Bioreaction engineering for the kinetic resolution of racemic epoxides by epoxide hydrolase
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General Introduction
The use of biocatalysts for the production of fine chemical and pharmaceutical intermediates is becoming increasingly common because of their ability to catalyse reactions with high stereo-, regio-, and chemo-selectivity. Biotransformation steps can either complement chemical steps or exist as alternative synthesis routes for a given compound, often with the advantage of reducing the number of reaction steps required (Cheetham, 1998). In some cases biocatalysis has provided new routes for the synthesis of high value compounds that cannot be produced by conventional chemo-catalysis. Synthesis of products with multiple chiral centres is one key area where the selectivity of biocatalysts can be exploited.

Biotransformations are generally recognised as being environmentally friendly compared to chemical processes since water is often the solvent of choice and reactions are catalysed at ambient temperatures and pressures. The high specificity of the reactions often also means that fewer side products are produced. Nevertheless, at the industrial scale, biotransformations must be economically competitive with chemical processes in terms of productivity and cost. It is precisely this lack of economic competitiveness which in some cases has led to the slow acceptance of bioprocesses as suitable alternatives to chemical processes. To be economically competitive a biotransformation must be operated at high volumetric productivity, which is defined as:

\[
\text{volumetric productivity} = \frac{\text{amount of product produced}}{(\text{reaction time}) \cdot (\text{reaction volume})}
\]  

Clearly, high productivity is achieved if the product is present in the reaction volume at a high concentration and if the time taken for reaction is low. It is also desirable to maximise the yield of the reaction to reduce the formation of waste by-products, especially when reactant costs and the cost of waste disposal are high. In addition, biocatalyst productivity (amount of product formed per amount of catalyst consumed) should be as high as possible since biocatalyst costs can be relatively important (Tischer and Kasche, 1999; Tramper, 1996). Good biocatalyst productivity requires high biocatalyst stability and effective recycle of the biocatalyst. Bioprocess development should be aimed at maximising these factors while satisfying key process requirements such as product (enantio)purity. Good integration between the biocatalytic step and downstream processing is also important in the overall optimisation of a bioprocess. In this respect, the use of high product concentrations is desirable to ease product recovery and purification. Reaction engineering for the production of optically pure epoxides by biocatalytic kinetic resolution, aimed at achieving high product and biocatalyst productivity, is the subject of this thesis.
Synthesis of optically pure epoxides

Optically pure drugs are increasingly being used since they are more target specific and have fewer side effects than racemic mixtures (Kloosterman, 1988). This is because different enantiomers of a compound can have different pharmacokinetic properties. The sale of single isomer drugs reached 32% of the total drug sales world wide in 2000 and further increases in market share are forecast (Stinson, 2001). This increase has resulted due to the requirement by the regulatory agencies for testing both enantiomers of a racemate in pharmacological studies during the drug approval process (Persidis, 1997), and also reflects the fact that drug companies are using chirality to extend the patent lives of block buste drugs by patenting single isomer versions of the racemates (Stinson, 2001).

Epoxides are extremely useful chemical and pharmaceutical intermediates since the epoxide functional group can easily undergo ring opening, accepting a wide variety of nucleophiles (De Vries and Janssen, 2003). For example, epoxides are attractive intermediates in the production of β-blockers, insect pheromone (Kloosterman, 1988) and the herbicide (S)-indofan (Tanaka et al., 2002). The need to introduce chirality in the earliest steps of a synthetic route has made the production of chirally pure epoxides all the more important.

Chemocatalytic routes for the production of optically pure epoxides include epoxidation of allylic alcohols and conjugated alkenes, reactions developed respectively by Sharpless/Katsuki and Jacobsen (Jacobsen, 2000), which make use of transition metal complexes as catalysts (Archer, 1997). High enantiomeric purity and reasonable yields can be achieved with these methods, however, they can be used only for specific substrate structures and require the undesirable use of heavy metals. New metal containing salen complexes which catalyse the kinetic resolution of terminal epoxides to high enantiopurity have also been developed (Furrow et al., 1998; Tokunaga et al., 1997; Yoon and Jacobsen, 2003). The most promising biocatalytic routes for the production of enantiopure epoxides include stereospecific epoxidation of alkenes by monooxygenases (Archer, 1997; Schmid et al., 2001), and the resolution of racemic epoxides by epoxide hydrolases (Steinreiber, 2001). More recently, the scope of producing optically pure epoxides by enantioselective kinetic resolution of racemic halohydrins by direct ring closure mediated by halohydrin dehalogenases has been investigated (Lutje Spelberg et al., 2002b).

Asymmetric synthesis of epoxides by monooxygenases is attractive because the maximum theoretical yield of enantiopure epoxide is 100%. Monooxygenases are cofactor-dependent enzymes, and therefore whole cells are usually employed for easy cofactor regeneration (Duetz et al., 2001). Since oxygenases often have low specific activities, research efforts on this biosynthetic route have concentrated on the formation of recombinant E. coli
Chapter 1

strains which express monoxygenases at high levels in order to achieve high productivity in preparative applications (Schmid, 2001).

Optically pure epoxides can also be obtained by kinetic resolution, whereby whole cells or enzymes degrade a racemic epoxide mixture stereoselectively. An inherent disadvantage of this method is that the maximum yield is limited to 50% since the unreacted enantiomer of the racemic mixture is the desired product. However, this route remains attractive since racemic mixtures are relatively cheap and since optically pure diols are also useful intermediates. Attempts to increase the yield of optically pure epoxide have made use of epoxide hydrolases with complementary enantiospecificities so that optically pure diols are obtained in high yields (92%) (Pedragosa-Moreau et al., 1993). In another example, acid catalysis was used to convert the remaining epoxide enantiomer to give enantiomerically pure diol in high yield (>90%) (Pedragosa-Moreau, 1997). To obtain enantiopure epoxide, chemical ring closure of the formed enantiomerically pure diol with retention of enantioconfiguration would be a necessary extra step. In the kinetic resolution of 4-isobutyl-α-methyl styrene oxide, Cleij et al. (1999) obtained enantiopure epoxide in high yield by recycling the produced unwanted diol to racemic epoxide by treatment with HBr/AcOH, followed by cyclization of the formed bromohydrin under basic conditions.

**Epoxide hydrolase from *A. radiobacter* AD1**

Epoxide hydrolases catalyse the addition of a water molecule to an epoxide (Fig. 1.1). Epoxide hydrolases are cofactor independent enzymes and are ubiquitous in nature, having been found in mammalian cells, bacteria, yeast, fungi, plants and insects (Archer, 1997). In mammalian cells, microsomal epoxide hydrolase activity is highest in organs such as the liver, kidney and lungs and the enzymes have the important function of catalysing the conversion of a broad array of xenobiotic substrates to more polar metabolites (Seidegard and De Pierre, 1983). This function is particularly important since the high reactivity of epoxides with nucleophiles makes them potent mutagens and carcinogens by modification of DNA by alkylation (Mlejnek and Kolman, 1999). Similarly epoxide hydrolases in bacteria can be part of a degradation route for xenobiotic compounds. Again, the toxicity of epoxides to cells in the degradation of chlorinated compounds has been attributed to the high reactivity of epoxides towards cellular nucleophiles such as proteins and nucleic acids (Oldenhuis et al., 1991).

The work described in this thesis concerns the use of an epoxide hydrolase of bacterial origin, originally isolated from the organism *Agrobacterium radiobacter* AD1 (van den Wijngaard et al., 1989) which can grow on and thus degrade epichlorohydrin to glycerol. The degradation is mediated by a haloalcohol dehalogenase and an epoxide hydrolase. The gene coding for the epoxide hydrolase has been cloned and brought to
overexpression in *E. coli*. This opened the possibility for its exploitation for preparative biocatalysis since typical enzyme production levels increased from 1-2 mg L\(^{-1}\) culture for the original organism to 200 mg L\(^{-1}\) culture (Rink et al., 1997).

![Figure 1.1. Reaction catalysed by epoxide hydrolase. The chiral centre is indicated with an asterix.](image)

This enzyme has a molecular mass of 34 kDa and is monomeric in solution. The X-ray structure has been solved and shows that this enzyme belongs to the \(\alpha/\beta\) hydrolase fold family of enzymes (Nardini et al., 1999). The catalytic triad residues are Asp107, His275, Asp246 and are located in a cavity between the core and cap domains of the enzyme molecule. The catalytic mechanism comprises two steps, the formation of an alkyl-enzyme intermediate by nucleophilic attack of a carboxylate oxygen of an aspartate residue on the least hindered carbon atom of the epoxide, followed by hydrolysis of the intermediate by a water molecule which is activated by deprotonation by a general base (His275) (Rink and Janssen, 1998) (Fig. 1.2). Two tyrosine residues, Tyr152 and Tyr215, which are found on the \(\alpha\)-helical cap domain, point into the active site and serve as proton donors in the opening of the epoxide ring. Both tyrosine residues help stabilise the alkyl-enzyme intermediate by hydrogen bonding of the epoxide oxygen (Rink et al., 2000).

![Figure 1.2. Reaction mechanism of the epoxide hydrolase from *A. radiobacter* AD1. (A) alkylation reaction and (B) hydrolysis of covalent intermediate.](image)
The wild-type enzyme hydrolyses a broad range of terminal epoxides. The hydrolysis of (substituted) styrene epoxides such as styrene oxide and \( p \)-nitrostyrene oxide, proceeds with moderate to high enantioselectivity (Lutje Spelberg et al., 1998, 2002a) yielding the optically pure (\( S \))-enantiomers of the epoxides. The hydrolysis proceeds with a high degree of regioselectivity favouring attack on the less sterically hindered carbon atom of the oxirane ring with retention of configuration. Mutation of one of the two active site tyrosines to phenylalanine results in active mutants which differ in conversion kinetics compared to the wild-type enzyme. The mutation affects primarily substrate binding and the alkylation half reaction (Rink et al., 1999). Overall, the single tyrosine mutants show a higher enantioselectivity to various epoxides than the wild-type enzyme and are therefore valuable for industrial biocatalytic application. The use of the Y215F mutant epoxide hydrolase is evaluated in Chapter 5.

Both the wild-type and active site mutants of this enzyme have a broad pH optimum between pH 6 and 10 (Rink et al., 2000) and the active site mutants were found to be more stable than the wild-type enzyme at higher pH. Further studies of operational stability of the enzymes are described in Chapters 3 and 4.

**Characterisation of kinetic resolution reactions**

The enantiopurity of a substrate or product is usually described in terms of enantiomeric excess (e.e.) which is calculated by,

\[
\text{e.e.} = \frac{([R]-[S])}{([R]+[S])},
\]

where \([S]\) and \([R]\) are the concentrations of the enantiomers. Another parameter used to quantify the selectivity of a kinetic resolution is the enantiomeric ratio, \( E \) (Chen et al., 1982). The enantiomeric ratio is defined as the ratio between the specificity constants for the conversion of the two enantiomers \( \left( \frac{k_{\text{cat}}/K_m}{R}/\frac{k_{\text{cat}}/K_m}{S} \right) \) and is an intrinsic property of the enzyme. The \( E \) value of a conversion can be calculated by knowing the substrate enantiomeric excess (\( \text{ee}_S \)) achieved for a certain conversion (\( X \)) using the equation:

\[
E = \frac{\ln \left[ (1-X)(1-\text{ee}_S) \right]}{\ln \left[ (1-X)(1+\text{ee}_S) \right]}
\]

The relationship between e.e. and conversion is shown in Figure 1.3 for different values of \( E \). The figure illustrates one of the advantages of kinetic resolution, namely that the desired e.e. can be achieved by setting the necessary conversion, even though this occurs at the expense of the yield of remaining enantiopure substrate. An enantiomeric ratio greater than 20 is a minimum prerequisite for industrial application (Sheldon, 1996).
Figure 1.3. Change in substrate enantiomeric excess with conversion during kinetic resolution shown for different values of the enantiomeric ratio, E. The figure shows that the conversion required to achieve high enantiomeric excess of substrate increases for decreasing values of E. For industrial application, an E value greater than 20 is required (Sheldon, 1996).

In a few cases, resolution reactions follow sequential conversion kinetics, whereby one enantiomer is converted before another. This type of kinetics has been reported for the conversion of tert-butyloxirane by microsomal epoxide hydrolase (Watabe et al., 1981; Wistuba and Schurig, 1992). An important example of this type of conversion kinetics is the hydrolysis of styrene oxide by the wild-type epoxide hydrolase (Rink and Janssen, 1998) (Fig. 1.4, Table 1.1). This epoxide is used as a model substrate for much of the work described in this thesis. Sequential conversion is due to the much higher affinity of the (R)-enantiomer for the active site, so that the conversion of the (R)-enantiomer inhibits the conversion of the (S)-enantiomer. For this substrate the (R)-enantiomer is first converted with a $k_{\text{cat}}$ of 3.8 s$^{-1}$ and then the (S)-enantiomer is converted with a $k_{\text{cat}}$ of 10.5 s$^{-1}$ so that once the (R)-enantiomer is depleted the (S)-enantiomer is converted at a much faster rate. The implications of this type of complex kinetics for the operation of large scale resolution processes are that the end-point of the reaction has to be well controlled to maximise the yield of remaining pure substrate enantiomer. If the reaction is stopped before the ideal end-point then the enantiomeric excess may be too low, if the optimum end point is passed then the yield of pure enantiomer decreases quickly. For more classical conversion kinetics (Fig. 1.4), for example in the conversion of styrene oxide by the Y215F epoxide hydrolase mutant, these
considerations are still valid but less critical since the enantiomer which is hydrolysed last is usually converted at a lower rate.

**Figure 1.4.** Simulated conversion curves for the kinetic resolution of styrene oxide by wild-type (□ (R)-enantiomer, ○ (S)-enantiomer) and Y215F mutant (■ (R)-enantiomer, ● (S)-enantiomer) epoxide hydrolase. The conversion profile for the wild-type enzyme is typical for sequential kinetics where the conversion of the remaining enantiomer follows the conversion of the first enantiomer. This effect is less dramatic for the conversion by the Y215F mutant enzyme since the second enantiomer is only slowly converted. Concentration profiles were obtained by equations describing competitive Michaelis-Menten kinetics of the type, 

\[
\frac{d[S]}{dt} = -\frac{k_{cat}^S [E][S]}{[S] + \left(\frac{K_{M}^R}{K_{M}^S} + 1\right)K_{M}^S} - k_c[S].
\]

Kinetic constants used are given in Table 1.1. Simulations were carried out using the software package Scientist™ (MicroMath Inc., Salt Lake City, UT).

**Table 1.1.** Steady state kinetic parameters of wild-type and mutant epoxide hydrolase for the enantiomers of styrene oxide (Rink et al., 2000)

<table>
<thead>
<tr>
<th></th>
<th>(R)-styrene oxide</th>
<th>(S)-styrene oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>(K_m) (mM)</td>
</tr>
<tr>
<td>wild-type</td>
<td>3.8</td>
<td>0.0005</td>
</tr>
<tr>
<td>Y215F</td>
<td>2.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Bioprocess Design

Bioprocess design involves identifying key characteristics of the reactants, products and the biocatalyst which impose constraints on the bioprocess performance (Lilly, 1997). The productivity of kinetic resolutions of racemic epoxides is constrained by several factors. Many epoxides, such as epoxyoctane, p-nitrostyrene oxide and styrene oxide have a solubility of less than 5 g/L at 30 °C, limiting the productivity of reactions which can be carried out in aqueous solutions. The use of organic solvents to reduce this limitation is described below. In addition, epoxides tend to be unstable in water and undergo spontaneous chemical hydrolysis to their diols. A typical value for the first order rate constant for spontaneous hydrolysis is $1 \times 10^{-5}$ s$^{-1}$. Since chemical hydrolysis occurs in a non-enantioselective manner, this side reaction results in a decrease in the yield of enantiomerically pure substrate and also a decrease in the enantiopurity of the formed diol. The calculated E-value of a resolution based on e.e. vs. conversion data (Fig. 1.3) will be lower than the intrinsic E-value for the enzyme if such a non-selective side reactions occurs. To minimise the loss of yield from spontaneous chemical hydrolysis, the rate of the enzymatic reaction has to be high relative to that of chemical hydrolysis (Chapter 2) and since chemical epoxide hydrolysis is favoured at extremes of pH, the operating pH should be selected appropriately. Diols, on the other hand, are stable in water and generally have a high aqueous solubility at 30 °C. In several different studies, however, inhibition of epoxide hydrolase activity by diols has been proposed as one of the causes for reduction in conversion rates during epoxide kinetic resolution reactions (Choi et al., 2000; Genzel et al., 2001a; Tang et al., 2001). None of these studies have explored the type and reversibility of this inhibition, and this is investigated in Chapter 4.

Multiphase systems

Water miscible organic solvents such as DMSO, acetone and ethanol are often added to reaction mixtures to increase the aqueous solubility of poorly water soluble reactants. At low solvent concentrations this strategy can be effective without adversely affecting enzyme activity and stability. At higher cosolvent concentrations, however, biocatalyst inhibition or inactivation may become prohibitive, thus limiting the maximum cosolvent concentration which may be used. A disadvantage of this approach is that the use of miscible cosolvents does not automatically simplify downstream recovery of the biocatalyst or product separation.

Alternatively, immiscible apolar organic solvents can be used to create a multiphase system where the organic solvent acts as a reservoir for the poorly water soluble substrate, allowing much higher substrate concentrations to be present in the reaction volume. In these biphasic systems, the substrate is transferred to the aqueous phase and is converted by
the enzyme contained therein. Multiphase systems can be used advantageously to facilitate downstream separation of substrates and products depending on partitioning of these species between the phases. Appropriate selection of the organic phase must take into account these partitioning effects so that inhibitory products can be removed and substrates can be fed at sub-inhibitory levels. Optimal partitioning can also minimise unwanted side reactions. Minimising unwanted epoxide chemical hydrolysis by keeping the concentration of epoxides low in the aqueous phase is a good example of this. Optimisation of solvent physical properties is also important with respect to downstream processing since separation of emulsified layers can be a problem on an industrial scale (Kloosterman, 1988). The advantages afforded by multiphase systems listed above, prompted us to investigate the use of a two-liquid-phase system for carrying out the kinetic resolution of epoxides at high substrate concentrations (Chapter 2).

Ideally, the use of organic solvents should be avoided since safety issues concerning solvent flammability and toxicity arise for bioprocess operation and formulation of the final product. This can be achieved if the substrate itself constitutes the separate phase. Both solid and liquid substrates have been used in this context (Straathof et al., 2002). Recently ionic liquids have emerged as potential replacements of organic solvents since they are non-flammable and non volatile, and are therefore less toxic to operators and the environment. The use of these liquids is reviewed by Kragl et al. (2002) and will not be described further here.

The advantages of using liquid-liquid systems to increase substrate concentrations can be offset by the inhibiting and inactivating effects that organic solvents can have on the biocatalyst. Toxic effects of solvents on the activity and stability of biocatalysts can be divided into two types: the effect which arises due to contact between the dissolved solvent and the biocatalyst on a molecular level (molecular toxicity), and the effect which arises due to direct contact between the biocatalyst and the aqueous/organic interface (interfacial toxicity). A rule of thumb has emerged for determining solvent biocompatibility which is based on the value of the LogP of the solvent, defined as the logarithm of the partition coefficient of the solvent in a water/1-octanol two-phase system. Broadly, a solvent is expected to be biocompatible if the LogP < 2 and incompatible if LogP > 4 (Laane et al., 1987) and for values between 2 and 4 the solvent effects are considered unpredictable. This rule tends to be more successful in predicting biocompatibility of miscible cosolvents. However, it may be still effective in determining the biocompatibility of an immiscible solvent since the LogP is inversely correlated with the saturation concentration of the solvents in water. Interfacial toxicity of solvents is dependent on other solvent properties such as polarity and interfacial tension (Ross et al., 2000). Despite the amount of research carried out in this area many exceptions
seem to exist to the general rules which have been proposed, and effects of solvents on biocatalysts have to be studied on a case by case basis. Although the direct relation between the amount of enzyme inactivated and total interfacial area in a biphasic system has been established (Ghatorae et al., 1994), a rigorous investigation of the main factors that influence the overall rate of interfacial inactivation of enzymes in emulsion reactors as a function of operating parameters is lacking. This is likely due to the difficulty in characterisation of emulsion systems, particularly in obtaining accurate measurements of droplet size distributions and droplet lifetime.

To reduce interfacial inactivation effects, membrane bioreactors can be used whereby the aqueous and organic liquid phases are kept separate by a hydrophilic or hydrophobic membrane. The advantage of such systems is that the biocatalyst in the aqueous phase does not directly contact the organic phase (Choi et al., 2000). However, membrane bioreactors tend to be complex to operate compared to emulsions since unwanted breakthrough of solvent through the membrane can occur and recycle rates through the two sides have to be carefully controlled. They are also not suited in cases where high interfacial areas are required for good mass transfer and are inflexible since bioreactors are usually designed for and dedicated to one reaction.

In multiphase systems, increasing the organic to aqueous phase ratio is an obvious way of increasing the volumetric productivity of a reaction that involves hydrophobic compounds since the concentration of substrate increases with respect to the total reaction volume. Limitations to the increase in phase ratio arise from practical difficulties in achieving sufficient mixing of the two phases. Furthermore, modifying phase ratios can change the concentration of product that accumulates in the aqueous phase, for example when the product is insoluble in the organic phase. The variation in interfacial area with phase ratio affects the overall rate of mass transfer of substrate to the aqueous phase, and thus also the substrate aqueous phase concentration. Because of this, the optimum enzyme concentration applied in the aqueous phase will almost certainly change with phase ratio as well as the overall rate of enzyme inactivation by the interfacial mechanism (Ghatorae et al., 1994). These types of interactions can be usefully studied by mathematical modelling of the biotransformation systems (Chapter 2). For liquid/liquid biphasic biotransformation systems, determination of model parameters and verification of model simulations can be carried using reactors such as the Lewis/stirred cell where interfacial areas are well defined (Woodley, 1991).

**Preparative scale production of optically pure epoxides.**

In recent years, large-scale preparation of optically pure epoxides has become an active field of research. Examples of the most successful preparative scale epoxide hydrolase mediated kinetic resolutions described
### Table 1.2. Selected examples of preparative scale production of enantiopure epoxides by biocatalytic kinetic resolution

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Epoxide Substrate</th>
<th>Epoxide conc.</th>
<th>[e.e. (%)] Yield (%)</th>
<th>Avg. Productivity † (Biocatalyst conc.)</th>
<th>Solvent/operating condition</th>
<th>Biocatalyst stability/inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger epoxide hydrolase (EH) (^{[1]}) (whole cells)</td>
<td>styrene oxide</td>
<td>0.08 M 10 g L(^{-1})</td>
<td>[96] 23 (S)</td>
<td>0.32 g L(^{-1}) h(^{-1}) (32 g cdw L(^{-1}))</td>
<td>27 °C, aqueous phase + 2% v/v ethanol</td>
<td>product inhibition for substrate conc. &gt; 0.02 M</td>
</tr>
<tr>
<td>A. niger EH (^{[2]}) (partially purified enzyme)</td>
<td>(p)-nitrostyrene oxide</td>
<td>0.33 M 54 g L(^{-1})</td>
<td>[99] 49 (S)</td>
<td>3.7 g L(^{-1}) h(^{-1}) (51 g L(^{-1}) at 0.02 U mg(^{-1}))</td>
<td>25 °C, aqueous phase + 20% DMSO (solid substrate as 2(^{nd}) phase)</td>
<td>enzyme inactivation for substrate conc. &gt; 0.35 M</td>
</tr>
<tr>
<td>A. niger EH (^{[3]}) (partially purified enzyme)</td>
<td>2-pyridyl oxirane</td>
<td>0.082 M 10 g L(^{-1})</td>
<td>[99] 27* (S)</td>
<td>0.33 g L(^{-1}) h(^{-1}) (0.5 g L(^{-1}) at 23 U mg(^{-1}))</td>
<td>4 °C, single aqueous phase</td>
<td>low temperature required for biocatalyst stability</td>
</tr>
<tr>
<td>A. radiobacter wild-type EH (^{[4]}) (cell free extract)</td>
<td>2-pyridyl oxirane</td>
<td>0.127 M 15.4 g L(^{-1})</td>
<td>[&gt;99] 34.5* (S)</td>
<td>2 g L(^{-1}) h(^{-1}) (0.07 g L(^{-1}) at 13 U mg(^{-1}))</td>
<td>28 °C, single aqueous phase</td>
<td>product inhibition for substrate conc. &gt; 0.2 M</td>
</tr>
<tr>
<td>A. radiobacter Y215F mutant EH (^{[5]}) (cell free extract)</td>
<td>2-pyridyl oxirane</td>
<td>0.127 M 15.4 g L(^{-1})</td>
<td>[98] 36* (S)</td>
<td>0.8 g L(^{-1}) h(^{-1}) (0.98 g L(^{-1}) at 0.11 U mg(^{-1}))</td>
<td>28 °C, single aqueous phase</td>
<td>product inhibition (K_i) (racemic) = 0.12 M</td>
</tr>
<tr>
<td>A. niger LCP521 EH (^{[6]}) (cell free extract)</td>
<td>(p)-bromo-(\alpha)-methyl styrene oxide</td>
<td>0.38 M 80 g L(^{-1})</td>
<td>[99.7] 39* (S)</td>
<td>0.16 g L(^{-1}) h(^{-1})</td>
<td>4 °C, biphasic system (liquid substrate as 2(^{nd}) phase)</td>
<td>low temperature required for biocatalyst stability</td>
</tr>
<tr>
<td>Rhodoturula glutinis ATCC 201718 EH (whole cells) (^{[7]})</td>
<td>1,2-epoxy hexane</td>
<td>1.1 M</td>
<td>[98] 24 (S)</td>
<td>2.5 g L(^{-1}) h(^{-1}) (22 g cdw L(^{-1}))</td>
<td>25 °C, biphasic membrane bioreactor (substrate in dodecane)</td>
<td>product inhibition (K_i) = 0.05 M (continuous diol removal by extraction across membrane)</td>
</tr>
<tr>
<td>Biocatalyst</td>
<td>Epoxide substrate</td>
<td>Epoxide conc.</td>
<td>[e.e. (%)]</td>
<td>Yield (%)</td>
<td>Avg Productivity † (Biocatalyst conc.)</td>
<td>Solvent/operating condition</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------------------------------------</td>
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<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>A. niger</em> EH [8] (cell free extract)</td>
<td>4-isobutyl-α-methyl styrene oxide</td>
<td>0.263 M</td>
<td>(S)</td>
<td></td>
<td>0.04 g L⁻¹ h⁻¹ (87 g L⁻¹)</td>
<td>4 °C, biphasic system (substrate as 2nd liquid phase)</td>
</tr>
<tr>
<td><em>S. tuberosum</em> EH [9] (cell free extract)</td>
<td><em>p</em>-chloro styrene oxide</td>
<td>0.2 M</td>
<td>[99]</td>
<td>47* (R)</td>
<td>0.84 g L⁻¹ h⁻¹ (33.6 g L⁻¹ at 0.037 U mg⁻¹)</td>
<td>0 °C, pH 6.7, biphasic system (substrate as 2nd phase)</td>
</tr>
<tr>
<td><em>A. niger</em> EH [10] (cell free extract)</td>
<td><em>p</em>-chloro styrene oxide</td>
<td>2 M</td>
<td>[99]</td>
<td>47* (S)</td>
<td>26g L⁻¹ h⁻¹ (255 g L⁻¹ at 0.23 U mg⁻¹)</td>
<td>0 °C, pH 7 biphasic system (substrate as 2nd phase)</td>
</tr>
<tr>
<td><em>A. radiobacter</em> EH [11] (partially purified enzyme)</td>
<td>styrene oxide</td>
<td>0.324 M</td>
<td>(S)</td>
<td></td>
<td>0.27 g L⁻¹ U⁻¹ (0.014 g L⁻¹ at 7.5 U mg⁻¹)</td>
<td>30 °C, biphasic system 30 % v/v octane/buffer</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em> EH [12] (whole cells)</td>
<td>Phenyl glycidyl ether</td>
<td>0.06 M</td>
<td>[99.5]</td>
<td>25.6 (S)</td>
<td>0.14 g L⁻¹ h⁻¹ (30 g cdw L⁻¹ at 0.003 U mg⁻¹)</td>
<td>30 °C, aqueous phase + 5 % v/v DMSO</td>
</tr>
</tbody>
</table>

† calculated on the basis of total reactor volume; *isolated yield

1 Pedragosa-Moreau et al., 1993; 2 Morrissette et al., 1997; 3 Genzel et al., 2001b; 4, 5 Genzel et al., 2001a, substrate solubility = 0.45 M (55 g L⁻¹); 6 Cleij et al., 1998; 7 Choi et al., 2000; 8 Cleij et al., 1999; 9, 10 Manoj et al., 2001; 11 Baldascini et al., 2001; 12 Tang et al., 2001
Table 1.3. Selected examples of preparative scale production of enantiopure epoxides by biocatalytic epoxidation and chemo-catalyzed hydrolytic kinetic resolution

<table>
<thead>
<tr>
<th>Bio/catalyst</th>
<th>Epoxide</th>
<th>Epoxide conc.</th>
<th>[e.e. (%)]. Yield (%)</th>
<th>Avg. Productivity† (Biocatalyst conc.)</th>
<th>Solvent/operating condition</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> JM101(pSPZ10) [13]</td>
<td>styrene oxide</td>
<td>0.184M</td>
<td>[99.5]</td>
<td>2.2 g L⁻¹ h⁻¹</td>
<td>organic/aqueous biphasic system 50 % v/v bis (2-ethylhexyl) phthalate</td>
<td>monooxygenase inhibition by styrene oxide. Cell growth inhibition by 2-phenylethanol (by-product). Toxicity to cells of styrene at organic phase conc. above 2 % v/v.</td>
</tr>
<tr>
<td><em>E. coli</em> JM101(pSPZ10) [14]</td>
<td>styrene oxide</td>
<td>0.27 M</td>
<td>[&gt;99] 49.7 (S)</td>
<td>1 g L⁻¹ h⁻¹</td>
<td>organic/aqueous biphasic system: 50 % v/v bis (2-ethylhexyl) phthalate</td>
<td></td>
</tr>
<tr>
<td>(salen)Co(III)(OAc) [15]</td>
<td>styrene oxide</td>
<td>(22.8 ml styrene oxide + 2 ml THF +2 ml water)</td>
<td>[&gt;99] 44 * (R)</td>
<td>0.15 g h⁻¹</td>
<td>8 % v/v THF, temp. increased from 0 °C to room temp. during reaction</td>
<td>no product inhibition, catalyst recyclable</td>
</tr>
</tbody>
</table>

† calculated on the basis of total reactor volume; *isolated yield
13 Panke et al., 2000 (2L scale); 14 Panke et al., 2002 (30 L scale); 15 Schaus et al., 2002
in the literature to date is presented in Table 1.2. The most successful biocatalytic epoxidation and chemical preparative scale hydrolytic reactions are given for comparison in Table 1.3. The tables report the average reaction productivity, substrate concentrations, enantioselectivity, yield, and reaction type (single or multiphase) and give indications of biocatalyst stability and inhibition effects noted for the reaction systems. In all examples of kinetic resolution a high enantiomeric excess of the desired epoxide enantiomer could be obtained by letting the reaction proceed to sufficient conversion. For these resolution reactions the yield is limited to 50% by definition and yields approaching this maximum value were achieved in some examples, though often using very high biocatalyst concentrations. In nearly all cases the hydrolysis of the (R)-enantiomer occurred preferentially, yielding the enantiopure (S)-enantiomer. Organic solvents have been employed when the substrates are poorly soluble in water and in this manner high epoxide concentrations have been applied (entries [1], [2], [7], [11], [12]). In some cases ([2], [6], [8], [9], [10]), the liquid substrate constitutes a separate organic phase. Pyridyloxiranes are unusual epoxides which have the advantage of being reasonably soluble in aqueous solution (0.5 M) and these have been used in single phase systems to concentrations of 0.13 M without addition of cosolvents (entries [3], [4], [5]). Such single phase systems, however, offer no advantages for downstream recovery of the biocatalyst or epoxide product. In nearly all cases product inhibition has been identified as a limitation to using high epoxide concentrations, and this effect is generally attributed to inhibition by the formed diol. The application of a membrane bioreactor to enable use of high substrate concentrations in an apolar solvent, avoiding inactivation of the biocatalyst by direct contact with the organic phase, and allowing removal of the inhibitory diol, showed high productivity although overall yield was low (entry [7]). In these preparative scale examples average productivities range from 0.16 to 26 g L\(^{-1}\) h\(^{-1}\), although direct comparison between the different systems is difficult since the biocatalyst concentrations used in the different examples vary greatly. In entry [11] a further increase in productivity would easily be possible by increasing the organic phase fraction containing substrate. Overall it seems that biocatalyst productivity remains low and appears not to have been optimised. Clearly, maintaining high biocatalyst activity during the biotransformation is a key issue in each of the examples listed. Biocatalyst inhibition is also a recurrent problem and in some cases the reactions are carried out at 4 °C to increase biocatalyst stability ([3], [4], [6], [8], [9], [10]), something which would likely be a disadvantage at an industrial scale. Before successful industrial application of these systems can occur, the stability and reusability of the biocatalyst needs to be determined.

Recently, the production of enantiopure styrene oxide has been carried out at the pilot-scale (30 L) (Panke et al., 2002) using recombinant \textit{E.coli} expressing a styrene monooxygenase. The biotransformation was carried out
in a two-phase system for the common reasons outlined above. The concentration of the substrate, styrene was limited to 2% v/v in the organic phase since it is not tolerated by *E. coli* cells. The organic solvent was chosen to keep the aqueous concentration of the formed styrene oxide and 2-phenylethanol (a by product) in the aqueous phase low, since they both have an inhibiting effect. A specific inactivating effect of epoxides on alkene monooxygenases has been reported in many other cases (Habets-Crutzen and de Bont, 1985; Prichanont et al., 1998; Stanley et al., 1992) suggesting that due to their extreme reactivity, epoxides can affect biocatalyst stability by enzyme covalent modification by a common mechanism. Despite these inhibition effects, reasonably high concentrations of the formed epoxide accumulate in the organic phase. Volumetric productivity is also good at optimal cell densities, but scale-up of the reaction to 100 L already shows that factors such as the rate of substrate transfer from the organic phase and oxygen supply to the cells can become limiting (Table 1.3 entries 13 and 14).

Schaus et al. (2002) have recently reported on the practical hydrolytic kinetic resolution (HKR) of terminal epoxides using a chiral (salen) Co^{III}(OAc) complex. In this application the epoxide concentration used was generally very high since water was added only as a reactant. Small amounts of organic solvents such as Tetrahydrofuran (THF) were added for poorly aqueous soluble epoxides. The catalyst complex could be recycled without appreciable loss of activity and no product inhibition was observed. From a practical point of view, a disadvantage of this method is that the reaction temperature is required to be initially 0 °C and reaches room temperature during the conversion. The HKR of propylene oxide by the same catalyst has apparently been carried out at the multi-hundred kilogram scale at ChiRex, yielding enantiopure epoxide and 1,2-diol (Jacobsen, 2000). Ultimately, the choice between chemical and biocatalytic resolution methods of epoxides will be dictated by the required enantioselectivity for a specific epoxide intermediate and overall process economics.

**Enzyme structure and stability**

Enzyme stability is of primary importance in bioprocesses in order to maximise biocatalyst productivity so as to operate biotransformations at low cost. Correct enzyme function requires a correctly folded enzyme molecule. Protein folding is determined by a balance between conformational entropy which opposes the folded state and enthalpic and other entropic factors which favour the folded state (Mozhaev, 1993). Enzymes fold to maximise favourable interactions forming tightly packed hydrophobic cores exposing hydrophilic groups on the surface. Hydrophobic interactions greatly contribute to formation of the compact folded state due to the increase in
entropy of the water molecules which are released from the hydrophobic side groups of the polypeptide chain upon folding. Hydrogen bonds also play an important role in the stabilisation of enzyme secondary structure and together with electrostatic and dispersive forces all these interactions act to determine the structure of enzymes in a given environment. Since the stabilising and destabilising interactions compensate one another to a large extent, the native structure of a protein is only marginally stable. The Gibbs free energy for the transition from native to denatured state is only about –20 kJ mol⁻¹ (Mozhaev, 1993) implying that even small changes in the environment of the enzyme can cause structural unfolding and loss of activity.

**Enzyme inactivation**

Causes of loss of enzyme activity include enzyme denaturation at high temperatures and extremes of pH, covalent enzyme modification by chemical species, enzyme adsorption to interfaces and inactivation due to shear which arises from structural damage from fluid forces in agitated systems. Studies of enzyme stability, particularly thermostability, are frequently conducted in the absence of substrate and product, under non-reacting conditions. However, it is known that enzymes are frequently stabilised in the presence of substrates, and similarly substrates can also interact with the enzyme to cause inactivation (Illanes et al., 1996). The increased application of enzymes in non-conventional media, for example in the presence of organic solvents, means that studying enzyme stability under representative process conditions is becoming increasingly important.

Enzyme stability is characterised by both thermodynamic and kinetic stability. Thermodynamic stability describes the reversible unfolding of enzymes molecules from the native state (N) to the unfolded state (U) under denaturing conditions (Mozhaev, 1993):

\[
N \leftrightarrow U
\]  

(1.4)

Differential scanning microcalorimetry (DSC) and circular dichroism (described below) can be used to determine the enthalpy of unfolding and the enzyme melting temperature \(T_m\) for such reversible unfolding. Assessment of the thermodynamic stability of enzymes using these techniques is particularly useful to compare the stability of different enzymes, or mutants of the same enzyme. However, thermodynamic stability can not easily be translated to kinetic enzyme stability, which concerns the time dependent irreversible enzyme inactivation under a set of conditions (Schein, 1990).

Irreversible inactivation involves a transition to an inactive form of the enzyme (I). In the simplest description, irreversible inactivation occurs by a first order irreversible unimolecular reaction,
and the rate of change of enzyme activity is described by the first order rate equation,

$$\frac{dN}{dt} = -k_{obs}N.$$  \hspace{1cm} (1.6)

Frequently, irreversible inactivation is assumed to proceed by a two step mechanism comprising reversible unfolding to an intermediate denatured state (D), followed by an irreversible transition to the inactive form (Mozhaev, 1993),

$$N \xrightarrow[k_u]{k_f} D \xrightarrow[k_i]{k_f} I$$  \hspace{1cm} (1.7)

During thermoinactivation, for example, the last step is usually aggregation of the unfolded enzyme form, and under other denaturing conditions may involve covalent modification of labile amino acid residues, loss of cofactor or incorrect refolding of the denatured state. The rate constant for overall inactivation is (Sterner and Liebl, 2001),

$$k_{obs} = \frac{(k_u k_i)}{(k_f + k_i)}$$  \hspace{1cm} (1.8)

Operational stability of enzymes is frequently quoted in terms of half-life ($t_{1/2}$) which is calculated by, $t_{1/2} = \frac{-\ln(0.5)}{k_{obs}}$. Determining the kinetics of enzyme inactivation is important to be able to take into account enzyme inactivation in bioprocess modelling.

Elucidation of the physical mechanism of inactivation is fundamental in order to devise targeted strategies to reduce inactivation. Enzyme inactivation can proceed by dissociation into subunits (quaternary structure), chemical modification (primary structure) and denaturation/conformational change (secondary and tertiary structure) which may then lead to enzyme aggregation and precipitation from solution. It is difficult to relate changes in activity to changes occurring in enzyme structure especially since the activity measured at any point in time can be the sum of contributions of any partially active enzyme forms (Polakovic, 1998). However, in systems where kinetics of substrate turnover are well-described, it is possible to differentiate between a change in substrate binding and a change in catalytic turnover rate and this can allow to distinguish between different causes of inactivation. In addition, following changes in enzyme structure by biophysical methods while measuring activity in parallel can be particularly informative about the inactivation mechanism.
Biophysical techniques for studying enzyme degradation

As described above, the loss of enzyme activity is always accompanied by a change in enzyme structure. This can be either a large conformational change, or a localised structural or chemical modification. Various biophysical techniques have been developed for monitoring these changes enabling fundamental studies of inactivation mechanisms. These techniques include UV absorption, circular dichroism (CD), fluorescence spectroscopy, mass spectrometry, peptide mapping and microcalorimetry (Schmid, 1997). Each technique is particularly suited for detecting specific enzyme structural modifications (Table 1.4) and can be used to determine enzyme thermodynamic stability by monitoring reversible unfolding under denaturing conditions. A brief description of the techniques used for work described in this thesis follows here.

CD measures the difference in adsorption between the two rotations of circularly polarised light by asymmetric molecules such as proteins, and so the CD spectrum is dependent on the conformation of the asymmetric molecule. The technique is non-destructive and requires only small amounts of material. CD in the far-UV region (190-240 nm) is dominated by adsorption by the peptide group linking the amino acid residues, so that features of secondary structure such as $\alpha$ helices and $\beta$ sheets give rise to characteristic spectra (Woody, 1995). The technique is frequently used to predict the secondary structure of proteins of unknown structure by comparison with reference spectra, and it is also a powerful technique for analysing changes in protein secondary structure. For example, far-UV CD can be used to follow thermal denaturation of proteins by following the change in CD signal at a fixed wavelength with temperature. We were particularly interested in using this technique to monitor large conformational changes in secondary structure for enzymes incubated in liquid/liquid biphasic systems (Chapter 3). Far-UV CD has also been a useful technique for studying the conformation of proteins adsorbed onto solid phases (Norde and Zoungrana, 1998). This has been possible since the solid phases can be made optically neutral. On the other hand, analysis of the structure of proteins adsorbed at liquid-liquid interfaces has been hindered by the physical instability of such surfaces and optical reflection at the interfaces. For this reason, in our experiments, we were limited to following changes in structure of soluble enzyme rather than enzyme adsorbed at the liquid/liquid interface. Recently external reflection circular dichroism has been presented as a novel technique to study the structure of proteins at the air/liquid interface (de Jongh and Meinders, 2002). This technique allows separation of the optical effects due to reflection and those due to molecular properties of the protein. The experimental set-up, however, is complex and sensitive to experimental imperfections so further improvements in the technique are necessary before unequivocal information on protein conformation can be obtained. In the near-UV range (250-300nm)
<table>
<thead>
<tr>
<th>Biophysical Technique</th>
<th>Structural features monitored</th>
<th>Information derived</th>
<th>Advantages (\text{Disadvantages})</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV Absorption</td>
<td>- Tertiary structure</td>
<td>- Protein conc.</td>
<td>- Non-destructive</td>
<td>Schmid, 1997</td>
</tr>
<tr>
<td></td>
<td>- Ligand binding</td>
<td>- Conformational changes</td>
<td>- Spectra can be difficult to interpret</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Interference by chromogenic species</td>
<td></td>
</tr>
<tr>
<td>CD Far-UV</td>
<td>- Secondary structure</td>
<td>- Large conformational changes</td>
<td>- Non-destructive</td>
<td>Woody, 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Thermostability ((T_m))</td>
<td>- Low enzyme conc. (~ 0.1mg ml(^{-1}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Low sensitivity to localised structural changes</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Interference by chromogenic species</td>
<td></td>
</tr>
<tr>
<td>CD Near-UV</td>
<td>- Tertiary structure</td>
<td>- Localised conformational changes</td>
<td>- Non-destructive</td>
<td>Woody and Dunker, 1996</td>
</tr>
<tr>
<td></td>
<td>- Ligand binding</td>
<td>- Region of ligand binding</td>
<td>- Localised changes identified</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Ligand binding constants</td>
<td>- High enzyme conc. required (~2mg ml(^{-1}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Interference by chromogenic species</td>
<td></td>
</tr>
<tr>
<td>Steady State Fluorescence</td>
<td>- Tertiary structure</td>
<td>- Conformational changes</td>
<td>- Non-destructive</td>
<td>Schmid, 1997</td>
</tr>
<tr>
<td></td>
<td>- Ligand binding</td>
<td>- Unfolding</td>
<td>- Low protein conc. sufficient (0.01mg ml(^{-1}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- \text{Interference by fluorescing species}</td>
<td></td>
</tr>
<tr>
<td>Peptide mapping</td>
<td>- Primary structure</td>
<td>- Covalent modifications</td>
<td>- Precise knowledge of the place of modification</td>
<td>Stone et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Region of modification</td>
<td>- \text{Labour intensive}</td>
<td></td>
</tr>
<tr>
<td>ESI-MS</td>
<td>- Ligand binding</td>
<td>- Molecular mass</td>
<td>- Accurate change in mass allows identification of ligand</td>
<td>Jardine, 1990</td>
</tr>
<tr>
<td>Microcalorimetry</td>
<td>- Unfolding</td>
<td>- Enthalpy of unfolding</td>
<td>- Determines relative protein stability in different environments</td>
<td>Wadso, 1997</td>
</tr>
</tbody>
</table>
the CD spectrum is dominated by contributions made by the aromatic side chains of Phe, Tyr and Trp residues and is sensitive to the spatial arrangement of these side chains, so spectra reflect the tertiary structure of proteins (Woody and Dunker, 1996). Small conformational changes and ligand binding can be studied by following spectral changes in this region.

The fluorescence of proteins originates from Phe, Tyr and Trp residues but is often dominated by Trp when present (Schmid, 1997). Fluorescence spectra are sensitive to changes in the environment of these fluorophores making it a useful technique to monitor global protein unfolding. By comparing fluorescence spectra of single tryptophan mutants more site specific structural information can also be derived. Changes in emission intensity and shifts in the wavelength of maximal emission closer to 350 nm, which is the maximum for free tryptophan in solution, are observed upon unfolding. Measuring changes in fluorescence is commonly used to follow protein denaturation at high temperatures and in the presence of denaturants.

Mass spectrometry by electrospray ionization (ESI) is a technique which allows the determination of protein molecular weights to high accuracy. Typically, relative masses in the range of 5 to 40 kDa can be obtained with an accuracy of 0.01% (Edmonds and Smith, 1990). The technique is particularly suited for identifying covalent and non-covalent protein-ligand complexes which may be formed upon inactivation (Pramanik et al., 1998). To be useful in identifying such complexes the technique must be optimised, especially regarding the sample preparation which can involve the use of cosolvents and acidic conditions, which may cause the complexes to be destroyed.

**Improving enzyme stability**

The simplest way of improving enzyme operational stability is by medium engineering. This involves optimising the operating temperature, choice of buffer and its concentration and working pH to achieve maximum enzyme stability. Various protective agents can also be added to increase stability. Commonly, glycerol is used to stabilise enzymes during storage, and 2-mercaptoethanol can be used to prevent intermolecular sulfydryl cross-linking, while EDTA can be used to chelate metal ions which can cause protein oxidation (Schein, 1990).

Many different strategies can be employed to further improve enzyme stability. The most common strategy used in industrial applications is immobilisation of the enzyme onto a solid carrier. Immobilisation usually functions by resisting protein unfolding and many examples in which enzyme operational stability has been successfully increased have been reported. Apart from increased stability, biocatalyst immobilisation onto a solid carrier offers the added advantage of facilitating the recovery of the biocatalyst from the reaction liquor so that it can be recycled. Immobilisation can be carried out by physical adsorption, gel entrapment, or
covalent attachment onto a carrier. The choice of immobilisation method will depend on the bioprocess conditions, taking into account the partitioning of substrate and product within the immobilisation matrix, and the perceived cause of inactivation. For example, in multiphase liquid systems, immobilisation of biocatalysts inside hydrophilic porous supports can help to reduce direct contact between the biocatalyst and the organic apolar liquid at the phase interface, thereby reducing interfacial inactivation. Disadvantages of immobilisation include the occurrence of diffusion limitations within the carrier porous particles. Mass transfer limitations result in an inefficient use of the biocatalyst and may cause a significant reduction in reaction selectivity (Barros et al., 2000), which is particularly relevant for kinetic resolution reactions. To reduce these negative effects the biocatalyst catalyst loading on the carrier particles has to be carefully optimised. An alternative approach could be to immobilise the biocatalyst on the outside of the carrier particles, but in this case the protection against interfacial inactivation would be less effective and the specific activity of the biocatalyst-carrier complex would be greatly reduced. Bioprocess modelling is invaluable in the study of the interaction between mass transfer and reaction in immobilised systems. Mateo et al (2003) described the covalent immobilisation of *Aspergillus niger* epoxide hydrolase onto an epoxy activated support. The immobilised biocatalyst could be reused for 12 successive batch epoxide resolution reactions with only a small loss of activity. The loading of enzyme onto the carrier was limited to 40 mg g\(^{-1}\) of (wet) support to avoid diffusional limitations and conversion kinetics were found to be changed with respect to the soluble enzyme, although this resulted in a favourable increase in enantioselectivity of the immobilised enzyme.

Recently, the use of cross linked enzyme crystals (CLECs) has emerged as a very promising method for increasing enzyme stability (Margolin, 1996). CLECs are prepared by crystallisation of the enzyme followed by cross-linking with a bifunctional reagent, usually glutaraldehyde, which results in an insoluble and porous solid catalyst. This technique exploits the stabilising effects of both inter- and intramolecular cross-links which are formed between the amine groups of lysines. Compared to the soluble enzyme forms, CLECs have been shown to possess higher thermostability, proteolytic stability and increased resistance to organic solvents (Govardhan, 1999). Additionally CLECs can easily be recovered from reaction media by filtration for reuse. Disadvantages in the use of CLECs are that the specific activity is often greatly reduced compared to the soluble enzyme. Compared to enzymes immobilised on solid supports, however, specific activity is often higher and the expensive carrier material is not required. Similarly to immobilised enzymes, mass transfer limitations may occur within the porous crystal matrix and this can have important consequences on the effective enantioselectivity of the biocatalyst (Tischer
Use of CLECs at an industrial scale (100 L) in a two-phase system for the kinetic resolution of sec-phenethyl acetate has been demonstrated (Collins et al., 1998). Reusability of the CLECs in successive batch reactions indicated that most of the activity that was lost between successive batches was due to loss of catalyst mass at the filtration stage in the recovery step, indicating that the size of the CLECs is an important physical characteristic for their use at the industrial scale.

Crystallisation of an enzyme with retention of activity is not always possible. An alternative method which also exploits cross-linking for enzyme stabilisation is the creation of cross-linked enzyme aggregates (CLEAs) (Cao et al, 2001). This method does away with the necessity of crystallisation since cross-linking is carried out on enzymes which are aggregated under non-denaturing conditions. CLEAs of penicillin acylase have been shown to be more stable in the presence of organic cosolvents than the conventionally immobilised enzyme, but the general applicability of this method remains to be demonstrated.

The preferred strategy for maintaining biocatalyst activity under reaction conditions would be to increase the intrinsic stability of the biocatalyst (enzyme). The use of enzymes from thermophilic organisms, which show high thermostability, has been advocated since higher thermostability has been correlated with increased resistance to other mechanisms of denaturation, such as cosolvent effects (Owusu and Cowan, 1989). However, there is often a trade-off between thermostability and activity, since enzymes from thermophilic organisms generally have low activities at room temperatures (Carrea and Colombo, 2000).

Site-directed mutagenesis (SDM) and more recently directed evolution are emerging as powerful techniques to improve enzyme characteristics (Bornscheuer and Pohl, 2001). The former technique requires in-depth knowledge of enzyme structure-function relationships in order to be able to predict mutations will have the desired effect on enzyme function (van den Burg and Eijsink, 2002). The mutation of one active site tyrosine residue of the epoxide hydrolase used for work in this thesis resulted in mutants (Y215F and Y152F) with much higher enantioselectivity for the conversion of some substrates (Rink et al., 1999), which is clearly highly desirable for industrial application. Interestingly, these mutations also increased enzyme stability against extremes of pH (Rink et al., 2000) which prompted us to also investigate the thermostability of the different enzyme forms (Chapter 5).

In contrast to SDM, directed evolution does not require much detailed knowledge of the enzyme structure-function relationships since mutations are introduced randomly. However, it does require a robust screening assay which accurately reflects the properties of interest (Arnold, 2001; Petrounia and Arnold, 2000). Directed evolution has successfully been used to improve enzyme substrate range and to increase, or alternatively invert,
enzyme enantioselectivity (Zha et al., 2001). The development of quantitative high throughput methods to screen for catalyst enantioselectivity remains a challenge, and recent examples of such methods are reviewed by Reetz (2002). Improvement of enzyme thermostability by directed evolution has also been demonstrated (Gonzales-Blasco et al., 2000) and again, screening for increased enzyme thermostability should be accompanied by screening for reasonable activity at low temperatures. Using the technique of DNA shuffling, Ness et al. (2001) successfully improved subtilisin activity at room temperature, thermostability and stability against organic cosolvents in a single enzyme clone. Analysis of the mutations in evolved enzymes show that these are often not situated in the active site and that mutations farther away seem to have long ranging structural effects that can affect enzyme performance (Petrounia and Arnold, 2000). For this reason, rational design of improved stability is particularly challenging since many different interactions contribute to the stability of proteins.

Although there is a certain correlation between increased enzyme thermostability and increased operational stability even in the presence of organic solvents, it is important to appreciate that resistance to inactivation due to different mechanisms will not necessarily be directly correlated. For example, a percentage increase in thermostability may not give a discrete improvement in the resistance to inactivation at a liquid/liquid interface. Nevertheless, it is expected that these two techniques will play a major role in improving enzyme properties especially in regards to their use as industrial catalysts, and in the future this will be focussed to a large extent on the increase of their operational stability.

The techniques which are used to stabilise enzymes should be evaluated in terms of their effects on the whole biocatalytic process. For example, while immobilisation may increase stability, the introduction of a large proportion of solid phase may cause unwanted problems in downstream separation. Moreover, the optimum operating pH, and temperature need to be redefined for an immobilised enzyme preparation. Even when mutants are created which have improved characteristics such as higher enantioselectivity – their use in the whole bioprocess must be re-evaluated. This is because while the desired characteristic of the biocatalyst might be improved, unexpected effects on other properties may emerge. The creation of the site directed mutants of the epoxide hydrolase used for the work described in this thesis is a good example of this. The active site mutant Y215F showed a much higher enantioselectivity than the wild-type enzyme. Although the enantioselectivity was higher, the $K_m$ values for the conversion of epoxide enantiomers were increased 100 to 1000-fold compared to the wild-type enzyme (Rink et al., 1999). The consequences of this for bioprocess design could be two-fold. Firstly, in a biphasic application maintaining high enough substrate concentration in the aqueous phase in order to maintain high overall conversion rates could become
problematic, thus necessitating higher mass transfer rates (*Chapter 2*). Secondly, it was found that although the $K_m$ values for substrate binding to the enzyme active site were increased, product binding (inhibition) constants did not vary much, thereby increasing the relative importance of product inhibition at lower diol product concentrations (*Chapter 4*), which is clearly undesirable. Koeller and Wong (2001) suggest that in a strategy for the development of effective biocatalysts, process development comes as the final step after the biocatalyst has been optimised. It can be argued that evaluation of biocatalyst characteristics in relation to bioprocess optimisation should be integrated with mutant screening as early as possible in bioprocess development, so that the evolution of the biocatalyst is directed towards optimising overall bioprocess performance rather than specific characteristics of the biocatalyst alone. Rapid screening facilities to combine high throughput evaluation of catalyst variants with regard to overall bioprocess performance have already been demonstrated for whole cell biocatalysts (Lye et al., 2003).

**Aim and Outline of this Thesis**

Research on the epoxide hydrolase from *Agrobacterium radiobacter* AD1 carried out in our and other research groups has included a detailed study of enzyme function (van den Wijngaard et al., 1989), catalytic and kinetic mechanism (Rink and Janssen, 1999) and structure by X-ray crystallography (Nardini et al., 2000). Recognition of the potential of the use of this enzyme in organic synthesis for the production of optically pure epoxides initiated the study of enzyme substrate range and an exploration of biocatalytic applications of the enzyme (Lutje Spelberg et al., 1998, 2002a). The combination of all these studies allowed the rational design of mutant epoxide hydrolases with greatly increased enantioselectivity for the kinetic resolution of various industrially interesting epoxides (Rink et al., 1999). The work in this thesis is focussed on the application of the wild-type and mutant epoxide hydrolases at a preparative scale, addressing the constraints which are identified in obtaining optically pure epoxides with high volumetric productivity, high enantiomeric excess and high biocatalyst productivity. The focus on epoxide hydrolase stability in this thesis is motivated by the fact that stability is a major determinant of bioprocess productivity. A fundamental study of the factors which affect epoxide hydrolase operational stability is carried out in order to formulate appropriate solutions for maximising enzyme stability.

In *Chapter 2* the use of a two-liquid-phase system composed of octane and buffer is demonstrated in order to carry out the kinetic resolution with high epoxide concentrations and to reduce the loss of yield caused by spontaneous hydrolysis of the epoxide. Styrene oxide was used as model
substrate for this biphasic biotransformation. A process model describing the bioconversion in the biphasic system was developed and validated using a stirred cell reactor. The model was used to study the effect of mass transfer limitations on the apparent enantioselectivity of the kinetic resolutions.

Results from Chapter 2 indicated that enzyme inactivation from dissolved octane in the aqueous phase was low, whereas inactivation due to contact between the enzyme and the organic/aqueous interface was greater. A study of enzyme interfacial inactivation occurring at the octane-buffer interface is the subject of Chapter 3. The influence of mixing intensity, enzyme concentration and presence of styrene oxide in the organic phase on the rate of interfacial inactivation are investigated. By measuring changes in the secondary structure of dissolved enzyme by CD, interfacial inactivation was proposed to involve the sequential steps of adsorption, enzyme structural rearrangement with loss of activity, desorption and aggregation and precipitation of desorbed enzyme molecules. Using a Lewis cell reactor, it was found that the rate of interfacial inactivation increased with increasing mixing intensity. This was attributed to an increase in the rate of eddy assisted desorption of inactivated enzyme from the interface which in turn allows enzyme in solution to adsorb and inactivate. The specific inactivation rate measured in an emulsion system was lower than in the stirred-cell even though interfacial area was higher. The presence of styrene oxide at concentrations above 250 mM in the octane liquid was shown to increase the rate of interfacial inactivation.

Chapter 4 describes a study of the effect of vicinal diols on epoxide hydrolase stability. Incubation of wild-type enzyme in aqueous solutions of diols caused irreversible enzyme inactivation. The inactivating effect was greater at higher diol concentrations. The inactivation occurred specifically with vicinal diols and was positively correlated with the inhibition effect of the vicinal diols, which are also competitive inhibitors of the enzyme. Active site specificity was demonstrated for the inactivation. Inactivation was not accompanied by large changes in enzyme structure but a smaller conformational change could be observed by near-UV CD spectroscopy. Interestingly, the Y215F active site mutant of this epoxide hydrolase was resistant to inactivation by diols.

The research described in Chapter 5 was aimed at investigating the possibility of using cross-linked epoxide hydrolase crystals (CLEhCs) to carry out kinetic resolution reactions with high enzyme stability. Crystals of the epoxide hydrolase active site mutant (Y215F) were obtained by batch crystallisation using PEG as the precipitating agent. The enzyme crystals were cross-linked using glutaraldehyde. The CLEhCs were resistant to dissolution in buffer, were active and enantioselective and could be reused for successive epoxide hydrolysis reactions. The apparent enantioselectivity of the CLEhC preparation was lower than that of soluble enzyme for the
conversion of two epoxides tested. Nevertheless, epoxides with high e.e. and reasonable yield could be obtained using the CLEhCs.

Chapter 6 presents a summary of the results and an outlook for the different possibilities for production of optically pure epoxides by kinetic resolution of racemic mixtures.