The stabilisation of epoxide hydrolase activity

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Summary

Biocatalysis and enzyme technology represent significant research topics of contemporary biotechnology. The immobilisation of these catalysts on or in static supports serves the purpose of transforming the catalyst into a particle that can be handled through effortless mechanical operations, while the entrapment within a membrane or capsule leads to the restraint of the enzyme to a distinct space. This confinement leads to a catalyst with a superior stability, and cell durability under reaction conditions.

Epoxide hydrolase is a widely available co-factor independent enzyme, which is known to have remarkable chemio-, regio- and stereoselectivity for a wide range of substrates. Recently it was found that certain yeasts, including *Rhodosporidium toruloides*, contain this enzyme and are able to enantioselectively catalyse certain hydrolysis reactions.

The objective of this project was four-sided: a) to immobilise *Rhodosporidium toruloides* in an optimised immobilisation matrix (calcium alginate beads), for the kinetic resolution of 1,2-epoxyoctane in order to obtain an optically pure epoxide and its corresponding vicinal diol, b) to determine the effect of immobilisation on activity as well as stability of the enzyme and gain better understanding of the parameters that influence enzyme activity in a support, c) to determine the effect of formulation parameters on some of the bead characteristics and, d) to gain some insight in the distribution of epoxide and diol in the water and bead phases and the formulation parameters that have an effect thereon.

Rhodosporidium toruloides was immobilised in calcium alginate beads consisting of different combinations of alginate and $CaCl_2$ concentrations. Best results were obtained with a combination of 0,5% (m/v) alginate and 0,2 M $CaCl_2$. The immobilised cells exhibited lower initial activity, but more than 40 times the residual activity of that of the free cells after a 12-hour storage period. Both the immobilised and free cells exhibited an increase in reaction rate (V) with an increase in substrate concentration.

An increase in the alginate concentration lead to the formation of smaller beads, but a decrease in enzyme activity, while an increase in the $CaCl_2$ solution concentration had no effect on bead diameter or enzyme activity.

Epoxide diffused preferentially into the beads (± 96 %), and the diol into the water phase, which leads to the natural separation of the epoxide and the diol. The $CaCl_2$ concentration affected epoxide diffusion with no effect on diol diffusion, which opens up the possibility to regulate the diffusion of epoxide into the beads.

Although only a very small fraction of the epoxide inside the beads could be extracted, the alginate proved to be chirally selective for the (R)-epoxide, improving the reaction efficiency by increasing the % ee_s of the epoxide extracted from the beads between 26 % and 43 %.

The possibility to develop a system where the product is formed, purified and concentrated in a one-step reaction by extracting the product from the bead phase was clearly demonstrated.

Keywords: 1,2-epoxyoctane, epoxide hydrolase, immobilisation, calcium alginate, kinetic resolution

Opsomming

Biokatalise en ensiemtegnologie is van die grootste fokuspunte in hedendaagse biotegnologie. Immobilisering van die katalisators op of in statiese materiaal vorm 'n maklik hanteerbare eenheid. Die inkapseling binne 'n membraan of 'n kapsule beperk ensiembeweging tot 'n spesifieke ruimte en lewer 'n katalisator met hoër stabiliteit en langer operasionele aktiwiteit.

Epoksiedhidrolase is 'n ko-faktor onafhanklike ensiem wat algemeen beskikbaar is en is bekend vir uiters goeie chemio-, regio- and stereoselektiwiteit teenoor 'n wye reeks verbindings. Epoksiedhidrolase is onlangs in sekere giste ontdek, waronder *Rhodosporidium toruloides*, en beskik oor die vermoë om sekere hidrolisereaksies enantioselektief te kataliseer.

Die doel van die projek was vierledig: a) om *Rhodosporidium toruloides* in `n geoptimiseerde medium te immobiliseer (kalsiumalginaatkapsules) vir die kinetiese resolusie van 1,2-epoksi-oktaan om optiese suiwer epoksied en die ooreenstemmende diol te lewer, b) om insig oor die effek van immobilisasie op ensiemaktiwiteit asook stabiliteit te verkry, asook oor die formuleringsfaktore wat ensiemaktiwiteit in 'n immobilisasiemedium beïnvloed, (c) om die effek van formuleringsfaktore op sekere kapsule-eienskappe te ondersoek, en d) om die verspreiding van epoksied en diol in die waterfase en kapsule te ondersoek asook die formuleringsfaktore wat 'n effek daarop het.

Rhodosporidium toruloides is geïmmobiliseer in kalsiumalginaatkapsules, bestaande uit verskillende kombinasies van $CaCl_2$- en alginaatkonsentrasies. Die beste resultate is verkry met 'n kombinasie van 0,5 % (m/v) alginaat en 0,2 M $CaCl_2$. Die geïmmobiliseerde selle het `n laer aanvanklike aktiwiteit vertoon, maar met uiters goeie stabiliteit, naamlik meer as 40 keer die oorblywende aktiwiteit van die nie-geïmmobiliseerde selle na 'n stoortyd van 12 uur.

`n Verhoging in alginaatkonsentrasie het kleiner kapsules tot gevolg gehad, maar met 'n laer aktiwiteit, terwyl die geïmplementeerde $CaCl_2$-konsentrasie geen effek op die deursnit van die kapsules of aktiwiteit van die ensiem getoon het nie.

Die grootste deel van die epoksied het in die kapsuulfase verdeel (96 %) terwyl die diol in die waterfase verdeel het. Dit het tot die natuurlike skeiding van die epoksied en diol gelei. Die $CaCl_2$ het diffusie van slegs die epoksied beinvloed en geen uitwerking op die diol gehad nie. Dit skep die moontlikheid om die diffusie van die epoksied in die kapsules in te reguleer.

Alhoewel slegs 'n klein fraksie van die epoksied uit die kapsule geëkstraheer kon word, was die alginaat selektief vir die (R)-epoksied, wat 'n verhoging in die effektiwiteit van die reaksie tot gevolg gehad het met `n verhoging van tussen 26 % en 41 % in die % *ee* (van die epoksied wat uit die kapsules geëkstraheer is).

Die moontlikheid om `n sisteem te ontwikkel waar die produk in een stap deur ekstraksie van die kapsules gevorm, gesuiwer en gekonsentreer word is duidelik gedemonstreer.

Sleutelwoorde: 1,2-epoksi-oktaan, epoksiedhidrolase, immobilisasie, kalsiumalginaat, kinetiese resolusie

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Chapter 1

Introduction

1. Introduction

1.1 Background

Optical activity was discovered by Jean-Baptiste Biot in the early 1800's. A few years later Louis Pasteur conducted the first resolution of a racemic mixture of D- and L-tartaric acid (Figure 1.1), from which he proposed that optical activity was caused by molecular asymmetry. Van't Hoff and Le Bel strengthened Pasteur's proposal by independently hypothesising that the chiral nature of compounds was due to the fact that carbon constituents could have a non-polar spatial arrangement giving rise to nonsuperimposable mirror images (Hyneck *et al.*, 1990:1). These nonsuperimposable mirror images are called enantiomers (Ahuja, 1997:1).

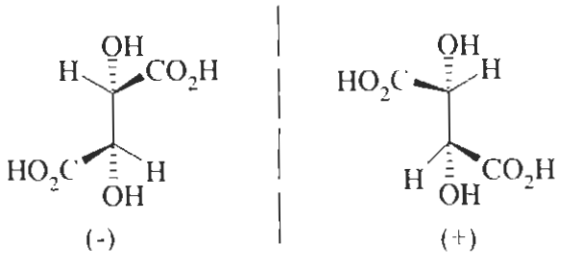


Figure 1.1 Tartaric acid enantiomers (Crossley, 1995:5)

The manufacture of enantiomeric pure chemical products applied for the promotion of human health or to combat pests and disease has become an important topic in industry due to the difference in biological effect of enantiomers (Crosby *et al.*, 1992:2).

Common observed effects of chirality include differences in (Maier *et al.*, 2001:4):

- bioavailability,
- distribution,
- metabolic and excretion behaviour, and
- biological action.

Furthermore, it was established that the greater the potency for the interaction in question, the greater the difference of activity between the two enantiomers and that the highest affinity and selectivity will be obtained from molecules with the greatest degree of chirality (Crossley, 1995:18).

1.2 Methods for obtaining optically pure compounds

The scientific and economic relevance of chiral substances has favoured developments in separation techniques of chiral racemates as well as the synthesis of single enantiomers (Maier *et al.*, 2001:7). Approaches to obtain optically active compounds include the utilisation of the chiral pool, separation of racemates and creation of chiral precursors (Crosby, 1992:5). All these methods are discussed in detail in Chapter 2.

1.3 Immobilisation

The immobilisation of biological substances or living cells has become a widespread tool in bioprocess technology. Immobilisation can be defined as any process that restrict substances or cells inside a given structure and limits its free diffusion or movement (Huebner & Buccholz, 1999:1785).

The use of immobilised microorganisms offer some interesting alternatives to bioprocesses conventionally carried out with free cells. Among the advantages are:

- ease in handling and cell separation from the liquid medium (Laca *et al.*, 1998:225),
- repeated and continuous use of biocatalysts, and
- stabilisation of the reaction conditions with a subsequent increase in the bioprocess efficiency (Tanaka *et al.*, 1999:504).

Methods of immobilisation can be classified by means of the mechanism of bonding used i.e., chemical or physical (Bommarius, 1993:433). Figure 1.2 illustrates these methods, which will further be discussed in Chapter 2.

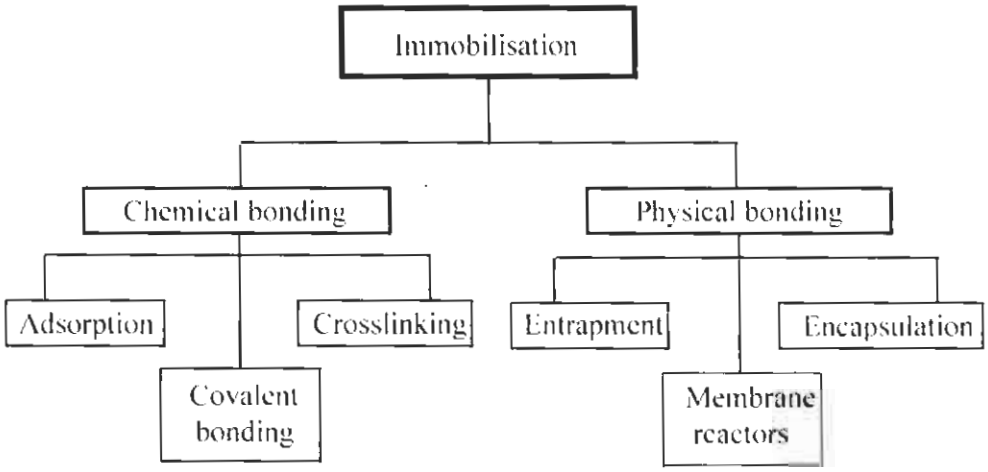


Figure 1.2 Methods of immobilisation

2. Motivation

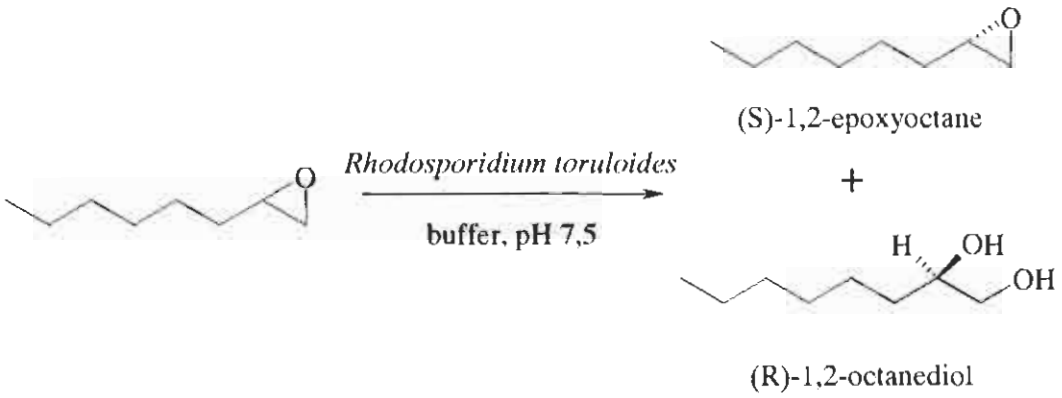
The wide range of advantages such as higher stability and the possibility of continuous use of biocatalysts in immobilised form make immobilisation one of the facets in biotechnology with the highest expansion rate. Many methods of immobilisation are currently under research and industrially implemented, but no supreme support material or immobilisation method exists for a given application. Although a few basic principals do exist in choosing an immobilisation method and matrix, immobilisation can affect the inherent and perceivable enzyme kinetics. Due to the complex nature of immobilised cell kinetics, comprehensive studies to understand the processes are necessary. By applying this technology it was found that the possibilities for immobilised biocatalysts are enormous and numerous.

3. Aims and objectives

The aims of this study were firstly to immobilise *Rhodosporidium toruloides* in an immobilisation matrix (calcium alginate beads), for the kinetic resolution of 1,2-epoxyoctane (Scheme 1.1), in order to obtain an optically pure epoxide and its corresponding vicinal diol. Secondly, it was to determine the effect of immobilisation on activity as well as stability of the enzyme. Thirdly, it was to determine the relation of the physical characteristics of the immobilisation matrix on the enzyme activity as well as the effect of formulation parameters on the physical characteristics of the alginate beads.

Furthermore the diffusion of epoxide and diol in the water and bead phase was investigated as well as parameters that have an effect on the diffusion to determine if the kinetics of an

immobilised system could be controlled by varying formulation parameters and initial substrate concentration.



Scheme 1.1 Stereospecific hydrolysis of 1,2-epoxyoctane (Botes, 1999:134)

4. Outline of this dissertation

Hydrolytic kinetic resolution of racemic epoxides offers a convenient route to obtain single enantiomer synthons for the preparation of fine chemicals. A broad overview is given on chirality, biocatalysis, and methods available to stabilise and prolong the lifetime of biocatalysts for industrial processes (Chapter 2).

Rhodosporidium toruloides was immobilised in calcium alginate beads. Parameters such as the concentration of the cationic and anionic solutions, the amount of immobilised biomass as well as the effect of these two parameters on the physical properties of the immobilisation matrix were investigated and utilised in the determination of the optimum immobilisation procedure (Chapter 3).

The effect of formulation conditions and initial substrate concentration on the partitioning of both epoxide and diol inside the water phase as well as the bead phase were investigated as well as the effect of initial substrate concentration on free and immobilised enzyme activity (Chapter 4).

The study is concluded with an overview of all the results obtained as well as the implications and future developments (Chapter 5).

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Chapter 2

Immobilisation of chiral biocatalysts

1. Introduction

In this chapter a broad overview is given on chirality, the implications of chirality as well as methods available for obtaining optically active compounds, with specific reference to optically active epoxides.

A broad overview is also given on biocatalysis, and methods available to stabilise and prolong the lifetime of biocatalysts for industrial processes.

2. Chirality and chiral compounds

2.1 Definitions

The word *chiral* is derived from the Greek word *cheir*, which means hand. Molecules that relate to each other like a pair of hands (non-superimposable mirror-images), are called chiral molecules. These non-superimposable stereoisomers are also known as enantiomers (Ahuja, 1997:1) and show optical activity. That is, they rotate the plane of plane-polarised light in opposite directions (Stevenson & Williams, 1988:1). The two enantiomers of a given compound have rotary powers of equal absolute value but of opposite sign. One is positive or *dextrorotary* and the other is negative or *levorotary*. The absolute designations of the signs are arbitrary inasmuch as they are wavelength, temperature, and solvent dependent, but the relative designations are always valid (Jacques *et al.*, 1981:4).

These molecules (Figure 2.1), generally have a tetrahedral carbon atom with 4 different substituents (Ahuja, 1997:1).

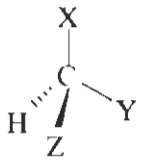


Figure 2.1 Tetrahedral carbon atom of type CHXYZ (McMurray, 1992:285)

The symmetric factor (Figure 2.2), classifies molecules with different spatial arrangement of atoms as either enantiomers or diastereomers (Aitken, 1992:16). Diastereomers are essentially stereoisomers with two or more centres of asymmetry that are not enantiomers of each other. Stereoisomers can occur when a molecule has one or more centres of chirality (Ahuja, 1997:1).

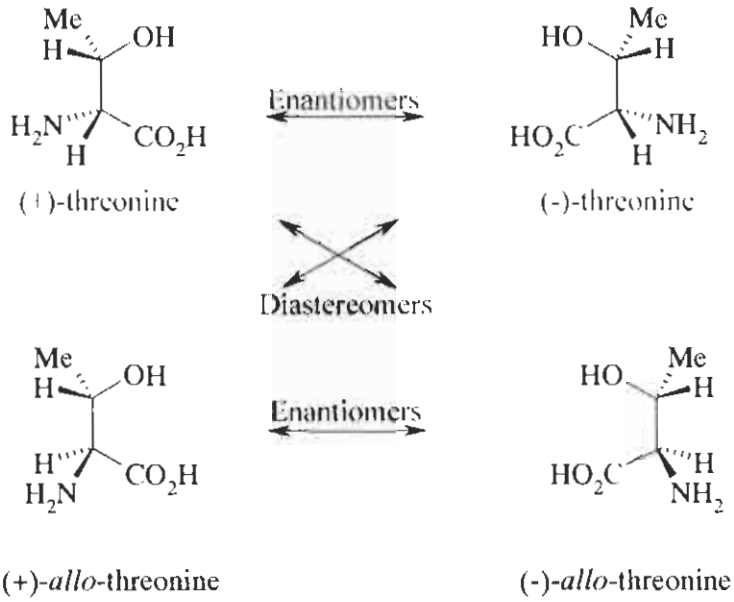


Figure 2.2 The four possible stereoisomers of a chiral compound with two asymmetric centres

An equimolar (50:50) mixture of two enantiomers is called a racemate, and the separation of such a mixture is called resolution or optical resolution. The expression "optically active substance" thus signifies a pure enantiomer or a mixture containing an excess of one of the two isomers (Jacques *et al.*, 1981:4).

2.2 Chirality and the implications thereof

2.2.1 Biological implications of chirality

It has been recognised for a long time that the shape (stereochemistry), of a molecule has considerable influence on its physiological action (Crosby, 1992:2), for example; biological systems which are largely constructed from chiral molecules such as L-amino acids or D-sugars. In this highly chiral environment it is not surprising that some chiral drugs exhibit a high degree of stereoselectivity in their interactions with biological molecules (Davies, 1990:45).

These stereochemical differences between isomers have lead to a number of reasons for obtaining optically pure pharmaceutical compounds:

- Biological activity is due to one isomer only, e.g.
  - The L-isomer of the antihypertensive drug α-methyldopa has all the desired activity.
- Enantiomers have similar activities but the potencies differ, e.g.

- The potency of (S)-(+)-warfarin is two to five fold greater than that of the (R)-(-)-warfarin. Predicting a therapeutic outcome can be complex with racemates having this activity profile. If the pharmacokinetic disposition of the isomers is different, the pharmacodynamic effect can be different between different patients and may even vary in the same patient over the course of drug treatment.

- Pharmacological activity differs qualitatively and quantitatively in each isomer, e.g.
  - The optical isomers of propoxyphene have completely different activities. D-Propoxyphene has analgesic properties, while L-propoxyphene has antitussive properties (Hyneck *et al.*, 1990:5-10).

- The pure enantiomer may display more than twice the activity of the racemate because of possible antagonism, e.g.
  - As little as 1% of the (S,Z)- 5-(1-decenyl)-dihydro-2(3H)-furanone isomer of the sex pheromone of the Japanese beetle *Popillia japonica* inhibits the activity of the (R,Z)-isomer of the same pheromone (Hiscox & Matteson, 2000:314, Crosby, 1992:2).

2.2.2 Economical implication of chirality

The use of racemic drugs is becoming increasingly unattractive owing to policy changes made by regulatory agencies. The FDA's policy on chiral drugs state that the stereoisomeric composition, as well as the quantitative isomeric composition of the material used in pharmacological, toxicological, and clinical studies should be known. Specifications for the final product should also assure identity, strength, quality, and purity from a stereochemical point of view. As a result, the practical preparation of optically pure drugs is a critical issue in the pharmaceutical industry were the activity or potency of the two stereoisomers differs (FDA, 1997). In 1990, 25% of the "synthetically" derived drugs on the market were chiral, but only 3% were marketed as pure enantiomers. Worldwide sales of chiral drugs in single-enantiomer dosage forms exceeded predictions to reach $133 billion in 2000 (Stinson, 2001:79). Although the market for resolved chiral intermediates used for the synthesis of these chiral drugs will be much smaller ($1-2bn), it is still a major opportunity for the chiral manufacturing sector (Crosby, 1997:1-2).

3. Obtaining optically pure compounds

Apart from the isolation of natural chiral pure molecules from the chiral pool, the production of optically pure compounds has generally presented a considerable challenge bearing in mind that the crude material which is initially produced ought to have an enantiomeric excess (ee_s) of at least 70% and, if at all possible greater than 80% to be of practical large-scale use (Crosby, 1992:5).

Current chiral drugs are mostly synthesised through a series of reactions from smaller, lower molecular weight precursors. Chiral centres are introduced at a suitable place in the reaction sequence by incorporating chiral precursors obtained from the chiral pool or by employing asymmetric reactions or resolution processes (van Eikeren, 1996:17).

3.1 The chiral pool

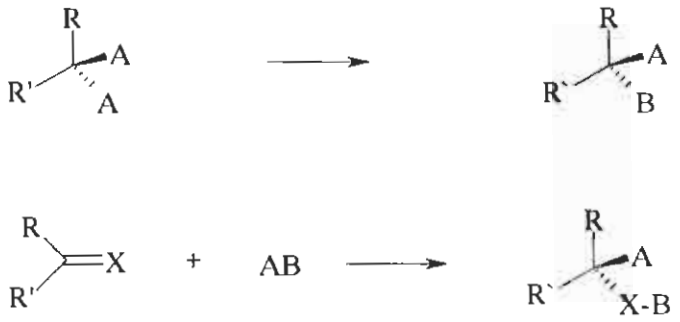
The chiral pool refers to fairly inexpensive, readily obtainable optically active natural product. (Crosby, 1992:5).

The major classes of chiral starting materials available from nature are (Aitken & Gopal, 1992:64-69):

- amino acids and amino alcohols,
- hydroxy acids,
- alkaloids and amines,
- terpenes, and
- carbohydrates.

3.2 Asymmetric synthesis

According to Aitken, asymmetric synthesis can be defined as a synthesis in which an achiral unit in a group of substrate molecules is converted to a chiral unit (Scheme 2.1), to form uneven amounts of the potential stereoisomers with the aim of producing the highest possible proportion of the wanted isomer (Aitken, 1992:5), which can be accomplished stoichiometrically or catalytically (Crosby, 1992:37).



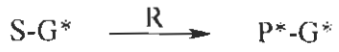
Scheme 2.1 Transformation of prochiral substrates (Crosby, 1992:39)

3.2.1 Non-enzymatic asymmetric synthesis

Aitken and Gopal (1992:72-77) distinguish between four different types of non-enzymatic asymmetric syntheses.

3.2.1.1 Substrate–controlled asymmetric synthesis

The synthesis is intramolecularly directed by a stereogenic unit present in the chiral substrate (Scheme 2.2), with the formation of another stereogenic unit after the reaction with an achiral reagent. The main drawback of this method is the need for an enantiomerically pure starting material (Aitken & Gopal, 1992:73).



Scheme 2.2 Substrate-controlled asymmetric synthesis (S = substrate; G = chiral directing group; R = reagent; P-G = product; * = chiral centre containing group)

3.2.1.2 Auxiliary-controlled asymmetric synthesis

This approach is similar to the first method in that a chiral group in the substrate achieve intramolecular control. The difference is that the directing group is attached to an achiral substrate and can be removed once it has served its function (Scheme 2.3). In this method the two possible products are diastereomers and not enantiomers as a result of the additional stereogenic centre of the auxiliary, which has the advantage of the easy removal of the undesired diastereomer (Aitken & Gopal, 1992:74).

$$S \xrightarrow{A^*} S\text{-}A^* \xrightarrow{R} P^*\text{-}A^* \xrightarrow{-A^*} P^*$$

Scheme 2.3 Auxiliary-controlled asymmetric synthesis (A = auxiliary)

3.2.1.3 Reagent controlled asymmetric synthesis

In contrast to the first and second methods, the control is intermolecular; an achiral substrate is directly converted to the chiral product (Scheme 2.4), through the use of a chiral reagent (Aitken & Gopal, 1992:75).

$$S \xrightarrow{R^*} P^*$$

Scheme 2.4 Reagent controlled asymmetric synthesis

3.2.1.4 Catalyst controlled asymmetric synthesis

In each of the previous mentioned classes, an enantiomerically pure substrate, reagent or auxiliary was required, which could be recovered in some cases for reuse. In this class a chiral catalyst is used to direct the conversion of an achiral substrate directly to a chiral product with intermolecular control (Scheme 2.5). By definition the catalyst can be recovered unchanged at the end of the reaction and be reused. This class includes enzymatic as well as non-enzymatic catalysts (Aitken & Gopal, 1992:77).

$$S \xrightarrow[cat^*]{R^*} P^*$$

Scheme 2.5 Catalyst controlled asymmetric synthesis (cat = catalyst)

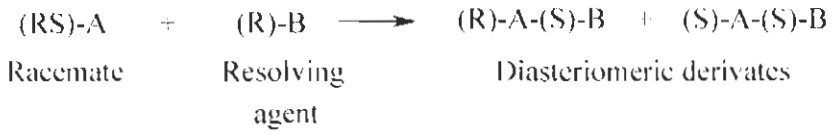
3.2.2 Enzymatic asymmetric synthesis

A growing number of syntheses of homochiral materials incorporate enzymic steps, due to higher selectivity under mild reaction conditions (Halgäs, 1992:1). Enzymatic methods in asymmetric syntheses include oxidation, hydrogenation, reductive amination, ammonia addition, transamination, hydration and cyanahydrin formation (Crosby, 1992:37-52).

3.3 Separation of racemates

3.3.1 Classical resolution

Classical resolution is the most widely used method for the production of enantiomerically pure compounds (Bruggink, 1997:81). This technique (Scheme 2.6), is based on the interaction of a racemic product with an optically active substance (resolving agent), to give two diastereomeric derivates (usually salts):



Scheme 2.6 Formation of diastereomeric salts during classical resolution

The salts formed are diastereomers with diverse physical properties and may be separated in a number of ways, for example by chromatography. However, the most efficient method of separating such diastereomers is by crystallisation (Bayley & Vaidya, 1995:70).

3.3.2 Resolution by direct crystallisation

Crystal science techniques provide powerful aid in the manufacture of chiral compounds (Wood, 1997:149). Direct crystallisation depends on the occurrence of some substances as crystalline conglomerates (racemic mixtures) rather than racemic compounds. Although in bulk, a conglomerate is optically neutral, individual crystals contain only one enantiomer, whereas in a racemic compound individual crystals contain equivalent amounts of both enantiomers (Figure 2.3). Conglomeration is thus a prerequisite for resolution by direct crystallisation (Crosby, 1992:24).

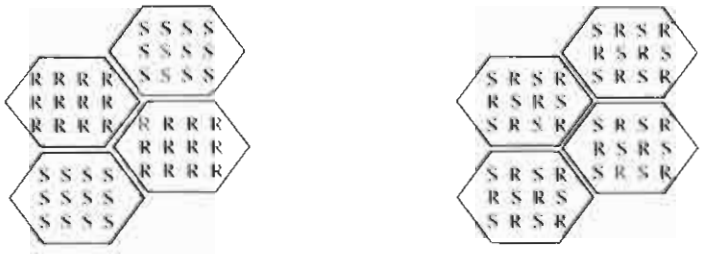


Figure 2.3 Crystal forms of a racemate (Wood, 1997:149)

3.3.3 Kinetic resolution

While diastereomers can be readily separated, enantiomers are physically and chemically identical in an achiral environment. This makes separation rather difficult requiring conditions under which they react at different rates, whereby ultimately their separation can be effected (Nógrádi, 1995:13). The term enantiomeric excess (Equation 2.1), gives an indication of the excess of predominant enantiomers expressed as a percentage (van Eikeren, 1996:21).

.

$$ee = \frac{(\text{predominant enantiomer}) - (\text{minor enantiomer})}{(\text{predominant enantiomer}) + (\text{minor enantiomer})} \qquad \textbf{(Equation 2.1)}$$

If only one enantiomer has reacted to leave the other enantiomer in its enantiopure form, a *complete kinetic resolution* has been accomplished (Atkinson, 1995:24).

The efficiency of resolution is stated by the enantiomeric ratio, E, (Equation 2.2 and 2.3), and is connected to the enantiomeric excess of the recovered reactant (ee_R) and of the product (ee_P) at a given extent of conversion (c) (Crosby, 1992:28):

$$E = \frac{\ln[(1-c)(1-ee_R)]}{\ln[(1-c)(1+ee_R)]} \qquad \textbf{(Equation 2.2)}$$

$$E = \frac{\ln[1-c(1+ee_P)]}{\ln[1-c(1-ee_P)]} \qquad \textbf{(Equation 2.3)}$$

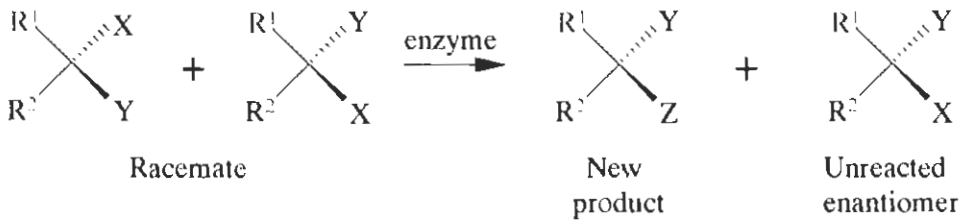
E can also directly be determined (Equation 2.4), from ee_R and ee_P (Rakels *et al.*, 1993):

$$E = \frac{\ln\left((1-ee_R)\left(1+\left(ee_R\, ee_P\right)\right)\right)}{\ln\left((1+ee_R)\left(1+\left(ee_R\, ee_P\right)\right)\right)} \qquad \textbf{(Equation 2.4)}$$

While the maximum yield of one product is 50 %, the *ee* varies as the reaction progresses due to the kinetics of the system. If, however, the reaction is carried out under conditions in which the enantiomers of the substrate can interconvert, the entire substrate can be converted to the enantiomerically pure product resulting in a product yield of 100 % (Aitken & Gopal, 1992:77).

3.3.3.1 Enzymatic kinetic resolution

The kinetic separation of a racemate with the help of an enzyme (Scheme 2.7), leads to the formation of two different, more or less pure enantiomers (Halgăs, 1992:45).

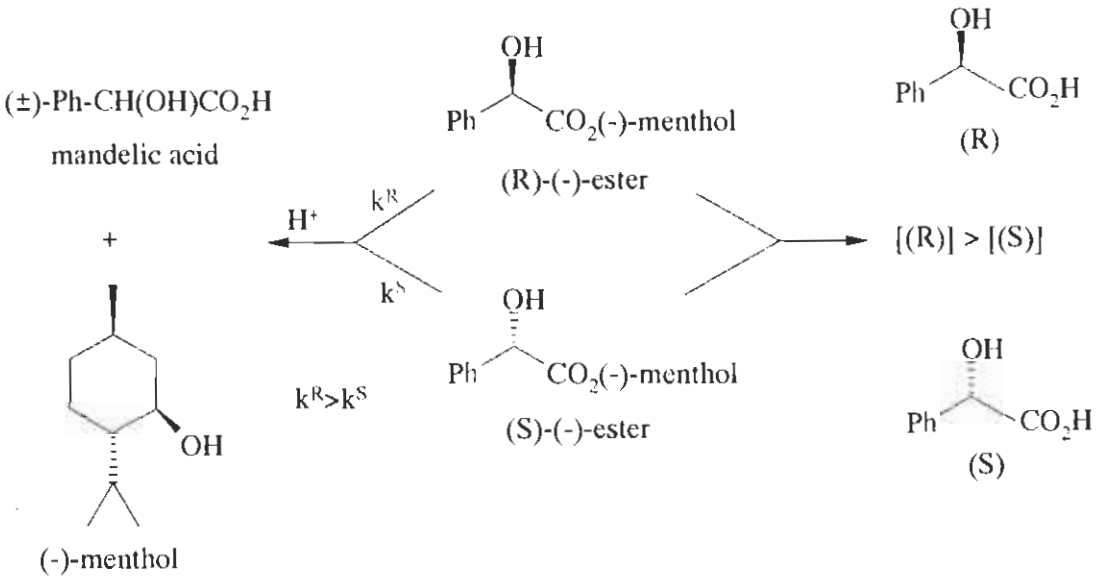


Scheme 2.7 Enzymatic kinetic separation of a racemate

With this method 50 % of the racemate, at the most, can be utilised for the synthesis of another product. Therefore, in industry the unaltered enantiomer is usually racemised and recycled (Halgăs, 1992:45).

3.3.3.2 Non-enzymatic chemical kinetic resolution

The chemical kinetic resolution of (±)-mandelic acid with (–)–menthol (Scheme 2.8), was the reaction leading to the discovery of kinetic resolution in 1899. Incomplete reaction leaves an excess of (S)-mandelic acid, while total hydrolysis of the esters give a mixture enriched in (R)-mandelic acid (Nógrádi, 1995:15-16).



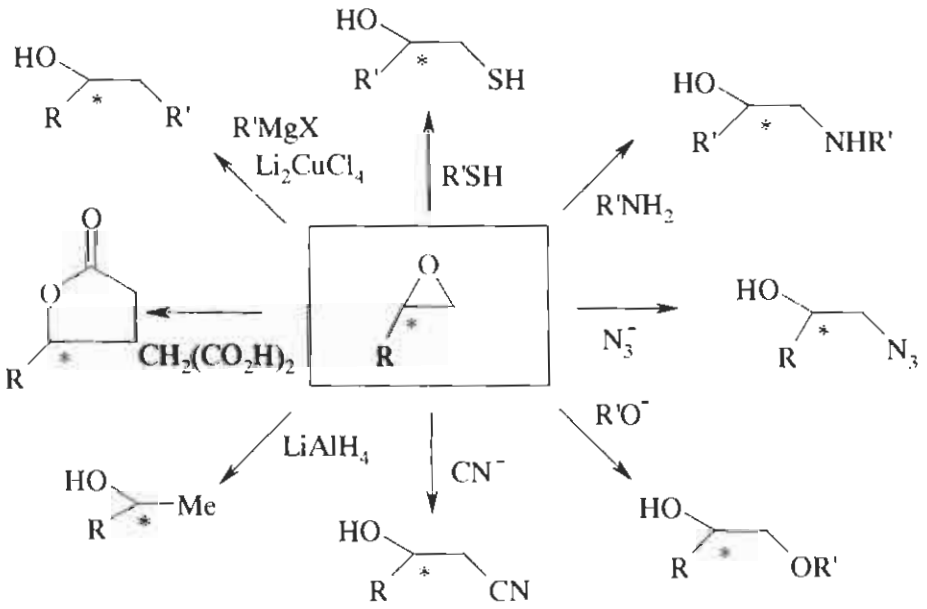
Scheme 2.8 Experiment of Marckwald and Mckenzie

Kinetic resolution is an inherently wasteful process for producing optically active compounds and can only compete with conventional resolution (of poor economy itself), when rate differences are extreme. With few exceptions this has so far only been realised with enzymes (Nógrádi, 1995:17).

4. Optically pure epoxides

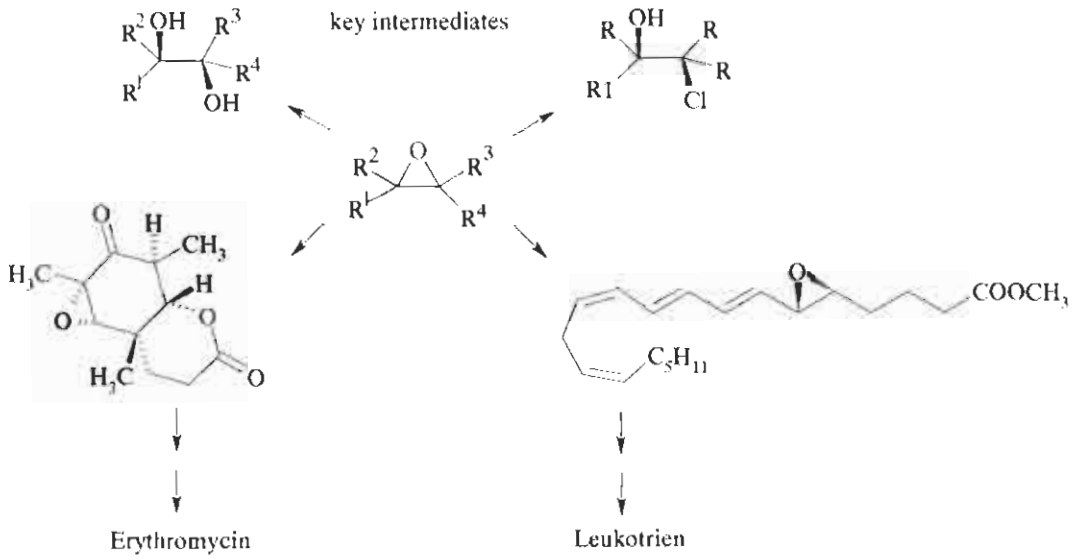
4.1 Industrial application and reactions

Epoxides are recognised as being highly valuable intermediates for the synthesis of organic fine chemicals due to the versatility of the oxirane function (Scheme 2.9), that can be chemically transformed into numerous, more complex intermediates through stereospecific ring-opening reactions with nucleophiles (Archelas & Furstoss, 1999: 160).



Scheme 2.9 Some intermediates from the stereospecific ring opening of epoxides

These compounds can be used in the preparation of more complex optically pure bioactive compounds (Scheme 2.10), such as leukotriene and erythromycin or as end products, which also have biological activities such as the gipsey moth pheromone (+)-disparlure (Besse & Veschambre, 1994:8886).



Scheme 2.10 Epoxides as key intermediates or as end products in organic synthesis

4.2 Methods for obtaining optically pure epoxides

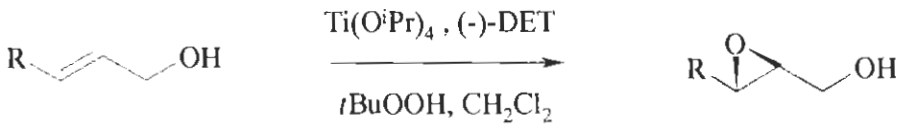
Considerable effort has been devoted to the synthesis of enantiomerically pure epoxides in recent years. Both chemical and enzyme-catalysed methodologies have been developed. The Katzuki-Sharpless asymmetric epoxidation method was the first conventional chemical approach, followed by the Sharpless dihydroxylation reaction. Both these approaches however, have the disadvantage of requiring the use of heavy metal-based catalysts, which are possible sources of industrial pollution (Archelas & Furstoss, 1997:492-493).

4.2.1 Asymmetric synthesis

4.2.1.1 Non-enzymatic asymmetric synthesis

- *Sharpless asymmetric epoxidation*

Sharpless and Katsuki developed the reaction in 1980. In the Sharpless asymmetric epoxidation reaction (Scheme 2.11), an allylic alcohol reacts with *tert*-butyl hydroperoxide (TBHP) in the presence of $Ti(O^iPr)_4$ and diethyl tartrate (DET) to form an epoxy alcohol of high enantiomeric purity (Gao *et al.*, 1987:5765).

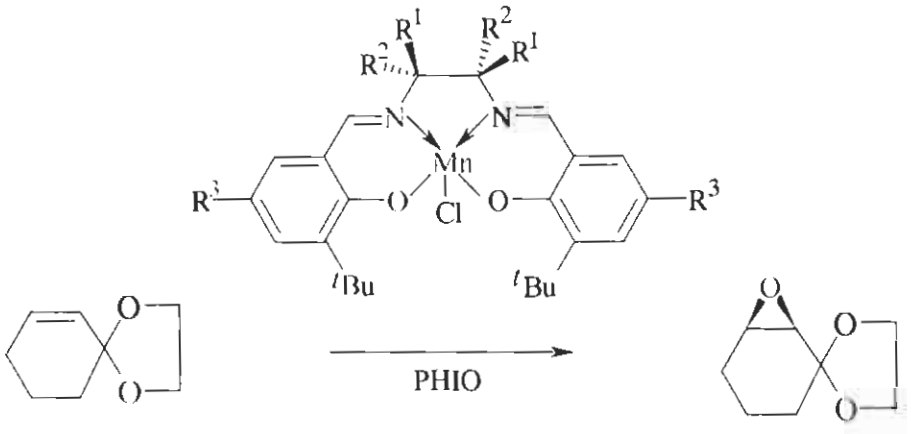


Scheme 2.11 Asymmetric epoxidation (Atkinson, 1995:25)

Although this reaction is possible for various substituent groups, large enantiomeric excess was only achieved with primary or secondary allylic alcohols (Besse & Veschambre, 1994:8893).

- *Jacobsen asymmetric epoxidation*

Jacobsen was the first to report asymmetric catalysis (Scheme 2.12) with chiral Mn(III)salen complexes (Pietikäinen, P. 2000:74). These catalysts are generally derived from chiral 1,2,3-diaminio-1,2-diphenylethane. Systematic variations of the steric and electronic nature of the different substituents on the Mn(III)-complex lead to the discovery of catalysts that are particularly effective for epoxidation (Besse & Veschambre, 1994:8897).

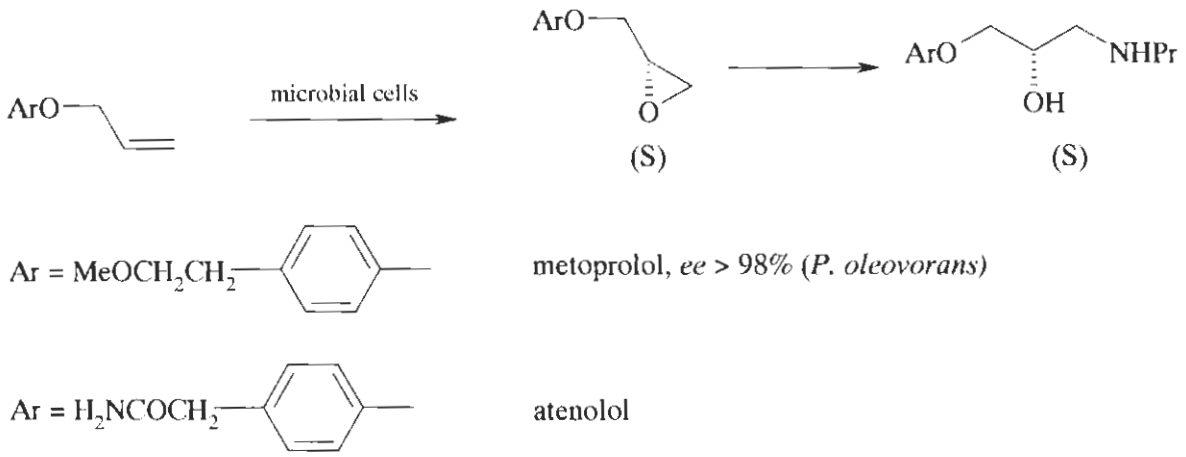


Scheme 2.12 Jacobsen asymmetric synthesis

4.2.1.2 Enzymatic asymmetric synthesis

Biocatalytic methods have been proven to provide useful alternative methods to the above mentioned chemical epoxidation techniques (Mischits *et al.*, 1995:1261). In 1974 the microbial epoxidation of olefinic compounds was first described. Since then, various microorganisms have been found to produce optically active epoxides from unfunctionalised olefins, which cannot be epoxidised in high optical purity by conventional chemical synthetic methods (Furuhashi, 1992:168).

An example of biocatalytic asymmetric synthesis is in the production of *β*-blocker intermediates. Shell and Gist-Brocades reported a route to a single enantiomer (Scheme 2.13), using stereoselective microbial epoxidation (Crosby, 1992:48).



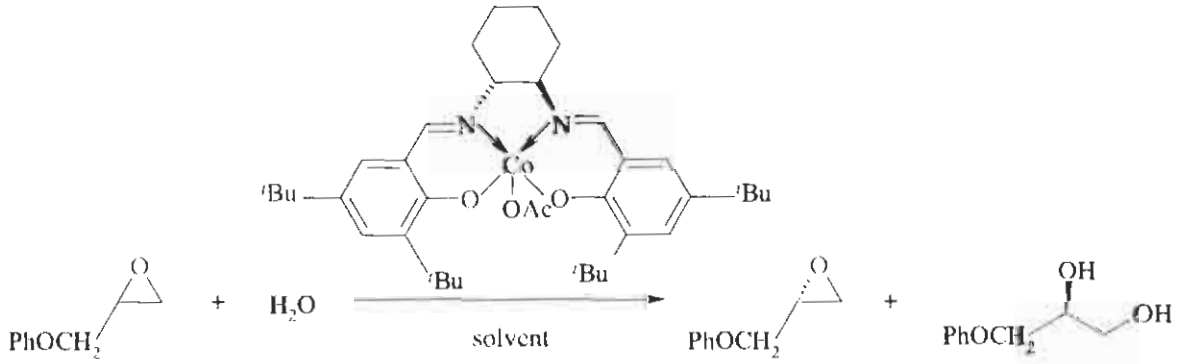
Scheme 2.13 Biocatalytic asymmetric synthesis of β-blockers

4.2.2 Kinetic resolution

4.2.2.1 Non-enzymatic kinetic resolution

- *Jacobsen kinetic resolution*

The Jacobsen's chiral poly-salen-Co(III) (Scheme 2.14) is used for the resolution of racemic terminal epoxides with the concomitant formation of enantiopure diols.



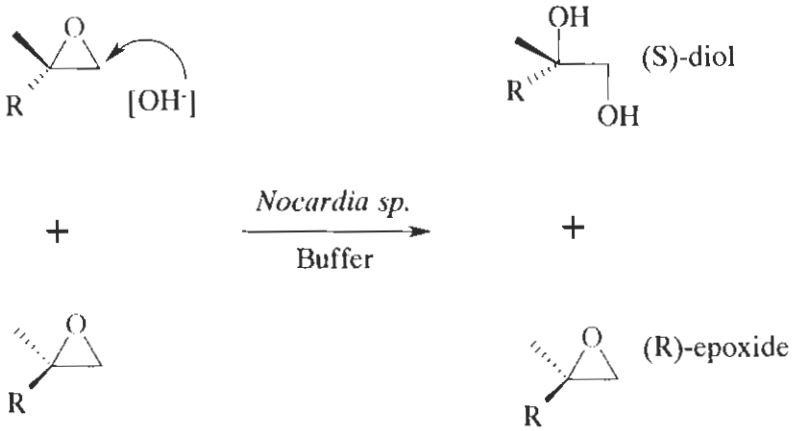
Scheme 2.14 Jacobsen kinetic resolution

This kinetic resolution presents a method for accessing terminal epoxides in high enantiomeric purity (Song *et al.*, 2002:6625), but with the disadvantage of by-product formation (Crosby, 1997:4)

4.2.2.2 Enzymatic kinetic resolution

Epoxide hydrolases are readily available enzymes that catalyse the hydrolysis of an epoxide to furnish the corresponding vicinal diol (Orru & Faber, 1999:16) and unreacted chiral epoxide (Goswami *et al.*, 1999:3167).

An example is the hydrolysis of 2,2-disubstituted epoxides (Scheme 2.15), by the bacterial epoxide hyrolase enzyme produced by a *Nocardia sp.* This reaction furnished the corresponding (S)-diol an (R)-epoxide (Strauss *et al.*, 1999:113).



(±)-2,2-disubstituted epoxides

Scheme 2.15 Kinetic resolution of 2,2-disubstituted epoxides (R = alkyl, alkenyl, (aryl)alkyl, haloalkyl)

5. Epoxide hydrolase enzymes

A number of reasons exist for the use of hydrolase enzymes in an increasing number of biotransformation reactions:

- hydrolase enzymes are co-factor independent enzymes (e.g. NAD(P)/NAD(P)H),
- the enzymes are widely available,
- they frequently show remarkable chemio-, regio- and stereoselectivity on a wide range of substrates (Archer, 1997:15617),

- Hydrolases can be partially purified and used as an enzymatic powder, and
- water-insoluble substrates can be handled due to the fact that these enzymes remain catalytically active in the presence of organic solvents/non-aqueous media (Archelas, 1998:84).

5.1 The occurrence of epoxide hydrolases

Epoxide hydrolase enzymes have been isolated from a wide range of organisms, including mammals (Bellucci *et al.*, 1993:1153), insects, plants and various microorganisms, such as yeasts (Weijers, 1997:639), fungi and bacteria (Mischitz & Faber, 1994:81). Epoxide hydrolases play an important role in the cleavage of reactive and toxic epoxides that are found *in vivo* as intermediates in catabolic pathways (Nardini *et al.*, 2001:1035).

5.1.1 Mammalian epoxide hydrolase

Mammalian epoxide hydrolase plays a major role in the detoxification process of drugs and other toxic compounds (Arand *et al.*, 1999:37). Mammals possess two key epoxide hydrolases, microsamal epoxide hydrolase (mEH), as well as soluble epoxide hydrolase (sEH). In general it is assumed that mono- and *cis*-disubstituted epoxides bearing a lipophilic substituent are good substrates for mEH. For sEH also tri- and tetra-substituted epoxides and in particular, several *trans*-substituted epoxides are excellent substrates (Weijers & de Bont, 1999:201).

5.1.2 Insect epoxide hydrolases

The role of the insect epoxide hydrolase is assumed to convert stimulatory pheromones (7,8-epoxy-2-methyloctadedecane) to nonstimulatory products, thus preventing sensory adaptation. Both the (7S,8R)- and the (7R,8S)-enantiomers, and as well two *meso* analogues are found to be hydrolysed with inversion of configuration at the (S)-configured carbon atom, yielding solely the corresponding *threo*-(R,R)-diols. Large-scale productions of insect enzymes are still fairly difficult, which strongly hampers biocatalytical applications of insect epoxide hydrolases (Weijers & de Bont, 1999:203).

5.1.3 Plant epoxide hydrolases

Plant epoxide hydrolase is specific for the hydrolysis of *cis* fatty acid epoxides. In principle these enzymes are useful for the synthesis of enantiopure epoxy fatty acids and dihydroxy fatty acids because of their stereochemical features and their relatively high activities (Weijers & de Bont, 1999:203).

5.1.4 Bacterial epoxide hydrolases

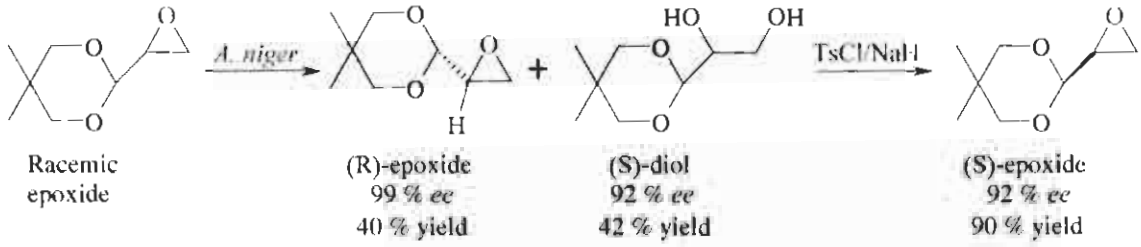
Bacterial epoxide hydrolases are easily accessible tools in organic synthesis because large-scale production of these biocatalysts is relatively easy. The constitutively produced bacterial epoxide hydrolases are useful in the resolution of di-substituted epoxides only. In addition the reported specific activities are not very high (Weijers & de Bont, 1999:203).

A few synthetic applications for obtaining biologically active compounds based on the use of these bacterial enzymes have been described, for instance, the synthesis of (S)-frontalin in a five step chemoenzymatic process which produced a 94 % *ee* (but rather low yield), using cells of *Rhodococcus equi* (Archelas & Furstoss, 1999:179).

5.1.5 Fungal epoxide hydrolases

Fungal epoxide hydrolases have relatively broad substrate specificity and high enantioselectivities, and are used for the hydrolysis of aryl- and substituted alicyclic substrates but not for aliphatic epoxides (Weijers & de Bont, 1999:206).

Archelas demonstrated the biohydrolysis of glycidyl acetal derivates using *Aspergillus niger* as biocatalyst (Scheme 2.16). The derivates are used in the synthesis of azasugars and in the preparation of glycosidase inhibitors. In comparison the optically pure glycidyl acetal derivates are very difficult to synthesise by chemical ways, and the best obtained yield was 50 % (Archelas, 1998:79).



Scheme 2.16 Biohydrolysis of glycidyl acetal derivates

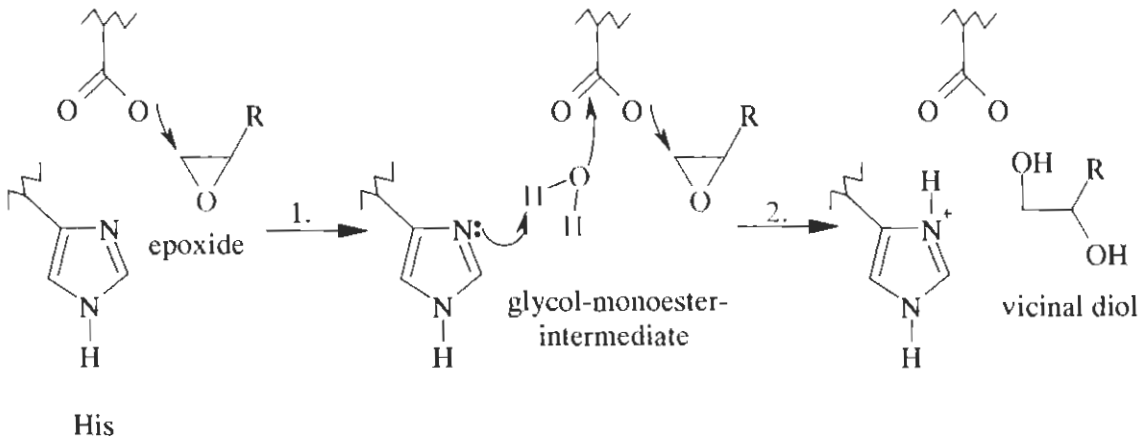
5.1.6 Yeast epoxide hydrolases

Yeasts with epoxide hydrolase activity have only recently been discovered. A *Rhodotorula glutinis* strain was shown to possess outstanding activity, with high preference for phenyl group containing compounds (Weijers, 1997:641). Monosubstituted epoxides are highly flexible and rather slim molecules, which make chiral recognition rather difficult. The only selective

enzymes for these molecules were found among red yeasts, such as *Rhodotorula araucarae*, *Rhodotorula glutinis* (Weijers, 1997:641)(Orru & Faber, 1999:19) and *Rhodosporidium toruloides* (Botes *et al.*, 1999:3327)

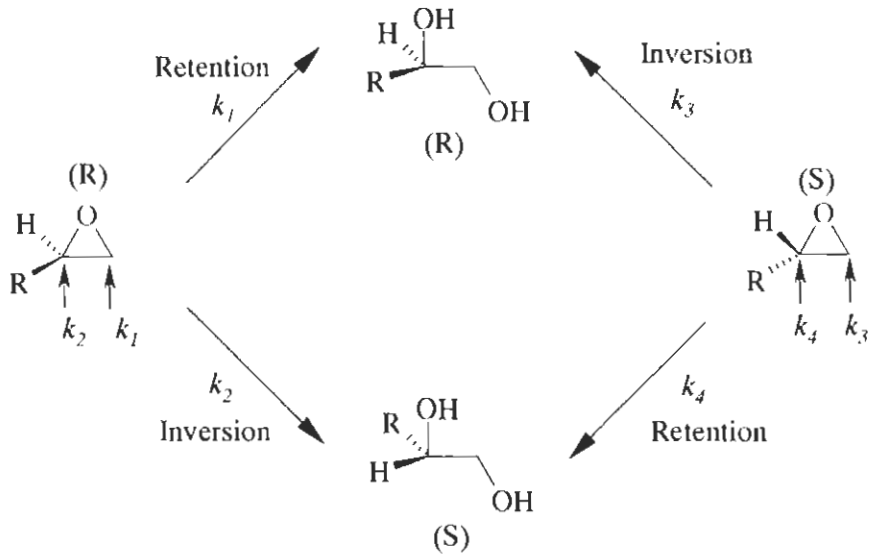
5.2 Mechanism of enzymatic hydrolysis

The enzymatic reaction is initiated by an S_N2 type nucleophilic attack (Weijers & de Bont, 1999). The epoxide is attacked by a carboxylate nucleophile (aspartic acid), leading to the formation of a covalent glycol-monoester-enzyme intermediate (Scheme 2.17). In the second step, the glycol-monoester-enzyme intermediate is hydrolysed through the attack of OH^- from water, thereby resulting in the release of the vicinal diol. The water is provided by the aid of a histidine residue of the catalytic amino acid (Orru & Faber, 1999:17).



Scheme 2.17 Mechanism of epoxide hydrolase

Considering that the mechanism involves a nucleophilic attack, the absolute configuration of the epoxide compound may be retained or it may be inverted (Scheme 2.18). The inversion or retention of configuration depends on the regioselectivity of the enzyme as well as on the substitutional pattern of the carbon atom under attack. For example, an attack on the most substituted carbon atom (k_2) of the (R)-enantiomer will lead to the inversion of the original stereochemistry and lead to the formation of the (S)-diol (Orru & Faber, 1999:16).



Scheme 2.18 Stereochemical pathways of epoxide ring opening (Steinreiber & Faber, 2001:553)

6. Immobilisation

Immobilised biocatalysts can be defined as enzymes, microbial cells or plant cells, physically confined or localized in a defined region of space for repeated or continuous use, with retention of their catalytic activities (Chibata *et al.*, 1992: 352).

One of the major advances in optimising biotechnological processes lies in immobilisation technology, which can result in increased productivity and product concentrations. The use of immobilized microorganisms for the production of biologically active substances has gained increasing interest because of its potential in a variety of industrial processes in (Konstantin *et al.*, 2000:1177).

Immobilisation of enzymes/whole cells firstly serves the purpose of transforming the catalyst into a particle that can be handled through simple mechanical operations (carrier-fixation or cross-linking), while the entrapment within a membrane or a capsule leads to the restraint of the enzyme to a defined space (Cruz *et al.*, 2001:419). This confinement leads to a catalyst with a greater stability (Rasor, 2000:99), and cell longevity under reaction conditions with the possibility of repeated and/or continuous use, in contrast to that of the free counterpart (Cruz *et al.*, 2001:419).

The materials used as well as the procedure of immobilisation should be compatible with the biocatalyst and the process that the biocatalyst is used for, i.e. the immobilisation procedure should be mild, diffusion of substrates and products in the support material should be possible and the support material should be stable during the reaction process (Bickerstaff, 1997:1).

Methods of immobilisation can be classified as carrier binding, cross-linking, entrapment (Chibata *et al.*, 1992:352), or encapsulation (Bickerstaff, 1997:3). These methods can also be classified (Figure 2.4), by means of the mechanism of bonding, i.e., chemical or physical (Bommarius, 1993:433).

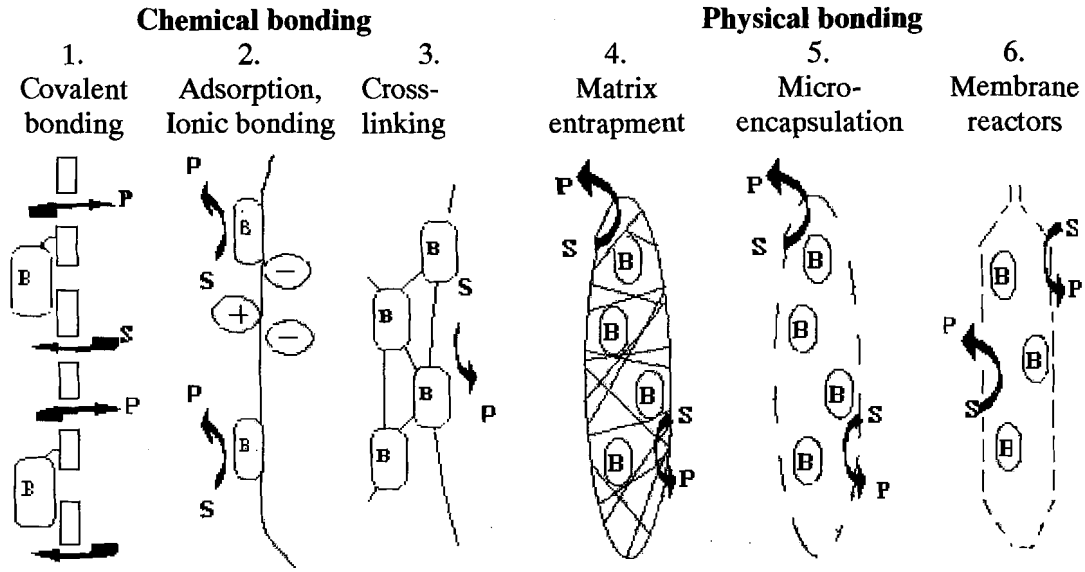


Figure 2.4 Methods of immobilisation (S = substrate; P = product)

6.1 Chemical bonding

6.1.1 Covalent bonding

This method involves the formation of covalent bonds between the cell and a support material. The bond is usually formed between functional groups present on the surface of the support and functional groups belonging to amino acid residues on the surface of the enzyme/cell. The amino acid groups most commonly involved in covalent bond formation are the amino group (NH_2), of lysine or arginine, the carboxyl group (CO_2H), of aspartic acid or glutamic acid, the hydroxyl group (OH), of serine or threonine, and the sulfydryl group (SH), of cysteine (Bickerstaff, 1997:3).

The immobilisation by covalent bonding is carried out under severe conditions, and an enzyme with relatively high activity will not be obtained (Chibata *et al.,* 1992:352). For instance; the covalent bonding of a partially purified β-galactosidase on Eupergit C only 55% of the catalytic activity was retrieved after immobilisation (Hernaiz & Crout, 2000:29).

Mineral carriers, e.g. pumice, silica gel, and titanium oxide, offer various possibilities for the binding of cells after their modification with chlorides of metals. The modified carrier can bind cells either directly by chelation, or after an added modification, by ionic or covalent bonds (Halgăs, 1992:38).

Other supports used for covalent bonding of enzymes and cells include nylon (Isgrove *et al.,* 2001:225), polyacrylamide (González-Sáiz & Pizarro, 2001:435), polytyramine (Situmorang *et al.*, 1999:211), and Eupergit C (Mateo *et al.*, 2000:509).

6.1.2 Adsorption

Immobilisation by adsorption is the simplest means of immobilisation and involves reversible surface interactions between the cell/enzyme and support material.

The forces involved are mostly electrostatic forces:

- Van der Waals interactions,
- Ionic bonding interactions, and
- Hydrogen bonding interactions.

Since yeast cells have a surface that is largely negatively charged, the use of a positively charged support will facilitate immobilisation. This approach has the advantage that the existing interactions between the cell and support are utilised and hence no chemical modification is necessary and little damage is done to the cells (Bickerstaff, 1997:3).

6.1.3 Cross-linking

This method is more frequently used for cells than for enzymes and different modifications are known (Halgăs, 1992:39). In the technique the use of a water-insoluble matrices is excluded (Chibata *et al.,* 1992:353). Cross-linking can be achieved by chemical (covalent bond formation between the cells), or physical (flocculation), methods and is most often used to enhance other methods to reduce cell leakage (Bickerstaff, 1997:10). Tanriseven & Dogan (2002:29), for instance, immobilised β-galactosidase from *Aspergillus oryzae* in fibers composed of alginate

and gelatine and cross-linked the alginate and enzymes with glutaraldehyde. This prevented leakage of the enzyme from the alginate structure.

An example is the flocculation of *Escherichia coli* with chitosan, and subsequent cross-linking of the cells with glutaraldehyde (a cationic biopolymer). The cross-linked cells can easily be collected by centrifugation at low speed, or filtration for reuse (Fan *et al.,* 1999:224).

6.2 Physical bonding

6.2.1 Entrapment

Entrapment into polymer materials is the most frequently used method of cell immobilisation (Halgăs, 1992:39). This method entails the confinement of cells in the lattice of a polymer matrix or semipermeable membrane. Entrapment differs from covalent binding or cross-linking methods in that the cell itself does not bind to the matrix or membrane (Chibata *et al.,* 1992:353).

The porosity of the gel lattice is controlled to ensure that the structure is tight enough to prevent leakage, yet at the same time allow free movement of the substrate and product. Inevitably the support will act as a barrier to mass transfer, and although this can have serious implications on kinetics, it can have useful advantages since harmful cells, proteins, and enzymes are prevented from interaction with the immobilised biocatalyst (Bickerstaff, 1997:8).

Many diverse gel matrices have been proposed as possible carriers. In these cases, either natural biopolymers e.g. alginate, carrageenan, gelatine and collagen or synthetic polymers such as polyacrylates and polyurethanes can be used as gel-forming agents (Lovinsky & Plieva, 1998:227).

6.2.2 Encapsulation

Encapsulation of cells can be achieved by enveloping the biological component within various forms of semipermeable membranes. It is related to entrapment in that the cells are free in solution, but restricted in space. Large proteins or enzymes cannot pass into or out of the capsule, but small substrates and products can pass unreservedly across the semipermeable membrane. Problems associated with diffusion may result in rupture of the membrane if products from a reaction accumulate rapidly (Bickerstaff, 1997:9).

6.2.3 Membrane reactors

Membrane reactors combine selective mass transport with chemical reactions. The selective removal of products from the reaction site increases the conversion of product-inhibited or thermodynamically, unfavourable reactions. Giorno & Drioli (2000:339), classified membrane reactors into two classes, the biocatalyst can be (1) suspended in solution and compartmentalised by a membrane in a reaction vessel or (2) immobilised within the membrane matrix itself (Figure 2.5).

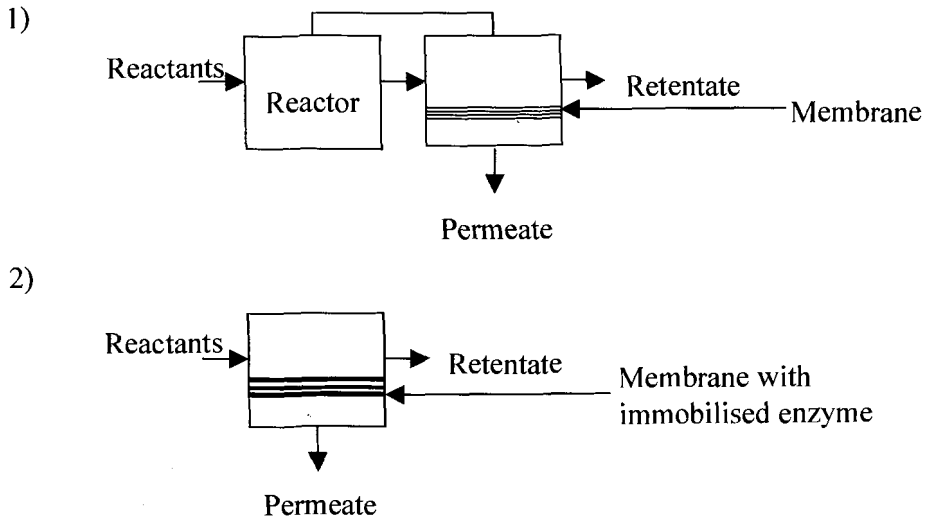


Figure 2.5 Main configuration of membrane reactors: a) reactor combined with membrane operation unit, b) reactor with the membrane active as a catalytic and separation unit.

These reactors are well suited for enzyme-catalysed reactions and implemented for the large-scale production of chemical compounds, but with fouling of the membrane as major drawback (Bommarius, Drauz & Groeger, 1992:372-374).

7. Conclusion

The search for and development of methods to obtain optically pure compounds have increased immensely over the past decade due to the different physiological effects of enantiomers of the same chiral compound. Biocatalysts, more specifically epoxide hydrolase ensymes, have proven to be very sufficient in the kinetic resolution of a wide range of substrates (Orru & Faber, 1999:16). However, some of these catalysts have the disadvantage of being inherently labile (Illanes, 1999:1) and repeated use is impaired due to difficulty in handling of the whole cells/ ensymes. One of the advances in optimising biotechnological processes lies in immobilisation technology, which can greatly increase productivity and product concentrations while simultaneously transforming the catalyst into a particle that can easily be handled (Laca, *et al.*, 1998:225) for repeated and/ continuous use (Tanaka, *et al.*, 1999:504).

In this chapter chirality, the implications and methods to obtain chiral compounds were described. More specifically there was focused on chemical and biocatalytic methods for obtaining enantiopure epoxides, and methods available for the immobilisation of biocatalysts.

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Chapter 3

Immobilisation of yeast cells in calcium alginate beads

1. Introduction

The production of enantiomerically pure compounds is of increasing importance in the fine chemical industry (Buque *et al.*, 2002:656). Optically active epoxides are useful chiral synthons in organic synthesis that have been used as intermediates in the synthesis of natural products and other biologically active substances. These compounds are easily derivatized in a regioselective and stereoselective manner (Furuhashi, 1992:167).

Enzymes catalyse chemical reactions with great specificity and at high reaction rates. While enzymatic reactions form the basis of the metabolism of all living organisms, they provide a tremendous opportunity for industry to carry out efficient and economical biocatalytic conversions (van Beilen & Li, 2002:1). Asymmetric hydrolysis of (±)-1 to (±)-4 terminal epoxides was previously achieved by employing a number of yeast strains. All the strains preferentially hydrolysed the (R)-epoxide to the (R)-diol. Strains belonging to the *Rhodosporidium* and the *Rhodotorula* genera displayed extremely high E-values and outstanding reaction rates and for C6 to C8 unbranched epoxides (Botes *et al.*, 1999:3328).

In Chapter 2 it was shown that one of the major advances in optimising biotechnological processes lies in immobilisation technology (Konstantin *et al.*, 2000:1177). Immobilisation can be defined as any process that restricts substances or cells inside a given structure and limits their free diffusion or movement (Huebner & Buccholz, 1999:1785).

Immobilisation thus offer an interesting alternative to bioprocesses conventionally carried out with free cells (Laca *et al.*, 1998:225). Among the advantages of immobilisation are the ease of handling, cell separation from the liquid medium (Laca *et al.*, 1998:225), the possibility of repeated and continuous use of biocatalysts as well as an increase in enzyme stability (Rasor, 2000:99), and longevity in contrast to that of free cells (Cruz *et al.*, 2001:419), with a subsequent increase in bioprocess efficiency (Tanaka & Kawamoto, 1999:504).

The methods of immobilisation can be classified (Figure 3.1), according to the mechanism of bonding i.e., chemical or physical (Bommarius, 1993:433). The materials used as well as the procedure of immobilisation should be compatible with the biocatalyst and the process that the biocatalyst is used for, i.e. the immobilisation procedure should be mild, diffusion of substrate and products in the support material should be possible and the support material should be stable during the reaction process (Bickerstaff, 1997:1).

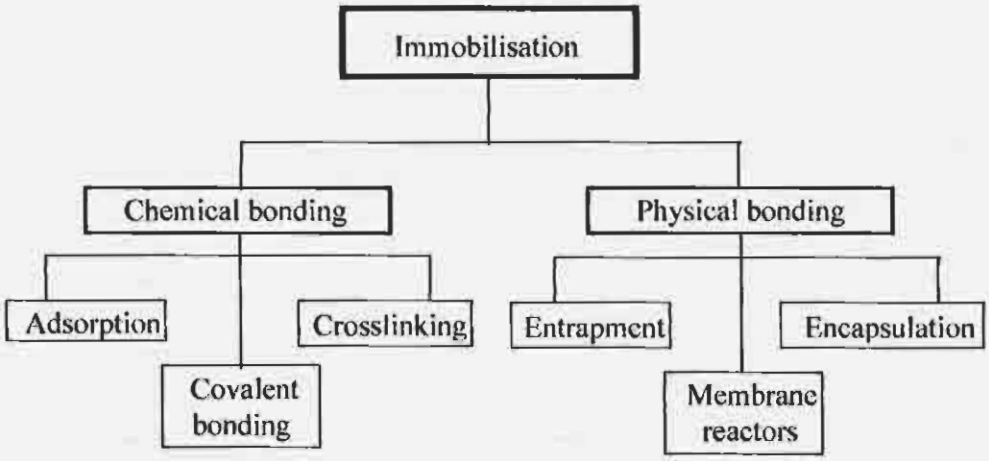

Figure 3.1 Methods of immobilisation

Entrapment of cells in calcium alginate is the most widely used immobilisation technique in the biocatalytic production of chemicals (Smidsrod & Skjak-Braek, 1990:71). Alginic acid (Figure 3.2), is a natural polyuronic acid extracted from brown seaweed and bacteria (Tanaka & Kawamoto, 1999:508). Chemically, alginates are block copolymers of 1,4-linked β-D-mannoronic acid and α-L-glucoronic acid arranged in block patterns, which polymerise when in contact with divalent cations such as Ca^{2+}, Cu^{2+} and Na^{+} (Heubner & Buccholz, 1999:1792).

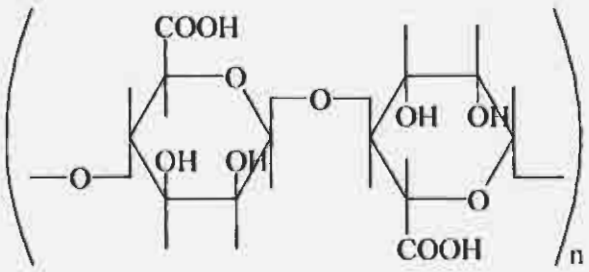

Figure 3.2 Structure of alginic acid

According to Buque *et al.* (2002:656), alginate is cheap and readily available, has a high affinity for water, and has the ability to form gels under mild conditions, which are suitable for most biological cells. Alginate has the further advantage of being non-toxic and non-pathogenic which makes it attractive for applications in the food and pharmaceutical industry (Tanaka & Kawamoto, 1999:508).

For pure enzymes to be used industrially, the extraction of crude enzymes from the cell broth and purification is a prerequisite. When whole cells are employed these steps are redundant, leading to a 70 % decrease in the price of the pure enzyme. The use of enzymes in whole cells also leads to a high enzyme recovery (Park & Chang, 2000:312). In this chapter the immobilisation of whole cells of *Rhodosporidium toruloides* (UOFS Y-0471), in calcium alginate beads for the stereoselective hydrolysis of 1,2-epoxyoctane is described. Investigating parameters such as the concentration of the cationic and anionic solutions, the amount of immobilised biomass as well as the effect of these two parameters on bead diameter were investigated and utilised in the determination of the optimum immobilisation procedure.

2. Experimental

2.1 Materials

Rhodosporidium toruloides (UOFS Y-0471), was obtained from the Yeast Culture Collection of the Department of Microbiology and Biochemistry of the *University of the Free State, Bloemfontein, South Africa.*

Alginic acid salt obtained from brown algae as well as a medium viscosity carboxymethylcellulose (CMC) sodium salt was purchased from *Fluka BioChemika*, South Africa. Pure granular anhydrous calcium chloride ($CaCl_2$) was obtained from Merck South Africa. Tris(hydroxymethyl)aminomethane for the preparation of tris buffer was purchased from *Saarchem, South Africa.* 1,2-Epoxyoctane (epoxide) and 1,2-octanediol (diol) were both obtained from Aldrich, South Africa. Double distilled deionised water was used. All solutions were prepared with a 20 mM tris buffer (pH 7,5) solution.

2.2. Methods

2.2.1 Analysis

Reactions where analysed and monitored by GC (ThermoFinnigan Focus equipped with FID). Chiral separation of the respective isomers of 1,2-epoxyoctane and 1,2-octanediol was attained on a β-dex 120 fused silica cyclodextrin capillary column (Supelco) using H_2 as carrier gas. Higher seusitivity was obtained when H_2 was used as carrier gas in comparison with N_2. The epoxide and diol were respectively analysed at 50 °C and 130 °C. Retention times were 63,24 min and 63,81 min for (R)- and (S)-1,2-epoxyoctane and 15,64 min and 16,06 min for (S)- and (R)-1,2-octanediol, respectively.

2.2.2 Preparation of frozen yeast cells

Cells of *Rhodosporidium toruloides* were grown at 27 °C for 72 hours from pre-cultures in 1L shake-flask cultures containing 200 ml of YM media. The media contained 1,5 % (m/v) glucose, 2,0% (m/v) malt extract, 1,0 % (m/v) peptone, and 0,5 % (m/v) yeast extract. A vitamin solution was added through a 0.2 μm filter to autoclaved media. The cells were centrifuged in Falcon centrifuge tubes, the supernatant discarded and the cells washed with a 50 mM phosphate buffer (pH 7,5). The cells were centrifuged for a second time, supernatant discarded and the cells resuspended (1:1 m/m) in phosphate buffer containing 15,0 % (v/v) glycerol. The yeast cells were stored in Falcon tubes at –15 °C. Yeast cells were thawed, centrifuged and washed with

tris buffer (pH 7,5) before immobilisation in alginate. Cells used for unimmobilised whole cell enzyme reactions were washed and resuspended in tris buffer (20,0 % cell solution).

2.2.3 Preparation of calcium alginate beads

Calcium alginate capsules were prepared by extrusion using a simple one step process as described by Fraser and Bickerstaff (1997:62). Sodium alginate solutions of various concentrations were prepared as an anionic solution. This process was performed by the stepwise addition of the alginate powder while rapidly stirring the buffer to avoid the formation of a lumpy precipitate. The solution was stirred for 12 hours. For the preparation of the cationic solutions, 0,5 % (m/v) CMC was dissolved in $CaCl_2$-solutions of varying concentration. CMC was used as a non-polar gelling polymer to modulate the viscosity and density of cationic solutions to ensure the spherical shape of the beads. Wet biomass was suspended in the alginate solution and stirred thoroughly to ensure a homogenous distribution of the cells in the alginate solution.

The alginate/cell solution was added drop-wise (using a peristaltic pump), to 40 ml of $CaCl_2$-solution through a silicone tube (Figure 3.3). The $CaCl_2$ solution was constantly stirred (130 rpm) using a magnetic stirrer to keep the droplets from sticking together. A dropping height of 10,0 cm was used to ensure the formation of spherical droplets. The inner diameter of the silicone tube was 3,0 mm. The alginate/cell suspension dropping time was kept to less than 1,6 % of the residence time of the bead in the cationic solution in order to ensure uniformity of the beads.

Once the alginate/cell solution has been dropped into the cationic solution, a capsular membrane immediately forms around each droplet due to the crosslinking of the interfacial alginate molecules in the presence of Ca^{2+} cations. Blandino *et al.* (2002:203), previously established that no significant changes in membrane thickness occur during contact of the anionic with the cationic solution after one hour. Thus, gelation time was restricted to one hour. The $CaCl_2$/CMC solution was discarded after the gelation time.

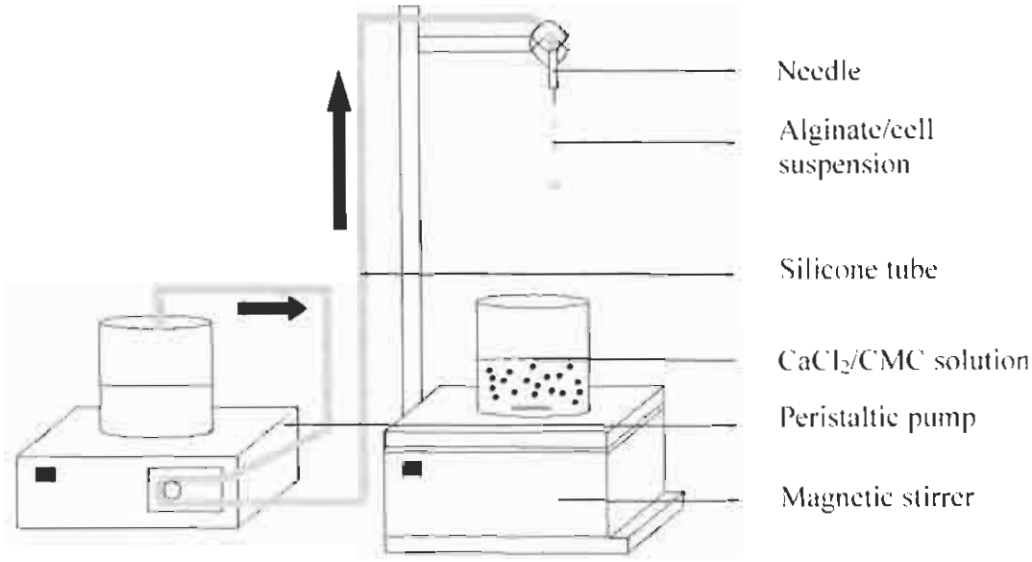

Figure 3.3 Experimental set-up for the preparation of calcium alginate beads

Subsequently the beads were washed three times and stored in 20 mM tris buffer (pH 7.5) at 4.0 °C. Alginate bead preparation was done at room temperature (25.0 °C).

2.2.4 Determination of optimum $CaCl_2$ and alginate concentration

Calcium alginate beads were prepared using three different alginate concentrations (0.5; 0.75 and 1.0 % m/v), and four $CaCl_2$ concentrations (0.1; 0.2; 0.4 and 0.5 M). 16.6 % wet biomass were immobilised. After gentle drying on filter paper the alginate beads were weighed. 1.0 gram of alginate beads was placed in 2.0 ml micro-centrifuge tubes and 0.5 ml tris buffer was added. The beads were stored at room temperature for 24 hours. The activity was measured directly after preparation (0 hours) and after storage at room temperature for one day (24 hours). The initial epoxide concentration was 20.0 mM 1,2-epoxyoctane in the liquid volume (0.5 ml). The reaction mixtures were incubated for 20.0 min at 30 °C while continuously being shaken in a water bath. The unreacted residual epoxide and formed diol in the water phase were extracted with a 1:1 volume of ethyl acetate and analysed by GC. Three beads from every $CaCl_2$ and alginate solution batch were used for the determination of the bead diameter.

2.2.5 Determination of bead properties

The beads were gently dried on filter paper and the diameter measured with a Nikon® 129114-Optiphot microscope fitted with a PS No 2 measuring graticule. All measurements were done in triplicate.

For the photographs of the alginate beads, the beads were dehydrated in acetone solutions of increasing concentration [50, 70 and 100 % (v/v)] and subsequently critically dried with liquid CO_2. The beads were then coated with a thin layer of carbon (C) and a gold (Au)/palladium (Pd) mixture. Photographs were taken with a Phillips® XL30 Scanning Electron Microscope.

2.2.6 Determination of the viscosity of alginate solutions

Several solutions of different alginate concentrations were prepared and viscosity determined at 25 °C with a RV WinGather V1.1 Brookfield viscosimeter equipped with a SC4-18 spindle.

2.2.7 Determination of optimum immobilised biomass

Calcium alginate beads were prepared as described in 2.2.3. Suspensions with three different cell/alginate ratios were prepared [20,0; 16,6 and 14,3 % cell/alginate solution (m/m)], using a 0,5 % m/v alginate solution in combination with 0,4 M $CaCl_2$. The alginate beads were weighed after gentle drying on filter paper. 0,6 g of alginate beads were placed in a 1,5 ml micro-centrifuge tube and 0,3 ml tris buffer was added. The beads were stored at room temperature for 24 hours, and activity was measured at three 12-hour intervals after bead preparation (0; 12 and 24 hours). The initial epoxide concentration was 20,0 mM 1,2-epoxyoctane in the liquid volume. The reaction mixtures were incubated for 20,0 min at 30 °C with continuous shaking in a water bath. After 20 minutes the unreacted epoxide and formed diol in the water phase was extracted with a 1:1 volume of ethyl acetate and analysed by GC.

2.2.8 Determination of the effect of $CaCl_2$ concentration on free cell enzyme activity

Frozen yeast cells were thawed and resuspended in tris-buffer (20 % cell suspension), and 500 μl dispensed in 1,5 ml micro-centrifuge tubes. $CaCl_2$ (40 mM and 100 mM), were respectively added. The reaction mixtures (total concentration of 20,0 mM epoxide), were incubated at room temperature for 10 miuutes and the remaining enzyme activities assayed over a period of 50 minutes at 30 °C while being continuously shaken. At different time intervals one micro-centrifuge tube of each $CaCl_2$ concentration was removed and analysed for epoxide and diol.

3. Results and discussion

3.1 Determination of optimum $CaCl_2$ and alginate concentrations

The cationic and anionic solution concentrations were varied to evaluate the effects and possible interactions of the three formulative parameters ($CaCl_2$ concentration, alginate concentration and the average bead diameter), on the enzymatic activity exhibited by alginate entrapped *Rhodosporidium toruloides*. The initial epoxide hydrolase activity (0 hours) as well as stability (decrease in the initial activity), after storage at room temperature (25 °C) for 24 hours after entrapment was assayed by determining both the epoxide utilisation and diol production after a 20-minute batch reaction. Data was studied macroscopically without valuing the intrinsic enzyme kinetics or the epoxide and diol transportation efficiency. The experiment was done in triplicate with an average deviation of 3,7 % for the epoxide values and 10,9 % for the diol values. The results attained are presented in Table 3.1

From the data it can be seen that neither an increase in the sodium alginate concentration nor the $CaCl_2$ concentration had a significant effect on the initial (0 hour) residual epoxide concentrations. The residual epoxide also remained constant after the 24-hour observation period where a 0,5 % m/v alginate solution was employed, relentless of the $CaCl_2$ concentration.

An increase in the $CaCl_2$ concentration in conjunction with the same alginate solution only had a major effect on the stability of the enzyme (looking at the residual epoxide concentration), when higher concentrations of sodium alginate [0,75 and 1,0 % (m/v)] were employed. After a 24-hour incubation time the effect of higher $CaCl_2$ concentrations can clearly be seen when a high alginate concentration was employed [1,0 % (m/v), where a substantial decrease in activity was prominent with an increase in the $CaCl_2$ concentration, looking at the epoxide concentration.

Table 3.1 Residual epoxide and formed diol under different gelation conditions

[Sodium alginate] 0,5 % m/v								
[$CaCl_2$]	0,1 M		0,2 M		0,4 M		0,5 M	
Time (hours)	0	24	0	24	0	24	0	24
[Epox] (mM)	0,43	0,44	0,47	0,40	0,46	0,45	0,46	0,62
[Diol] (mM)	4,50	3,46	6,21	3,46	5,68	3,12	5,73	2,35

[Sodium alginate] 0,75 % m/v								
[$CaCl_2$]	0,1 M		0,2 M		0,4 M		0,5 M	
Time (hours)	0	24	0	24	0	24	0	24
[Epox] (mM)	0,51	0,68	0,51	0,74	0,47	0,60	0,50	0,70
[Diol] (mM)	6,38	1,81	5,85	0,86	6,38	0,86	6,50	0,33

[Sodium alginate] 1,0 % m/v								
[$CaCl_2$]	0,1 M		0,2 M		0,4 M		0,5 M	
Time (hours)	0	24	0	24	0	24	0	24
[Epox] (mM)	0,41	0,57	0,47	0,66	0,46	0,71	0,45	0,74
[Diol] (mM)	7,10	2,28	6,31	0,90	8,24	0,45	6,13	0,03

An increase in the initial diol production (0 hours) was eminent with an increase in alginate concentration, but stayed relatively constant with an increase in $CaCl_2$ concentration.

It was found that the diol concentration in the liquid phase did correlate with the residual epoxide concentration when comparing the whole range of varying $CaCl_2$ and alginate concentrations (for example a higher diol concentration did resemble a lower epoxide concentration when looking at two different $CaCl_2$ and alginate concentration combinations), but that the produced diol concentration did not correlate with the residual epoxide concentration for every individual experiment (for example in the combination 0,1 M $CaCl_2$ and 1,0 % m/v alginate, with a residual epoxide concentration of only 0,41 mM epoxide in the liquid phase and only 7,10 mM diol). Due to this discrepancy, the concentration of diol in the liquid phase was used to determine the relative % activity loss (Figure 3.4) after incubation of the beads at room temperature for 24 hours.

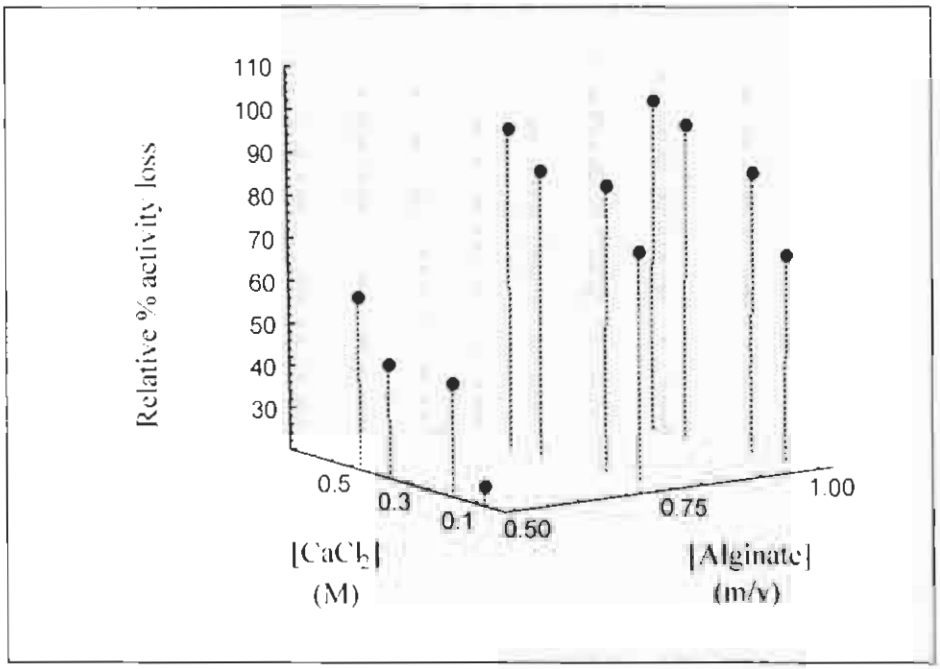

Figure 3.4 The influence of $CaCl_2$ and alginate concentrations on relative % activity loss after 24 hours

It can be seen that an increase in the $CaCl_2$ concentration for each alginate concentration, and an increase in alginate concentration for each $CaCl_2$ concentration lead to an increase in the relative % activity loss, with no major difference for the transitional $CaCl_2$ solutions (0.2 M and 0.4 M).

3.2. Bead properties

3.2.1 Photomicrograph of alginate beads

Electronmicroscopic pictures were taken of the beads to ensure the total spherical form of the beads as well as to confirm studies previously done on the morphological characteristics of alginate beads. When looking at a surface micrograph (Figure 3.5) of the beads it can be seen that perfectly spherical beads were formed, and the sponge-like structure reported by Ouwerx *et al.* (1998:402), can clearly be observed from a micrograph taken of a cross section through the bead.

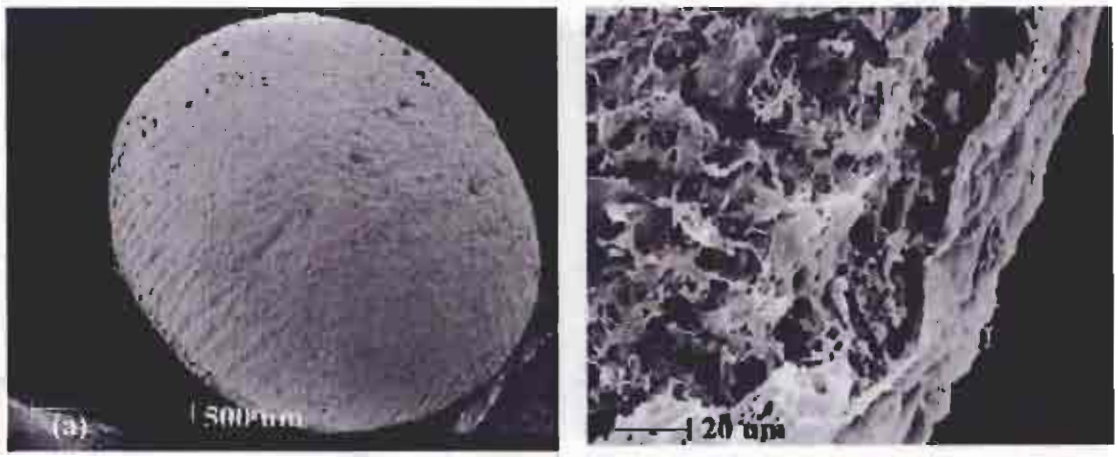

Figure 3.5 Photomicrographs of alginate beads

3.2.2 Determination of bead diameter

The bead diameter of each combination of $CaCl_2$ and alginate concentration was measured in triplicate and was obtained with an average standard deviation of 0,028. The average bead diameter for each scenario (Figure 3.6) showed a definite increase in bead diameter with increasing alginate concentrations, irrespective of the $CaCl_2$ concentration, while no major influence of $CaCl_2$ concentration on bead diameter was observed.

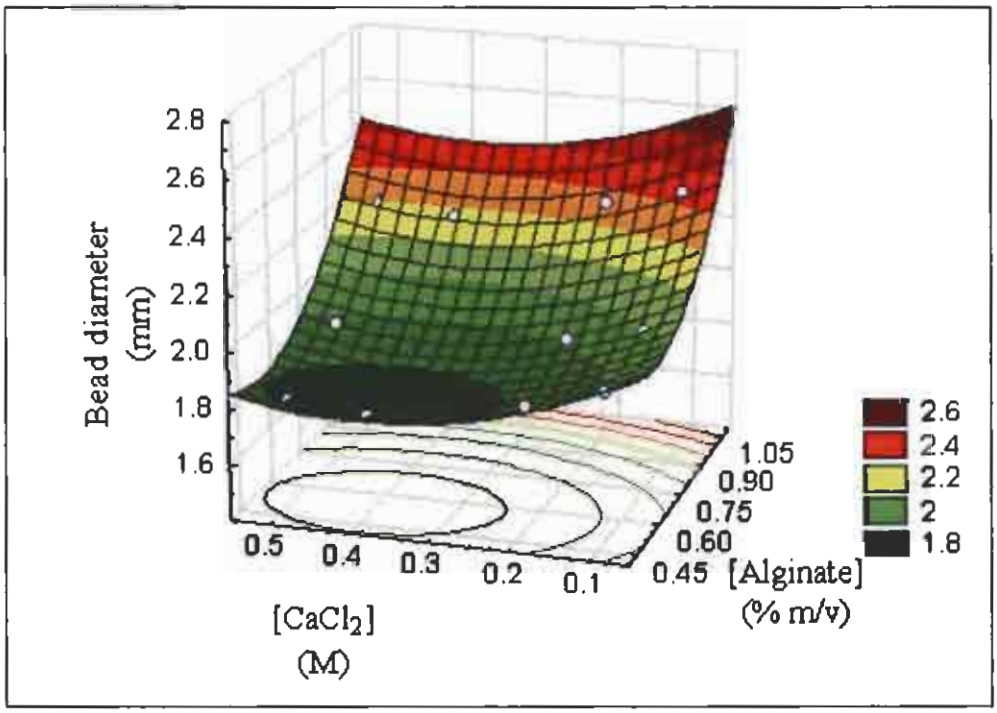

Figure 3.6 The influence of $CaCl_2$ and alginate concentrations on bead diameter

The two main factors influencing drop size when making use of a simple dropping method for the preparation of microcapsules are the force of gravity pulling the solution down and the resisting interfacial tension force (Huebner & Buccholz, 1999:1787). Thus, a droplet will only fall if the gravitational force (the droplets mass under gravitational acceleration), exceeds the resisting interfacial force.

When measuring the viscosity of alginate solutions as a function of alginate concentration (Figure 3.7) it can clearly be seen that viscosity increases with an increase in alginate concentration. This viscosity increase leads to the increase of interfacial forces between the formed droplets and the needle tip. This interaction necessitates a larger volume of alginate solution to reach the critical droplet mass for the gravitational force to overcome the interfacial forces, explaining the larger bead diameter found with an increasing alginate concentration.

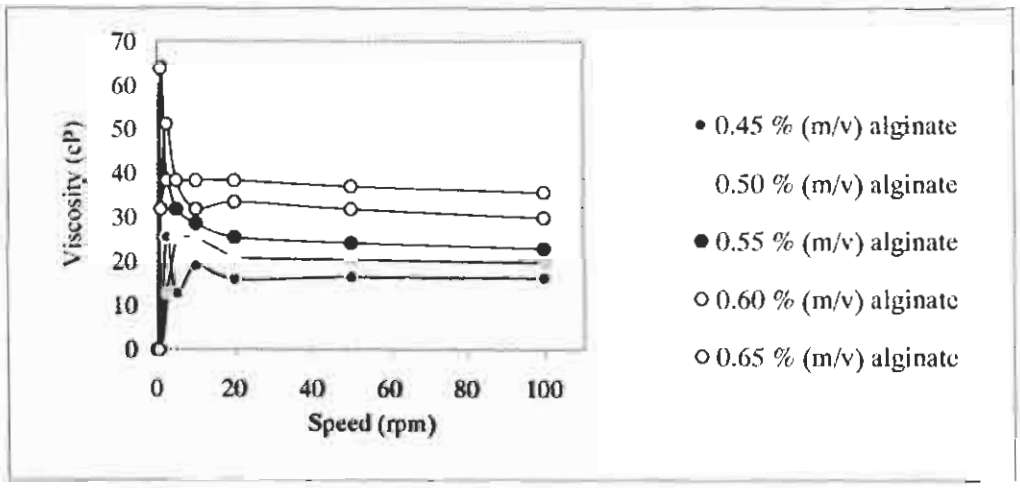

Figure 3.7 The relation between alginate concentration and solution viscosity

In a previous study by Ouwerx *et al.* (1998: 407), it was found that the alginate beads' properties were greatly influenced by the concentration and nature of the polymer type as well as by the concentration of the cation in the maturation medium. These results are in accordance with their findings, also showing the major effect of $CaCl_2$ and alginate solutions employed in bead formation on bead properties as well as the effect on enzyme activity and stability.

According to Kragl *et* al. (1999:1068), employing particles with a diameter larger than 1,0 mm, film diffusion would be the limiting factor in the enzyme reaction rate (the diffusion of the epoxide into the bead and the diol out of the bead are limited), due to mass-transport resistance. It was thought that whole cells immobilised in alginate beads with a larger bead diameter would

lead to an enzyme with lower activity, due to mass-transport limitations, but would provide a more stable environment for the whole cell enzymes and therefore lead to enzymes with higher stability. However, when comparing this to the results in Table 3.1 and Figure 3.6 (the corresponding bead diameters), it can be seen that bead diameter either did not have any effect on the resultant activity, or that other factors such as the Ca^{2+}-ions had a larger influence on the enzyme activity and stability.

3.3 Determination of optimum immobilised biomass

Three different cell/alginate suspensions were prepared [25,0; 16,6 and 14,3 % cell/alginate solution (m/m)], using a 0,5 % m/v alginate solution in combination with 0,4 M $CaCl_2$ solution. The initial epoxide hydrolase activity (0 hours) as well as stability (decrease in the initial activity), after storage at room temperature (25 °C) for 12 and 24 hours after entrapment was assayed by chiral GC analysis for both the residual epoxide and diol produced after a 20-minute batch reaction. This was also repeated with a 20 % cell/buffer suspension (1:0 alginate/cell suspension). The experiment was done in duplicate with an average deviation of 15 % for the epoxide concentrations and 5 % for the diol concentrations. The results attained are portrayed in Table 3.2.

It can be seen that immobilisation does affect the % ee_p (diol). While the (S)-diol and (R)-diol is usually produced in a 1:3 ratio for free cells where a % ee_p of 65 at 0 hours is found. This is not observed with the alginate immobilised cells where the initial (0 hour), % ee_p was between 5 and 8, where a % ee_p of 65 can be observed for the free cells. Although kinetic resolution of epoxides is primarily used to obtain enantiopure epoxide while the enantiomeric ratio of the diol is of lesser importance (Botes *et al.*, 1998:425), this is still a very interesting phenomenon, which cannot be explained.

The increase in % ee_s after immobilisation in comparison with free cell enzymes can be attributed to lower enzyme activity of the cells after immobilisation in comparison with free cell enzymes.

Although the initial activity of free cells is much higher than that of the immobilised cell enzymes the relative % activity loss (a maximum of 9 % after 12 hours in comparison with 98 % with free cells), did reinforce the stabilisation effect that immobilisation has on whole cell enzyme activity.

The amount of immobilised biomass did not have a significant effect on the amount of diol produced, but a 4:1 (cell: alginate), ratio did lead to the formation of the most spherical beads.

The total epoxide and formed diol concentration did not add up to a total concentration of 20 mM, which is the initial epoxide concentration. This is due to absorption of the epoxide and diol in the bead that seems to be preferential towards the epoxide.

Table 3.2 The effect of different immobilised biomass on the enantioselectivity and stability after 24 hours.

Alginate/ biomass (m/m) ratio			Time			Relative % activity loss	
			0 hr	12 hr	24 hr	12 hr	24 hr
	0:1	[Epoxide]	4.25	19,58	20	-----	-----
		% ee_s	67	n.d.*	n.d.*	-----	-----
		[Diol]	12,55	0,20	0,069	> 98	> 99
		% ee_p	65	79	12	-----	-----
	4:1	[Epoxide]	0,07	0,27	0,45	-----	-----
		% ee_s	> 98	>98	58	-----	-----
		[Diol]	6,37	5,98	3,21	6	49
		% ee_p	5	11	36	-----	----
	5:1	[Epoxide]	0,11	0,27	0,45	-----	-----
		% ee_s	> 98	88	72	-----	-----
		[Diol]	6,48	6,61	2,94	-----	54
		% ee_p	7	10	16	-----	-----
	6:1	[Epoxide]	0,15	0,20	0,28	-----	-----
		% ee_s	> 98	> 98	65	-----	-----
		[Diol]	6,09	5,53	3,70	9	39
		% ee_p	8	10	13	-----	-----

* not determined

3.4 The effect of calcium chloride concentration on enzyme activity

Botes (1999:173), previously demonstrated the inhibitory effect of the chlorides of a number of metal ions on the activity of an enzyme extract of *Rhodosporidium toruloides*, but it was not known if Ca^{2+} ions would have the same adverse effect on whole cells, and subsequently an effect on the immobilised cells.

To determine the effect of $CaCl_2$ on the whole cell enzyme activity, whole cells were incubated with and without $CaCl_2$ for 10 minutes. Calcium chloride has a negative effect on enzyme activity (Figure 3.8), and an increase in $CaCl_2$ concentration leads to a decrease in the activity of free cells.

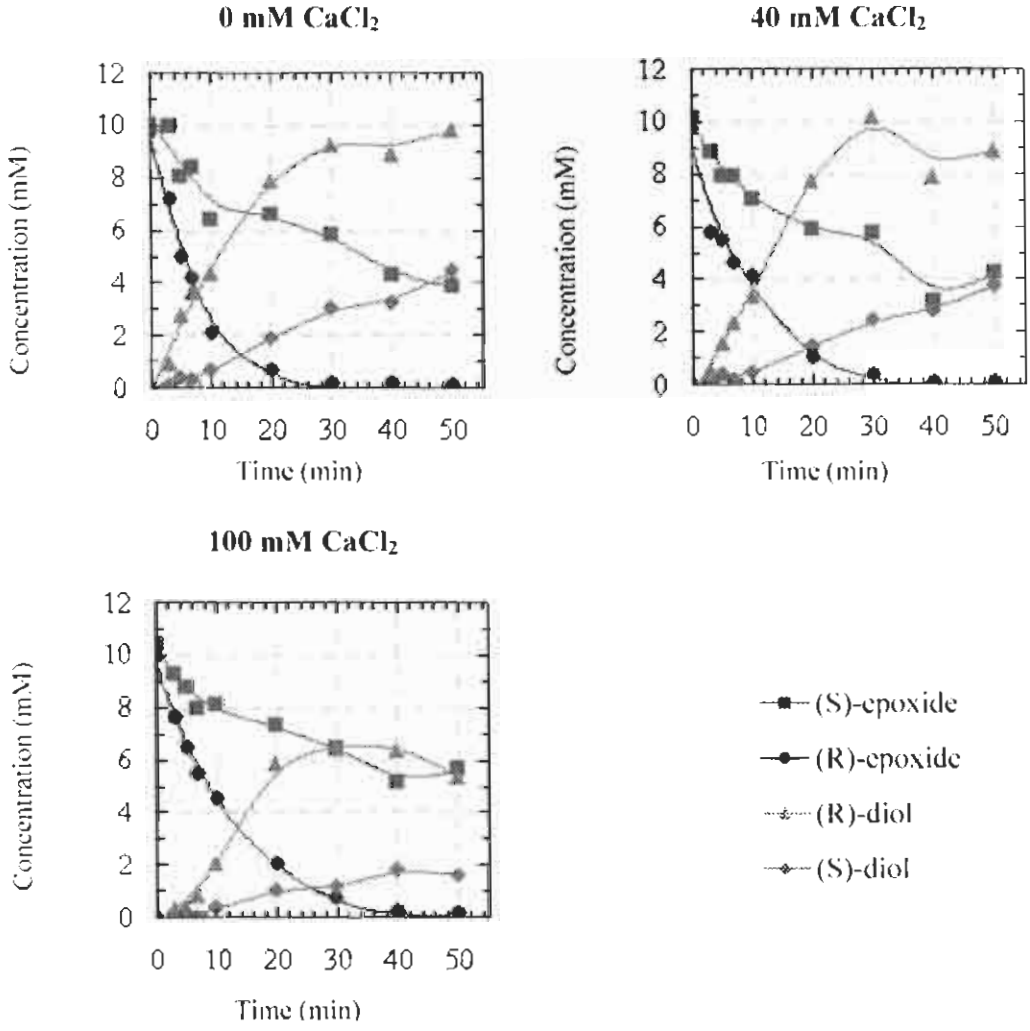

Figure 3.8 The effect of $CaCl_2$ on enzyme activity

When a polyvalent metal ion comes in contact with an alginate sol, a primary membrane will be formed immediately on contact. Khairou *et al.* (2002:447), previously determined the stoichiometry of the gelation process (Equation 3.1), which can be expressed by the following exchange reaction:

$$2(\text{Na - Alg})n + M^{2+} = (\text{M} - \text{Alg}_2)n + 2\,\text{Na}^+ \qquad \textbf{Equation 3.1}$$

where Na-Alg denotes the sodium alginate, M-Alg_2 is the metal alginate gel complex and M is the metal ion.

The Na^+ ions produced from the dissociation of alginate sol migrate from the alginate sol outward into the electrolyte solution through the primary membrane and the metal ions (Ca^{2+}) go inward and occupy the places left by Na^+ ions. During this formation process the initial bead volume is reduced due to crosslinking of the macromolecules and the expulsion of water (Huebner & Buccholz, 199:1785).

A decrease in stability was observed when a higher alginate solution was employed for bead preparation (Table 3.1). From Equation 3.1 it can clearly be seen that the number of Ca^{2+} ions included in the polymer structure is directly proportional to the number of alginate molecules. Thus, a higher alginate concentration would lead to a higher calcium concentration in contact with the cells.

4. Conclusion

Rhodosporidium toruloides was immobilised in calcium alginate beads. The immobilised cells did exhibit lower initial activity, but with exceptional stability in comparison with free cells, which showed a 98 % activity loss after a 12-hour storage period at room temperature in comparison with a maximum of 9 % activity loss for immobilised cells. Although the effect of $CaCl_2$ on the immobilised enzyme activity was eminent, the immobilisation matrix still stabilised the enzyme.

An increase in the alginate solution implemented, lead to the formation of beads with a smaller diameter, but a decrease in enzyme activity. This is contrary to what would be expected if mass-transfer were the limiting factor. A higher alginate concentration utilises a higher number of Ca^{2+} ions, which leads to a higher Ca^{2+} concentration in close contact with the immobilised cells. This explains the lower stability with higher alginate concentrations.

No relation was found between the enzyme activity and $CaCl_2$ concentration used as curing medium. If taking the adverse effect of only 100 mM $CaCl_2$ after 10 minutes on free cells compared to a contact time of 60 minutes during bead preparation with concentration of up to 400 mM $CaCl_2$ into consideration, this also demonstrates that the bead matrix protects against environmental factors even immediately after the primary membrane of the bead is formed.

The % ee_s and % ee_p from the immobilised cells only reflect the enantiomeric ratios in the water phase and no conclusion can be drawn from these results in terms of absolute enzyme activity and selectivity. Contrary to expectations, the epoxide seemed to be preferentially absorbed by the alginate beads, which cannot be explained yet.

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Chapter 4

Distribution of epoxide and diol in calcium alginate beads

1. Introduction

Sodium alginate is an anionic linear polysaccharide that consists of β-(1,4)-linked D-mannuronic acid units and some L-guluronic acid units where the macromolecular chains are chelating polyvalent metal ions (Khairou *et al.*, 2002:445). Entrapment of cells in hydrophilic calcium alginate is the most frequently used immobilisation method in the biocatalytic production of chemicals (Smidsrod & Skjak-Braek, 1990:71, Tanaka & Kawamoto, 1994:508). Alginate immobilisation has the benefit that it is possible to modify some of the capsule features by varying appropriate formulation conditions such as gelation time and alginate and $CaCl_2$ concentrations (Blandino *et al.*, 2001:601).

Epoxide hydrolases that are found in a wide range of living organisms including yeast cells, are co-factor independent enzymes utilised in the kinetic resolution of racemic epoxides. Enantiopure epoxides and their corresponding vicinal diols are important chiral building blocks in the preparation of more complex enantiopure bioactive compounds (Weijers & de Bont, 1998:200).

In Chapter 3 results were presented that seemed to indicate that the residual epoxide might be preferentially absorbed in the alginate beads while the produced diol accumulates in the water phase during the kinetic resolution of 1,2-epoxyoctane by the yeast *Rhodosporidium toruloides*. A recurring setback in biocatalytic processes is the production of dilute and complex product streams, requiring concentration and purification for the recovery of the desired product (Buque *et al.*, 2002:656).

Therefore, in this chapter the effect of formulation conditions and initial substrate concentration on the partitioning of both epoxide and diol inside the water phase as well as the bead phase is presented. The effect of initial substrate concentration on free and immobilised cell activity was also investigated.

2. Experimental

2.1 Materials

Rhodosporidium toruloides (UOFS Y-0471) was obtained from the yeast culture collection of the department of Microbiology and Biochemistry of the University of the Free State, Bloemfontein, South Africa.

Alginic acid salt obtained from brown algae, as well as a medium-viscosity carboxymethylcellulose (CMC) sodium salt, was purchased from Fluka BioChemika, South Africa. Pure granular anhydrous calcium chloride ($CaCl_2$) was obtained from Merck South Africa. Tris(hydroxymethyl)aminomethane for the preparation of tris buffer and potassium phosphate (K_2HPO_4) were purchased from Saarchem, South Africa. 1,2-Epoxyoctane (epoxide) and 1,2-octanediol (diol) were both obtained from Aldrich, South Africa. Double distilled deionised water was used. All solutions were prepared with a 20 mM tris buffer (pH 7,5) solution.

2.2 Methods

All analyses, the preparation of the frozen yeast cells and the calcium alginate beads were done as described in Chapter 3.

2.2.1 Partitioning of epoxide and diol into alginate beads

A 20 % cell/alginate suspension was prepared using a 0,5 % m/v alginate/tris buffer solution. The cells were thermally deactivated before immobilisation. Two concentrations of $CaCl_2$ (0,2 M and 0,4 M) were used as curing medium. Initial concentrations of 20,0 and 100,0 mM epoxide and 30 mM diol were used in the determination of the partitioning of epoxide and diol into the alginate beads. 2g Beads were placed in a closed glass reaction vessel and 15,0 ml tris buffer/epoxide or tris buffer/diol mixture added. The mixture was constantly shaken at 30 °C for 16 hours and samples were taken at regular intervals and analysed for the residual epoxide and diol respectively.

2.2.2 Effect of immobilisation and substrate concentration on enzyme activity

Beads were prepared using a 20,0 % (m/m) cell/alginate suspension prepared with a 0,5 % m/v alginate/ tris buffer solution cured in 0,2 M $CaCl_2$-solution. All the alginate beads attained from 10 ml alginate/cell suspension (± 2,5 g beads), were placed in a closed glass reaction vessel containing 10 ml tris buffer. The initial epoxide concentration was 20, 50, 75 and 100 mM 1,2-epoxyoctane respectively in the liquid volume (10 ml). The reaction mixtures were incubated at

30 °C while continuously being shaken in a water bath. At different time intervals samples from each concentration experiment were taken from the water phase and analysed for epoxide and diol.

For the free cell reactions frozen yeast cells were thawed and resuspended in tris-buffer (20 % cell suspension) and 500 µl dispensed in 1,5 ml micro-centrifuge tubes. Epoxide was added to give a final concentration of 20, 50, 75 or 100 mM. The reaction mixtures were incubated at 30 °C and continuously shaken. At different time intervals one micro-centrifuge tube of each substrate concentration was removed and analysed for epoxide and diol.

2.2.3 Epoxide and diol distribution the water and bead phase

Beads were prepared as described in 2.2.3. All the alginate beads obtained from a 2 ml alginate/cell suspension (± 0,5 g beads), were placed in a closed glass reaction vessel with 2 ml tris buffer. The initial epoxide concentration was 20, 50, 75 and 100 mM 1,2-epoxyoctane respectively in the liquid volume (2 ml). The reaction mixtures were incubated at 30 °C while continuously being shaken in a water bath. At different times (established from data attained in the previous experiment), samples were drawn from the liquid phase, the beads washed with 2 ml tris buffer for one minute and the beads dissolved by adding 0,2 g K_2HPO_4 and 500 µl ethyl acetate. The epoxide and diol were analysed in all three phases (water, wash and bead phase), for each initial epoxide concentration.

3. Results and discussion

3.1 Partitioning of epoxide and diol into alginate beads

To determine the partitioning of epoxide and diol into alginate beads, as well as the effect of $CaCl_2$ concentration on the partitioning, thermally deactivated cells were immobilised in alginate beads. The epoxide and diol in the liquid phase were determined.

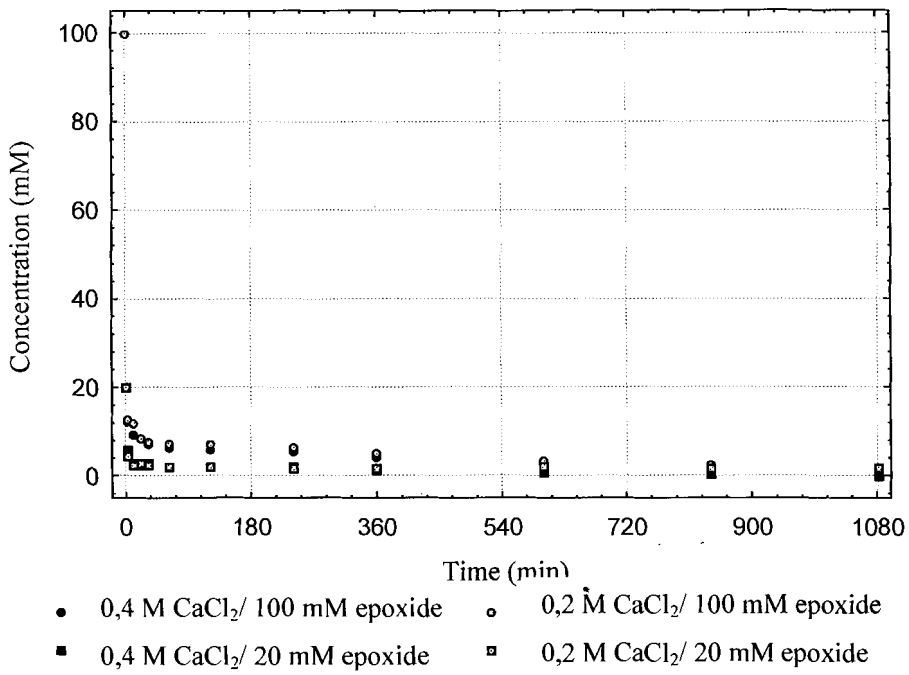

Figure 4.1 The effect of initial substrate concentration on epoxide diffusion into the beads

As can be seen in Figure 4.1, the initial extracted concentration in the liquid phase is much lower than the added epoxide concentrations, 100 mM and 20 mM respectively. This could either be because of the very low substrate solubility (± 6 mM) causing the epoxide to form a layer on top of the liquid phase (seeing that samples are taken from the same reaction vessel, it might be that the total concentration is not extracted), or due to a very fast rate of initial substrate diffusion into the bead. The first option is very unlikely since even at a 100 mM initial epoxide concentration the final concentration is below 4 mM i.e., below the saturation concentration of epoxide (± 6 mM), suggesting that no top layer of epoxide is still present. Furthermore, the initial epoxide concentration does not have a large effect on the residual epoxide concentration in the water phase, implying that the alginate beads are not yet saturated with epoxide, and that alginate has a very high epoxide saturation concentration.

When taking a closer look at the effect of $CaCl_2$ concentration used for curing, it can be seen that the concentration of the cation solution used as curing medium has an effect on the amount of epoxide absorbed by the beads.

An average of 97 % of the initial extracted epoxide was absorbed after 600 minutes (10 hours) where a 0.4 M $CaCl_2$ solution was implemented in comparison with an average of 92 % with the 0.2 M $CaCl_2$ solution. It therefore seems that higher $CaCl_2$ concentration results in higher epoxide absorption.

As was suspected from data produced in Chapter 3, it can be seen that the diffusion of diol into the beads is much less than that of epoxide. After 120 minutes only 18 % of the diol diffused into the beads where a 0.4 M $CaCl_2$ solution was implemented and 20 % with a 0.2 M $CaCl_2$ solution (Figure 4.2). The $CaCl_2$ solution concentration implemented in bead preparation does not seem to have a prominent effect on the amount of diol absorbed into the beads.

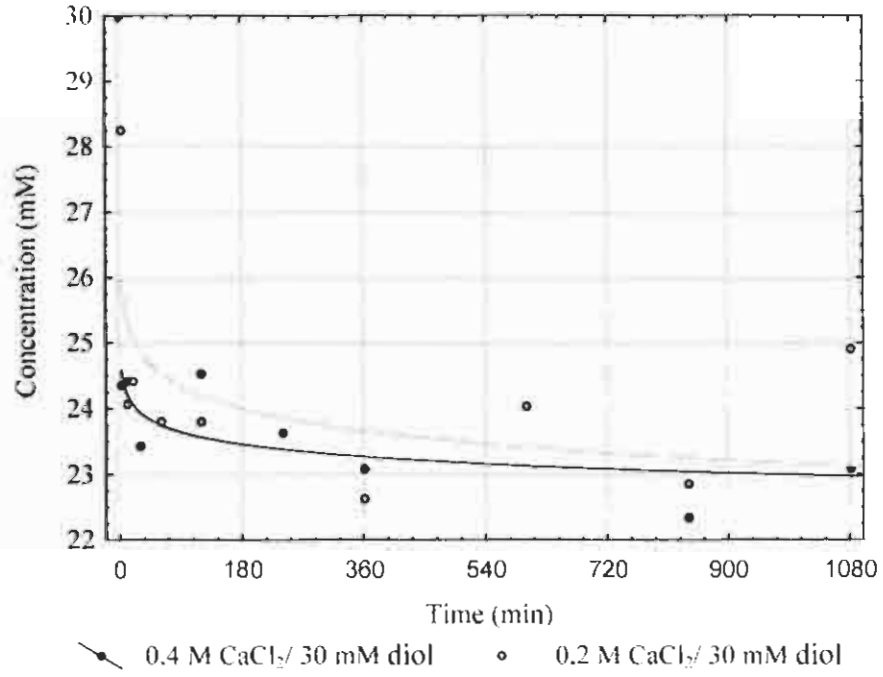

Figure 4.2 Partitioning of diol into calcium alginate beads

The preferential diffusion of epoxide into the alginate beads is contrary to the initial expectations considering the hydrophilic character of alginates (Tanaka & Kawamoto, 1994:508). If taking into consideration that the epoxide of a long carbon chain is more hydrophobic than the diol it

was assumed that the diffusion of epoxide and diol into a hydrophilic structure such as alginate would rather be preferential for the more hydrophilic diol.

Other studies have shown that the aqueous solubility of some hydrophobic compounds can be increased when using hydrophilic compounds such as cyclodextrin. Cyclodextrin is a 6-membered chiral ring that forms a hydrophobic cavity in aqueous surroundings (Stevenson & Williams, 1988:3). However, when studying the three-dimensional structure of a left-handed helix of calcium poly-α-L-glutoromate (Figure 4.3) it seems unlikely that there could exist any primarily hydrophobic region that would support this hypothesis (Chaplin, 2002:1).

However, in spite of the absence of any specific visual hydrophobic regions it can be assumed that the alginate in its totality is more hydrophobic that the aqueous phase which might help to explain the preferential absorption of the epoxide.

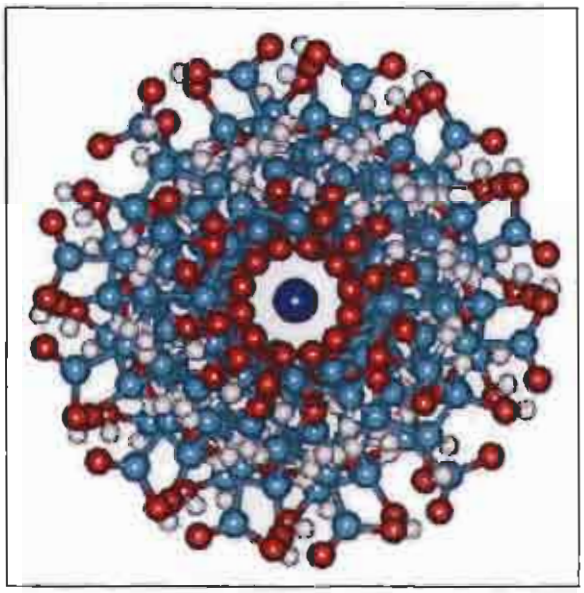

Figure 4.3 Calcium poly-α-L-glutoromate left handed helix (Chaplin, 2002:1).

3.2 The effect of immobilisation and substrate concentration on enzyme activity

To determine the effect of immobilisation and the increase of substrate (epoxide) concentration on enzyme activity as well as on reaction rate, time coarse reactions were done with different concentrations of epoxide (20, 50, 75 and 100 mM) with immobilised as well as free cells, and the epoxide and diol were determined at certain time intervals.

Contrary to previous research done on the effect of an increased substrate concentration on purified enzymes (Botes, 1999:177) an increased specificity for the production of the (R)-diol was found with free cells and not an increase in the specific utilisation of the (R)-epoxide. This was not noticed for the immobilised cells (Figure 4.4).

It can be seen that the epoxide concentration extracted after 6 hours from the liquid phase of immobilised cells is more or less the same independent of the initial epoxide concentration. The low epoxide concentration found for all the immobilised cell reactions, confirm the absorption of epoxide by alginate beads, underlining the results in the previous section.

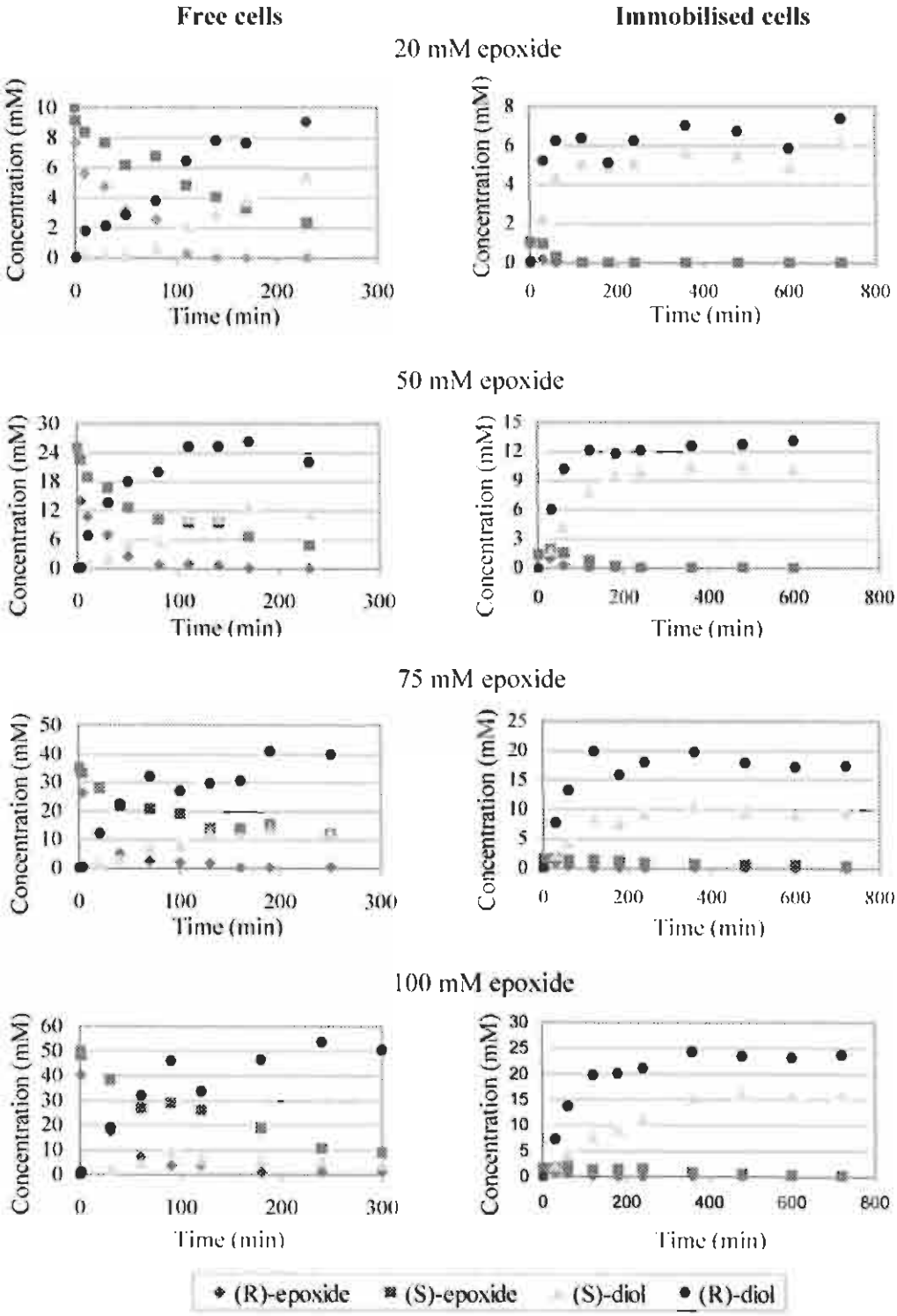

Figure 4.4 The effect of concentration and immobilisation on enzyme activity

Even though not all of the diol could be recovered from the immobilised cell reactions, the relative recovery is still much higher than that of the recovered epoxide and hence the diol values were used to determine the initial reaction rates (V) (Figure 4.5).

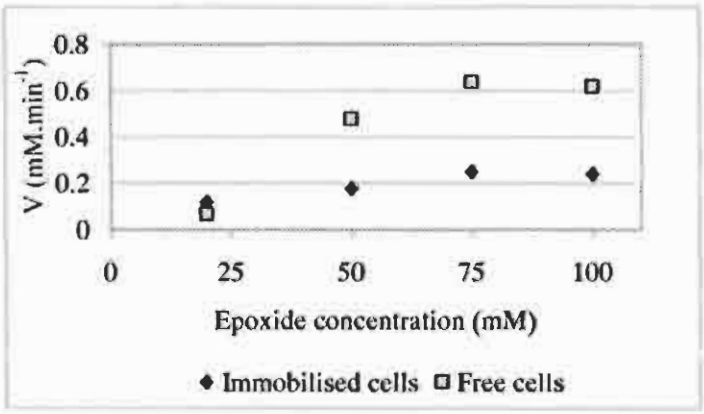

Figure 4.5 Reaction rates and corresponding concentrations for the hydrolysis of 1,2-epoxyoctane by free and immobilised cells

For both the immobilised cells as well as the free cells an increase in the initial substrate concentration lead to an increase in the initial reaction rate up to an initial epoxide concentration of 75 mM after which the initial reaction rate was independent of the substrate concentration. Maximum reaction rate (V_m) was in both cases observed with a substrate concentration (S_m) of 75 mM.

At the S_m concentration it can be assumed that all the active enzyme-binding sites are saturated, therefore additional epoxide molecules would not render higher reaction rates, or it might be attributed to the low solubility of the epoxide (± 9 mM) in aqueous solutions. Due to the very high saturation concentration of the alginate for epoxide (which has been shown in section 3.1), it can be assumed that the unaltered V at substrate concentrations higher than 75 mM is due to the saturation of the enzyme-binding sites.

The lower reaction rate observed for immobilised cells is probably due to the fact that some of the diol is trapped inside the bead (see section 3.1) and thus cannot be extracted from the water phase making it quite difficult to establish the effect of immobilisation on the reaction rates. To establish the activity of the immobilised biocatalyst with greater accuracy, it is crucial to determine the concentration of both the epoxide as well as the diol in the water phase and inside the beads.

3.3 Distribution of epoxide and diol in the water and bead phases

To determine the distribution of epoxide and diol at different substrate concentrations a liquid volume (2 ml) was added to the same amount of beads (0,5 g). The reaction time was chosen according to the results attained in the time coarse reactions (section 3.2), (where an *ee* of 1 for the epoxide was attained in the water phase). The epoxide and diol was extracted from the water phase as well as from the bead phase after being washed once. The experiment was done in duplicate. The average standard deviations were 13 % for the epoxide values, 18 % for the diol values, and 6 % for the % ee_p and % ee_s. The results are presented in Table 4.1.

Only between 34 % and 46 % of the total epoxide and diol of the respective concentration experiments was recovered, clearly showing that the total concentration of epoxide and diol could not be recovered. Taking into account that all calibrations were done for the extraction of epoxide and diol from the water phase, it can be concluded that all the epoxide and diol was not extracted from the bead phase.

It has to be kept in mind that most of the recovery is contributed to the diol while only small fractions of the epoxide were recovered. This again supports the results suggesting that the beads preferentially absorb the epoxide. Interesting from the results, however, is that in spite of dissolving the beads in K_2HPO_4 and ethyl acetate the epoxide (and probably a smaller fraction of the diol) is still not completely released from the alginate.

In spite of the quantification difficulties auother observation can be made from the % *ee*. For all the concentrations a higher % ee_s was exhibited from the data obtained from the extraction from the bead phase (between 26 % and 43 % for the respective concentration experiments), in comparison to that extracted from the water and the wash phases, with no real difference between the % ee_p.

The (R)-epoxide and the (S)-epoxide both exhibit the same chemical and physical properties in an achiral environment (as do the (R)-diol and the (S)-diol). Thus, the molecular configuration could be the only parameter affecting the diffusion of the epoxide and diol. It can only be concluded that the alginate gel is chirally selective, i.e. it not only retains the epoxide better than the diol, it furthermore has a preferential selectivity for the (R)-epoxide enantiomer, further increasing the % ee_s obtained for the catalyst, thus improving the process efficiency further.

Initial Substrate *	Reaction time (h)	Bead phase				Water phase				Wash phase				Total % recoverd
		Epoxide (residual)		Diol (product)		Epoxide (residual)		Diol (product)		Epoxide (residual)		Diol (product)		
		Total *	Ee (%)	Total *	Ee (%)	Total *	Ee (%)	Total *	Ee (%)	Total *	Ee (%)	Total *	Ee (%)	
20	1	0,24	100	0,30	56	1,22	70	4,68	61	0,13	100	0,30	55	34
50	2	0,42	75	1,26	66	2,17	44	11,13	49	0,40	65	0,36	100	31
75	3	0,28	89	1,80	63	3,93	46	23,63	58	0,73	62	2,43	77	44
100	4	1,66	81	6,52	58	2,96	55	30,04	55	0,65	82	3,97	73	46

* Concentration (mM)

Table 4.1 Epoxide and diol extracted respectively from the bead-, water- and wash phases

The chiral selectivity of the alginate gel is quite understandable considering that the alginate itself is a chiral molecule and hence the alginate would have a preferential absorption for one of the enantiomers.

The results obtained from washing the beads after they were removed from the water phase, clearly shows that even after only one minute washing almost as much diol as was extracted out of the beads diffuses from the bead. The results again also show that the epoxide diffused from the bead has a higher *% ee*, i.e. the bead is preferential for the (R)-epoxide

4. Conclusion

The preferential diffusion of epoxide into the bead was clearly shown in all the data presented. Furthermore the effect of the $CaCl_2$ solution concentration used as curing media was also shown. The fact that the $CaCl_2$ concentration only had an effect on epoxide and not on diol diffusion could make it possible to regulate/influence the amount of epoxide that does diffuse into the bead by modifying the preparation method ($CaCl_2$ and alginate solution concentration combination).

The preferential binding of the (R)-epoxide to the alginate is another advantage. As was demonstrated in Table 4.1, effective and repeated washing could probably be used to remove most of the diol from the bead. Simultaneously, however, a more effective extraction method has to be found to remove more of the remaining epoxide from the beads and solvents such as dichloromethane can be tried.

Removing the beads from the liquid, extracting the diol from the water phase and extracting the optically pure epoxide from the bead phase could easily separate the substrate and product. These results clearly show the possibility to develop a system where the product is formed, purified and concentrated from an initial volume of 2 ml to a final volume of only 500 µl.

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Chapter 5

Concluding remarks

1. Introduction

Selectivity is an essential requirement in medicinal organic chemistry (Rasor & Voss, 2001:145). Enzymes catalyse chemical reactions with great specificity (Van Beilen & Li, 2002:338). In all chemical processes, the separation of reactants from products and the recovery of the catalyst from the reaction mixture are an important step, which significantly add to the cost of the process (Prazares & Cabral, 1994:738). The immobilisation of biocatalysts inside an inert support leads to a much more stable enzyme. The method of immobilisation and immobilisation matrix are generally chosen to have no interaction with any of the entities involved in the reaction so that the substrate diffuses easily into and the product out of the support where it can be collected in the liquid phase (Park & Chaug, 2000: 304). Membraues in a chiral bioreactor are on the other hand chosen to either separate phases or to act as a chiral selector by adsorption or by liquid-liquid partitioning systems (Pirkle & Bowen, 1994:773).

2. Effect of formulation parameters on alginate bead characteristics

Botes *et al.* (1999:3327) previously demonstrated the kinetic resolution of unbranched 1-epoxyalkanes using resting cells of a number of yeasts. Due to the growing importance of epoxides as chiral building blocks, cells of *Rhodosporidium toruloides* were immobilised in calcium alginate beads for the kinetic resolution of 1,2-epoxyoctane. The effect of formulation parameters such as alginate and $CaCl_2$ concentration on bead properties as well as on enzyme activity, and the interaction between physical and chemical properties of the immobilisation matrix were investigated.

The combination of alginate and calcium chloride had a diverse effect on enzyme activity. Although there was no immense difference in initial activity between the different combinations of $CaCl_2$ and alginate concentrations implemented for bead preparation, the difference in stability was prominent. A combination of 0,5 % (m/v) alginate and 0,2 M $CaCl_2$ rendered the best results, although not in initial activity, but in terms of stability.

Beads with a smaller diameter were prepared when higher alginate concentrations were used in bead preparation, but with lower stability due to the higher Ca^{2+} concentration in close contact with the cells.

Although immobilisation did render lower initial activity in comparison to free cells, a major increase in stability was eminent for immobilised cells (98 % activity loss after 12 hours for free cells, in comparison to less than 9 % after 24 hours for immobilised cells).

It was demonstrated that all factors influencing free cell and enzyme activity (including initial substrate concentration and formulation parameters of the immobilised system) had to be taken into consideration before an immobilisation method is chosen.

Contrary to expectations the epoxide seemed to be preferentially absorbed by the alginate beads, which could not be explained yet, and no conclusion could be drawn from the results obtained from the epoxide and diol concentrations in the water phase in terms of absolute enzyme activity or selectivity (Chapter 3).

3. Distribution of epoxide and diol in calcium alginate beads

The distribution of epoxide and diol inside the bead phase and the water phase was investigated under different conditions and under the influence of different initial substrate concentrations.

The epoxide preferentially distributed/diffused into the bead phase, and the diol into the water phase. Higher $CaCl_2$ concentrations promoted the diffusion of epoxide into the beads with no effect on diol. Higher concentrations of (R)-epoxide could be extracted from the bead phase in comparison to the (S)-enantiomer. This clearly demonstrated selectivity of the alginate for the (R)-epoxide

An increase in initial substrate concentration had an increasing effect on the initial reaction rate (V) for both the immobilised cells as well as the free cells up to a critical concentration of 75mM (Chapter 4).

4. Implications

The higher affinity of alginate for epoxide and the preferential diffusion of the diol out of the alginate beads into the water phase lead to a natural separation of the residual substrate and the formed product. The added preferential interaction between the (R)-epoxide and the alginate lead to an increase in the % ee_s and a subsequent increase in the reaction effectivity. Thus, not only did immobilisation lead to increased biocatalyst stability, but alginate beads also played the role of final chiral separator and removed some of the unreacted (R)-epoxide that was still be present in the reaction mixture.

If more of the epoxide could be extracted from the bead phase, it could lead to the development a system where the product is biocatalyticly synthesised by a stabilised enzyme leading to a one-

step process, where the product is produced, purified and concentrated to a final volume of only a quarter of that, that was initially started with.

5. Future research

Although the future prospects for the utilisation of this system for the production of enantiomerically pure epoxides are eminent, there are a few shortcomings that will have to be addressed:

- The type of interaction (physical or chemical), that exists between the epoxide and the alginate has to be investigated to determine if the epoxide that is absorbed into the bead is recoverable.

- The investigation of several extraction methods (i.e. supercritical CO_2 in combination with various organic solvents), to find an extraction method that removes more of the epoxide from the alginate.

- The prospective use of this system with other substrates and biocatalysts.

6. References

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