.2
24 (2) ‡	13.7

† Total epoxide concentration sampled

‡ Run "24 (2)" is the 24 hour run, after a second 24 hours of shaking with EtOAc

According to the data (Table 3-9), after only 3 hours about 12 mM epoxide has been adsorbed by the large Epps. It is also evident that after 24 hours only 2.2 mM of the epoxide is still in the buffer. After extraction of the 24 hour run, and washing of the Epp, the Epp was again shaken for 24 hours, this time with pure EtOAc (run 24(2)). While only pure EtOAc was added to the Epp the second time, the epoxide concentration measured after 24 hours was 13.7 mM, which is convincing evidence that the epoxide was actually adsorbed by the plastic of the large Epp. Adding the concentrations of the first 24 hours and the second 24 hour runs, the combined concentration obtained is approximately 16.0 mM. Although this does not total 20 mM, it is possible that the rest of the epoxide is still adsorbed by the Epp. The closed system in the Epp had most probably reached a state of equilibrium between the epoxide in EtOAc and the epoxide adsorbed to the Epp. As a consequence it was decided not to use any Epps in the experiments (to eliminate the adsorption by Epps) and 2 ml glass vials were hence used.

3.1.6. Efficiency of sampling method from 2 ml vials

It was expected that of the 10 mM epoxide solution, 6 mM epoxide (epoxide's solubility) could be extracted. No full volume extractions were done, i.e. only samples were taken, and extracted with EtOAc.

Table 3-10. Concentrations of sampling done in 2 ml glass vials

Run No.†	Total epox conc. (mM)
1	3.82
2	3.92
3	3.48
4	5.28

† See Table 3-3 for experimental conditions

From Table 3-10 it can be seen that two problems were encountered with this experiment. The first one is that the concentrations are low compared to the initial 6 mM. The second setback was that although the experimental methods for runs 1 to 4 differed slightly (Table 3-3), a 19.2 % variation in the total epoxide concentration was observed. This might have been due to the formation of an emulsion that had not yet separated when the samples were taken. As a consequence emulsion droplets might have been sampled, which would have increased the concentrations, causing significant variations between runs. This might explain why the two runs done without centrifugation (No. 2 and 4) had higher concentrations than the two done with centrifugation (No. 1 and 3). In a final attempt to explain the low values of the concentration of the runs done, the solubility of epoxide was investigated yet again [8, 16].

3.1.7. Solubility of 1,2-epoxyoctane

From the data of the experiments examining

- the extraction method (section 3.1.1) and
- the efficiency of sampling method from the 2 ml glass vials (section 3.1.6),

it can be seen that for all the runs done, where samples had been extracted after it had been taken from the test solution (Table 3-5 and Table 3-10), the epoxide concentrations were in the range of 3.4 to 3.9 mM (except one run, which gave a concentration of 5.3 mM). This seems to indicate that the previously found solubility of 1,2-epoxyoctane (6 mM) [16] was not correct. It was hence decided to test the solubility of the epoxide in 50 mM phosphate buffer at 30 °C.

Table 3-11. Solubility of 1,2-epoxyoctane in phosphate buffer at 30 °C.

Run No.	Total conc. (mM)	Average conc. † (mM)	Std. Dev.‡ (mM)	Error (%)
1	3.77			
2	3.94	3.849	0.085	2.21
3	3.83			

† Average concentration

‡ Standard deviation

The lower value of the solubility (3.85 instead of 6 mM) explains the low concentrations obtained by sampling through extraction and it correlates very well with the sampling concentrations obtained previously (sections 3.1.2 and 3.1.6). For full volume extractions the solubility of epoxide is irrelevant since the EtOAc not only extracts the epoxide solubilised in the buffer, but also the emulsified epoxide. With sampling, however, only the solubilised (in buffer) epoxide can be measured.

The lower solubility value also explains the concentrations that were acquired when testing the 2 ml glass vials and their efficiency (section 3.1.6). The two solutions that were centrifuged for 3 minutes (Table 3-10 – run no 1 and 3) gave epoxide concentrations of 3.82 and 3.48 mM, respectively. A possible explanation for the high epoxide concentrations of the other two runs (Table 3-10 – 3.92 and 5.28 mM) is that an emulsion formed and that 10 minutes was not enough for the emulsion droplets to descend to the bottom of the vials.

A possible explanation for the low concentrations for the experiments done to test the adsorption of epoxide to the Epps and the effectiveness of using McCartneys (Table 3-8) could be as follows. Although the actual runs were done in glass bottles (amber bottles and McCartneys), the extractions and the drying of the samples were still done in Epps, which have been shown to adsorb epoxide.

3.1.8. Adsorption of epoxide on 0.45 µm nylon membranes and chitosan

Experiments were done to see if the epoxide would adsorb to 0.45 µm nylon membranes and chitosan (Table 3-12), since these two compounds are used in the next chapter (see Chapter 4) of this study.

Table 3-12. Adsorption of epoxide to nylon membrane and chitosan

	Average conc. (mM)	Std. Dev. (mM)	Error (%)
Control	2.95	0.062	2.11
Membrane †	2.78	0.084	3.02
Chitosan ‡	2.84	0.259	9.12

† 1 x 47 mm membrane (0.06 g) used

‡ 0.6 g chitosan used

A possible reason for the high % error that was encountered with the chitosan, could be that the chitosan disturbed the descend of the epoxide's emulsion droplets. This problem might be solved by letting these runs stand for a longer period of time, before taking a sample. Still, from these data, it looks as though, compared to the control, there was only a 3.75 % decrease in the epoxide concentration when using chitosan, and a decrease of 5.66 % in epoxide concentration, when using the nylon membrane, i.e. both can be used (see Chapter 4) without fear of excessive adsorption of the epoxide.

3.2. OPTIMISATION OF STIR SPEED AND BIOMASS CONCENTRATION

In this part of the study, the possible effect of cell concentrations and stir speed on a batch reaction was investigated. Before presenting the interrelatedness of the variables tested, the effect of stirring speed is illustrated for the study where 3 % biomass was used, since 5, 10 and 20 % biomass showed similar tendencies.

It can be seen that as the reaction time increases (Figure 3-3) more diol is formed as the reaction progresses. It is also evident that as the stir speed increases, the reaction rate in terms of diol production increases, with the biggest improvement in the stir speed region between 240 and 600 rpm. It is evident that there is little difference in diol production between 600 and 960 rpm. The increase in reaction rate with an increase in stir speed can be attributed to the fact that more effective collisions occur between the substrate and the biocatalyst (cells) resulting in an improved reaction rate.

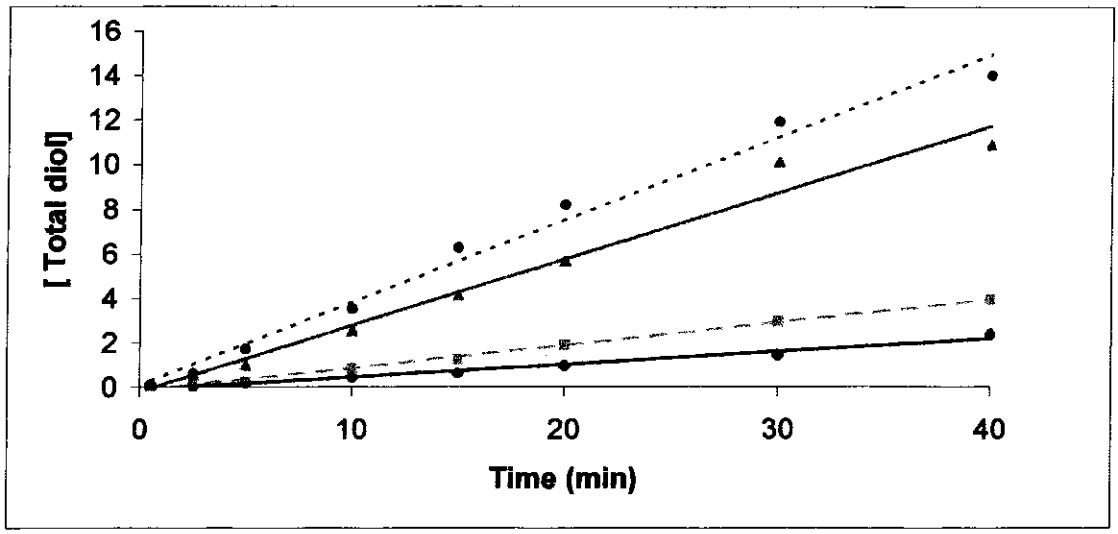

Figure 3-3. Total diol concentration as a function of reaction time. The runs were done at four different stir speeds: 72 (—◆—), 240 (- -■- -), 600(—▲—) and 960 rpm (···●···).

Plotting the calculated % ee_{diol} values as a function of time (Figure 3-4) shows that for 3 of the 4 runs done (72, 600 and 960 rpm), the % ee_{diol} decrease with time, while the run done with 240 rpm shows a steady increase. For all runs, the samples taken at t = 0.5 and 2.5 minutes, have very fluctuating % ee_{diol} values. A % ee_{diol} value of 100, for example, seems unlikely but when the very low initial diol concentrations are considered, it is possible to get such high % values, as the concentration of one enantiomer is 0 mM, while the other is only slightly higher. Thus, when calculating the % ee_{diol} value, 0 is subtracted from the low number, and divided by the sum of these two numbers (i.e. only low number), and a % ee_{diol} of 100 is obtained.

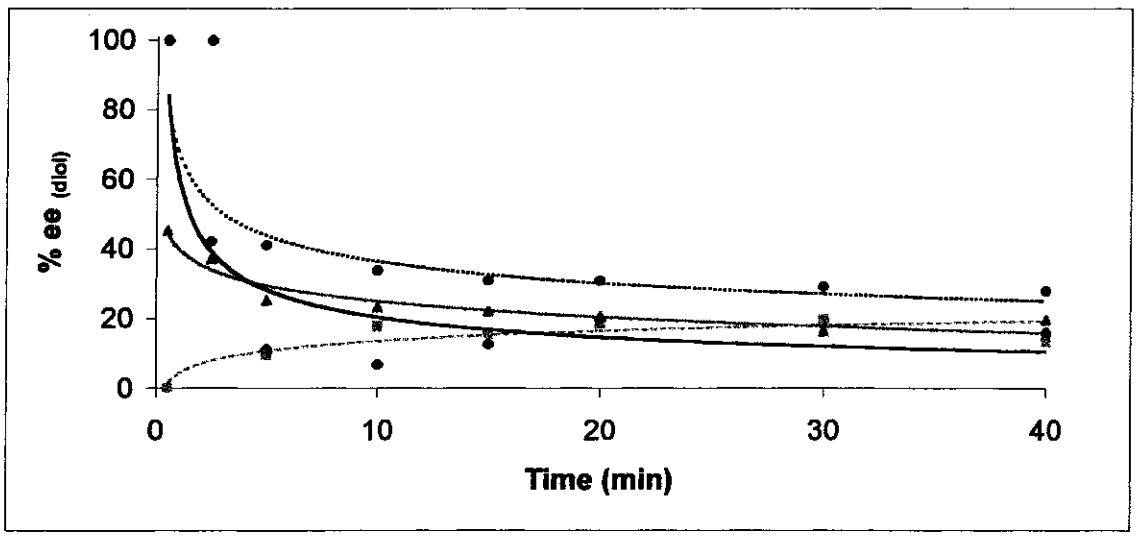

Figure 3-4. % ee_{diol} as a function of reaction time. Runs were done at stir speeds of 72 (—◆—), 240 (- -■- -), 600 (—▲—) and 960 rpm (···●···).

Further, it can be seen that steady state is reached after about 20 minutes for the 72 rpm run and 10 to 15 minutes for the other runs. This could be explained by the slower reaction rate at 72 rpm caused by reduced collisions of biomass and substrate at such low stir speeds (see Figure 3-3). It can also be seen that at 960 rpm a higher steady state is reached for % ee_{diol} than for the other 3 runs, however this is probably not statistically significant.

It has been shown previously [4, 8, 14, 15] that the operating conditions do not influence the diol concentrations as much as that of the epoxide. This is in good correlation with the data found in this study (Figure 3-4), since all four runs reached a steady state at an ee_{diol} value of between 15 and 30 %.

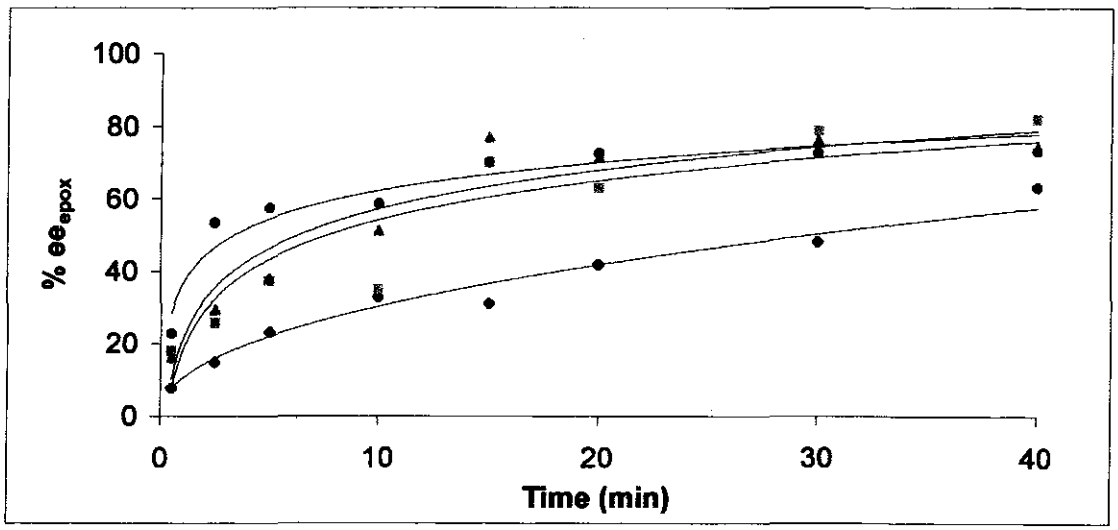

Figure 3-5. % ee_{epox} as a function of reaction time. Runs were done at stir speeds of 72 (—◆—), 240 (- -■- -), 600 (—▲—) and 960 rpm (···●···).

While most of the % ee_{diol} showed an initial decrease, the % ee_{epox} increased with time, irrespective of stir speed. While not statistically significant, the % ee_{epox} decreased as the stir speed decreased, irrespective of time.

If the biocatalyst is very selective for only one enantiomer, the difference between the two enantiomer concentrations will increase, resulting in a high % ee_{epox} value. From this graph it can be seen that the stir speed only adversely affects the % ee_{epox} at very low stir speeds (72 rpm). Care should be taken that too high stir speeds are not used, at higher cell concentrations, as this could cause abrasion between the cells, which could result in lower % ee_{epox} values.

In Figure 3-6 the difference in diol production as a function of time for two different stir speeds is presented. It is clear that while the % ee_{diol} differs slightly between 72 and 960 rpm, the effect is much more dramatic when comparing total diol concentration, confirming that much fewer collisions between epoxide and biocatalyst occur at low stir speed, thus causing a substantial decrease in reaction rate. It should therefore be kept in mind that ee as obtained from the ratios of the two enantiomers are not necessarily only indicative of the enantioselectivity of the biocatalysis, but also the time of sampling due to differences in reaction rates.

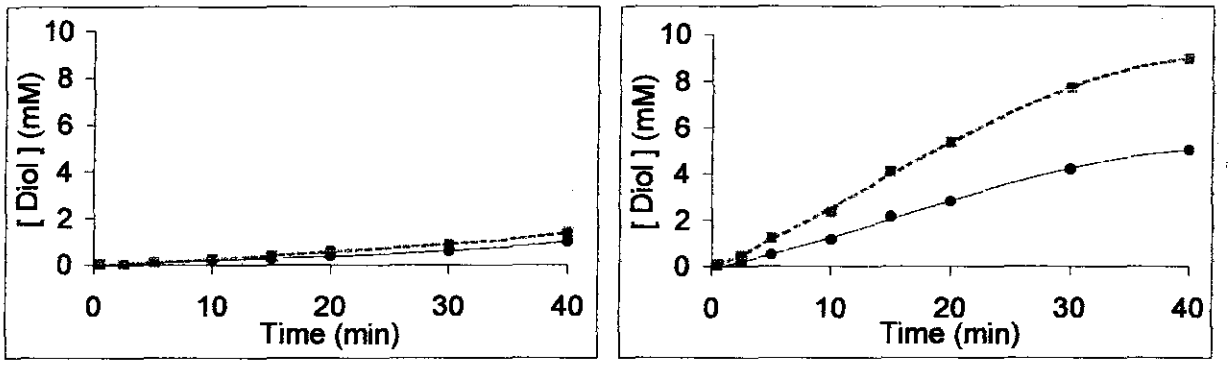

Figure 3-6. Effect of stir speed on production at 72 rpm (left) and 960 rpm (right). Diol concentration consists of concentration of 2 enantiomers, R (--■--) and S (—●—).

To compare all the variables for all conditions as well as their interrelatedness, three-dimensional graphs were drawn. Each of these shows the effect that two of the three variables (reaction time, stir speed and biomass concentration) have on both the % ee_{diol} and % ee_{epox}.

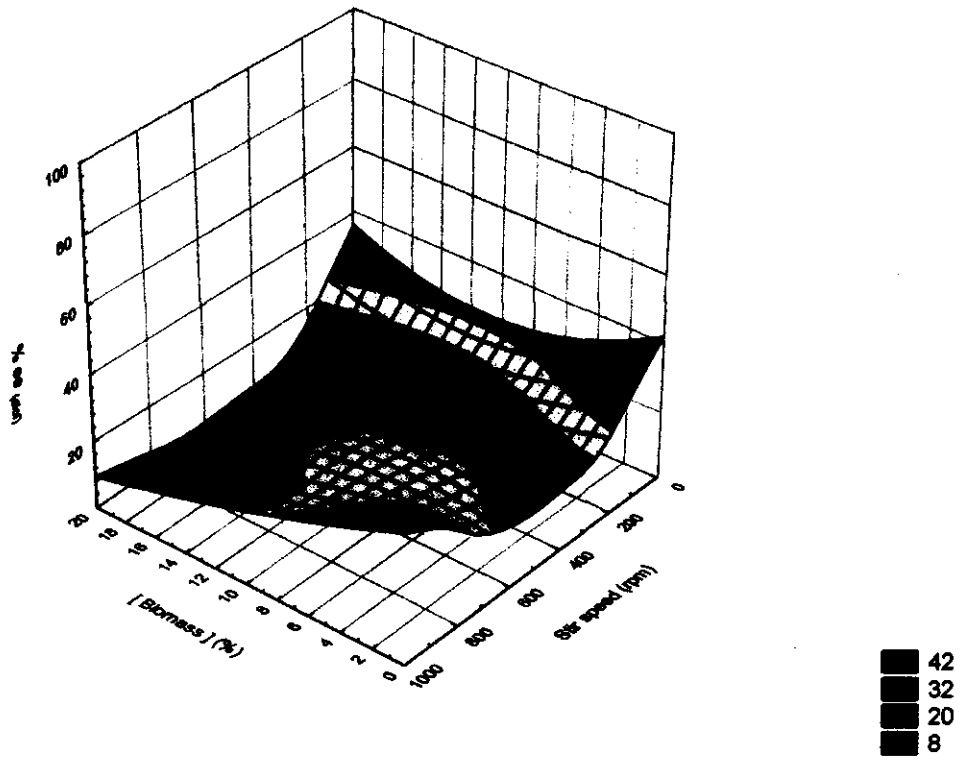

Figure 3-7. 3D graph of the effect of stir speed (rpm) and biomass concentration (%) on % ee_{diol}.

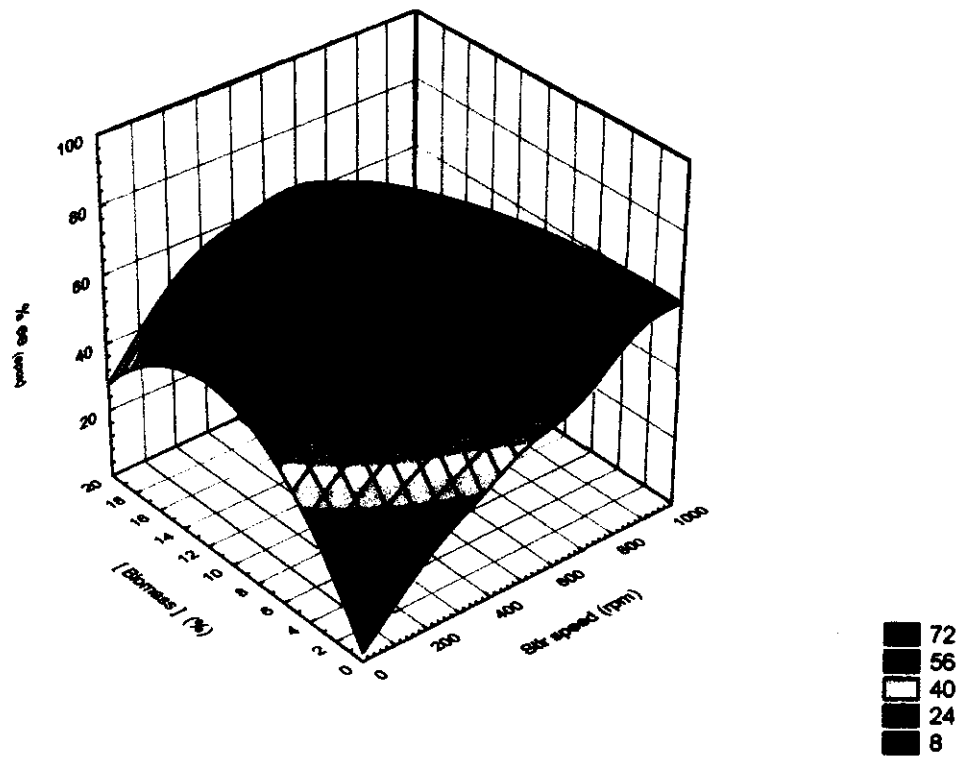

Figure 3-8. 3D graph of the effect of stir speed (rpm) and biomass concentration (%) on % ee_{epox}

Care should be taken when comparing the 3D-graphs of epoxide and diol, in terms of the direction of the axes, since the axes of all the % ee_{diol} graphs had to be rotated around the z-axis, so as to ensure the best view of the whole graph.

The effect of stir speed and biomass on % ee_{diol} and % ee_{epox} is presented in Figure 3-7 and Figure 3-8, respectively. At high stir speeds there is an increase in % ee_{diol} as the biomass concentration decreases. This could be caused by abrasion between the biomass at high stir speed, increasing as the biomass concentrations increase, causing cell damage at biomass concentrations above 12 %. Both at lower and higher biomass concentrations, an increase in the % ee_{diol} is observed. A higher % ee_{diol} is obtained when using low biomass concentrations, irrespective of stir speed – except at very low stir speeds. In general, the % ee_{diol} values are the highest at the low stir speeds (up to 250 rpm), with a slight increase at stir speeds above 700 rpm. The increase at stir speeds above 700 rpm is most visible at the low biomass concentrations. This supports the reasoning that at high stir speeds the cells grind against each other causing cell damage, reducing the % ee_{diol} values. However, at low biomass concentrations there is less biomass to rub against each other, thus less friction between the cells can take place and the % ee_{diol} is less negatively influenced by the higher stir speed.

At low biomass concentrations there is a steady increase in % ee_{epox} as the stir speed increases (Figure 3-8). Since there is very little biomass present, stir speed is very important to ensure effective contact (collisions) between the biomass and the substrate. At high biomass concentrations the same holds, however, only for stir speed below 400 rpm. At the high biomass concentrations, higher stir speeds are needed to ensure that the substrate is brought into contact with the biomass, as the biomass is a slurry (thick mixture of biomass and buffer), which is difficult to mix when using low stir speeds. At 400 to 700 rpm, the % ee values level off, and thereafter they decrease as the stir speed increases. This decrease can again be explained by abrasion between the cells at these high stir speeds and high biomass concentrations.

At low stir speed, the % ee_{epox} values are the lowest when low biomass concentrations are used. When the biomass concentration is increased, the % ee_{epox} values increase. However, at biomass concentrations of above 12 %, a further increase of biomass concentration only leads to a decrease in the % ee values. This can again be explained by the higher abrasion between cells at these higher cell concentrations, even at low stir speeds. At the lower concentrations an increase in the biomass concentrations leads to a better reaction between the cells and the substrate, thus an increase in % ee_{epox} is seen. At high stir speeds there is a gradual decline in the % ee values, as the biomass concentration is increased, again due to abrasion.

In general, the % ee_{epox} increases (at all stir speeds) as the biomass concentration increases, up to 12 to 14 %, and thereafter decreases again. At all biomass concentrations the % ee_{epox} increases as the stir speed is increased, up to 500 rpm, thereafter decreasing. All of this can again be explained by more abrasion taking place between the cells if there is a higher stir speed, and/or more cells used.

From this graph (Figure 3-8) it can be concluded that the maximum % ee_{epox} is achieved by using stir speeds of 450 to 500 rpm, and about 12 to 14 % cells. For highest % ee_{diol} however, the optimum conditions were a low biomass concentration with either very high or very low stir speeds (Figure 3-7). When comparing % ee_{epox} and % ee_{diol} (Figure 3-7 and Figure 3-8), it can be seen that the trends are opposite.

The % ee_{diol} increases at the extreme conditions (high or low biomass and/or stir speed), while % ee_{epox} values are higher in the middle regions of both stir speed (400 – 600) and biomass concentration (10 – 16 %). % ee is always a function of time (Figure 3-6) and variations in ee values are probably due to differences in reaction rates (% ee_{diol} decreases with time and % ee_{epox} increases with time) as was illustrated previously.

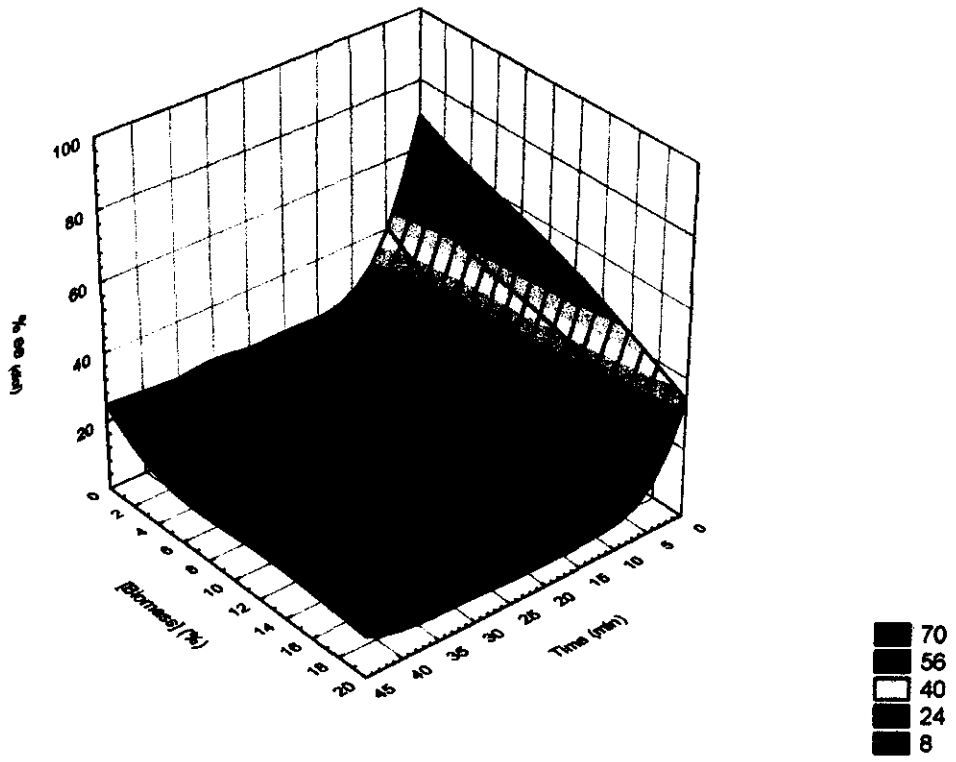

Figure 3-9. 3D graph of the effect of reaction time (min) and biomass concentration (%) on % ee_{diol}.

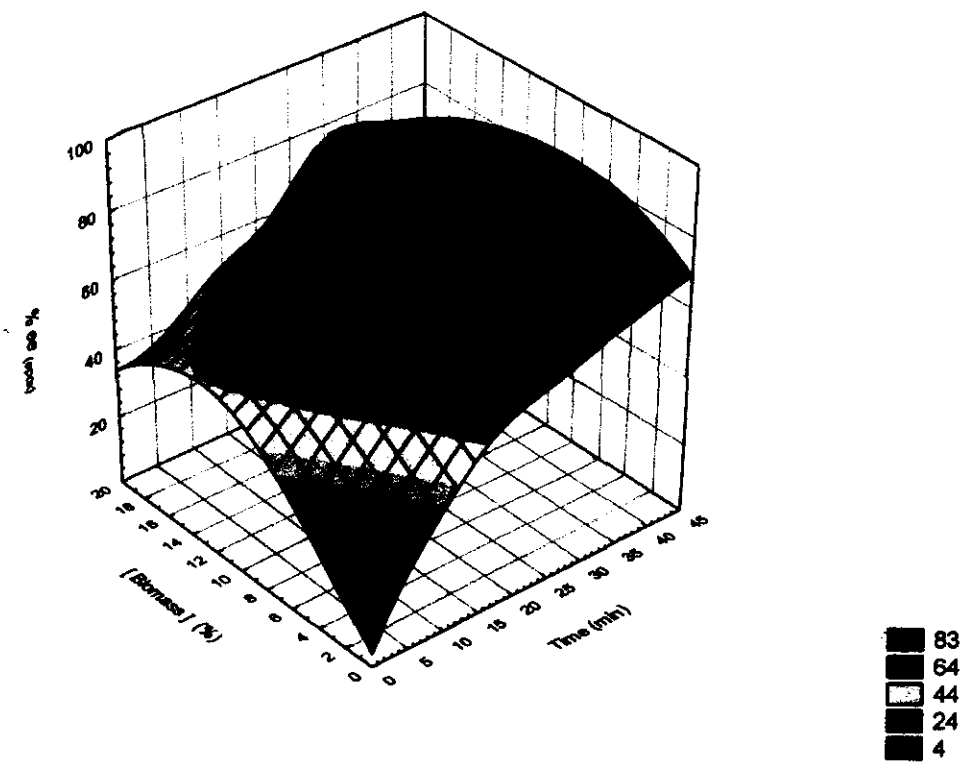

Figure 3-10. 3D graph of the effect of reaction time (min) and biomass concentration (%) on % ee_{epox}.

It was noted that the variations in % ee as a function of stir speed and biomass concentration is the result of differences in reaction rate, thus the effect of both biomass concentration and reaction time on ee values were determined.

In can clearly be seen (Figure 3-9) that at reaction times above 5 to 10 minutes, a steady state in % ee_{diol} (about 20 %) has been reached in the reactor, irrespective of biomass concentration. Between 10 and 45 minutes, there is a general decrease in % ee_{diol} as biomass concentrations increase to 4 %. The very high % ee_{diol} values obtained before 5 minutes can be explained by the very low concentrations of diol present at the start of the reaction.

For all biomass concentrations, the % ee_{epox} increases as the reaction time increases (Figure 3-10). At low reaction times, the maximum % ee is accomplished with biomass concentrations of 10 to 16 %. As the concentration of biomass increases or decreases above these levels, the % ee_{epox} decreases. At biomass concentrations above 16 %, the % ee_{epox} decreases, because of too many cells that are present, causing the formation of a thick slurry. At biomass concentrations below 10 %, the % ee decreases, because there is not enough biomass to ensure effective collisions and contact time. This again confirms that % ee is time dependant, because the effect of the biomass concentration on % ee decreases with increasing reaction times. However, the decline of % ee can be seen more prominently at the biomass concentrations below 10 %. At high reaction times the variations in % ee_{epox}, as the biomass concentrations are varied, are significantly less.

At low biomass concentrations there is a steep increase in % ee_{epox} as the reaction time increases, up to 15 minutes. Then there is a more gradual increase in % ee_{epox}, as the reaction time increases above 15 minutes. At high biomass concentrations there is a gradual increase in % ee_{epox} as the reaction time increases.

According to Figure 3-10 maximum % ee_{epox} values are attained by using reaction times of 35 to 45 minutes and biomass concentrations of about 10 %, while maximum % ee_{diol} is obtained in the region of about 13 % biomass and reaction times of about 30 minutes. As illustrated in Figure 3-7 and Figure 3-8, the inverse relation between the % ee values for epoxide and diol is observed with the highest % ee_{diol} at low biomass and short reaction times, while the highest % ee_{epox} is obtained after long reaction times and with high biomass concentrations.

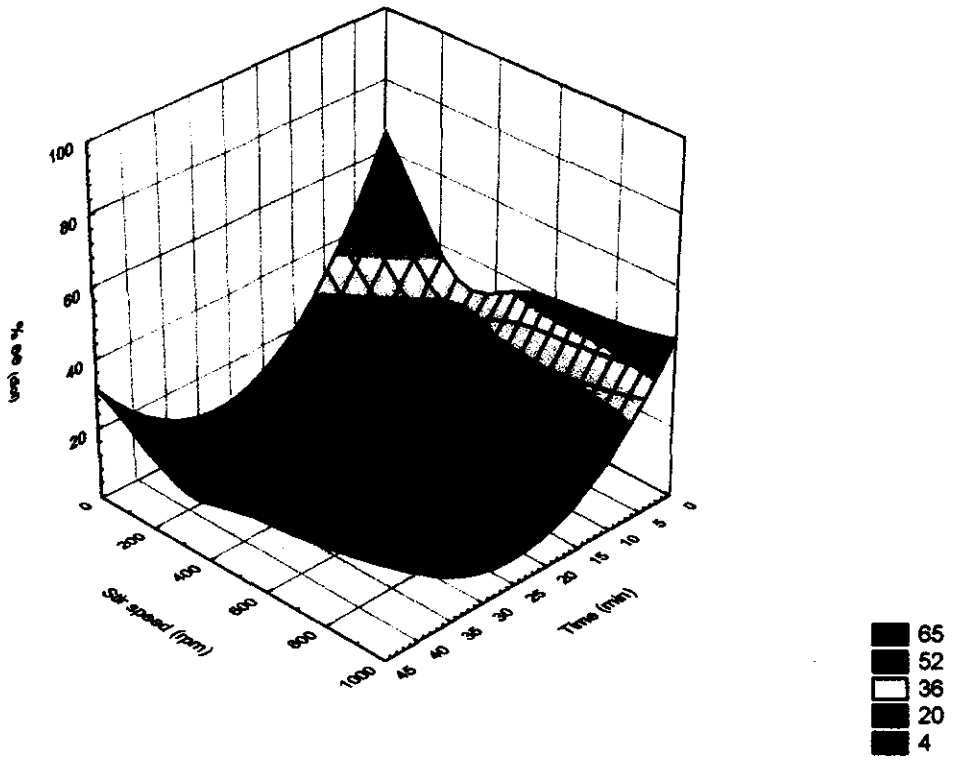

Figure 3-11. 3D graph of the effect of reaction time (min) and stir speed (rpm) on % ee_{diol}.

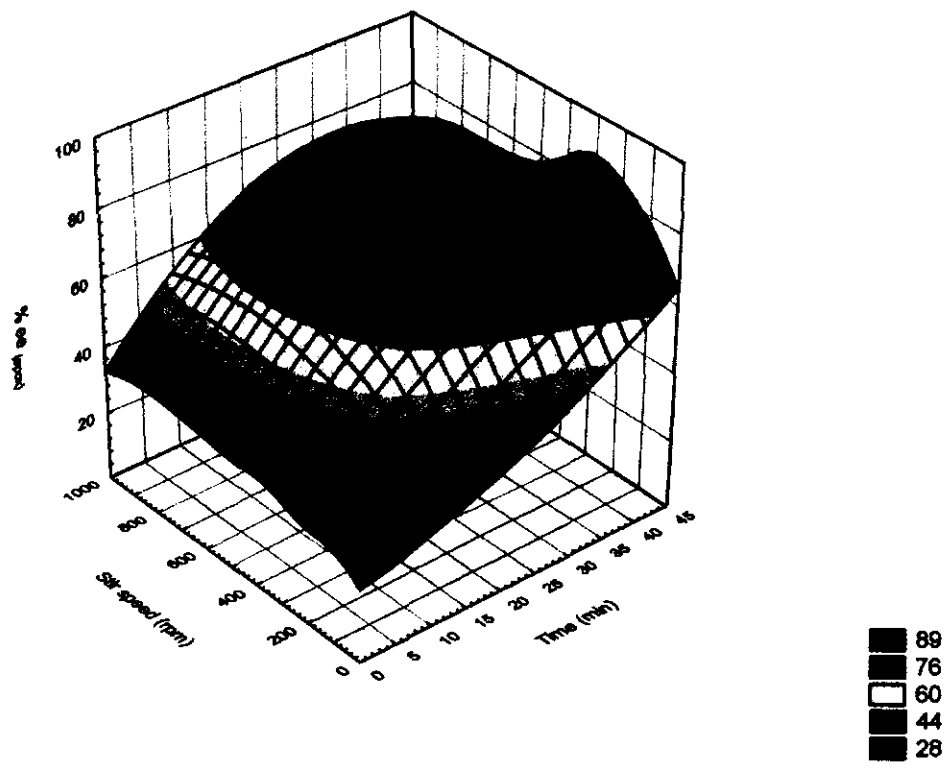

Figure 3-12. 3D graph of the effect of reaction time (min) and stir speed (rpm) on % ee_{epox}.

When comparing % ee_{diol} over time as a function of stir speed (Figure 3-11), minimum % ee_{diol} values were obtained at 25 to 30 minutes, irrespective of the stir speed. Before and after these times, an even increase in % ee is seen. The high % ee_{diol} values, at the low reaction times, can again be misleading (see Figure 3-9) due to the increased experimental error at low diol concentrations. Irrespective of reaction time, a stir speed of 450 rpm gives the lowest % ee_{diol} value and as the stir speed is increased, or decreased, the % ee_{diol} increases. The increase in stir speed facilitates the collisions between substrate and biomass, while the decrease in stir speed lowers abrasion between the biomass (cells). Both of these result in higher % ee_{diol} values.

Irrespective of stir speed there is an increase in % ee_{epox} as the reaction time increases. However, when using low stir speeds, there is a gradual increase in % ee as reaction time increases. While with high stir speeds a maximum is reached at about 30 to 35 minutes and a slight decrease in % ee_{epox} follows as the time increases above 35 minutes. This could perhaps be because there is so little of the preferred enantiomer [(R)-epoxyoctane] left that the enzyme starts to catalyse the reaction with the remaining [(S)-epoxyoctane] enantiomer. This decrease in the (S)-enantiomer will cause a decrease in % ee_{diol}.

At short reaction times the % ee_{epox} values slightly increase with increasing stir speed, while at high reaction times a maximum % ee is encountered at stir speeds of about 300 rpm. According to this figure (Figure 3-12) a maximum % ee_{epox} is achieved using a stir speed of 300 rpm and a reaction time of 45 minutes, while the highest % ee_{diol} is obtained at short (< 10 minutes) and long reaction times (45 minutes) irrespective of the stir speed.

Although there are various methods of determining E (enantiomeric ratio), only one equation gives reliable results for very low and very high conversions (equation 3-2). In such cases, the following equation is recommended, because only values for the optical purities of the substrate and product need to be measured. The latter are relative quantities, in contrast to the conversion, which is an absolute quantity [20].

In Figure 3-13 the E values for the three variables are presented.

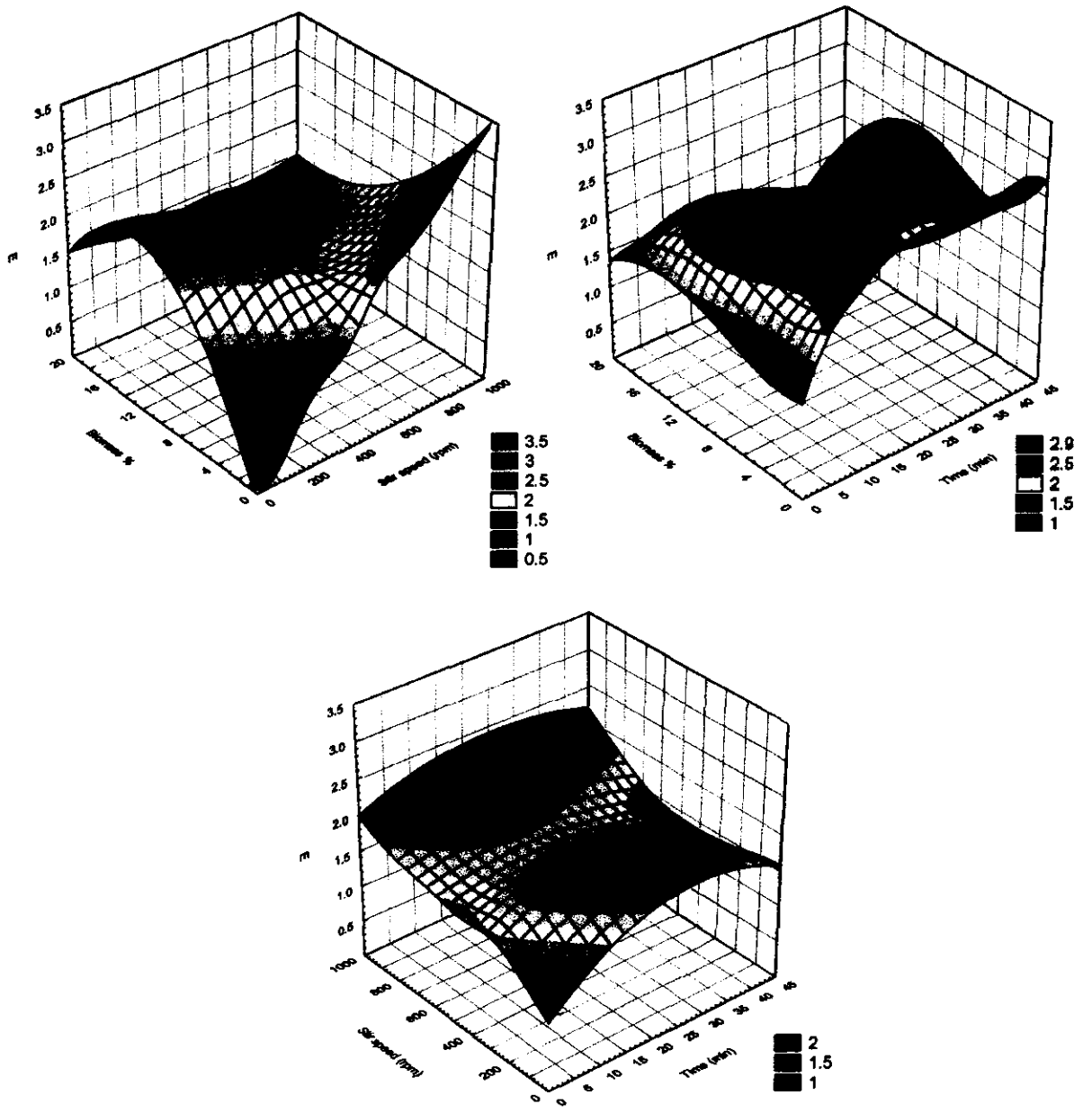

Figure 3-13. 3D-graphs to show the effect that stir speed (rpm), biomass concentration (%) and time (min) have on the E values.

When correlating the graphs in Figure 3-3 (E values) with those in Figure 3-11 and Figure 3-12 (% ee_{epox} and % ee_{diol}), it can be seen that % ee_{epox} plays a greater role than the % ee_{diol} value in determining the E value (as can be expected when considering equation 3-2). From the graphs it can be seen that the optimum biomass concentration is again 13 %, however, there is also a tendency of high E values at low biomass concentrations, if this is combined with high stir speeds. It can be seen that high stir speeds give the highest E values. Although time does not have too much of an effect on the E value, there is a slight increase in E at about 25 minutes.

4. CONCLUSION

From the results of the initial study done on 1,2-epoxyoctane, it can be seen that Epps (small and large), should not be used in the sampling or extraction processes. It was found that the epoxide adsorbs to the Epps resulting in lower concentrations. It was also found that the solubility for this substrate, at 30 °C, and in 50 mM phosphate buffer is 3.85 mM instead of 6 mM reported previously. Thus, when working with epoxide concentrations above 3.85 mM, full volume extractions should be done to ensure that the right concentrations of epoxide are extracted. However, at concentrations below this value, sampling can be used.

From all the graphs where % ee_{diol} is plotted, it can be seen that the % ee_{diol} is less influenced by variations of the conditions than the % ee_{epox}. This also implies that the regioselectivity of the reaction is not significantly influenced by operating conditions like stir speed or cell concentrations. However, from all the graphs where % ee is presented it could be seen that the % ee values were greatly influenced by the varied operating conditions.

The optimum conditions in terms of the highest enantioselectivity (% ee_{epox}) for the batch process used are stir speeds of about 400 rpm (Figure 3-8 and Figure 3-12). From Figure 3-8 and Figure 3-10, it can be seen that at 13 % biomass the maximum % ee_{epox} is achieved, while a reaction time of about 40 minutes is most favourable in terms of the % ee_{epox} (Figure 3-10 and Figure 3-12).

At extreme values for biomass concentrations as well as stir speed, the optimum conditions for % ee_{diol} are achieved. At short time intervals maximum % ee_{diol} is achieved, regardless of biomass concentration. At reaction times longer than 40 minutes, ee_{diol} also increases irrespective of stir speed.

A correlation between the % ee_{epox} values and the E values was obtained. Maximum E values were also obtained with 13 % biomass. For optimum E, high stir speeds should be used, as well as reaction times of about 25 minutes.

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CHAPTER 4

Use of chitosan as antifouling agent in a continuous bioreactor

1. INTRODUCTION

Fouling is a major problem encountered with most filtration processes as it reduces productivity and impairs the capabilities of the membrane [1], and if the fouling effect is not controlled, membrane processes can become nonviable.

One of the problems encountered with the flow-through reactor previously used in our laboratory [2], is the fouling caused by the cake formation of the biomass on the membrane. It was thus the objective of this study to lower the fouling on the membrane of the flow-through reactor, by using a specific spacer (antifouling agent), namely chitosan. Secondary objectives included maintaining, i.e. stabilising, this higher flow rate over time. Furthermore, the optimum operating conditions for the spacer containing bioreactor were sought. Lastly, a possible correlation between flow rate and enantiomeric excess (ee) was investigated.

A decline in flux (flow rate per area of membrane) is usually an indication of concentration polarisation and/or fouling of a membrane. Permeate flux usually decreases with time because of retained molecules that accumulate on, or within, the membrane pores. Fouling, compared to concentration polarisation, is when the flux decline is not reversible by simply altering operating conditions such as direction of flow (backflushing) or gas sparging [1,3]. Based on the terminology introduced by IUPAC fouling is "a process resulting in loss of performance of a membrane due to the deposition of suspended or dissolved substances on its external surfaces, at its pore openings or within its pores" [4]. Cake formation, sometimes also called external fouling, can be reversible, while internal fouling, i.e. the blocking of the internal pore structure of the membrane, is usually irreversible. Both of these, however, cause additional membrane resistance, which in turn causes a decline in flux [3].

In most cases the flux-flow relationship can be described by Darcy's law [5]:

$$J = L_P \frac{\Delta P}{\Delta x} \qquad (4\text{-}1)$$

where J is flux ($m^3.m^{-2}.s^{-1}$ often expressed as $\ell.m^{-2}.h^{-1}$), L_P is called the permeability constant ($m^3.m^{-2}.s^{-1}.Pa^{-1}$) and $(\Delta P/\Delta x)$ is the driving force, comprising of the transmembrane pressure ΔP (Pa) and the membrane thickness Δx (m).

To simplify equation (4-1) all the factors, except pressure, can be combined in one constant (B):

$$J = B\,\Delta P \tag{4-2}$$

Volume flux (J) can also be described by the Hagen-Poiseuille equation (4-3) [5]:

$$J = \frac{\varepsilon\, r^2}{8\,\eta\,\tau}\ \frac{\Delta P}{\Delta x} \tag{4-3}$$

J is again the (water) flux ($m^3.m^{-2}.h^{-1}$) of the system, while r is the membrane's pore radius (m) and η the viscosity (Pa.s) of the liquid. The tortuosity factor (τ) and the surface porosity of the membrane (ε) are also included in this equation. Surface porosity (ε) is the fraction of the pore surface area divided by the total surface area of the membrane. This can be calculated by multiplying the amount of pores (n) with their surface area ($\pi\, r^2$), which gives the total surface area of the pores. This value is then divided by the total surface area (m^2) of the membrane.

By combining equations (4-2) and (4-3), B can be defined as

$$B = \frac{\varepsilon\, r^2}{8\,\eta\,\tau\,\Delta x} \tag{4-4}$$

However, all the factors in equation (4-4), except η, are membrane related. η is the only variable that is associated with the liquid (liquid's viscosity). To include only the membrane related variables, B' can be defined as

$$B' = \frac{\varepsilon\, r^2}{8\,\tau\,\Delta x} \tag{4-5}$$

From equations (4-2) and (4-5) it follows that

$$J = \frac{B'\,\Delta P}{\eta} \tag{4-6}$$

According to equation (4-5) B' is a permeability factor depending only on the membrane related properties. Since permeability (B') is an indication of how easy a substance permeates through the membrane, the inverse of ease of permeability thus gives an indication of the resistance of the membrane. Accordingly

$$B' = \frac{1}{\Sigma R} \tag{4-7}$$

with ΣR as the sum of the resistances in the membrane system (m^{-1}).

By combining equations (4-6) and (4-7), the following is obtained [5, 6].

$$J = \frac{\Delta P}{\eta \Sigma R} \tag{4-8}$$

Equation 4-9 can be used to calculate the individual resistances in the system, where R_{Tot} is the total resistance of the reactor, R_m is the resistance caused by the membrane and R_c the resistance due to fouling by the yeast cells (biomass).

$$R_{Tot} = R_m + R_c \tag{4-9}$$

Accordingly, the different values of R, i.e. membrane resistance and resistance caused by fouling, can be calculated.

$$R_m = \frac{\Delta P}{\eta J_{bi}} \tag{4-10}$$

where J_{bi} is the flux before fouling (only bufffer). Subsequently

$$R_{Tot} = \frac{\Delta P}{\eta J_c} \tag{4-11}$$

where J_c is the flux after fouling had occurred (buffer and cells used). From equation (4-9), (4-10) and (4-11) the fouling resistance (R_c) can be calculated.

To calculate the efficiency of a spacing agent (antifouling material) the percentage resistance removal ($\%RR$) can be calculated [6]:

$$RR\,(\%) = \left(\frac{R_c - R_{cCh}}{R_c}\right) \times 100 \qquad (4\text{-}12)$$

where R_{cCh} is the resistance caused by fouling (cells) in the presence of chitosan (Ch). Equation (4-13) can be used to compare the flux before and after fouling. Since this equation gives an indication of flux improvement in the presence of chitosan, this ratio was defined as the percentage fouling prevention ($\%FP$):

$$FP\,(\%) = \left[1 - \left(\frac{J_{cCh} - J_{bi}}{J_c - J_{bi}}\right)\right] \times 100 \qquad (4\text{-}13)$$

where J_{cCh} is the flux of the buffer solution, in the presence of cells and chitosan.

Although there are many strategies that can be used to help reduce fouling, they can be divided into 3 main groups [1, 3]:

1. Changing operating conditions
2. Membrane modification
3. Modification of the feed

Higher cross-flow velocities, pulsating flow, back washing, air back flushing [1, 3] and generation of turbulent flow within the reactor [7], are a few methods that can be used to change the operating conditions to assist in the reduction of fouling. By adding additional prefiltration or centrifugation steps, the fouling can also be reduced. However, all of these strategies have cost implications and can cause problems for downstream separation processes.

In terms of membrane modification, the most frequently used method includes the pre-treatment of membranes with hydrophilic surfactants and polymers [8, 9]. Resistance to fouling can usually be improved by increasing the hydrophilicity of the membrane surface, which inhibits non-specific binding between the membrane surface and retained molecules [10 – 12]

Feed modifications are usually done by adding a substance, for example spacers, to the feed of the reactor. A spacer (also known as filter aid or antifouling agent), can include any material that can be utilised to help reduce fouling. Although there is a wide range of spacers that have been used previously, the emphasis is on mineral filter aids and organic, self-replenishing materials. Mineral filter aids include diatomaceous earth (DE) (also known as Kieselguhr) [13 – 17] and perlite [14, 18 – 20]. The second group includes mostly fibrous materials (plant and wood fibres). Well known examples of these include kenaf core fines [16], cellulose [13, 21] and hexamethylenediamine (HMDA) [22 – 24]. The most commonly used spacers are however DE, perlite and kenaf core fines.

DE is commonly used in the beer and beet sugar production industry as a precoat and/or a filter aid [17]. Studies have shown that if the filter aid dose is increased, the average cake resistance decreases [17]. However, other studies done on DE as a filter aid (spacer), and as a filter precoat, found that the coating on the membrane often did not form properly, or cracked [13 – 16].

Perlite can also be used either as a precoat, or as a body feed, i.e. added to the feed of the reactor [14, 18]. A comparative study was done between perlite and DE, and the highest flow rate was achieved using a higher grade perlite (Dicalite 4258).

Another comparative study was done, this time comparing kenaf core fines with DE [16]. When filtering larger molecules, like silica, the two substances showed the same filtration characteristics. However, with smaller molecules, like yeasts and bacteria, DE gave better results, i.e. higher fluxes.

However, there are some limitations and problems that are encountered when working with DE and perlite. Although DE (Kieselguhr) is conventionally used for filtration of beer and sugar beet, it causes health hazards and difficulties are encountered with its waste disposal [25]. Perlite is a natural form of glass (silicous rock). Its inertness makes it ideal to be used as a spacer, and it has been safely used for over 50 years in a variety of applications. However, as with all workplace dusts, perlite can be an irritant. Exposure can result in temporary physical irritation, discomfort and impaired vision [26].

There are two factors that have to be taken into account when comparing traditional spacers to the system that was used in this study. Most research have been done with cross-flow filtration (tangential flow) systems [13 – 15], which usually yield a higher flow rate than dead-end

processes, as was used in this study. In ultrafiltration it is recommended that the cross-flow velocity should be as high as possible to diminish the effect of concentration polarisation. It also helps to sweep the surface of the membrane, reducing the chances of cake-formation.

When selecting a spacer material, the ideal would be to find a substance that has good antifouling characteristics, is chemically stable and inert. Possible disadvantages of using a substance should also be considered, for instance, whether it is an environmentally hazardous material or whether it can cause health problems.

A material which is currently finding many new applications is chitosan [27 – 30]. Chitosan is a polycationic polysaccharide that is produced by the thermochemical alkaline deacetylation of chitin [31, 32]. Chitosan is inexpensive, environmentally benign (non-toxic), harmless to humans and biodegradable [28, 31, 33, 34]. Chitosan has also been shown to have good chemical resistance, excellent film forming properties, a large capacity to fix molecules such as proteins and a high permselectivity for water [35]. Commercial chitosan flakes consist of loose, inert particles which have the potential to be a useful filter aid.

Chitosan can be used as a course ground powder (as obtained from the suppliers). However, chitosan can also be modified, by making beads to immobilise enzymes or cells. Alternatively, pre-fabricated porous chitosan beads can be bought (Chitopearl®) and the enzymes subsequently immobilised on it by adsorption [36]. Chitosan beads can be made in three basic steps, where the third step is optional [37]:

1. chitosan dissolution,
2. bead formation and
3. chemical cross-linking.

Chitosan is used in various applications, including immobilisation of enzymes [36, 38 – 42], the removal of dyes [37, 38] and heavy metals [43 - 45], and the cleaning of wastewater [42].

Since chitosan is a weakly basic, cationic water-soluble polymer with good binding properties, it is suitable for the removal of ions from water [46]. In a study using Amicon YM10, YM30 (regenerated cellulose) and PM10 (polysulfone) membranes for the ultrafiltration of brackish water softening, it was found that chitosan formed gelatine-like "flexible" light blue cakes on the membrane surface. The formed layers of chitosan were easily removed from the membrane by flushing the membrane with water immediately after ultrafiltration (UF). However, it was also

found that pH plays a very important role during UF filtration using chitosan, as the pH affects the degree of protonation of the amino groups in chitosan [45, 46].

It was thus the aim of this study to evaluate the use of chitosan as a spacer in a biocatalytic dead-end reactor, where yeast cells of *Rhodosporidium toruloides* were used for the biocatalytic chiral hydrolysis of 1,2-epoxyoctane to 1,2-octanediol (Figure 3-1) for which the yeast *Rhodosporidium toruloides* is highly stereoselective [47, 48].

2. EXPERIMENTAL

2.1. MATERIALS

Rhodosporidium toruloides (UOFS Y-0471) was obtained from the Yeast Culture Collection of the Department of Microbial, Biochemical and Food Biotechnology of the University of the Free State (South Africa). The vitamin solution was also purchased from the same department at the University of the Free State. The chemicals for the growth medium were purchased from Biolab (South Africa). 1,2-Epoxyoctane and high molecular weight chitosan flakes (coarse ground powder) were obtained from Aldrich (South Africa). Buffer materials (K_2HPO_4 and KH_2PO_4) and glycerol were purchased from Saarchem (South Africa). Analytical grade ethyl acetate (EtOAc) was bought from Acros Organics. Deionised water (pH 7.0) (Millipore Milli-Q Plus® CPMQ004R1) was used throughout. All reagents were of analytical grade and used without further purification.

2.2. METHODS

2.2.1. Analysis

For the analysis and quantification of the reactions a Thermo Finnigan Focus GC, equipped with FID and AS 3000 autosampler, was used. The chiral separation was achieved by using a fused silica β-cyclodextrin column (CP Chirasil-DEX CB, 25 m x 0.25 mm, 0.25 µm film). H_2 was used as carrier gas, as it yields a higher selectivity than N_2 or He. The epoxide and diol were analysed at 70 °C and 140 °C, respectively. The retention times were Rt (70 °C) = 21.5 min and 21.8 min respectively for the (R)- and (S)-1,2-epoxyoctane and the retention times were Rt (140 °C) = 11.9 min and 12.3 min respectively for the (S)- and (R)-1,2-octanediol. The absolute configuration of the epoxide and diol had been established previously [49]. Butanol was used as an internal standard and its retention times were Rt (70 °C) = 4.0 min and Rt (140 °C) = 1.65 min. Quantification was done by using calibration curves.

2.2.2. Preparation of frozen yeast cells

Growth media consisting of 20 % (w/v) malt extract, 15 % (w/v) glucose, 5 % (w/v) peptone and yeast extract was used for the cultivation of the yeast cells. 2 % (v/v) of a filter sterilised vitamin solution was added to the growth media. After inoculation the cells were grown at 27 °C for 4 days on a Labcon® rotary shaker (180 rpm), before being harvested by centrifugation (5000 g, 5 min, 4 °C). The cells were washed twice with phosphate buffer (50 mM, pH 7.5) and

resuspended (50 % m/m) in phosphate buffer containing 20 % glycerol. The cells were frozen in 50 ml centrifuge tubes and stored at –18 °C. Cells were thawed prior to each experiment.

2.2.3. Flow optimisation

The experiments were done in a dead-end reactor as illustrated in Figure 4-1. The reactor, manufactured by the Department of Instrument Making, Potchefstroom University, had been used in previous studies [2, 47, 48]. It has a feed tank capacity of 400 ml and a reaction chamber with a diameter of 11.8 mm and a height of 43 mm.

For the flow optimisation study the feed consisted of 300 ml phosphate buffer (50 mM), consisting of 40 mM K_2HPO_4 and 10 mM KH_2PO_4, which contained 20 mM racemic 1,2-epoxyoctane. Nitrogen gas was utilised to apply pressure to the feed phase. A regulator was used to control the pressure applied to the feed, which was stirred at 1000 rpm to ensure emulsification and maximum solubilisation of the epoxide in the buffer. The temperature was maintained at 30 °C by a temperature-controlling mantle connected to a circulating water bath.

On the feed side a 0.5 mm pore diameter polyethylene support was used to help ensure that the stirring of the feed would not affect the cells or chitosan in the reaction chamber. On the permeate side a 0.45 µm pore diameter nylon membrane (47 mm diameter from Osmonics), supported on a 0.5 mm pore diameter copper support, was used to ensure that the cells were not washed from the reactor during permeation. The surface area of the membrane used was 1.21×10^{-3} m^2.

Before each experiment the specified amount of chitosan was measured and placed into the reaction chamber. The specified amount of thawed cells was then added to the chamber. Subsequently the reaction chamber was filled with phosphate buffer before being connected to the feed tank.

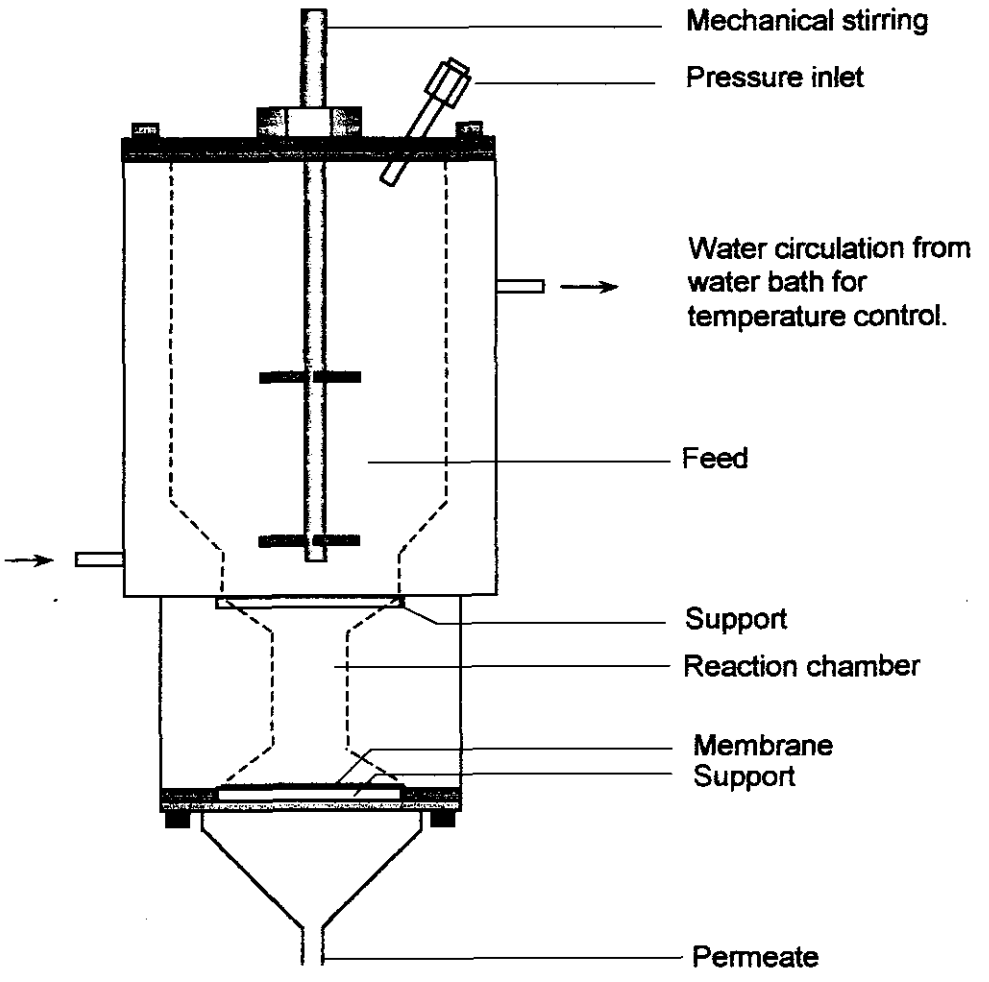

Figure 4-1. The dead-end reactor used for the reactions.

2.2.4. Feasibility study

A preliminary study was done to determine which variables affect the flow and thus fouling. Firstly the effect of pressure on fouling was investigated in the absence of chitosan. In a second set of runs the effect of chitosan was studied. In Table 4-1 the various experimental conditions of the preliminary runs are given.

Table 4-1. The experimental conditions for the preliminary study.

No.	Biomass (g)	Chitosan (g)	Pressure (kPa)
1	1.0	0.0	50
2	1.0	0.0	100
3	0.0	0.0	100
4	0.0	0.5	100
5	0.0	1.0	100
6	1.0	0.0	100
7	1.0	0.5	100
8	1.0	1.0	100

From the feasibility study the flow rate (Q), resistance (R), resistance removal (% RR) and fouling prevention (% FP) were calculated.

2.2.5. Effect of pressure, chitosan and biomass concentrations on flow rate

After the preliminary study a statistical design was used to optimise the flow. In this design the effect of the variables investigated in the preliminary study (pressure and spacer amount), as well as the biomass concentration on the flow rate was determined. The statistical software program Statistica 6.0 was used for the design modelling and entailed a response surface statistical method with a central composite design [50 – 52]. Two extra centre points (numbers 17c and 18c) were added to the model to increase the confidence level (Table 4-2). The ranges for the three variables were:

Pressure	25 – 125 kPa
Chitosan amount used	0 – 1.5 g
Cell amount used	0.5 – 1.5 g

The flow rate was measured in terms of the volume of permeate per time unit ($ml.min^{-1}$). Since the flow rates reached steady state within 8 hours for all the runs, all flow rates were determined at 480 minutes.

Table 4-2. The randomised design used in the experiments.

Run No.	Pressure (kPa)	Wet cell weight (g)	Chitosan (g)
8	100	1.3	1.202
7	100	1.3	0.304
15 (c)	70	1.0	0.755
11	75	0.5	0.755
3	50	1.3	0.304
5	100	0.7	0.303
17 (c)	75	1.0	0.755
10	117	1.0	0.755
16 (c)	70	1.0	0.753
6	100	0.7	1.202
18 (c)	75	1.0	0.755
9	33	1.0	0.754
4	45	1.3	1.205
2	45	0.7	1.204
13	70	1.0	0.000
1	50	0.7	0.305
14	75	1.0	1.504
12	70	1.5	0.754

2.2.6. Effect of flow rate on enantiomeric excess (ee) of epoxide and diol

The effect of the flow rate on the % ee was determined by combining the data of runs done previously [2] with the present study. All the runs were done with 1.2 g of cells. Samples (500 µl) were taken and extracted with 500 µl ethyl acetate. The extracts were then dried over Na_2SO_4 and analysed by GC.

3. RESULTS & DISCUSSION

3.1. FEASIBILITY STUDY

In the feasibility study, the effect of pressure and chitosan on the flow rate was determined. The influence of the pressure applied on the flow rate is shown in Figure 4-2.

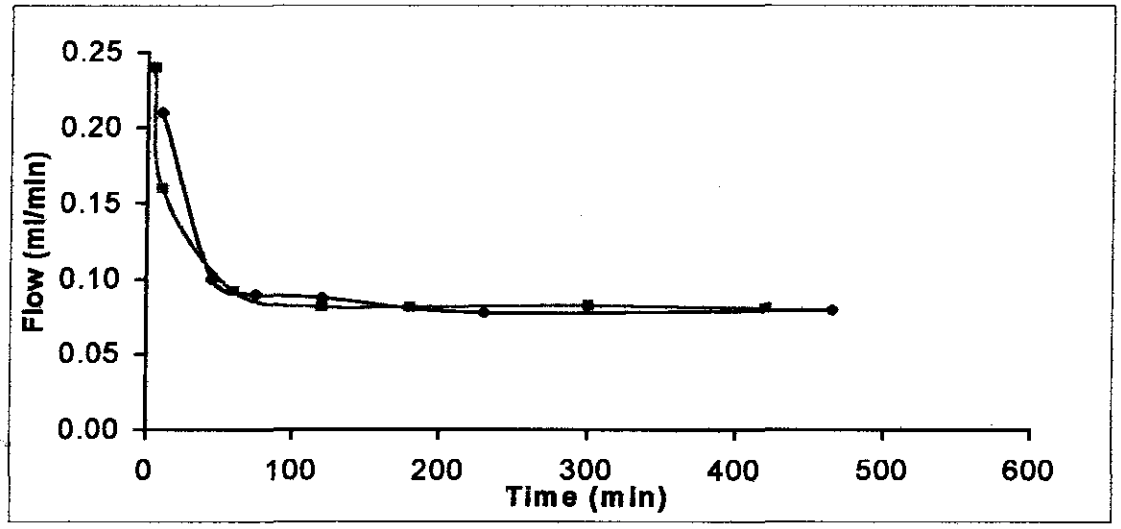

Figure 4-2. Flow rate of reactors run using 1 g of cells, no chitosan and pressures of 50 (—◆—) and 100 kPa (—■—).

An initial decrease in the flow rate can be seen in the first 45 minutes for both the 50 and 100 kPa runs. After this initial decrease, a state of equilibrium was however reached. Interestingly, both pressures yielded the same flow. To understand the effect of pressure and resistance on the flow rate, the following must be considered. It is known [5] that

$$Q = J \times A \tag{4-14}$$

where Q is the flow of the reactor (ml/min) and A is the surface area (m^2) of the membrane used in the reactor. By combining equations (4-8) and (4-14) it follows that

$$Q = \frac{\Delta P}{\eta \Sigma R} \times A \tag{4-15}$$

Since both the viscosity and surface area remain constant for all the experiments in this study equation (4-15) can be further simplified to:

$$Q \propto \frac{\Delta P}{\Sigma R} \qquad (4\text{-}16)$$

Since the flow remained constant with increasing pressure (Figure 4-2), it can safely be assumed that the resistance (R) increased proportionally to the pressure. This could be due to the higher compaction of the biomass at higher pressures resulting in the net flow remaining constant irrespective of pressure. It was decided to study the effect of the second variable (amount of chitosan used) at a pressure of 100 kPa. The results are shown in Figure 4-3.

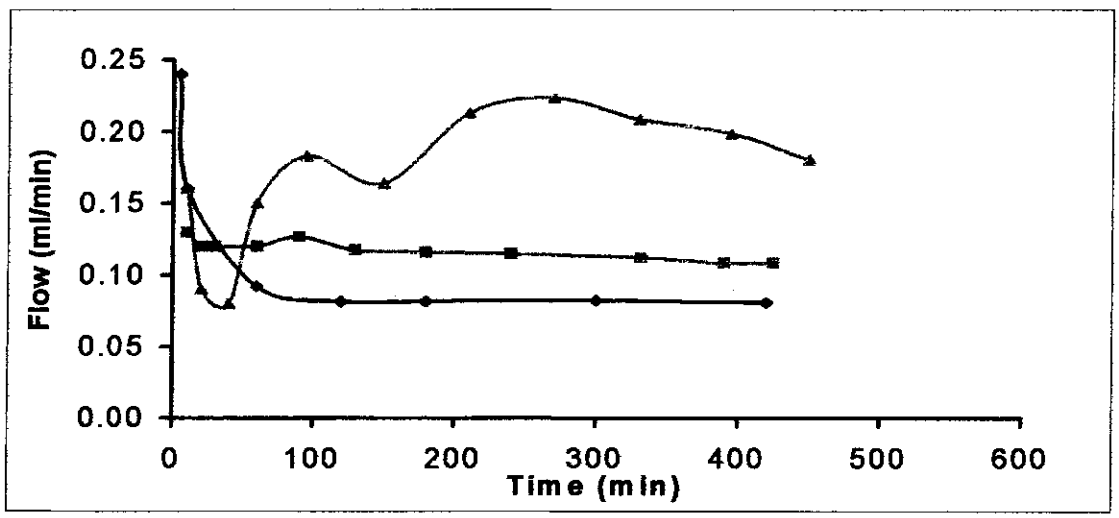

Figure 4-3. Flow rate with 1 g of cells, 100 kPa pressure and different amounts of chitosan. Chitosan amounts were 0 g (—◆—), 0.5 g (—■—) and 1 g (—▲—).

From these results it is clear that the spacer improves the flow and thus reduces fouling. With just 0.5 g of chitosan a higher flow rate (± 34 % increase) was obtained and maintained. With the addition of 1 g of chitosan, fluctuations in the flow rate occurred. Although these changes in the flow rate can not be fully explained, it could be due to air trapped in the reaction chamber. Another possible reason for the decrease in the fluctuations of the flow rate was that the cells in the reaction chamber might not have been evenly distributed throughout the chitosan matrix, which could then cause areas of higher resistance, with a subsequent decrease in flow rate. To ensure equilibrium of the 1 g experiment, the reactor setup was run for $32^1/_2$ hours. It was confirmed that equilibrium had in fact been reached at 420 min. It is thus important to note that

the runs done with chitosan not only had increased average flow rates, but the chitosan was further able to maintain the higher flow rates for extended periods of time.

To quantify the effect that each of the variables had on the reactor system, the flow (Q), resistance (R), percentage resistance removal (% RR) and percentage fouling prevention (% FP) for runs 3 to 8 (Table 4-1) were determined (Table 4-3). According to equation (3-13) the initial flux (J_{bi}) of the system, i.e. flux of buffer only, is part of the equation used to determine % FP. Since this value (J_{bi}) is much higher than the other fluxes (J_{cCh} and J_c) the % FP values are very low and not very useful in the assessment of the different variables. Runs no 7 and 8 (Table 4-3), for example, have % FP values of 0.016 and 0.066 %, respectively. Thus only the % RR is further used as an indication of chitosan efficiency.

Table 4-3. Flow, resistance and resistance removal at 100 kPa.

Variables	Q	R_c †	RR (%)
	(ml.min^{-1})	(x10^{10} m^{-1})	(%)
3. Buffer	162	0	-
4. Buffer and 0.5 g of chitosan	150	0.36	-
5. Buffer and 1 g of chitosan	53.25	9.16	-
6. Buffer and 1 g of biomass	0.081	8980	-
7. Buffer and 1 g of cells and 0.5 g of chitosan	0.107	6784	24.5
8. Buffer and1 g of cells and 1 g of chitosan	0.188	3868	56.9

† Resistance of added components, i.e. cells and chitosan ($R_{Tot} - R_m$)

From the data of runs 3 to 5 it can be seen that chitosan on its own reduces the flow rate, thus acting as a resistance in the system. However, the flow decrease and resistance increase are negligible compared to the decrease in flow and increase in resistance, after the addition of cells to the reactor setup (runs 6 to 8). It is clear from runs 6 to 8 that chitosan does reduce the fouling, and thus the cake formation of the biomass. At 1.0 g of chitosan the resistance removal was 57 %, compared to the 25 % RR when using 0.5 g of chitosan, indicating a near linear proportionality between % RR and amount of chitosan used.

Figure 4-3 and Table 4-3 again illustrates that as the chitosan concentration increases, the resistance decreased, and higher flow rates are achieved, confirming that chitosan does reduce fouling according to equation (3-16), which states that $Q \propto \frac{1}{R}$ [5].

3.2. EFFECT OF PRESSURE, CHITOSAN AND BIOMASS CONCENTRATIONS ON FLOW RATE

The flow was measured (ml.min^{-1}) at 480 minutes for each of the conditions set out by the statistical design. The table showing the flow rates at 480 minutes, is given in Appendix 2.

It was mentioned in the experimental procedure that four centre points (c), i.e. 4 runs with exactly the same conditions (Table 4-2), were used to ensure reproducibility. This ensures the validity and reliability of the design. The flow versus time plots of the 4 centre point runs are given in Figure 4-4. It can be seen that at equilibrium, the flow rates for all 4 runs are approximately 0.077 ± 0.005 ml.min^{-1}.

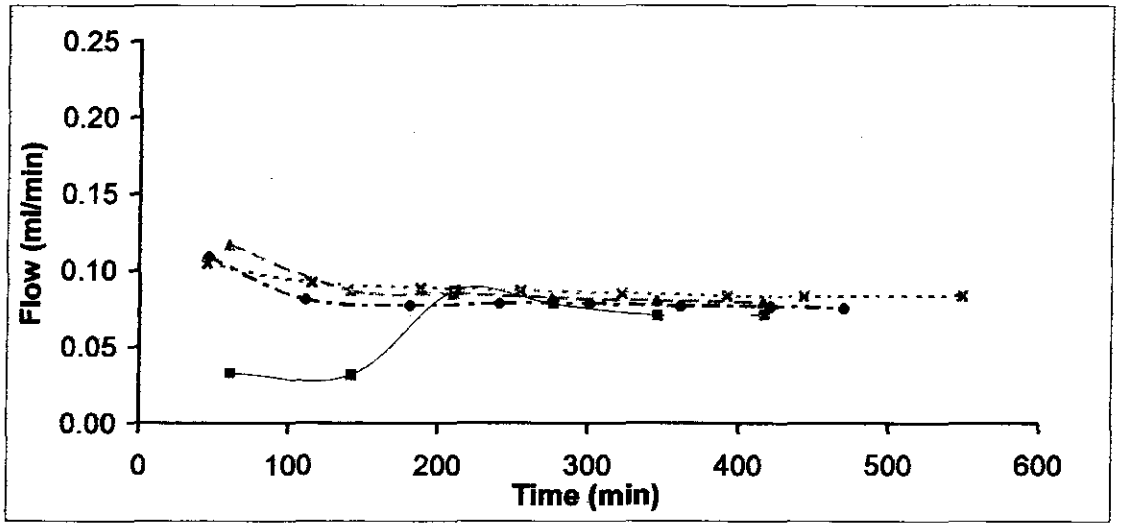

Figure 4-4. Flow of the four centre point runs, 15 (– ◆ –), 16 (—■—), 17 (– ▲ -) and 18 (···✱···) of the central composite design.

The standard deviation (*sd*) is only 5.1 x 10^{-3} ml.min^{-1}. Since the standard deviation is an indication of the distance of the individual values from the arithmetic mean, the experimental error can thus be calculated as approximately 6.6 %. This is within acceptable limits, considering that both chitosan and yeast cells (biological material) contribute to this variation.

Before analysing the 3D-surface plots, all data related from the statistical design were grouped and presented in Figure 4-5 (100 kPa) and Figure 4-6 (50 kPa) respectively. They illustrate the effect of chitosan and the influence of the amount of chitosan used on flow and fouling.

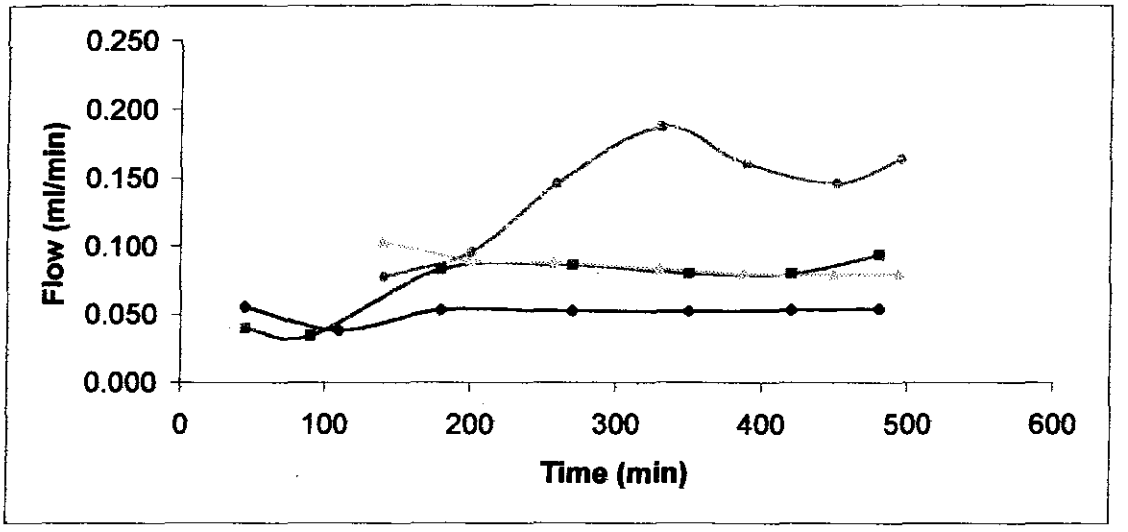

Figure 4-5. Flow at 100 kPa, with the following cell and chitosan combinations: 0.7 g of cells with 0.3 g of chitosan (—▲—), 0.7 g of cells with 1.2 g of chitosan (—●—), 1.3 g of cells with 0.3 g of chitosan (—◆—) and 1.3 g of cells with 1.2 g of chitosan (—■—).

From Figure 4-5 it can be seen that if the runs with the same amount of cells are compared, the two runs done with 1.2 g of chitosan had higher flow rates than the runs done with 0.3 g of chitosan. This confirms the results obtained in the preliminary study (Figure 4-3 and Table 4-3).

It has been shown previously (Table 4-3) that chitosan effectively reduces the fouling, i.e. cake formation (resistance), and therefore flow is higher. Both the runs done with 1.2 g of chitosan showed fluctuations in the flow. This confirms that the fluctuations seen in the preliminary study (Figure 4-3) was not due to experimental error, but rather that it is related to the dense packing of chitosan in the reaction chamber.

It can also be seen that the reactors run with 0.7 g of cells had higher flow rates that the ones run with 1.3 g of cells. If more cells are used the cake thickness and thus R_c increases, which causes the flow to decrease.

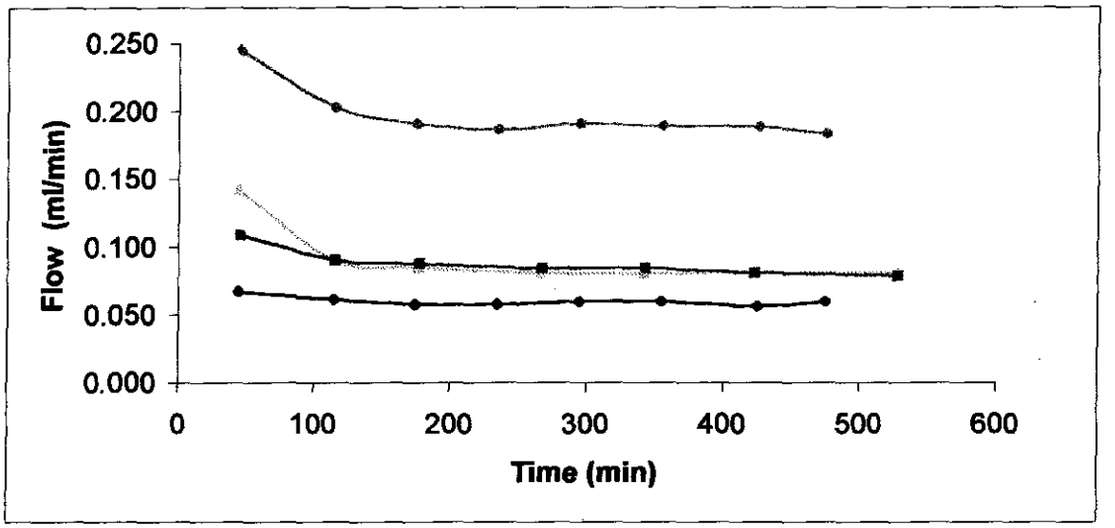

Figure 4-6. Flow of runs done at 50 kPa with the following cell and chitosan combinations: 0.7g of cells with 0.3 g of chitosan (—◆—), 0.7 g of cells with 1.2 g of chitosan (—●—), 1.3 g of cells with 0.3 g of chitosan (—◆—) and 1.3 g of cells with 1.2 g of chitosan (—■—).

In Figure 4-6 a similar pattern to that of the runs done at 100 kPa (Figure 4-5) can be seen. The higher chitosan concentrations cause a more effective decrease in fouling (cake formation) than the low chitosan concentrations. It can also be seen that at the higher cell concentrations fouling (cake thickness) increases.

Further comparison of Figure 4-5 and Figure 4-6 shows that the highest flow is obtained with high chitosan (1.2 g) and low cell concentrations (0.7 g), and vice versa. It can also be seen that, in general, flow at 50 kPa (Figure 4-6) was higher than flow at 100 kPa (Figure 4-5). This means that flow increases with a decrease in pressure. This confirms (according to equation 4-16) that the *effect* of less compaction (lower R_c) has a larger influence on flow than the increase in the driving force (higher ΔP).

Thus it would seem as though there is a relation between the amount of chitosan, the amount of cells that reach the membrane, the flux in the reactor and the amount of fouling that takes place. To show that the three variables (pressure, chitosan concentration and biomass concentration) are interconnected, three-dimensional graphs were drawn where the flow was plotted as a function of the three variables and their various combinations. This was done by plotting flow against the corresponding values of two of the three variables. The distance weighed least squared method was used to draw the graphs using the Statistica program. In all of the graphs the z-axis represents the flow at 480 minutes.

In the fourth phase (> 90 kPa) cake formation, but most probably cake compaction, increases causing a drastic increase in resistance.

The same trend can be seen at the high biomass concentrations. Three distinctive flow stages can be identified. The first phase is at pressures below 50 kPa, the second is between 50 and 90 kPa and the last one is at pressures above 90 kPa.

According to Figure 4-9 the optimum conditions in terms of pressure and biomass are achieved when using a pressure of about 40 kPa (medium-low) and 0.4 g of biomass (lowest). The maximum flow rate achieved under these conditions was 0.34 $ml.min^{-1}$.

It is important to keep in mind that with all the 3D plots one variable, in each instance, is not shown, although it has an effect on the flow rate. The variables are the amount of cells (Figure 4-7), pressure applied (Figure 4-8) and the amount of chitosan used (Figure 4-9). It is thus interesting to combine some of the results obtained, specifically the influence that the chitosan to biomass ratio has on flux (Figure 4-10).

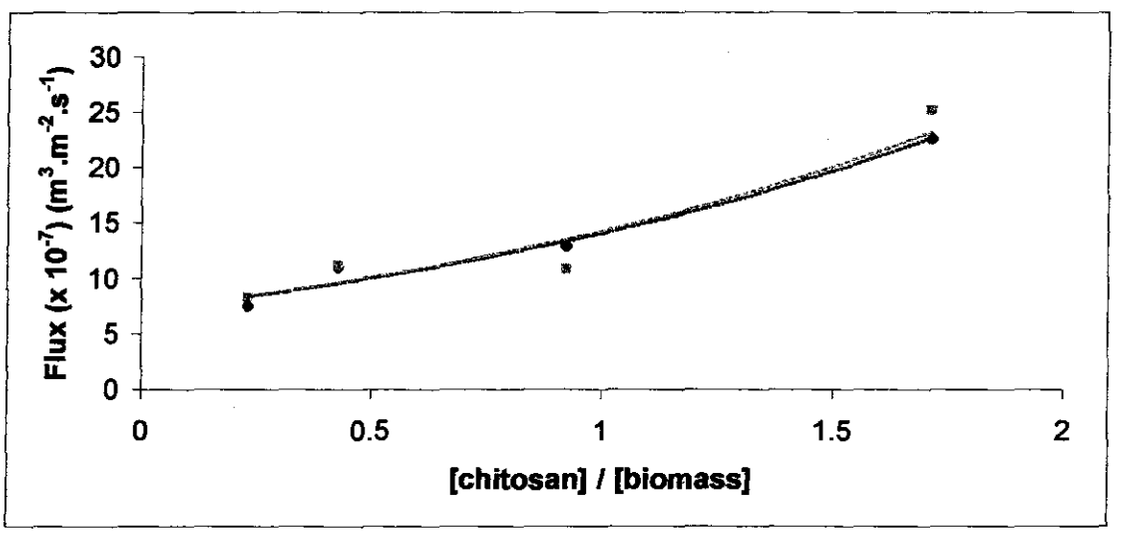

Figure 4-10. Flux of runs done at 100 kPa (···◆···) and 50 kPa (– ■ –), vs. the ratio of chitosan used (g) to biomass used (g). Runs were performed with 0.7 and 1.3 g of biomass in combination with 0.3 and 1.2 g of chitosan.

From Figure 4-10 it can be seen that irrespective of the pressure used, the flux increases as the ratio of chitosan to biomass increases. According to Equation 3-8, this implies that as the chitosan concentration increases, its ability to prevent fouling increases (resistance decreases and flow increases). A second reason is that the biomass concentration decreases with

increasing chitosan : biomass ratio, which also helps reduce resistance (and increase flux). When resistance is plotted as a function of the chitosan : biomass ratio (Figure 4-11) it can be seen that the resistance at 100 kPa is slightly higher than the resistance obtained at 50 kPa.

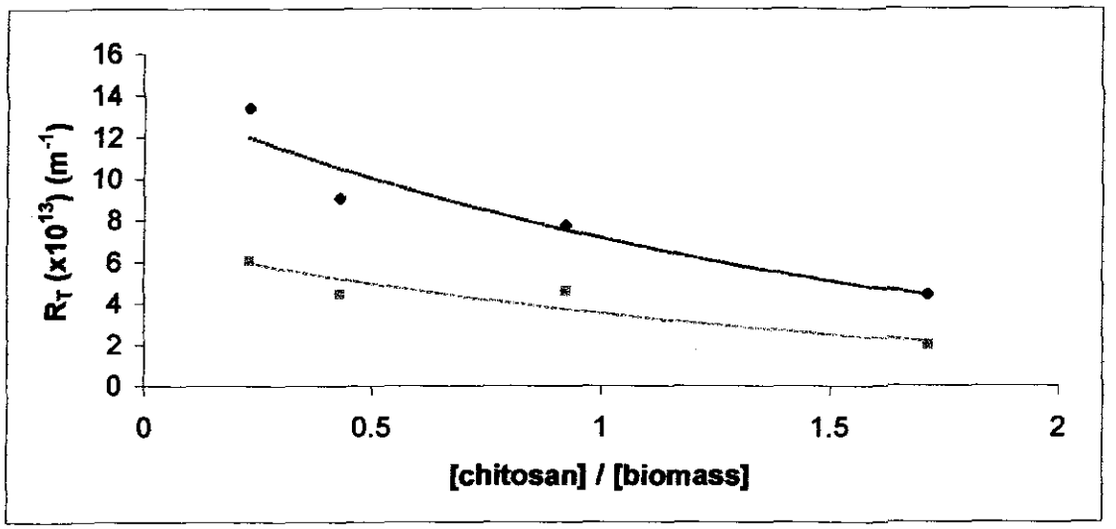

Figure 4-11. Total resistance of reactor setup, i.e. resistance caused by buffer, cells and chitosan vs. the ratio of chitosan used (g) to biomass used (g). Runs were done at 100 kPa (···◆···) and at 50 kPa (– ■ –). Runs were performed with 0.7 or 1.3 g of biomass in combination with 0.3 or 1.2 g of chitosan.

It is clear that for both pressures (50 and 100 kPa), the resistance decreases as the chitosan concentration in relation to the biomass increases. In the region where the chitosan : biomass ratio is low (high biomass concentrations), the relative resistance at 100 kPa, compared to 50 kPa, is much higher compared to the region where the chitosan : biomass ratio is high. In the low ratio region the biomass concentration is high, causing a high resistance to begin with. The higher pressure (100 kPa) increases the cake formation and compaction on the membrane, which further increases the resistance of the system. Both lines show a steady decrease, which can be explained by the higher ratio of chitosan to biomass. The higher concentrations of chitosan can more easily prevent cake formation while the lower biomass concentrations used cause less resistance. It has to be taken into account that chitosan also has a resistance (see Table 4-3). This might also contribute to the higher resistance, specifically at the high chitosan : biomass ratios. At higher pressures there are more compaction of both biomass and chitosan.

A viable conclusion as to what conditions will present the optimum run conditions, can only be made after all the data had been combined. From both Figure 4-7 and Figure 4-8 it can be

concluded that the optimum pressure for maximum flow rates would be between 40 and 43 kPa. This is also confirmed from the graphs in Figure 4-5 and Figure 4-6. The flow rates at 50 kPa (Figure 4-6) were slightly higher than the flow rates at 100 kPa (Figure 4-5). This further correlates with Equation 3-16. When the pressure increases above 45 kPa, the resistance increases and flow decreases while at pressures below 40 kPa, the pressure (driving force) is too low and flow decreases as pressure decreases.

According to both Figure 4-8 and Figure 4-9, the optimum biomass concentration is the lowest amount of biomass that can be used and in this study 0.4 g was used. This can again be explained in terms of Equation 3-16. As the biomass concentration decreases the resistance decreases and flow rates improve.

The optimum chitosan concentration would be the maximum amount of chitosan that can fit into the reaction chamber (Figure 4-7 and Figure 4-8). Figure 4-3 also verifies that an increase in chitosan concentration yields higher flow rates. The maximum amount of chitosan that can be packed into the reaction chamber is about 1.9 g. As the chitosan concentrations increase, resistance is lowered and flow rates improve.

3.3. EFFECT OF FLOW RATE ON ENANTIOMERIC EXCESS (EE) OF EPOXIDE AND DIOL

Although maximum flow was achieved using the least amount of biomass (cells) and the highest concentration of chitosan, this might not necessarily be the optimum conditions for the reactor. An important factor that has to be kept in mind is that the flow rate will affect the contact time of the substrate with the cells.

It has previously been shown in batch studies that contact time has a substantial influence on % ee (see equation 3-1) of both epoxide and diol [2, 48, 53, 54].

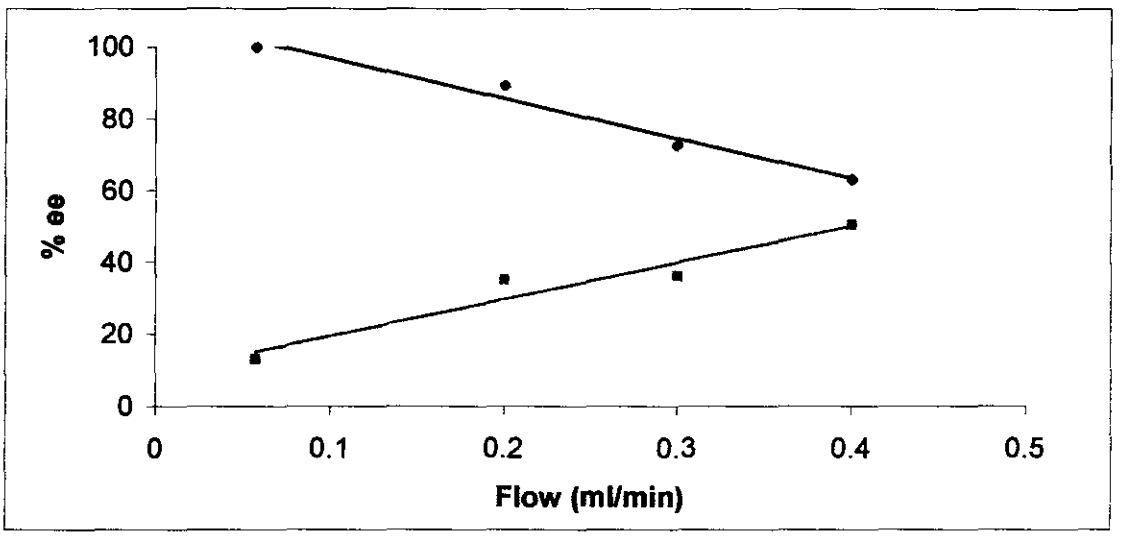

Figure 4-12. Calculated ee (%) values of epoxide (—●—) and diol (—■—). The runs were done with 1.2 g of cells and the ee were calculated after steady state had been reached.

From Figure 4-12 it is clear that there is a correlation between the flow rate and the enantiomeric excess of both the epoxide and the diol. Consequently it was shown that this correlation not only exist in batch processes, but also in continuous processes as was used in this study. If an epoxide with high % ee value is needed, a low flow rate would be more constructive to use. However, if a diol with high % ee value is required, a high flow rate would yield better results. Nonetheless, in both cases the chitosan would help to stabilise the flow and thus contribute to the optimisation of the reactor setup, especially at low pressures and high chitosan concentrations.

4. CONCLUSIONS

In this study the feasibility of using chitosan as a spacer in a bioreactor to help decrease fouling was successfully demonstrated. It was shown that the flow rate could be improved by using chitosan in the reactor. As the flow rate could be increased and maintained at this higher level, it is a clear indication of the fouling reducing properties of chitosan. It was further shown that the maximum flow (0.233 $ml.min^{-1}$) for this reactor was achieved by using as small amount of cells as possible, in the region of about 0.4 g. The minimum amount of biomass will, however, be determined by the effective hydrolysis of 1,2-epoxyoctane.

A higher flow rate would not necessarily mean that the overall performance of the reactor has improved - a balance has to be found between the flow rate (increase) and the (minimum) amount of catalyst required for an effective conversion.

The optimum amount of chitosan will be about 1.9 g, which is basically the maximum amount of chitosan that can be packed into the reaction chamber. The optimum pressure for this reactor setup is approximately 40 to 43 kPa. If lower pressures are used the flow rate decreases as the driving force decreases. If pressures above 45 kPa are used, cake formation and compaction on the membrane surface increases (porosity decreases) and the resistance will increase, causing a decrease in the flow rate of the reactor. The chitosan not only increased, but also helped in stabilising the flow rate in the reactor. The importance of a stable flow rate was illustrated by showing the linear relationship between flow rate and % ee of both the epoxide (substrate) and the diol (product).

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1. INTRODUCTION

Since enantiomers are so difficult to separate it is very common to find drugs that are sold as racemic mixtures. This means that at least half of a racemic drug could be inactive, or even cause side effects [1]. It is thus important to find or optimise ways of separating enantiomers.

Enzymes are chiral and frequently react almost exclusively with only one of the two enantiomers and are hence used as biocatalysts for the separation of enantiomers [2, 3]. The biocatalyst used in this study is *Rhodosporidium toruloides,* which contains an epoxide hydrolase enzyme. This enzyme facilitates the resolution of 1,2-epoxyoctane (Figure 3-1).

2. DISCUSSION

In this thesis the reaction system was evaluated and further optimised in both a batch process (Chapter 3) and a continuous process (Chapter 4).

2.1. BATCH PROCESS

Due to continual inconsistencies observed when measuring epoxide concentrations, initial experimental work was done to find possible explanations for this behaviour (Chapter 3). It was found not only that epoxide adsorbs to different Epps (0.5 and 1.5 ml), but also adsorbs to them differently. It was also shown that the method of sampling i.e. full volume extraction or sample extraction contributes to deviations in epoxide concentrations, since sample extraction is limited to the amount of epoxide in the buffer phase. Finally it was shown that the solubility of epoxide in buffer at 30 °C is in fact 3.85 mM, which is lower than previously reported (6 mM) [4].
After elimination of the epoxide concentration inconsistencies, the batch process reactor previously used [4] was optimised in terms of stir speed and cell (biomass) concentration. It was found that as the stir speed increases, the diol (product) concentration increase over time is higher. Furthermore the % ee_{diol} stabilised after about 10 minutes of reaction time, except at the lowest stir speed (72 rpm), which only reached steady state after about 20 minutes. The % ee_{epox} stabilised after only 20 minutes, while the % ee_{epox} at 72 rpm had not reached steady state after 40 minutes.

It was found that as the stir speed increases the % ee_{epox} increases. However, as the stir speed is increased above 500 rpm the % ee_{epox} decreases again. A similar trend was observed for the cell (biomass) concentrations. As the biomass concentration increases, the % ee_{epox} increases, but above 13 % the % ee_{epox} decreases again. Both of these tendencies can be explained by the fact that the increases in stir speed and biomass concentration cause more collisions between the substrate and the biocatalyst increasing the reaction rate. However, if the stir speed and biomass concentration are increased beyond 500 rpm and 13 % respectively, abrasion of the cells occur and the % ee_{epox} of the cells decrease. It is also evident that as the reaction times increase, the % ee_{epox} of the reaction increases initially (up to 40 minutes) before decreasing again. It is possible that as the time increases the % ee_{epox} increases, due to the progression of the reaction. However, after 40 minutes, all of the (R)-epoxyoctane has been hydrolysed and thus the enzyme starts to convert the (S)-epoxyoctane to (S)-octanediol. This in turn causes a decrease in the % ee_{epox} of the reaction. Lastly it can be seen that the optimum conditions for % ee_{epox} and % ee_{diol} are opposing. For high % ee_{diol} values, reaction times (contact times) of less than 15 minutes are needed, while high % ee_{epox} needs reaction times (contact times) of more than 30 minutes.

2.2. CONTINUOUS PROCESS

Before further optimisation of the continuous process, a preliminary study (Chapter 4) was done to evaluate whether chitosan can help to lower fouling during filtration in a continuous bioreactor. The resistance removal (% RR) was calculated to be 24.5 and 56.9 % for 0.5 g and 1 g chitosan respectively (at 100 kPa and 1 g biomass). From the statistical design (Chapter 4) it could be seen that, especially at medium pressures (45 kPa), increased and stabilised flow rates are achieved. At the lower pressures flow increased with an increase in pressure, as the resistance nearly remained constant with an increase in pressure. For maximum flow rates the highest chitosan amount (1.6 g) and lowest biomass amount (0.4 g) should be used. The high chitosan amounts prevent cake formation and/or compaction on the membrane, thus resulting in a decrease in fouling. If less cells (biomass) are used, less cake formation occurs. This was confirmed by the obtained flux improvements when increasing the chitosan : biomass ratio. Resistance decreases as this ratio increases, irrespective of the pressure (50 or 100 kPa) used. At 100 kPa the resistance is, however, much higher than at 50 kPa when using a low chitosan : biomass ratio. As the ratio increases, the difference between the resistance at the two pressures decrease.

Nevertheless, a higher flow rate does not necessarily mean that the overall performance of the reactor has improved – a balance has to be found between the flow rate and the % ee_{epox} or % ee_{diol}. When using low flow rates, a high % ee_{epox} is achieved, while a low flow rate results in a high % ee_{diol}. Thus the optimum flow rate will be determined by which of the two enantiomers are to be purified, the substrate or the product.

3. SUMMARY

It can be seen that reaction time, i.e. the effective contact time between the enzyme (biomass) and the substrate (epoxide), plays a very important role in the % ee of both the substrate and product. It should be decided which of these two compounds are needed, prior to determining the optimum operating conditions. High % ee_{epox} values can be achieved by using long contact times. In a batch process this can be achieved by allowing the reactor to run for longer times, while a low flow rate in a continuous reactor process would achieve the same goal. The opposite is true for high % ee_{diol} values, which can be achieved by short contact times in a batch process, or high flow rates in a flow-through reactor.

Up to date most of the studies with this specific reaction were done in a batch process. However, before optimising the batch process, the problem of inconsistent epoxide concentrations had to be resolved. After optimising the stir speed and biomass concentration in a batch process, a continuous reactor process was optimised in terms of flow rate and % ee (of both epoxide and diol). Both batch and continuous processes have certain advantages and disadvantages (Table 5-1) that have to be considered.

Other problems encountered with the batch and continuous system was that for the batch process a full volume extraction with ethyl acetate was necessary, resulting in the deactivation of the enzyme, thus implying that the cells can only be used once. Another problem, typical of all batch processes, are related to the process interruptions. After adding all the components (cells, buffer and substrate) the reaction starts. After about 40 minutes an extraction is done. Subsequently, the reaction vessel has to be cleaned before a second run can be started. Disadvantages for the flow-through reactor, however, include the very low concentrations of epoxide that were recovered from the reactor. This could have been due to the low solubility of 1,2-epoxyoctane, or the evaporation from the politop in which the samples were collected. A second disadvantage was that the standard deviation for repeated runs were rather high.

Table 5-1. Advantages and disadvantages of batch and continuous processes [4].

	Advantages	Disadvantages
Batch	• Setup is simple • Substrate solubility is not important	• Substrate concentration decreases over time • Enzyme inhibition by product • Work-up can be tedious • Upscaling is more difficult
Continuous	• Continuous process • Easy product recovery • Constant substrate supply • Upscaling is easy	• Substrate solubility problems • More complex design • Fouling

Advantages of using chitosan in the flow-through reactor include the prevention of fouling as well as higher and more stable flow rates. The reactor process has the added advantage that although the product (diol) and the remaining enantiomer (epoxide) are not separated in the reactor, they have different boiling points and can immediately be separated using vacuum distillation, since the biomass is retained in the reactor.

4. RECOMMENDATIONS

- Evaluate the effect of not previously tested co-solvents, to increase the low solubility of 1,2-epoxyoctane. Determine their effect on the stability of the biocatalyst and the % ee of both the product and substrate.

- Find an improved sampling method. Instead of taking samples in a politop (evaporation can take place) a closed system, from which samples can be taken, should be investigated.

- Further investigation is required to establish the possible causes for the fluctuations in the flow rate obtained with the flow-through reactor.

- Optimise the flow rate in terms of % ee_{epox} or % ee_{diol}. This should be done after deciding which of the two compounds are targeted for optimisation.

- Establish whether similar optimum patterns are obtained when using another biocatalyst and racemic mixture. Lastly, it would be interesting to investigate enantioselective reactions with longer reaction times.

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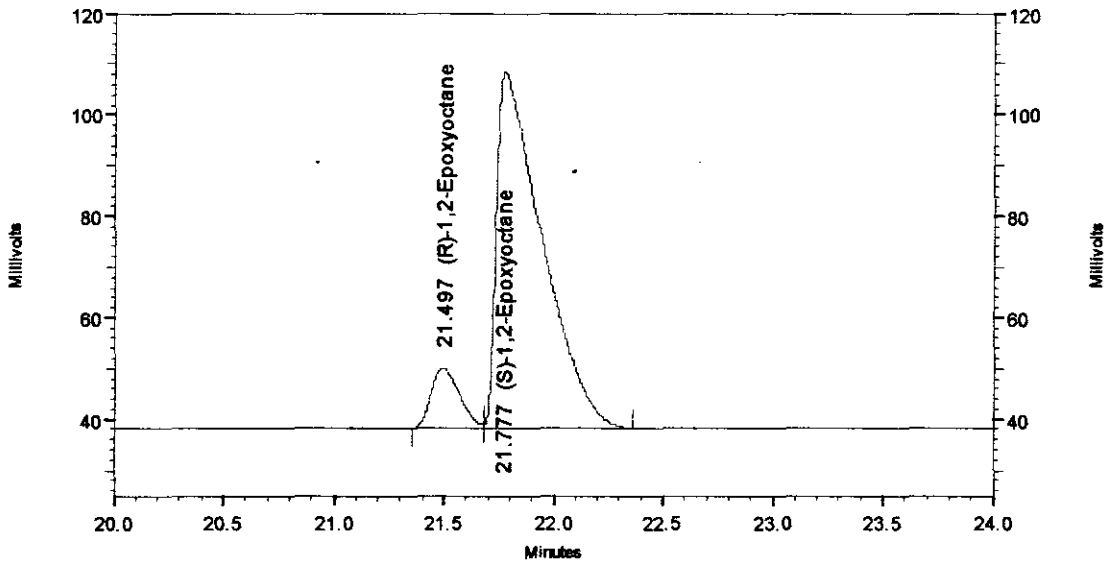
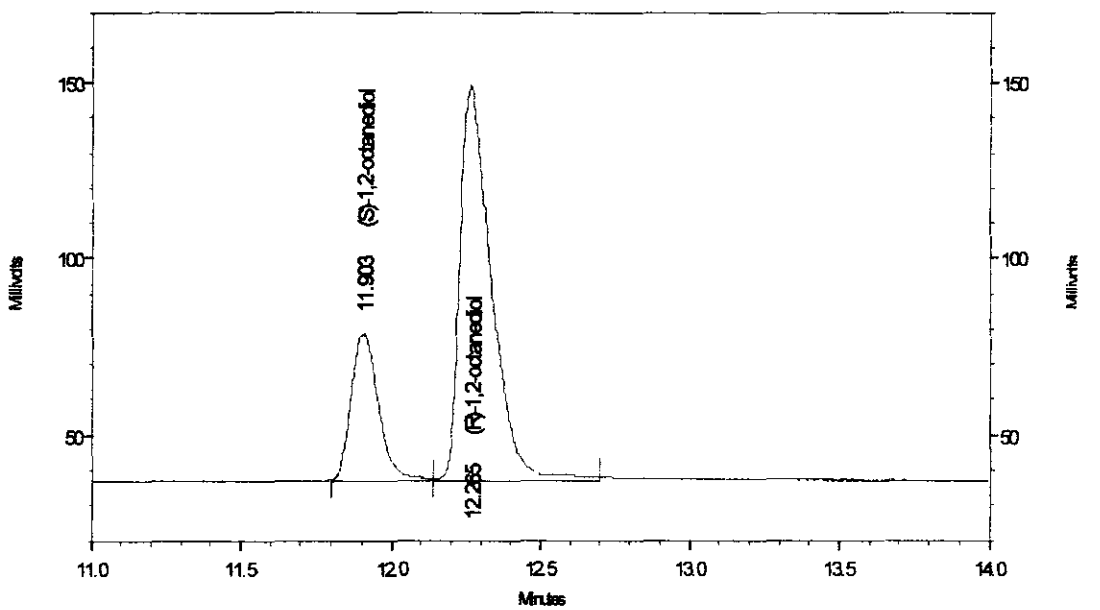
APPENDICES

APPENDIX 1. GC-Chromatogram of the chiral analysis of 1,2-epoxyoctane and 1,2-octanediol.

Chiral separation was achieved by using a fuse silica β-cyclodextrin column (CP Chirasil-DEX CB, 25 m x 0.25 mm, 0.25 µm film). H_2 was used as carrier gas. The epoxide was analysed at 70 °C. The retention times were Rt (70 °C) = 21.5 min and 21.8 min respectively for the (R)- and (S)-1,2-epoxyoctane.



The diol was analysed at 140 °C. The retention times were Rt (140 °C) = 11.9 min and 12.3 min for the (S)- and (R)-1,2-octanediol respectively.



APPENDIX 2. Statistical design with flow rates at 480 minutes.

Run No.	Pressure (kPa)	Wet weight cells (g)	Chitosan (g)	Flow at 480 min. ($ml.min^{-1}$)
8	100	1.3	1.202	0.089
7	100	1.3	0.304	0.054
15*	70	1.0	0.755	0.075
11	75	0.5	0.755	0.233
3	50	1.3	0.304	0.059
5	100	0.7	0.303	0.079
17*	75	1.0	0.755	0.079
10	117	1.0	0.755	0.087
16*	70	1.0	0.753	0.071
6	100	0.7	1.202	0.149
18*	70	1.0	0.755	0.083
9	33	1.0	0.754	0.011
4	45	1.3	1.205	0.080
2	45	0.7	1.204	0.185
13	75	1.0	0.000	0.083
1	50	0.7	0.305	0.081
14	75	1.0	1.504	0.072
12	75	1.5	0.754	0.067