Integration of Biocatalysis with Product Separation

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This thesis is submitted to the University of London for the degree of doctor of philosophy in biochemical engineering .

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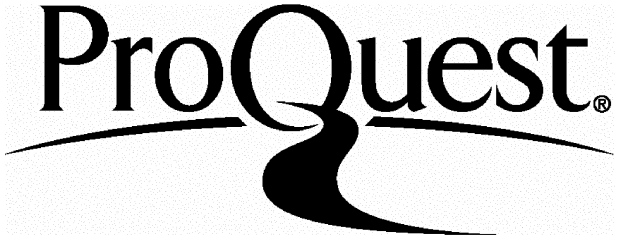

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Contents

Table of Figures

Table of Schemes

Index of Tables

Table of Equations

Abstract

This thesis deals with the research carried out to develop a column system for the continuous production of optically pure esters of 2-chloropropionic acid (CPA) and 2-phenoxypropionic acid (PPA).

Chapter 1 entitled introduction, explores the available literature. This chapter highlights the advantages and disadvantages of currently available methods for the resolution of CPA and PPA.

Chapter two explains our strategies for the resolution of CPA and PPA. We highlight the possible routes and steps we envisage to be important for the efficient resolution and continuous production of CPA and PPA.

Rest of the thesis deals with our attempts to establish the column reactor. The remaining thesis is divided into the following sections.

Analytical section: development of chiral assay to determine the optical purity of the products. Methods developed to measure the progress of reaction, enzyme activity and protein contents.

Ester synthesis: highlights the advantages and disadvantages of the various processes used to synthesis the substrates.

Lipase search: all commercially available lipases were tested for their activities against the substrates and optimal combination were explored in more detail.

Adsorption support: several macro reticular resin were tested for their adsorption affinity towards CPA, PPA and their esters and lipase. Conditions were optimised for lipase activity and substrate adsorption.

Development of a column reactor: column reactor was set-up according to the conditions listed in above chapters. Column was characterised according to the purity, optical purity and yield of the products and residual substrate isolated.

Discussion: results from all the research carried out are summarised in this chapter

Future work: this chapter highlights the additional points seemed necessary to successfully establish the continuous process.

Abbreviations

b.p.	boiling point
c	conversion
cat	catalyst / catalytic amount
$CDCl_3$	deuterated chloroform
CLECs	cross-linked enzyme crystals
CMPP	2-chloro-4-methylphenoxypropionic acid
CPA	2-chloropropionic acid
CRL	*Candida rugosa* lipase
CSP	chiral stationary phase
d.e.	diastereomeric axcess
DCC	dicyclohexylcarbodiimide
DEGME	di-ethylene glycol methyl ether
DEX	2-haloacid dehalogenase
DMF	dimethylformamide
DMSO	dimethylsulphoxide
ε	molar absorbance coefficient
e.e.	enantiomeric excess
ee_p	enantiomeric excess of the product
ee_s	enantiomeric excess of the substrate
E_R	enantiomeric ratio
Et_2O	diethyl ether
EtOAc	ethyl acetate
g	grams
GC	gas chromatography
h	hours
HCPA	hexyl-2-chloropropionate
HPLC	high performance liquid chromatography
HPPA	hexyl-2-phenoxypropionate
I.D.	internal diameter

IPA	iso propyl alcohol
i-PrOH	iso-propyl alcohol
J	coupling constant
kg	kilograms
LDA	lithium diisopropylamine
LiAlH	lithium aluminium hydride
M	mole / molar
m	multiplet
MCP	methyl-2-chloropropionate
MeCN	acetonitrile
MeOH	methanol
MES	morpholino ethane sulphonic acid
μg	microgram
mg	milligram
MHz	mega hertz
min/s	minute/s
μL	micro litre
mmol /mM	milli moles
mol	moles
MPD	2-methyl-2,4-pentanediol
NBS	*N*-bromosuccinimide
NCS	*N*-chlorosuccinimide
nm	nano meter
NMR	nuclear magnetic resonance spectroscopy
°C	degree Celsius
petrol	petroleum spirit
pNPA	pera nitro phenyl acetate
ppm	parts per million
Psi	pound per square inch
re	below the plane of page
s	singlet

se	above the plane of page
sec	secondary
SEL	*S*-ethyllactate
ser	serine
SMB	simulated moving bed
S_N2	bimolecular nucleophilic substitution
Td	tetrahedral
THF	tetrahydrofuran
tlc	thin layer chromatography
U	activity units
UV	ultra violet
vis	visible
w/v	weight/vol

1 Introduction

(2*S*)-Chloropropionic acid is a key chiral intermediate required for the synthesis of a range of important herbicides as their single active isomers. Derivatives of propionic acid such as chloropropionic acid and lactic acid, remain important building blocks for a wide range of herbicides. The simplest of these herbicides is 2,2-dichloropropionic acid, which has been used for the control of annual and perennial grasses. A characteristic of most of these products is the presence of a chiral centre at the α-carbon of the propionic acid moiety. Some of the most widely used herbicides are shown in **Fig 1** (*Taylor, 1990*).

Additions to the propionic acid group in these products varies from a simple chlorinated phenyl group (dichloroprop) through to quite complex heteroaromatic systems (napropamide).

The chirality of the molecule is important and in most cases the desired herbicidal activity resides only in one enantiomer, usually the *R*-enantiomer (*Buser, 1997*). Although these herbicides have traditionally been produced as their racemates, there is now a strong move in the industry to produce the products as their single active isomer form. This is happening both for reasons of reduced synthesis costs, particularly important with the complex heteroaromatic molecules, and to reduce the load on the environment of these widely used chlorinated compounds (*Taylor, 1990*).

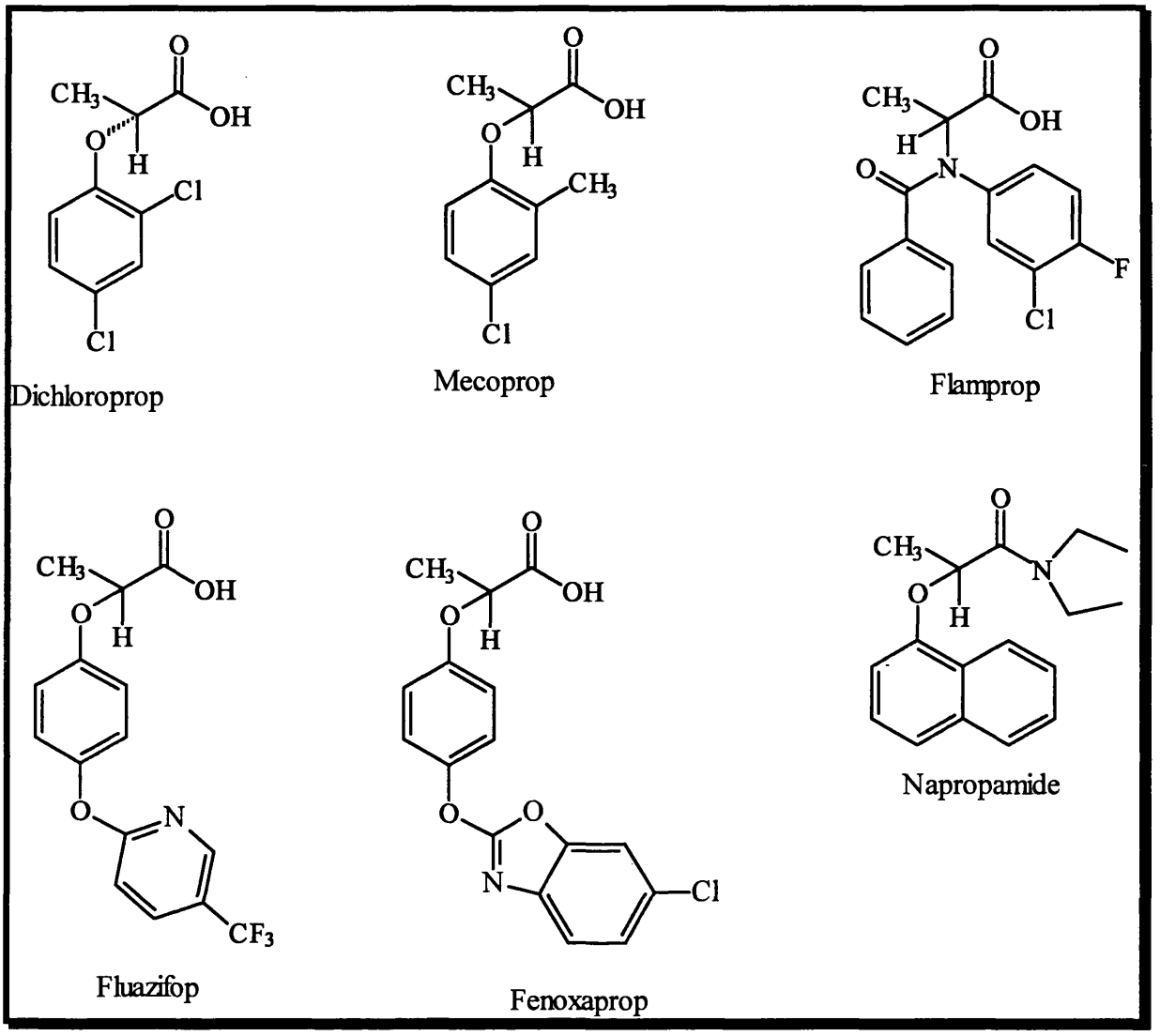


Figure 1: Examples of α-chloropropionic acid based herbicides

In a typical synthesis of the above herbicides, the phenol is deprotonated using a strong inorganic base and the phenoxide ion formed then displaces the chlorine at the α-carbon of the CPA (**fig 2**), attacking from the opposite face. The nucleophilic attack results in an inversion of configuration at the α–carbon (S_N2 mechanism), therefore the herbicides have the *S*-configuration at the α-carbon.

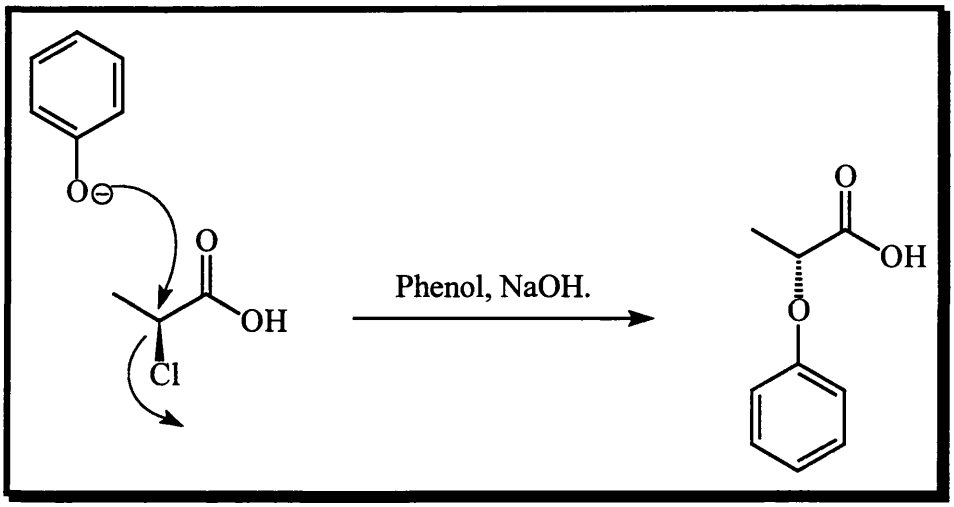


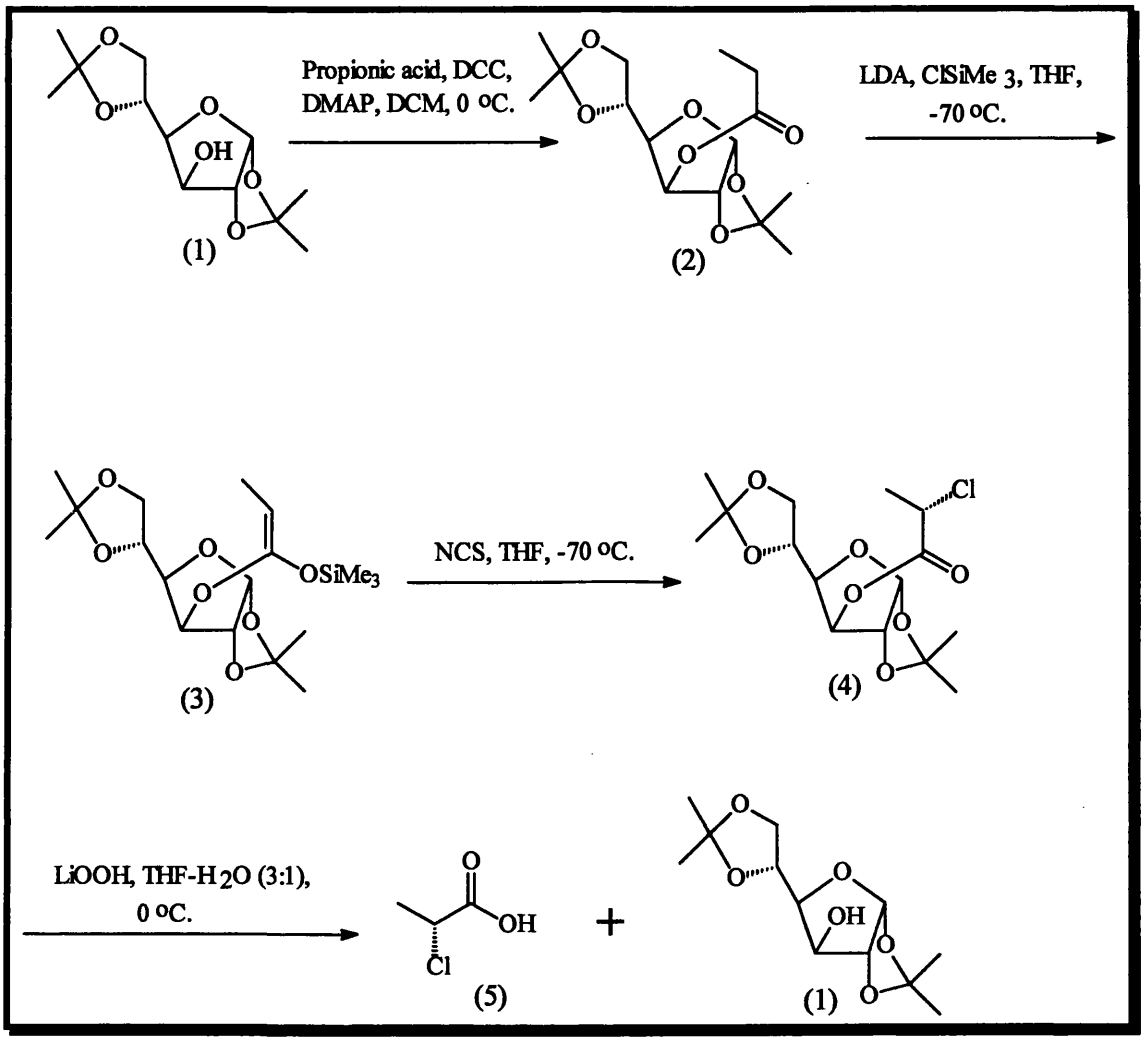
Figure 2: Formation of 2-phenoxypropionic acid from CPA

1.1 Chemical Synthesis of (2S)-CPA.

This section discusses the chemical methods available for the synthesis of optically pure CPA.

1.1.1 Chiral auxiliary method

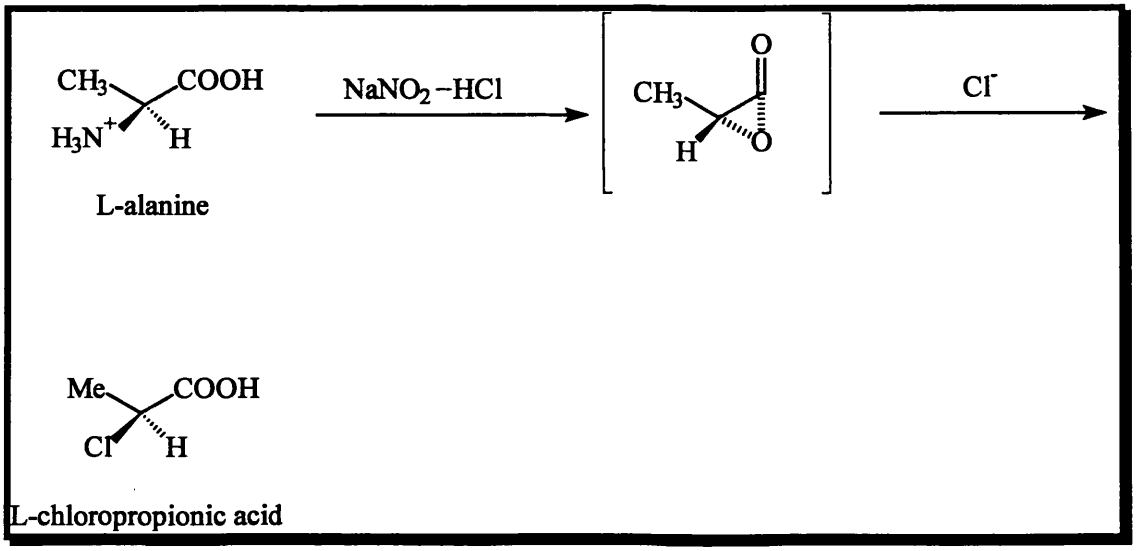
Dhuamel and co-workers synthesised (2*S*)-chloro and (2*S*)-bromopropionic acid in a four step strategy (**scheme 1**). Their synthesis started with the readily available chiral auxiliary, 1,2,5,6-di-*O*-(1-methylethylidene)-α-D-glucofuranose **1**, which was coupled to the propionic acid using a standard DCC coupling method to give the ester **2**. The silyl keteneacetal **3** was formed by the deprotonation of the α-proton from the ester **1**, using LDA as a base and the enolate formed was trapped by trimethylchlorosilane at -70 °C. The *E* configuration (9:1) was assigned to the major product of the reaction. Halogenation of the crude silyl ketene acetal **3** with NCS or NBS proceeded rapidly in THF at -70 °C leading to the bromoester or chloroester **4** in d.e. 89-96% depending on the solvent used. Hydrolysis of the chloroester **4** using lithium hydroperoxide in THF-water (3:1) at 0 °C furnished the (2*S*)-chloropropionic **5** acid in 99% e.e. The auxiliary **1** was retrieved in 80% yield (*Dhuamel, 1995*).



Scheme 1: Chiral auxiliary, 1,2,5,6-di-O-(1-methylethylidene)-α-D-glucofuranose (**1**) based enantioselective synthesis of (2S)-CPA

1.1.2 Amino acid based synthesis

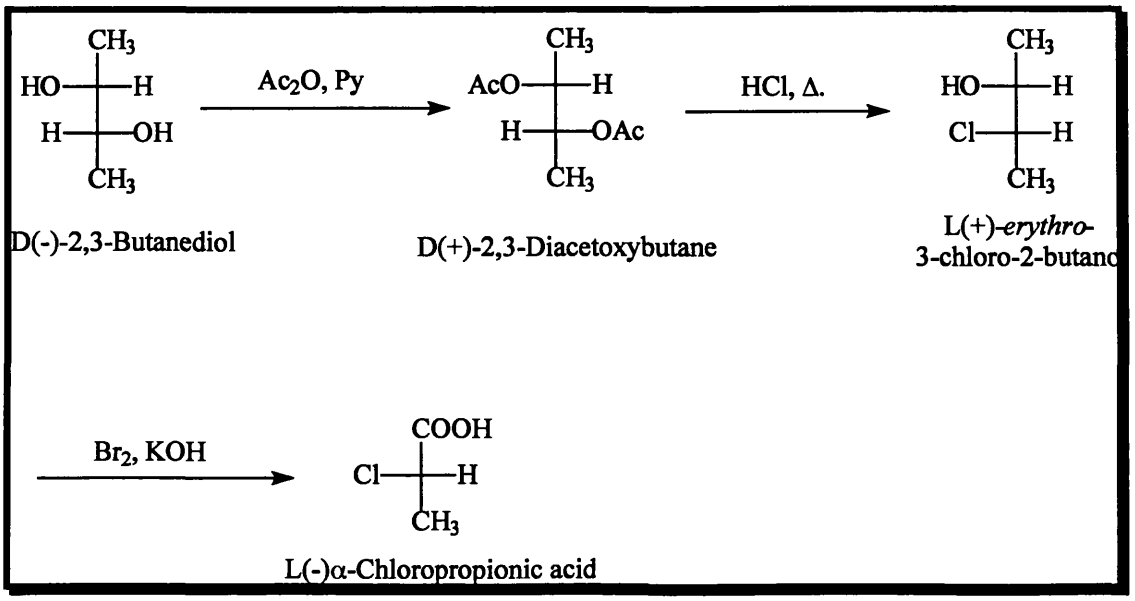
Aasen and *et.al* have synthesised (2*S*)-chloropropionic acid in a one step synthesis from L-alanine (**Scheme 2**). The amino acid was treated with nitrous acid in the presence of hydrochloric acid. The reaction intermediate was a three membered lactone formed with inversion of configuration. The lactone was opened with inversion of configuration by a chloride attack at α-carbon, to give (2*S*)-chloropropionic acid in 71% yield and 85% e.e. (*Aasen, 1984*).



Scheme 2: One step enantioselective synthesis of (2*S*)-CPA from amino acid

1.1.3 Diol based synthesis

Garner and co-workers used D(-)-2,3-butanediol as a starting material for their three step synthesis of (2*S*)-chloropropionic acid (**scheme 3**). Butanediol was protected with acetate protecting groups using pyridine or sulphuric acid as a catalyst. The diacetoxybutane was converted to L (+)*erythro*-3-chloro-2-butanol with hydrochloric acid and the configuration at C-3 was inverted by an S_N2 displacement of the acetoxy group by chlorine (Walden inversion). The chlorohydrin was transformed to *S*-chloropropionic acid using bromine and potassium hydroxide in 16% yield and 80% e.e. (*Garner, 1948*).



Scheme 3: Synthesis of (2*S*)-CPA from D(-)-2,3-Butanediol

None of the above methods are considered suitable for the industrial manufacture of (2*S*)-CPA because the method which uses chiral auxiliary requires:

- Very low temperatures for most of the transformations.
- Silyl protecting groups, which are expensive and air sensitive.
- Anhydrous conditions because of the nature of the reagents (LDA, trimethylchlorosilane) employed for the transformations.

The synthesis from butanediol is very inefficient because the last step of the synthesis only yields 16% (2*S*)-CPA with an 80 % enantiomeric excess. Finally the method based on the amino acid as a starting material yields (2*S*)-CPA in moderate yields and enantiomeric excess (71%, 85% respectively), but the reaction time is long (25 h) and the temperature has to be maintained at 0 °C.

In short none of these chemical methods provides an economical route to (2*S*)-CPA production which has a high enantiomeric excess.

1.2 Biochemical methods for the manufacture of (2S)-CPA

This section highlights four dehalogenase enzymes available for the potential production of CPA. The mechanisms of their action are explored and their specificity is discussed.

1.2.1 2-Haloacid Dehalogenases

2-Haloacid dehalogenases (DEX) catalyse the dehalogenation reaction (**fig 3**) of α-halo substituted carboxylic acids to produce the corresponding 2-hydroxy acids (*Soda, 1994*).

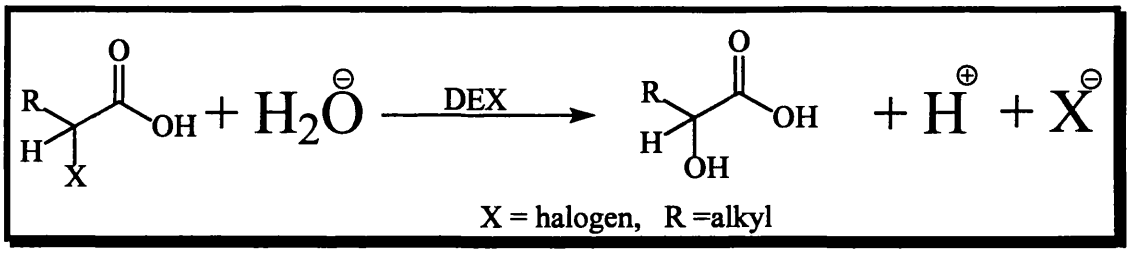


Figure 3: Dehalogenation of α-halo carboxylic acids using dehalogenase

DEX have been isolated and purified from several strains of *Pseudomonas*. The enzymes are classified into four types based on their substrate specificities and the configuration of the products. L-Dex specifically acts on *S*-isomers of 2-chloropropionate (2-CPA) and several other 2-haloacids and forms D-lactate (inversion) and other corresponding D-2-hydroxy acids. D-DEX acts exclusively on D-2-haloacids (e.g., 2D-CPA) and forms the corresponding L-2-hydroxy acids (e.g., L-lactate) (inversion). The two types of DL-DEX catalyse the dehalogenation of both isomers of 2-haloacids such as 2-CPA. The reaction of one DL-DEX proceeds with inversion of the configuration, and that of the other proceeds with a retention of configuration (*Soda, 1995*).

1.2.2 Mechanism of L-2-DEX of *Pseudomonas* sp. YL:

The carboxylate group of Asp^{10} acts as a nucleophile to attack the α-carbon of L-2-haloacid, leading to the formation of an ester intermediate, with inversion. This is hydrolysed by the attack of a water molecule activated by a basic amino acid residue of the enzyme to give 2-hydroxy acid as a final product with the inversion of configuration. (**fig 4**) (*Soda 1995*).

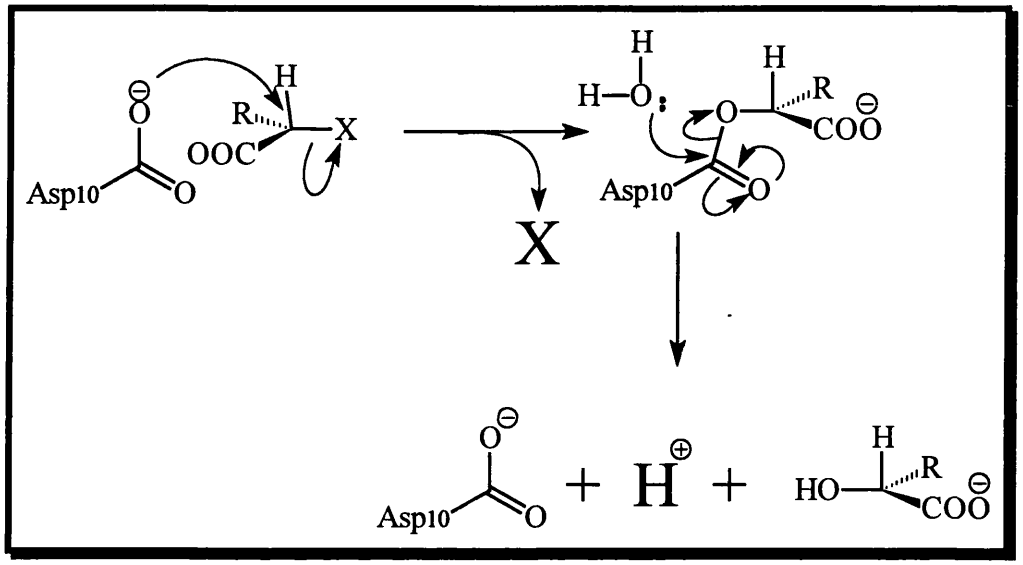


Figure 4: Dehalogenation mechanism of L-2-DEX from *Pseudomonas* sp.YL

1.2.3 Mechanism of DL-2-DEX retention type ($DL\text{-}DEX_r$)

The DL-DEXr reaction proceeds with retention of the C2-configuration of the substrate (**fig 5**). The enzyme catalyses the hydrolytic cleavage of the carbon-halogen bond of 2-haloalkanoic acids with C2-C4. The reaction involves a double inversion resulting in the retention of the C2-configuration of the substrate (*Soda 1996*).

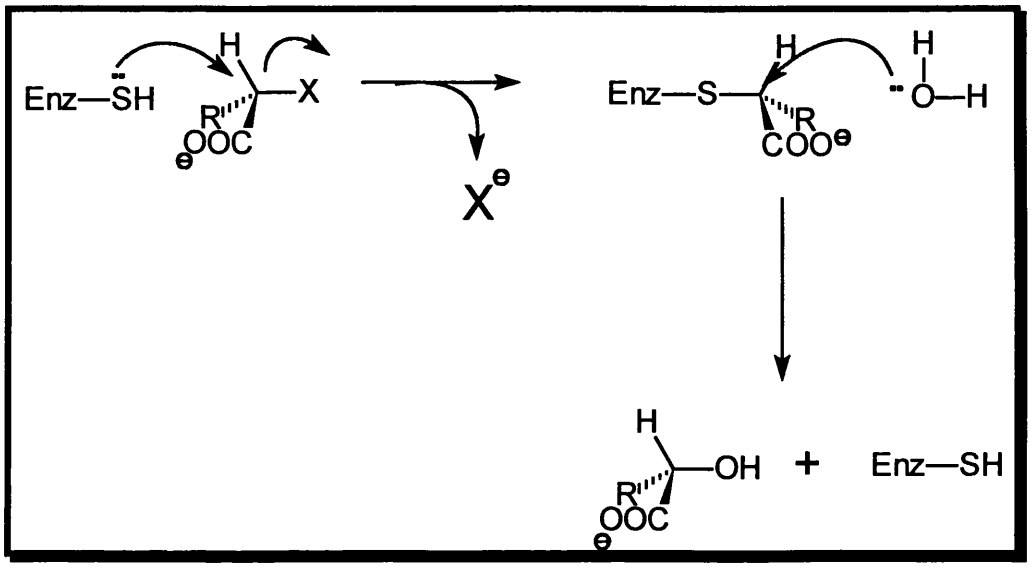


Figure 5: Dehalogenation mechanism of DL-2-DEX from *Pseudomonas sp.*YL (retention type)

1.2.4 Mechanism of DL-2-DEX (inversion type)

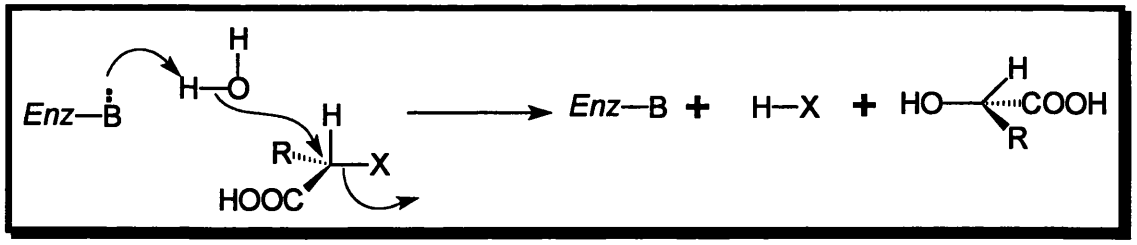
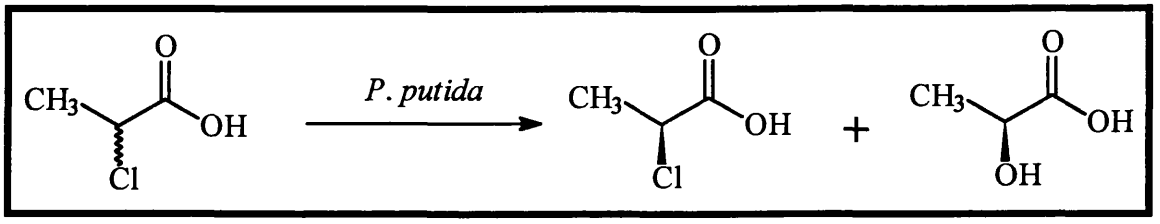


Figure 6: Mechanism of dehalogenation using DL-2-DEX (inversion type)

In this mechanism water is activated by a catalytic base (Asp^{10}) of the enzyme and directly attacks the α-carbon of L-haloalkanoic acid to displace the halogen atom leading to an inversion of configuration at the α-carbon (**fig 6**).

1.2.5 Current process for the production of (2*S*)-CPA

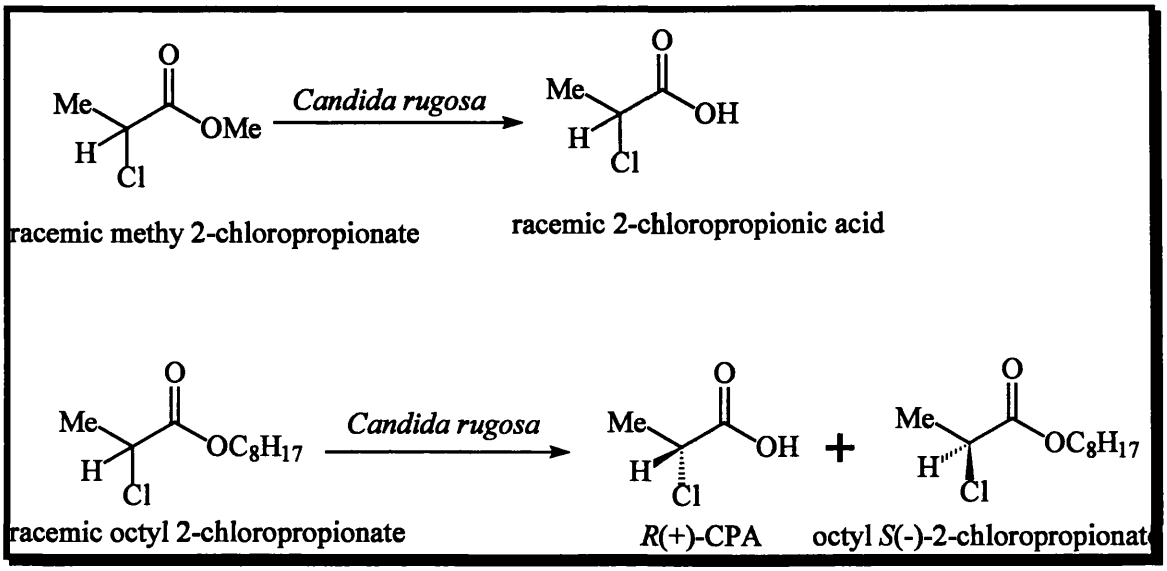
Avecia are currently manufacturing (2*S*)-CPA using dehalogenase technology. The whole cell biotransformation is carried out using *Pseudomonas putida* NCIMB 12018, which shows specificity towards *R*-CPA. In the process *R*-CPA is preferentially dehalogenated with inversion of stereochemistry to produce L-lactic acid (**scheme 4**). The residual (2*S*)-CPA is solvent extracted with an enantiomeric excess of >98 % and in 48% overall yield (*Taylor, 1990*).



Scheme 4: Resolution of racemic CPA using dehalogenase technology to produce (2*S*)-CPA and (2*S*)-lactic acid

1.3 Resolution of CPA as an Esterase process.

Klibanov and coworkers have found that the lipase from *Candida rugosa* selectively hydrolyses octyl 2-*R*-chloropropionate while, the octyl (2*S*)-chloropropionate is not hydrolysed (E_R = 196) (**scheme 5**). At the same time, the enzyme exhibits no appreciable stereospecificity in the hydrolysis of the methyl ester of the same acid. Solubility determination experiments showed that at the concentrations used, methyl 2-chloropropionate was completely soluble in water, whereas the octyl ester existed as an emulsion in water. It has been speculated that in order to express its stereospecificity the lipase needs to adsorb on the substrate-water interface (*Klibanov, 1984*).



Scheme 5: Resolution of CPA by enzymatic hydrolysis of its racemic esters

Dahod *et al.* (Dahod, 1987) found that some stereospecificity was displayed when excess insoluble methyl-2-chloropropionate (MCP) was present in the reaction mixture. They concluded that the stereospecificity would improve if the ester was made less soluble in water. The solubility can be reduced in various ways. An organic solvent can be used to dissolve MCP and then the organic phase can be dispersed in the aqueous phase. This will partition MCP away from the aqueous phase. A reduction in temperature will also reduce the solubility. Salts can also be used to salt out the MCP from the aqueous phase.

Several solvents were tested for their effect on reaction rates, stereospecificity, enzyme stability, and emulsion formation properties. Heavily chlorinated solvents gave better results than light organic solvents. Carbon tetrachloride and perchloroethylene gave superior results compared with other chlorinated solvents. Reactions with carbon tetrachloride gave better stereospecificity at 4 °C compared with 22 °C. As expected, the reaction rates were lower at 4 °C (*Dahod, 1987*).

In theory the resolution of chloropropionate esters ought to provide a convenient route to (2*S*)-CPA. If successful it should allow the *R*-enantiomer to be recycled so that there is no loss of the chlorine which is so wasteful in the chlorohydrolase process.

1.4 Hydrolysis of esters:

Ester hydrolysis is usually catalysed by acids or bases (**fig 7**). Since the group OR is a much poorer leaving group than halide or OCOR, water alone does not hydrolyse most esters. When the hydrolysis is base catalysed, the attacking species is the more powerful nucleophile OH^-. This reaction is called *saponification* and gives the salt of the acid. Acid catalyses the reaction by making the carbonyl carbon more electrophilic and therefore more susceptible to attack by the nucleophile. Both reactions are equilibrium reactions, so they are practicable only when there is a way of shifting the equilibrium to the right. Since the formation of the carboxylate anion does just this, ester hydrolysis is almost always done for the preparative purposes in basic solution, unless the compound is base sensitive (*March, 1992*). For example ester hydrolysis can also be catalysed by metal ions, by enzymes (*Zhu, 1990*), by cyclodextrins (*Saenger, 1980*) , and nucleophiles (*Satchell, 1969*).

The mechanism of action of ester hydrolysis in enzymes (**fig 8**) is similar to that found in base catalysed hydrolyses (**fig 7**). It involves the addition of the nucleophile to the carbonyl group with the formation of a tetrahedral intermediate, which subsequently collapses with the liberation of the free alcohol. The acyl enzyme intermediate formed then collapses by the nucleophilic attack of water to yield the carboxylic ester (*Hanson, 1995*).

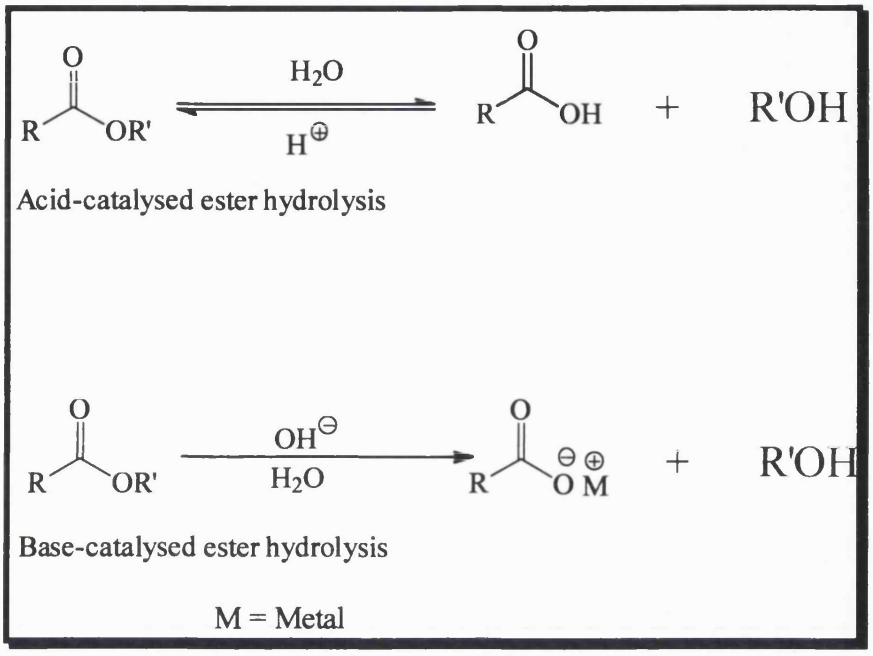


Figure 7: Acid and base catalysed chemical hydrolysis of carboxylic esters

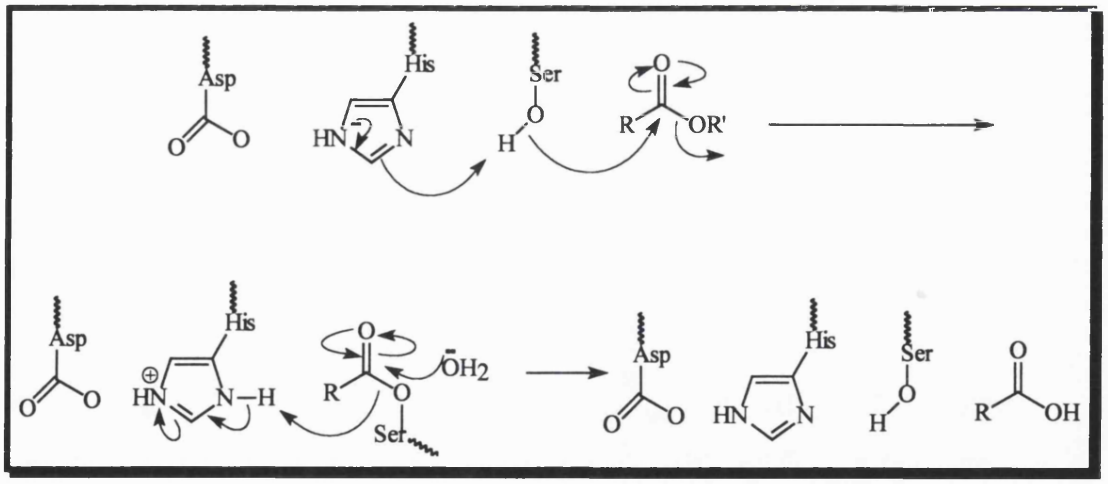


Figure 8: General mechanism of enzyme catalysed hydrolysis of carboxylic esters

1.5 Enzymatic hydrolysis of esters

Lipases and esterases have been used for the preparative resolution of racemic alcohols and carboxylic acids. The approach involves the chemical conversion of a racemic alcohol or acid to the corresponding ester, followed by a lipase catalysed asymmetric hydrolysis of the later.

Lipases catalyse the hydrolysis of esters in two phase systems (*Klibanov, 1984, Cipiciani, 1998, Caglar, 1981*). Lipases bind at the interface of the aqueous and organic phases, and catalyse hydrolysis at this point. This binding not only places the lipase close to the substrate, but also increases the catalytic power of the lipase, a phenomenon called interfacial activation (*Brockman, 1973*). Most lipases are poor catalysts in the absence of an interface such as that which occurs with an organic droplet or a micelle (Faber, *1992*).

1.5.1 Interfacial activation

The molecular rationale for this phenomenon has been thought to be a rearrangement process within the enzyme. The serine residue at the active site is protected by a flap (or α-helical lid) which opens when lipase comes in contact with an interface and thus leads to restructuring of the lipase (**fig 9**) by creating an electrophilic region around the serine residue, by exposing hydrophobic residues, and by burying hydrophilic ones, all of which increase the affinity of the complex for the lipid substrate and help stabilise the transition state intermediate during catalysis (*Hunziker, 1990, Camillau 1993*).

Lipases that also catalyse hydrolysis of unnatural esters are mostly enantiospecific, thus enabling chemists to generate enantiomerically rich starting materials. These starting materials are required for the next generation of pharmaceuticals, which will be enantiomerically pure to maximise their potency and to minimise side effects (*Faber, 1992, Olivo, 1993, Casey, 1994*).

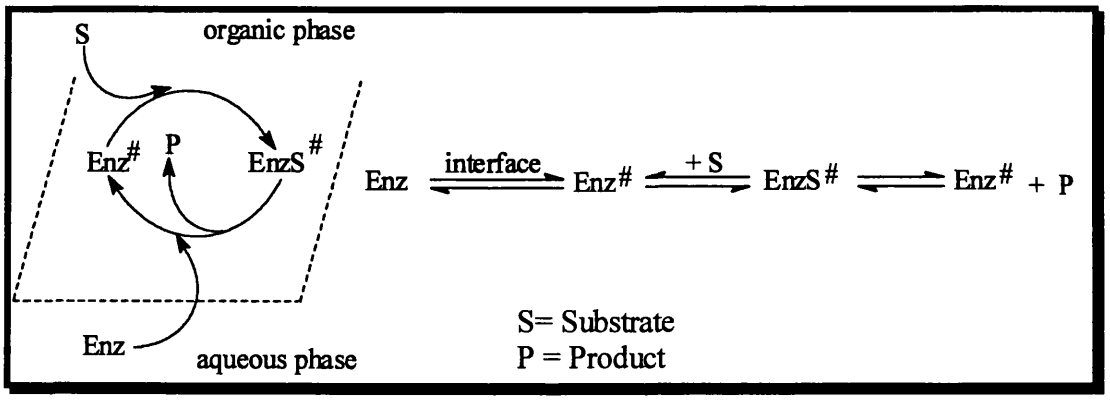


Figure 9: Graphical representation of interfacial activation

1.5.2 Substrate-types for esterases and proteases

The structural features of more than 90% of the substrates which have been transformed by esterases and proteases can be reduced to the following general formulae given in **fig 10** (*Faber, 1992*).

For both esters of the general type 1 and 2, the centre of chirality (as indicated with an asterisk [*]), should be located as close as possible to the site of the reaction, that is the carbonyl group of the ester. Thus, α-substituted carboxylates and esters of secondary alcohols are usually more specifically hydrolysed than their β-substituted counterparts and esters of chiral primary alcohols, respectively. Both substituents R_1 and R_2 can be alkyl- or aryl- groups, but they should differ in size and polarity to aid the chiral recognition process of the enzyme. They may also be joined together to form cyclic structures. The alcohol moieties (R_3) of type 1 esters should be as short as possible preferably methyl, cyanomethyl, ethyl or 2-haloethyl since carboxylates bearing long chain alcohols are usually hydrolysed at reduced reaction rates with esterases and proteases. The same is true for acylates of type 2, where acetates, haloacetates or propionates are the preferred acyl moieties (Faber, *1992*).

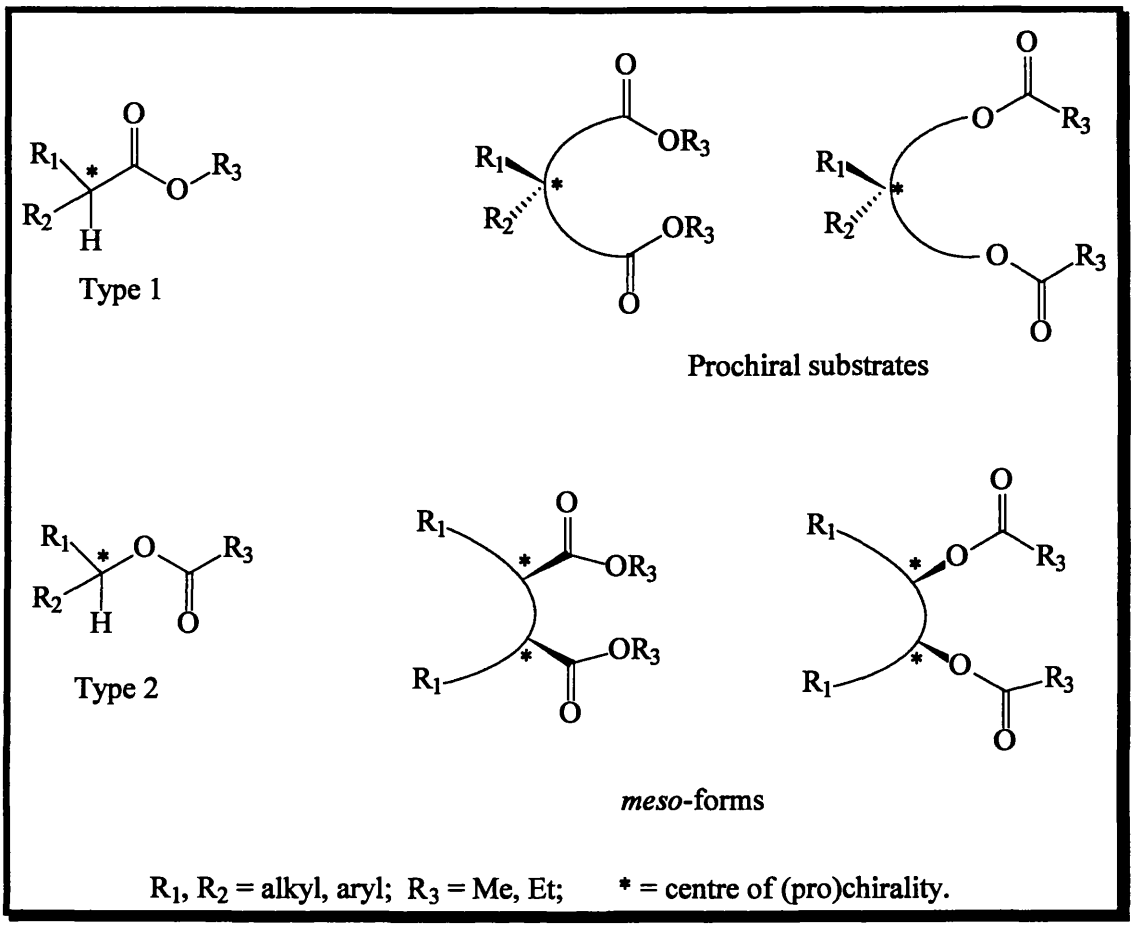


Figure 10: Substrate types for esterases and proteases

1.5.3 Substrate-types for lipases

Some of the general rules for substrate type are the same as those for esterase-substrate, such as the preferred close location of the chiral center and the necessity of having a hydrogen atom on the carbon atom bearing the chiral or prochiral centre. Other features are different. The acid moiety R_3 of the lipase-substrate of type I (**fig 11**) should be a straight-chain possessing at least three to four carbon units to ensure a high lipophilicity of the substrate. The majority of lipases show the same stereochemical preference for esters of secondary alcohols. Assuming that the sequence rule order of substituents R_1 and R_2 is large > small, the preferably accepted enantiomer lipase-substrate of type I possesses the (*R*)-configuration at the alcoholic center. Many proteases and in particular pig liver esterase exhibits a stereochemical preference opposite to that of lipases. Thus

the stereochemical outcome of an asymmetric hydrolysis can be directed by choosing a hydrolase from a different class.

Substrate-type 2 (**fig 11**) represents the general structure of a smaller number of esters which are hydrolysed by lipases. When type-2 substrates are hydrolysed by a lipase, the alcohol moiety R_3 should preferentially consist of a long straight-chain alcohol such as *n*-butanol (*Faber, 1992*).

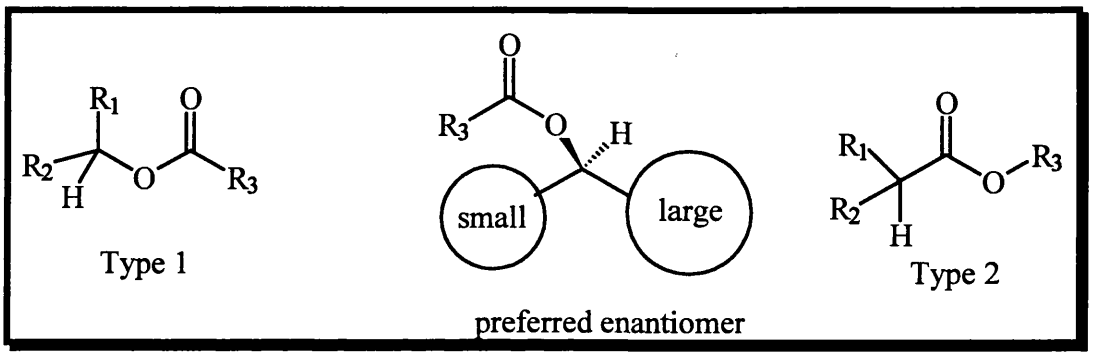


Figure 11: Substrate types for lipases

1.5.4 Mechanism of lipase catalysed hydrolysis of esters

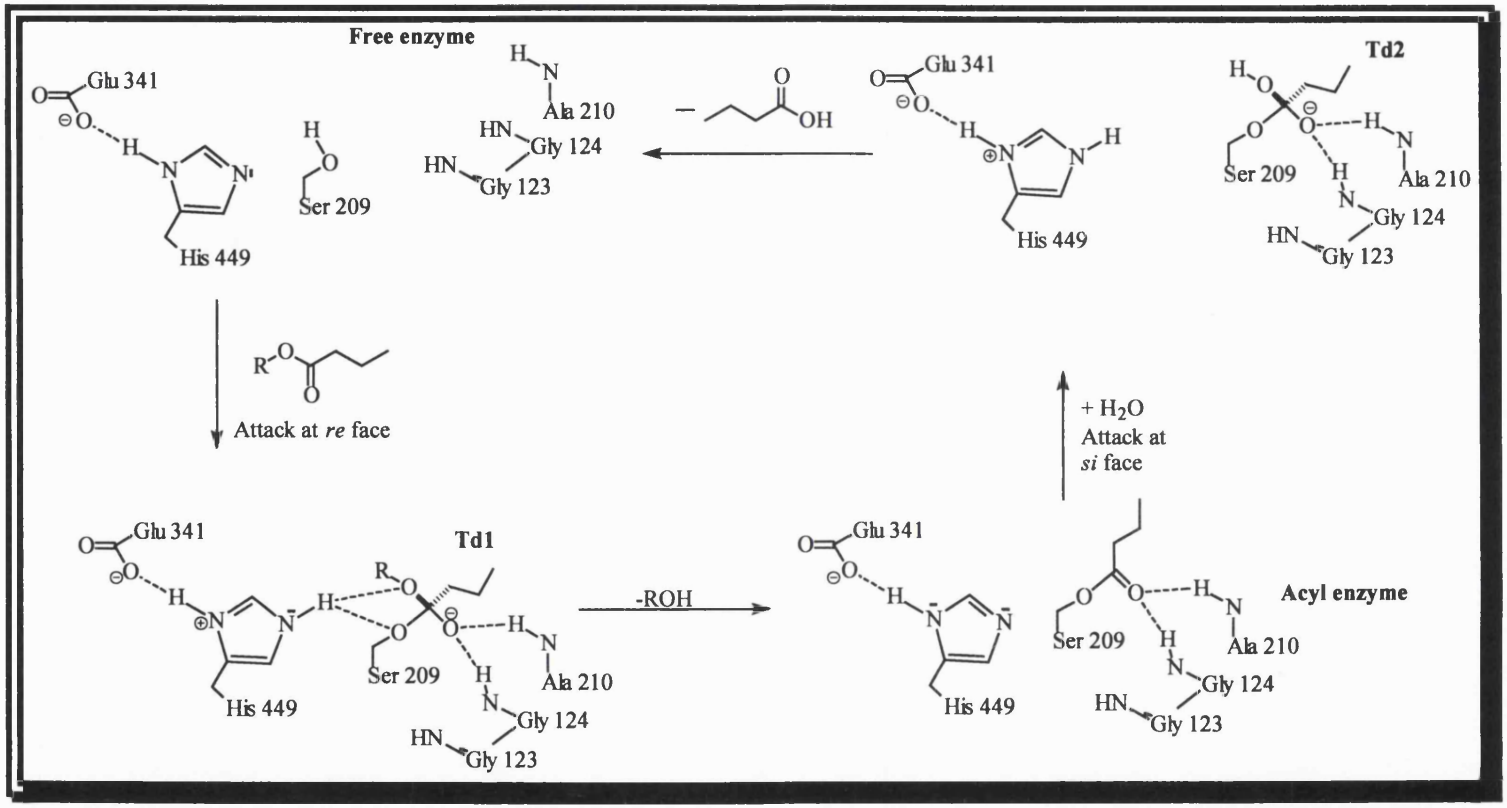
The hydrolysis of an ester (e.g. butyrate ester) catalysed by a lipase involves an acyl enzyme and two different tetrahedral intermediates (**scheme 6**). If the transition state for the reaction resembles the first tetrahedral intermediate, T_d1, then acylation limits the rate, and if the transition state resembles the second tetrahedral intermediate, T_d2, then deacylation limits the rate. During the formation of T_d1 in the reaction catalysed by the lipase from *Candida rugosa* (CRL) ser 209 attacks the ester at the *re* face (below the plane of page); however, during the formation of T_d2, water attacks at the *si* face (above the plane of page) of the acyl enzyme as shown in **scheme 6** (*Romas, 1994*).

The transition state that resembles T_d1 determines the specificity of the lipase towards alcohols, because the alcohol leaves as the acyl enzyme forms. On the other hand, a

transition state that resembles either T_d1 or T_d2 can determine the selectivity of the lipase towards carboxylic acids, because both transition states contain the carboxylic acid moiety. Thus, the specificity of a lipase towards carboxylic acids may depend on whether the formation, or the collapse, of the acyl enzyme limits the rate of reaction (*Romas 1994*).

1.5.5 Substrate binding site in lipase

X-ray crystal structures of transition state analogues bound to the active site of lipases have identified distinct binding sites for the alcohol and acid portion of the esters. The alcohol binding site is similar in all lipases. It is a crevice containing two regions- a large hydrophobic pocket which is open to the solvent and a small pocket that faces the floor of the crevice. The shape of the pocket sets the stereoselectivity of the lipases towards the secondary alcohols. The binding site for the acid portion of the ester varies considerably among the lipases. In *C. rugosa* the acyl chain binds in a tunnel long enough to accomodate at least an 18 carbon chain. The α-carbon of an acyl chain binds just below the large hydrophobic region of the alcohol binding site. The substituents at the α-carbon can extend into the hydrophobic pocket (*Romas 1994*).



Scheme 6: Mechanism of *Candida rugosa* lipase catalysed hydrolysis of butyrate

1.5.6 Optimisation of Selectivity

Many enantiospecific enzymatic hydrolyses of non-natural esters do not show a perfect selectivity, but are often in the range of 50-90% e.e. which is considered as being 'moderate' to 'good'. There are some methods which are used to improve the selectivity of an enzyme transformation itself.

Substrate-modification is one of the most promising techniques. This is applicable to all types of enzymatic transformations. The ability of an enzyme to 'recognise' the chirality of a given substrate strongly depends on its steric shape. Thus by variation of the substrate structure, most easily performed by the addition or removal of protective groups of different size and/or polarity, a better fit of the substrate may be achieved which leads to an improved selectivity of the enzyme.

Increasing the enantioselectivity of the lipase with the addition of organic solvents is a widely used method (*Klibanov, 1985, Cipiciani, 1998*), but finding a suitable solvent still remains a trial and error process. Nonpolar solvents are usually better than polar solvents. The choice of solvent is divide into three groups according to their log *P* values. Lipase shows low activity in solvents with log *P* values less than 2, which includes polar solvents like methanol, acetone, pyridine, and diethyl ether. Biocatalytic activity is difficult to predict for solvents of moderate polarity (log*P* 2-4) such as octanol, toluene and hexane. Lipases are usually active in nonpolar solvents (log *P* >4) such as decane. It is believed that lipase must retain an essential shell of water to remain active in organic solvents. Nonpolar solvents do not affect this shell of water, while polar solvents remove this necessary shell thereby inactivating the lipase (*Kelley, 1998*).

Variation of the solvent system by the addition of water-miscible organic cosolvents such as methanol, *tert*-butanol, acetone, dioxane, acetonitrile, dimethyl formamide (DMF) and dimethyl sulphoxide (DMSO) is a frequently used method to improve the selectivity of hydrolytic enzymes, in particular with esterases.

Optimisation of the reaction conditions such as adjustment of the *p*H or variation in the temperature usually counts for only minor changes in selectivity (*Faber, 1992*).

1.5.7 Immobilisation of lipase

Lipase powders are insoluble in organic solvents and can be recovered by simple filtration at the end of reaction. Unfortunately, even after optimising the solvent and water content, catalysis is often very slow when compared to the rate in water or water-organic solvent mixtures. One reason for the drop in activity is diffusional limitations, that is, the substrate cannot reach the lipase molecules in the centre of the particle. The simplest solution to the problem is found by adsorbing the lipase on an insoluble support. Adsorption both increases the surface area and also avoids lyophilisation of the lipase.

1.5.7.1 Adsorption

Adsorption, the longest established technique for the noncovalent immobilisation of enzymes, is based on physical adsorption or ionic binding, or both, of the enzyme to the surface of the support (*Basri, 1995*).

The nature of the enzyme and the carrier influence both the amount of the enzyme bound and the activity of the final preparation. The greater the surface area of the carrier and, in general, the higher its hydrophilic/hydrophobic group ratio, the greater is the amount of the enzyme that may be bound. Immobilisation by physical adsorption or ionic binding, both low cost procedures, are simple and effective, and usually bring about little change in the overall conformation of the protein or its active site. During operational use noncovalently immobilised enzymes, whether physically adsorbed or ionically bound, may leak from the carrier because the binding force (s) between the protein and the carrier is weak.

In the case of physical adsorption, the forces responsible for immobilisation include hydrogen bonding, Van der Waals forces, and, importantly, hydrophobic interactions. Immobilisation via ionic binding involves salt linkage formation between charged groups on the protein and the opposite charges on the carrier.

Due to the nature of the forces involved in the noncovalent immobilisation of proteins, it is relatively simple to reverse the immobilisation process and so to effect desorption of the protein from the support. This can be problematic if the enzyme is to be reused or if the loss of protein into the reaction mixture is to be avoided (*Mojovic, 1998*).

1.5.7.2 Covalent immobilisation

The immobilisation of proteins on solid supports by covalent coupling (*Lopez, 1996, Wong 1992*) usually leads to very stable preparations with an extended active life when compared with immobilised protein preparations obtained from physical adsorption and ionic binding.

The covalent binding method is based on the covalent attachment of proteins to water-insoluble matrices. The selection of conditions for immobilisation by covalent binding is more difficult than in the other carrier binding methods. The reaction conditions are relatively complicated and not usually mild. The immobilisation of an enzyme by covalent attachment to the support involves functional groups that are not essential for its catalytic action, and thus the active site of the enzyme remains unaffected by the various reagents that are used.

Three main factors have to be taken into account for covalent immobilisation of proteins by a specific method :

- The functional group of proteins suitable for covalent binding under mild conditions.

- The coupling reactions between the protein and the support.

- The funcationalised supports suitable for protein immobilisation.

Covalent immobilisation of protein on an insoluble carrier allows repeated use of protein without any loss of protein into the solution even at the high ionic strength during the reaction, however, some enzyme activity is lost upon adsorption and then cross-linking (chapter 7). The covalent immobilised protein can be reused much more extensively, than the protein which is physically adsorbed onto the carrier (chapter 7).

1.6 Use of lipase in synthetic organic chemistry

Many organic molecules could be synthesised by a route which uses the catalytic activities of the enzymes. In recent years, interest in the use of lipases as synthetic chiral catalysts has risen rapidly due to their broad substrate specificities and no coenzyme requirement for the catalysis. Although the natural substrates of lipases are acylglycerols, they can also catalyse the hydrolysis of a wide range of artificial water-insoluble esters with a high degree of enantiospecificity (*Sih, 1990*).

The following are a few examples where lipases have been used in synthetic organic chemistry to obtain single enantiomer in high purity.

1.6.1 Kinetic resolutions:

Kinetic resolutions of selected esters have been used in the preparation of a number of important compounds including a synthon for the hypocholestemic *delta*-lactones (*Olivo, 1993*), anti-AIDS agents (*McCague, 1994*) and the anti-fungal agent called brefeldin-A (*Carnell, 1994*).

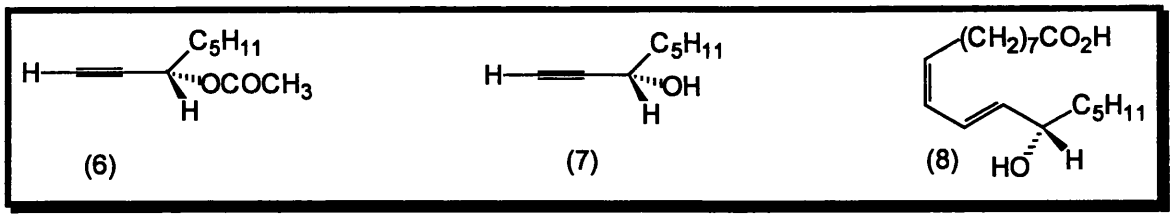


Figure 12: Intermediates formed during the synthesis of coriolic acid

The acetate **6** is hydrolysed enantioselectively using *Mucor miehei* lipases or lyophilised yeast cells (**fig 12**). The alcohol **7** is produced in 80-100% enantiomeric excess and this synthon is used in the preparation of 13-HODE (coriolic acid) **8**, a naturally occurring biologically active compound.

1.6.1.1 Asymmetrization of Meso-Esters:

Meso-diacetates such as compound **9** are interesting substrates in enzyme-catalysed transformations. "Asymmetrisation" can be observed (**fig 13**), where the dimethyl cyclopentane-dicarboxylate **9** is hydrolysed with excellent selectivity using pig liver esterase to give the mono-ester **10** in high yield and optical purity (96% yield, 98% e.e.) (*Cotterill, 1991a*). This type of enzyme catalysed transformation gives the possibility of forming optically pure products in quantitative yield from the chosen substrates, a process that is difficult to emulate using conventional catalysts.

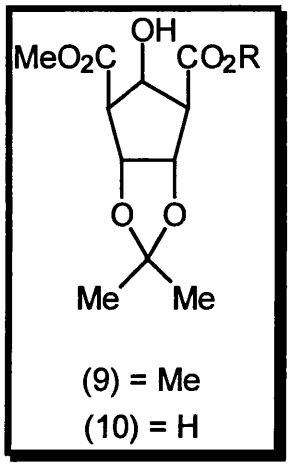


Figure 13: Meso diacetate

1.6.1.2 Esterification of racemic esters:

The racemic alcohol **11** in hexane was converted into the optically active ester **13** (90% e.e.) and optically enriched bicyclo[3.2.0]hept-2-en-6-*endo*-ol (**17, fig 14**) was recovered using cyclohexane carboxylic acid and the catalyst lipozyme (*Mucor meihei* lipase attached to an inert solid support) (*Cotterill, 1988a*)

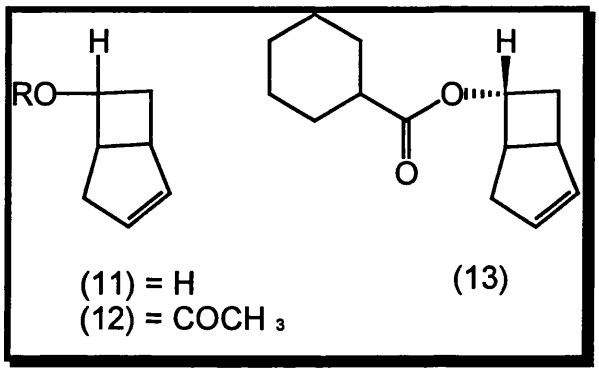
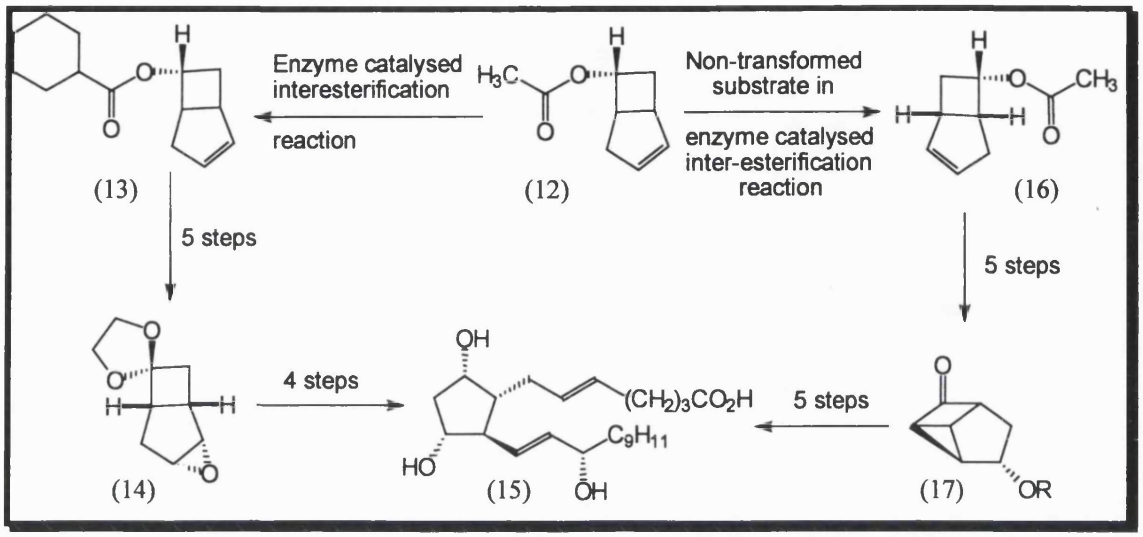


Figure 14: Intermediates in the synthesis of postaglandin-F2a

1.6.1.3 Inter-Esterification:

The ester **13** (**fig 14, scheme 7**) can be obtained in even higher optical purity (>99% e.e.) when employing an inter-esterification reaction involving the acetate **12** and cyclohexane carboxylic acid. The separated esters can be used in syntheses of postaglandin-F2a, a naturally occurring material with many biological activities (*Macfarlane, 1990*)



Scheme 7: Synthesis of postaglandin-F2a

The inter-esterification process has been extended to provide an example of double enantioselection (**fig 15**) by using the ester (±)-**12** and racemic 2-(*para*-chlorophenoxy)propanoic acid. One of the four possible diastereomeric esters, compound **18**, is formed almost exclusively (*Fowler, 1991*). Two stereoselective enzyme catalysed processes are in operation, namely the preferential hydrolysis of the 6(*R*)-acetate **12**, and selective acylation of the product alcohol by 2(-)-(*para*-chlorophenoxy)propanoic acid.

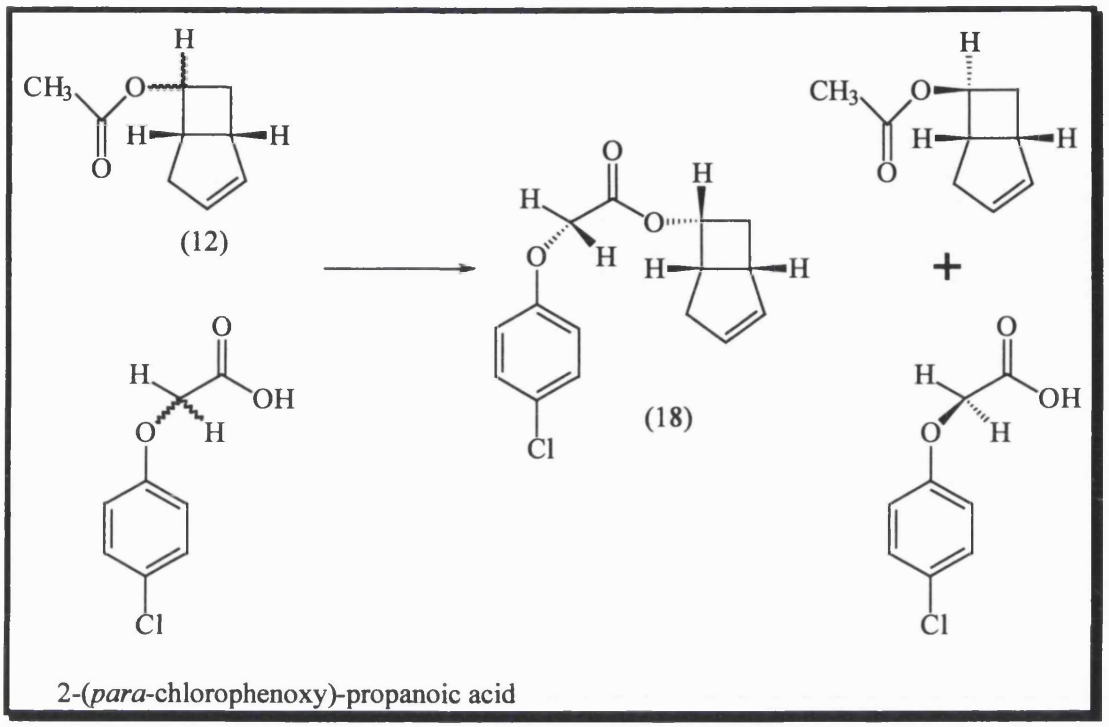
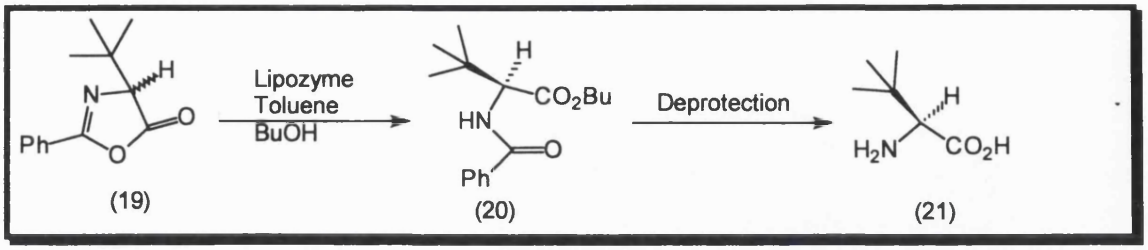


Figure 15: An example of double enantioselection

1.6.2 Dynamic resolution:

Substituted racemic azalactones e.g. **19** can be converted to the corresponding optically active amino acid derivatives **20** using a lipozyme under almost anhydrous conditions (**scheme 8**). In this process the azlactone starting material is able to racemise *in situ* leading to a high yield (94%) of the homochiral (99.5% e.e.) product **20**. A subsequent two step hydrolysis of **20** leads to L-(*S*)-*tert*-leucine **21**, an important amino acid that is used in therapeutic peptides and as a chiral auxiliary (*Turner, 1995*).



Scheme 8: Chemo-enzymatic synthesis of L-(S)-tert-leucine

1.7 Enzyme Kinetics

As outlined above, the current methods available for the chemical production of CPA are regarded as uneconomical. However, CPA has been commercially synthesised by Avecia using dehalogenase technology. We regard this method of production highly unsuitable for the synthesis of CPA for two main reasons. Firstly, this method converts the *R* enantiomer of CPA into D-(S) lactate, which presents us with a BOD (biological oxygen demand) problem, and also a large amount of chlorine is released as chloride. Secondly, the reaction can never produce CPA in more than 50% yield, as the *R* enantiomer is converted to D-lactate and cannot be recycled.

In the light of the above conclusions we plan to devise a column based system for the continuous production of enantiomerically pure CPA or one of its carboxylic ester. This process will be based on the lipase catalysed hydrolysis of CPA esters with a simple racemisation step incorporated into the system. The following section deals with the kinetics of lipase catalysed hydrolysis.

1.7.1 Irreversible case:

When an enzyme binds to both enantiomers (*R* & *S*) of a racemic mixture to form two diastereomeric complexes Enz-*R* and Enz-*S*, if the non-covalent bonding interactions with the enzyme surface are optimal for the *R*-enantiomer, it will be poised for efficient catalysis to give the product, *P*. In contrast, the non-covalent bonding interactions of the *S*-enantiomer with the enzyme will not be as favourable, and inefficient or slow catalysis of the Enz-*S* complex to Q follows (**fig 16**).

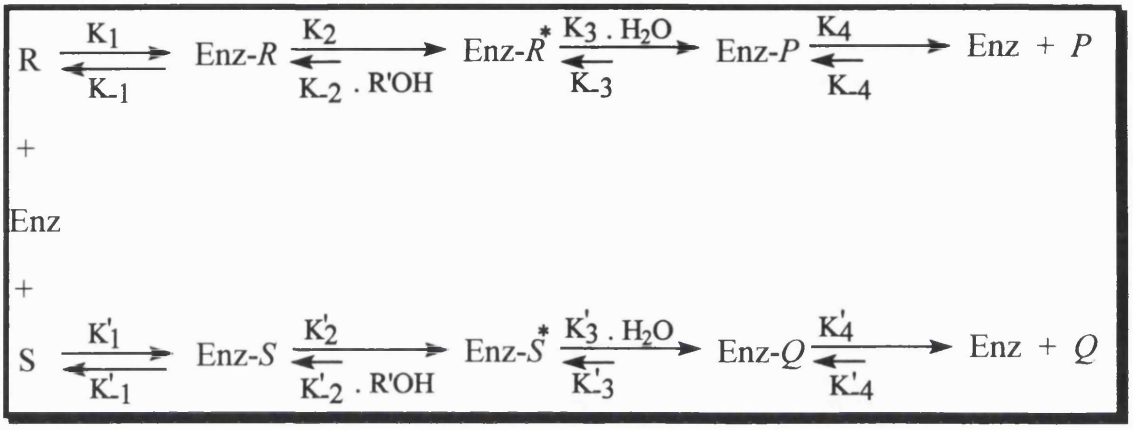


Figure 16: Irreversible binding of the substrate to the enzyme

The *R* and *S* enantiomers compete for free enzyme, Enz, to form the complexes Enz-*R* and Enz-*S*; they then undergo catalysis to give their respective acyl-enzymes, Enz-R^* and Enz-S^*. In turn, hydrolysis of the acyl-enzyme intermediates yields the enzyme-product complexes, Enz-*P* and Enz-*Q*, that dissociate to afford free enzyme, Enz, and the products, *P* and *Q*. In aqueous systems when the concentration of R'OH is low, the conversions of Enz-*R* → Enz-R^* and Enz-S → Enz-S^* becomes virtually irreversible (*Sih, 1989*).

1.7.2 Quantitative treatment of kinetic resolution data:

Consider a typical kinetic resolution experiment using two different ester hydrolyses, where one of the enantiomers is preferentially hydrolysed (**fig 17**).

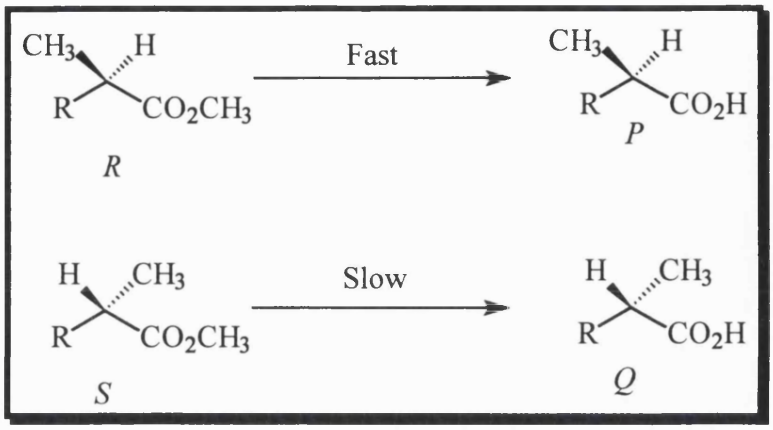


Figure 17: Kinetic resolution of two enantiomers by means of an enzyme catalysed hydrolysis of carboxylic esters

If the reaction for enzyme 1, is terminated at 69% conversion (c = 0.69) and the reaction for the enzyme 2 is terminated at 35% (c = 0.35). Then the following equation (**eq 1**) can be used to determine which of the above two enzymes is the more enantiospecific (*Sih, 1982*).

$$\frac{\ln\left(\left[1-c\right]\left[1-ee_s\right]\right)}{\ln\left(\left[1-c\right]\left[1+ee_s\right]\right)} = E_R$$

Equation 1: Equation to calculate the E_R of the enzyme for the starting material

Thus substituting the values of ee_s and c for the two enzymes, the E_R values for enzyme 1 and 2 can be calculated. A similar expression (**eq 2**) can be derived to relate ee_p (enantiomeric excess of the product), c, and E_R (*Sih 1982*):

$$\frac{\ln\left(1-c\left[1+ee_p\right]\right)}{\ln\left(1-c\left[1-ee_p\right]\right)} = E_R$$

Equation 2: Equation to calculate the E_R of the enzyme for the starting material

The value of c is usually determined experimentally by methods such as GC or HPLC, it is more accurate and convenient to calculate c by taking advantage of the following relationship (**eq 3**).

$$c = \frac{ee_s}{ee_s + ee_p}$$

Equation 3: Equation to calculate the degree of conversion

Hence, in kinetic resolution experiments, if the values of ee_s and ee_p are defined, the values of c and E_R may be calculated accurately from equations **1, 2, 3** (*Sih, 1989 and 1982*).

A theoretical plot of ee_s (enantiomeric excess of the residual substrate) and ee_p (enantiomeric excess of the product formed) as a function of *c* for various values of E_R is shown in **Fig 18**. The value of ee_s increases with the extent of conversion, c. Hence, highly enantiomerically enriched residual substrate may be obtained with enzymes of low E_R value by simply extending reaction times. However, this is at the expense of chemical yield.

Fig 18 shows the effect of a decrease in ee_p for values of *c* beyond 0.5. Therefore, if the product fraction is of interest, the reaction should be terminated before the conversion (*c*) is 0.5 irrespective of the value of E_R.

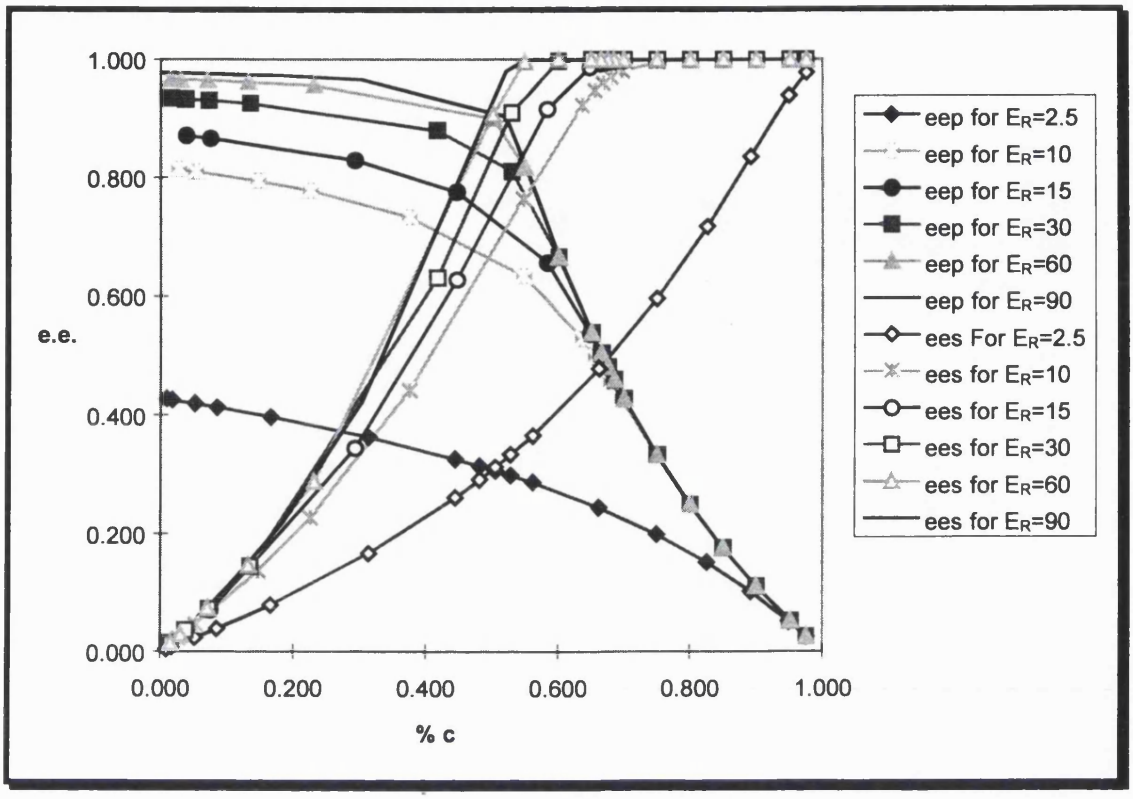


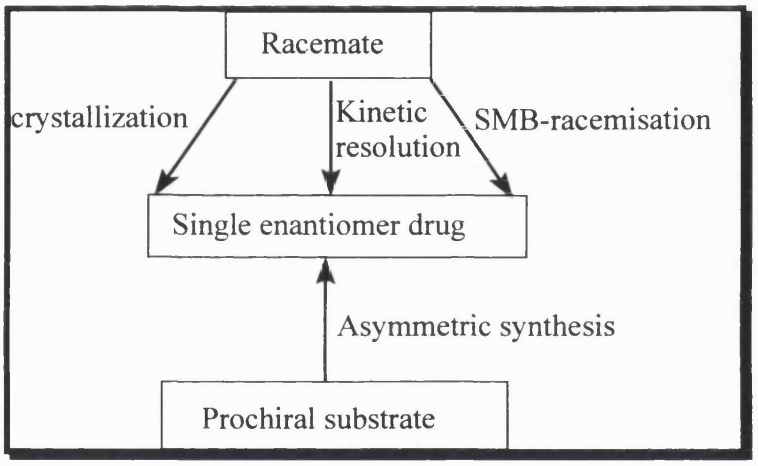
Figure 18: Plot of ee_s and ee_p during the enzyme catalysed hydrolysis of carboxylic esters

The plot of E_R for product was generated by relating variables *c* and ee_pin eq 3 to a function of x for values of $0 \leq X \geq 1$; $c = 1\text{-}X/2\text{-}X^E/2$; and $ee_p = (X\text{-}X^E)/(2\text{-}X\text{-}X^E)$. E_R for residual substrate is $ee_s = (c \times ee_p)/(1\text{-}c)$. (*Sih, 1982*)

CPA has been resolved by hydrolysis of its octyl ester (Klibanov, *1984)* using *C. rugosa* lipase. The only drawback of using this lipase is the lack of reproducibility of the results. This irreproducibility is attributed to the contaminating hydrolases with different specificities and activities present in the commercially available *C. rugosa* lipase. The contents of commercially available *C. rugosa* lipase very much depends on the source and the fermentation conditions, however, the specificity of the crude lipase can be enhanced by treatment with organic solvents (chapter 6)

1.8 Alternative methods of resolution

In recent years, there has been an increasing trend towards restricting the use of chiral drugs as racemates. Amounts ranging from 1 g up to 5 kg and more of optically pure compounds as chemical intermediates or for pharmacological and toxicological testing are now needed (*Cavoy, 1997*).



Scheme 9: Synthetic routes to the single enantiomer drugs

A number of synthetic strategies (**scheme 9**) are available for the preparation of single enantiomer drugs (*Gattuso, 1996*).

Chiral chromatography has become the most important analytical tool for determining the optical purity of organic molecules. Due to the development of a wide range of chiral stationary phases (CSPs) and new instrumentation available, the chromatographic separation of enantiomers on a preparative scale is also gaining acceptance as a simple, rapid and generally applicable method for supplying pure enantiomers of bioactive compounds and chiral synthons.

In elution batch chromatography, large scale separations require large amounts of CSP, hence, it is considered uneconomical because of the high costs of CSPs and the consumption of large amounts of the mobile phase.

Taking these problems into consideration Universal Oil Products (UOP) has developed Sorbex ® technology which employs the simulated moving bed (SMB) concept (**sec 1.8.2**), also known as countercurrent chromatography (**sec 1.8.1**). The advantages of continuous operation are increased flexibility, unattended operation and constant product quality. The technique can be readily scaled up from analytical HPLC to process and commercial scale. With SMB technology high purity can be obtained simultaneously with high recovery, a result which is difficult to achieve with preparative HPLC.

1.8.1 Continuous countercurrent process

A hypothetical moving bed system and a liquid phase composition profile is shown below (**fig 19**). The adsorbent circulates continuously as a dense bed in a closed cycle and moves up the adsorbent chamber from bottom to top. Liquid streams flow down through the bed in a countercurrent direction to the solid. The feed is assumed to be a binary mixture of A and B, with component A being adsorbed selectively. Feed is introduced to the bed as shown (**fig 19**).

Desorbent D is introduced to the bed at a higher level. This desorbent is a liquid of different boiling point from the feed components and can displace feed components from the pores. Conversely, feed components can displace desorbent from the pores with the proper adjustment of relative flow rates of solid and liquid.

Raffinate product, consisting of the less strongly adsorbed component B mixed with desorbent, is withdrawn from a position below the feed entry. Only a portion of the liquid flowing is withdrawn at this point; the remainder continues to flow into the next section of the bed. Extract product, consisting of the more strongly adsorbed

component A mixed with the desorbent is withdrawn from the bed again; only a portion of the flowing liquid in the bed is withdrawn, and the remainder continues to flow into the next bed section.

The positions of introduction and withdrawal of the net streams divide the bed into four zones, each of which performs a different function:

Zone 1. The primary function of this zone is to adsorb A from the liquid. The solid entering at the bottom carries only B and D in its pores. As the liquid stream flows downward, countercurrent to this solid, component A is transferred from the liquid stream into the pores of the solid. At the same time, component D is desorbed (transferred from the pores to the liquid stream) to make room for A.

Zone 2. The primary function of this zone is to remove B from the pores of the solid. When the solid arrives at the fresh feed point, the pores contain the quantity of A that was adsorbed in *zone 1.* However, the pores also contain a large quantity of B, because the solid has just been in contact with the fresh feed. The liquid entering at the top of *zone 2* contains no B, only A and D. As the solid moves upward, countercurrent to this stream, B is gradually displaced from the pores and is replaced by A and D. By proper regulation of the liquid rate in *zone 2*, B can be desorbed completely from the pores. This B desorption can be accomplished without simultaneously desorbing all of A, because A is more strongly adsorbed than B.

Zone 3. The function of this zone is to desorbe A from the pores. The solid entering the zone carries A and D in the pores; the liquid entering the top of the zone consists of pure D. As the solid rises. A in the pores is displaced by D.

Zone 4. The purpose of this zone is to act as a buffer to prevent component B, which is at the bottom of *zone 1*, from passing into *zone 3*, where it would contaminate extracted component A. When the adsorbent leaves *zone 3*, the pores are completely filled with desorbent. The liquid entering the top of *zone 4* is of raffinate composition

and B and D. If the flow rate in *zone 4* is properly regulated, component B will be readsorbed completely from the liquid, preventing its entry into *zone 3*, where it would contaminate the product A.

The use of the moving bed introduces the problem of mechanical erosion of the adsorbent. Obtaining uniform flow of both solid and liquid in beds of large diameter is also difficult. The performance of this type of operation can be greatly impaired by nonuniform flow of either phase (*Genbicki, 1991*).

1.8.2 Simulated moving bed operation

Simulated Moving Bed (SMB) chromatography is a continuous method of separation in which the sample is continuously injected and two product streams are continuously collected. SMB is thus a binary separation technique in which the original sample is split into two parts, the two product streams. If the sample contains many components, generally the component of interest is collected in one of the product streams, and all the other components are collected together in the other stream. If the sample contains only two components, as in chiral separations, each component can be collected in essentially pure form. The elution profile can be held in place if the stationary phase moves in one direction and the mobile phase moves in the other direction (**fig 20**) (*Gattuso, 1996[b]*).

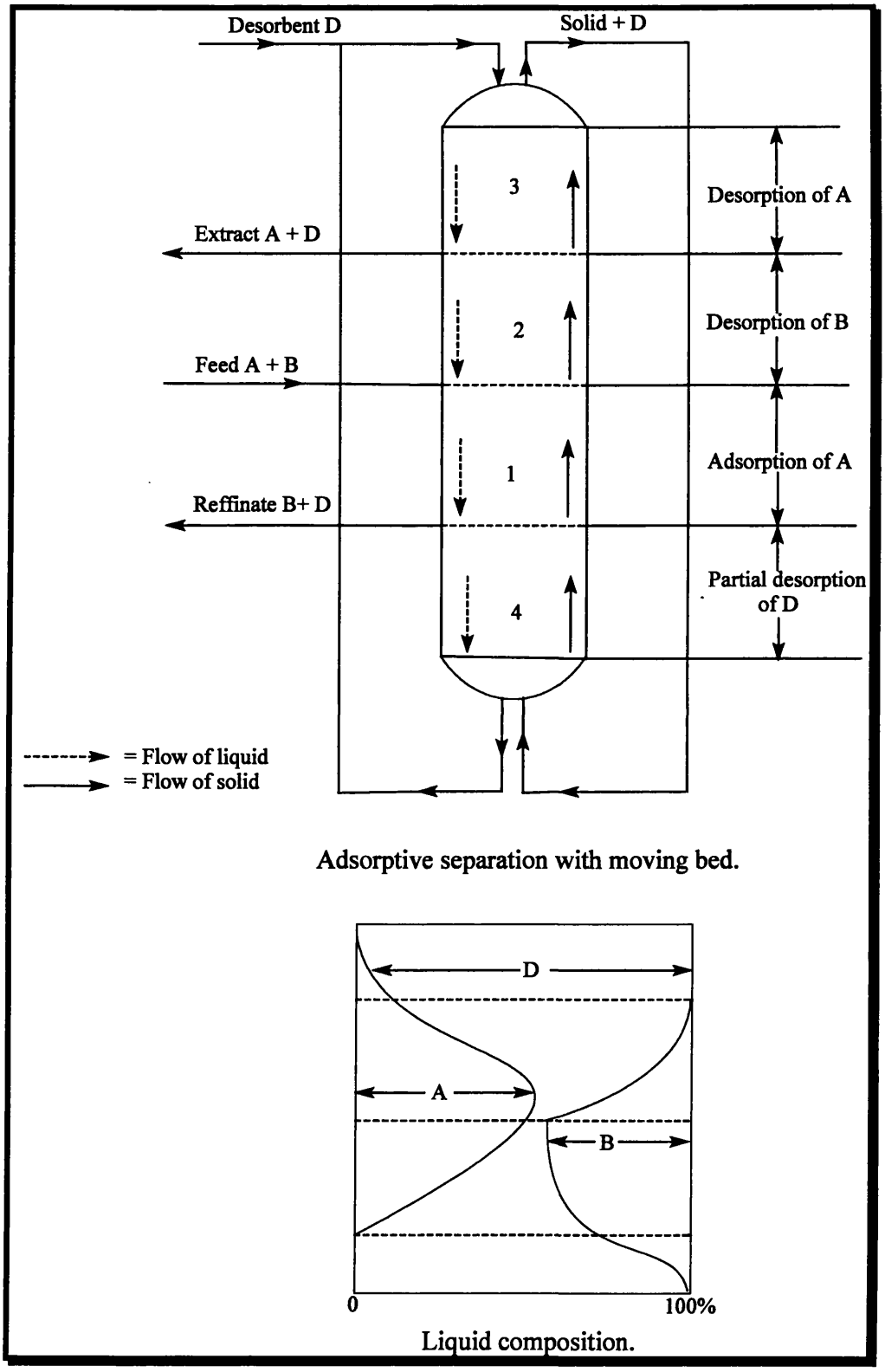


Figure 19: Continuous countercurrent process

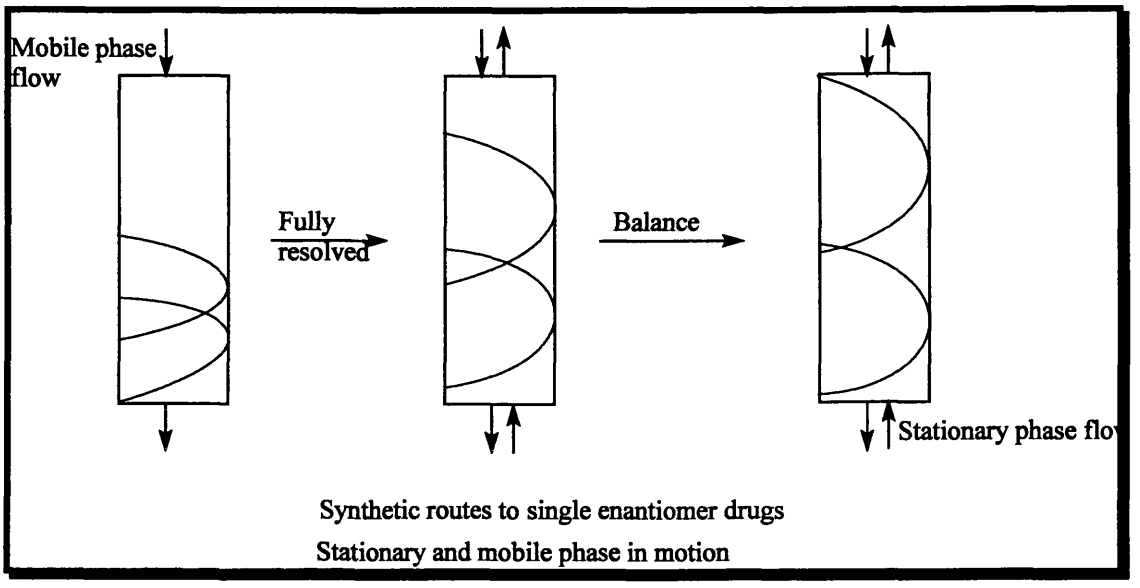


Figure 20: Simulated moving bed technology

If the feed is continuously introduced at the midpoint of the column, the concentration profile can be maintained, and the bands are spread out over the entire column. Although the profiles are only partially resolved, high purity products can be collected at either end (**fig 21**) (*Gattuso, 1996*).

For practical purposes, maintaining the movement of the stationary phase is difficult. If the movement of the stationary phase is stopped, the profile moves around the column, and the feed and withdrawal points are no longer in the correct position. To compensate, the feed and withdrawal points must be moved continuously, or indexed, to keep up with the profile. To avoid moving the feed and withdrawal points continuously, the feed and withdrawal points are indexed at discrete points. For ease of operation, the column is divided into sections, which consist of a number of individual columns arranged in series (**fig 22**). Now if the injection and collection points are regularly changed, the net result is the same as it would be if the stationary phase was moving. The number of column beds within each section and the total number of columns are adjustable.

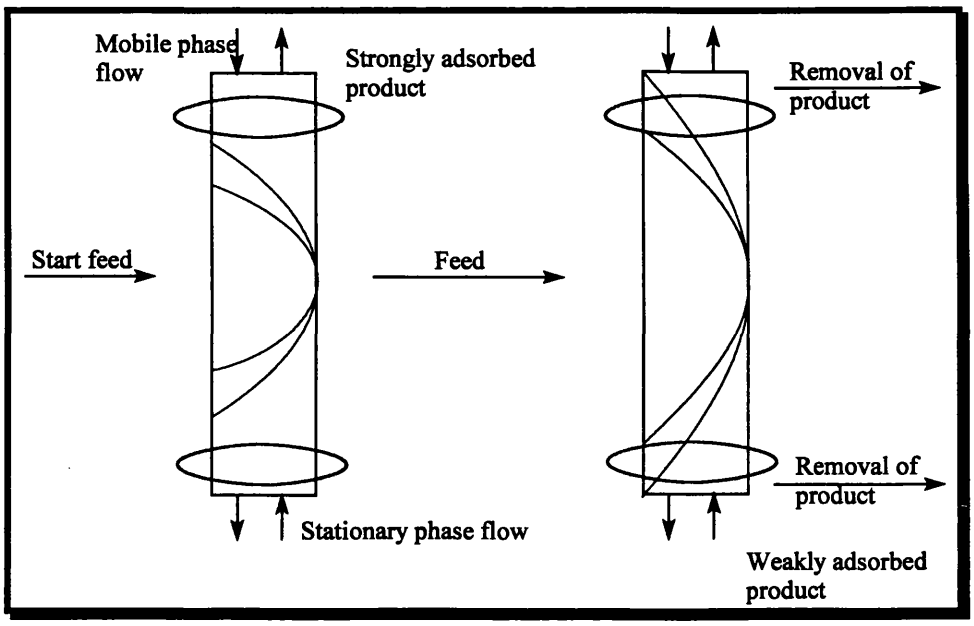


Figure 21: Continuous introduction of the feed in the midpoint of the simulated moving bed column

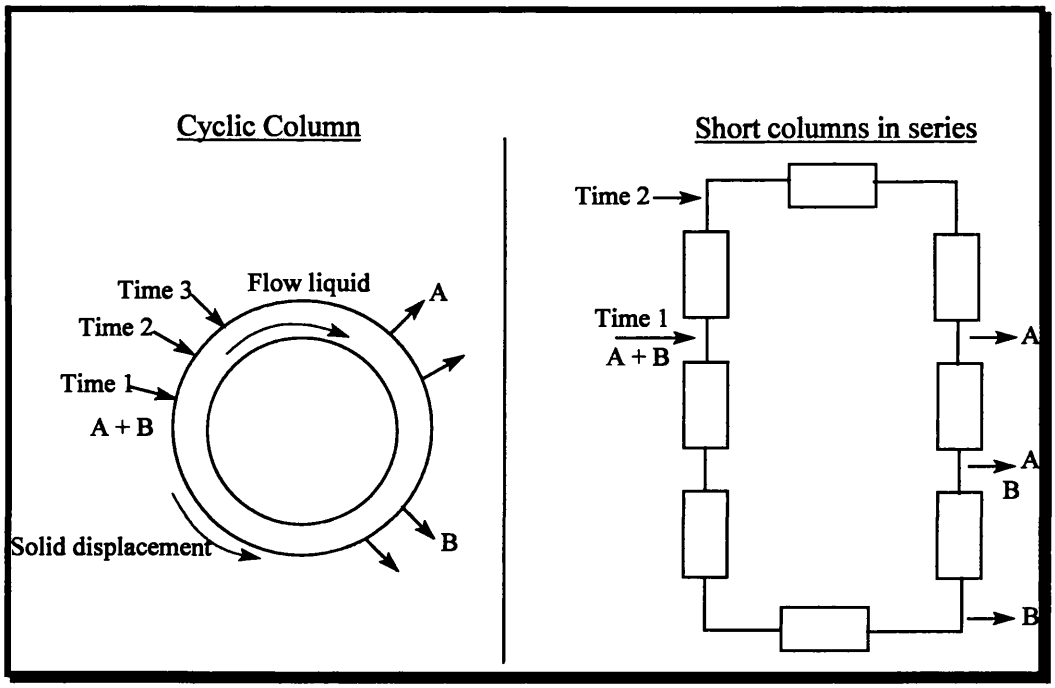


Figure 22: Series of columns connected in simulated moving bed chromatography

The commercial application of simulated moving bed is portrayed in **fig 23,** which shows the adsorbent as a stationary bed. A liquid circulating pump is provided to pump liquid from the bottom outlet to the top inlet of the adsorbent chamber. A fluid directing device known as a rotary valve is provided. The rotary valve functions on the same principle as a multiport stopcock in directing each of several streams to different lines. At the right hand face of the valve, the four streams to and from the process are continuously fed and withdrawn. At the left hand face of the valve, a number of lines are connected that terminate in distributors within the adsorbent bed (*Gembicki, 1991*).

At any particular moment, only four lines from the rotary valve to the adsorbent chamber are active. **Fig 23** shows the flow at a time when lines 2, 5, 9, 12 are active. When the rotating element of the rotary valve is moved to its next position, each net flow is transferred to the adjacent line; thus, desorbent enters line 3 instead of line 2, extract is drawn from 6 instead of 5, feed enters 10 instead of 9, and raffinate is drawn from 1 instead of 12.

In the moving bed (**fig 19**) operation, the liquid flow rate in each of the four zones is different because of the addition or the withdrawal of the various steams. In the simulated moving bed (**Fig 23**), the liquid flow rate is controlled by the circulating pump. The pump is between the raffinate and desorbent ports, and therefore should be pumping at a flow rate appropriate for *zone 4.* However, after the next switch in position of the rotary valve, the pump is between the feed and the raffinate ports, and should therefore be pumping at the rate appropriate for *zone 1.* The circulating pump must be programmed to pump at four different rates. The controlled point is altered each time an external stream is transferred from line 12 to line 1.

To complete the simulation, the liquid flow rate relative to the solid must be the same in both the moving bed and simulated moving bed. Because the solid is physically stationary in the simulated moving bed operation, the liquid velocity relative to the vessel wall must be higher than in an actual moving bed operation.

The primary control variables at a fixed rate (**fig 23**), are the cycle time, which is measured by the time required for one complete rotation of the rotary valve (this rotation is the analogue of adsorbent circulation rate in an actual moving bed system), and the liquid flow rate in zones 2, 3, 4. When these control variables are specified, all other net rates to and from the bed and the sequence of rates required at the liquid circulating pump are fixed. An analysis of sequential samples taken at the liquid circulating pump can trace the composition profile in the entire bed. This profile provides a guide to any changes in flow rates required to maintain proper performance before any significant effects on composition of the products has appeared (*Genbicki, 1991*).

Temperature and pressure are not considered as primary operating variables: the temperature is set sufficiently high to achieve rapid mass transfer rates, and the pressure is sufficiently high to avoid vaporisation. In liquid phase operation, as contrasted to vapour-phase operation, the required bed temperature bears no relation to the boiling range of the feed, an advantage when heat sensitive stocks are being tested (*Genbicki, 1991*).

1.8.3 Comparison

Using SMB, products can be isolated in purities in excess of 99% e.e. In addition, consistent optical purity can be readily achieved as a result of the continuous nature of the operation. If desired, both enantiomers can be recovered in high purity. In most cases, only one enantiomer is required, and the unwanted enantiomer can be racemised and recycled. If the SMB unit is operated on a once-through basis (as opposed to the recycling mode), a high recovery of one enantiomer will be required. When the SMB unit is operated in a recycle mode, however, a lower recovery of an enantiomer is acceptable (*Cavoy, 1997*).

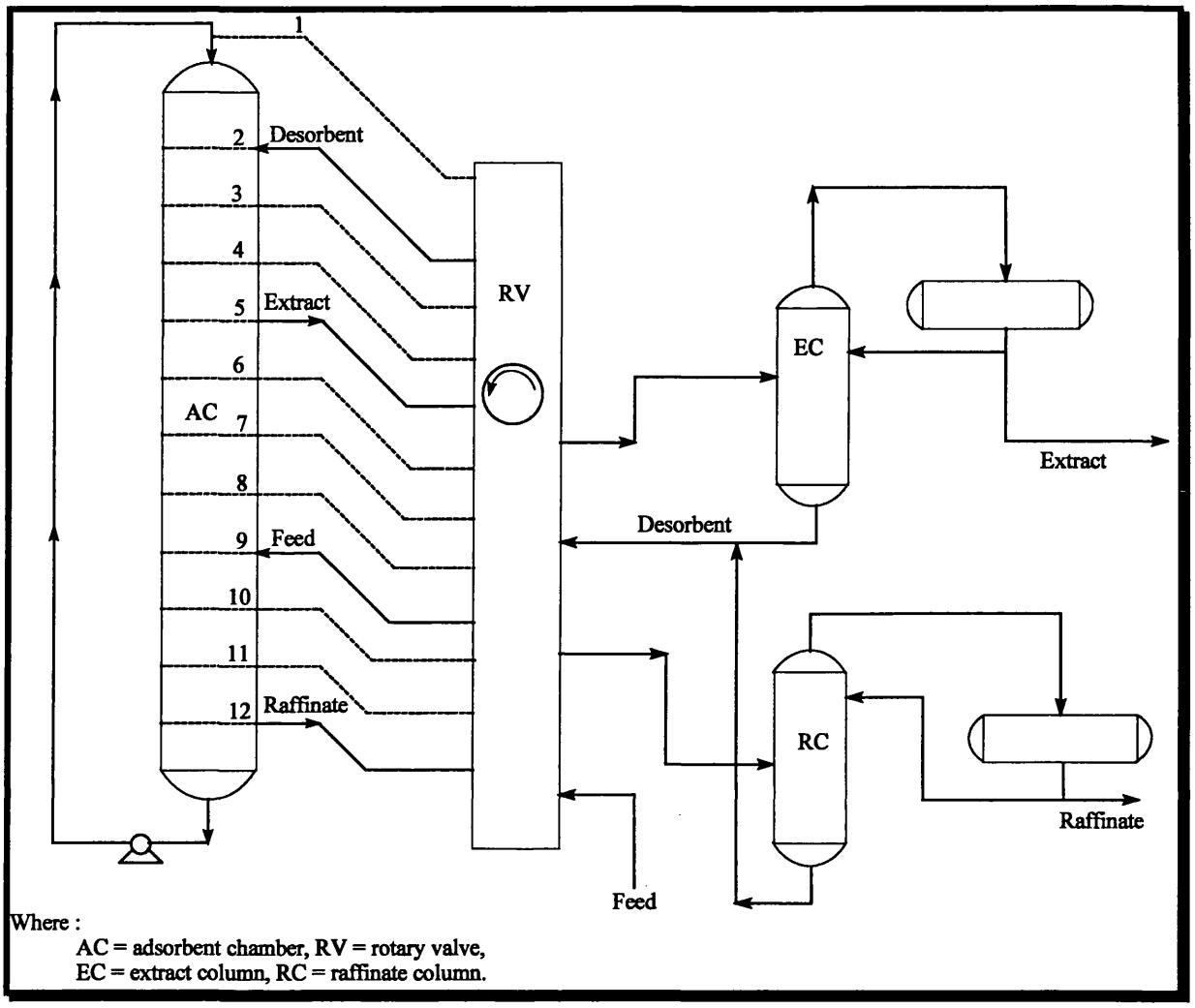


Figure 23: Commercial set-up of the simulated bed technology

1.8.4 Racemisation:

The SMB operation can be readily integrated with a racemisation process in which the unwanted enantiomer is racemised and recycled for re-use in the SMB unit (**fig 24**) (*Gattuso, 1996[b]*).

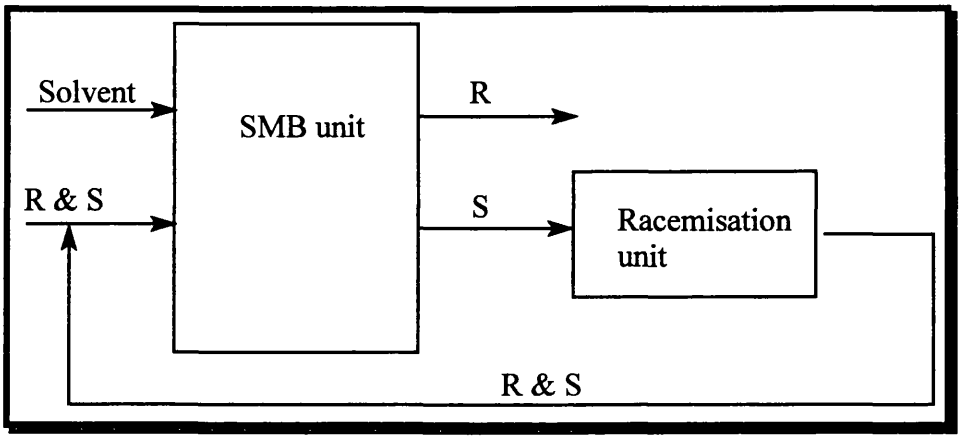


Figure 24: Integrated SMB-racemisation process

2 Proposed research

2.1 AIMS

To provide an integrated reaction scheme for the resolution of esters of CPA, and recycling of the unwanted enantiomer. In pursuit of this aim we expect to

- Hydrolyse higher esters of CPA (C4 - C12) using purified enzymes and available CLEC's to obtain $E_R > 10$.

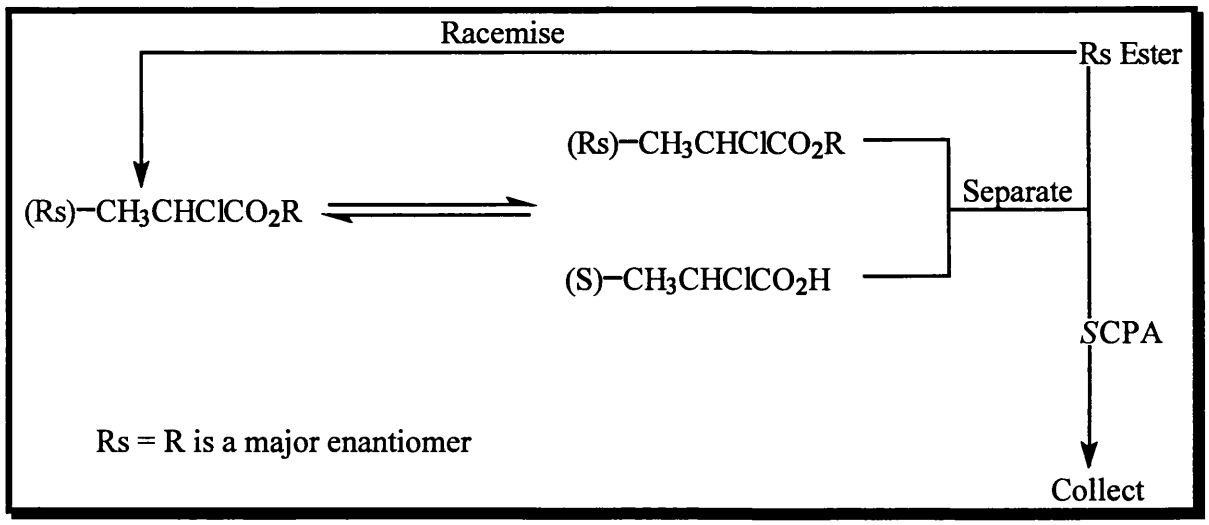
- Investigate the conditions for the separation of higher esters of CPA (the substrate) from the free carboxylic acid (the product).

- Define conditions which allow the hydrolysis of esters of CPA to be integrated with the separation of the acid, product, from the ester, substrate.

Several possible schemes are summarised below.

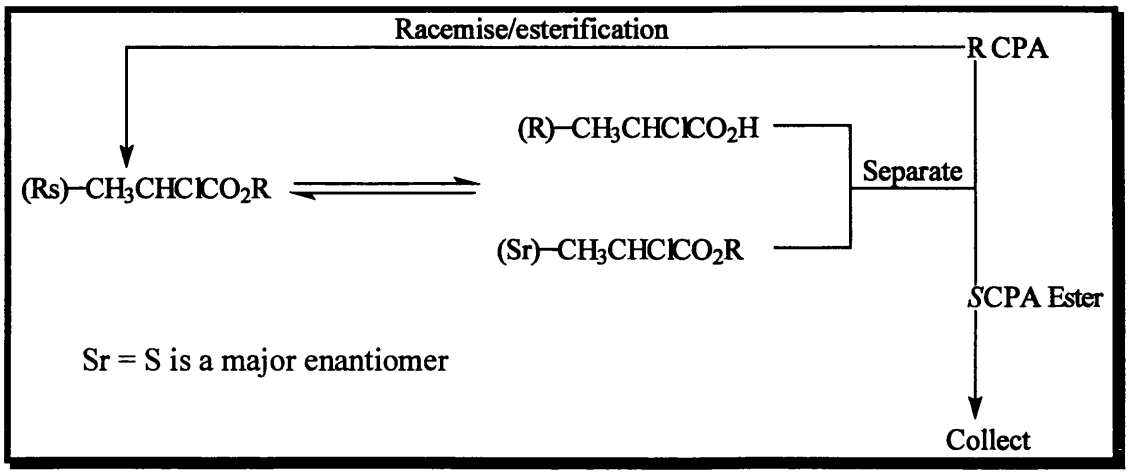
2.2 1 Enantiospecific resolution

The racemic CPA ester could be hydrolysed using commercially available lipases. The mixture of acid and ester will then be separated and the remaining *R*-ester racemised, before being recycled back into the system. The (2*S*)-CPA produced would be taken forward to synthesise Mecoprop and Dichloroprop (**scheme 10**), although the latter step is outside of the scope of this thesis.



Scheme 10: Enantiospecific hydrolysis of racemic hydrolysis with the recycling of residual ester.

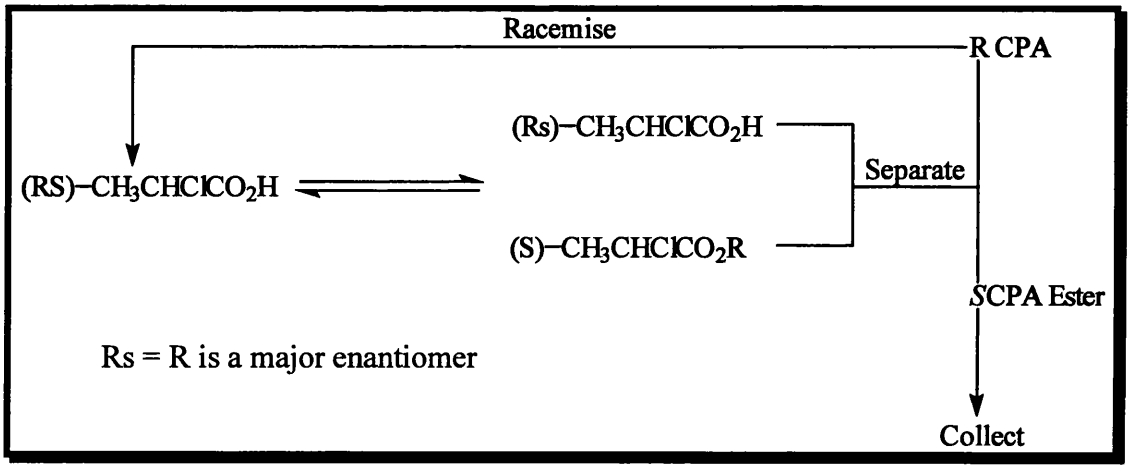
An enzymatic hydrolysis of racemic CPA ester will be carried out. In this case the ester and acid will be separated. The (2*S*)-CPA ester will be transformed into Mecoprop and Dicloroprop by a series of steps. The racemisation would need to be followed by an esterification step before the unwanted *R*-CPA could be recycled. However, where it is possible to invert the *R*-CPA, it could either be cycled directly into the phenoxypropionate synthesis (assuming it had a sufficiently high e.e.) or used to boost the e.e. of the ester substrate in favour of the *S*-enantiomer (**scheme 11**).



Scheme 11: Enantiospecific hydrolysis of racemic hydrolysis with the recycling of the acid produced.

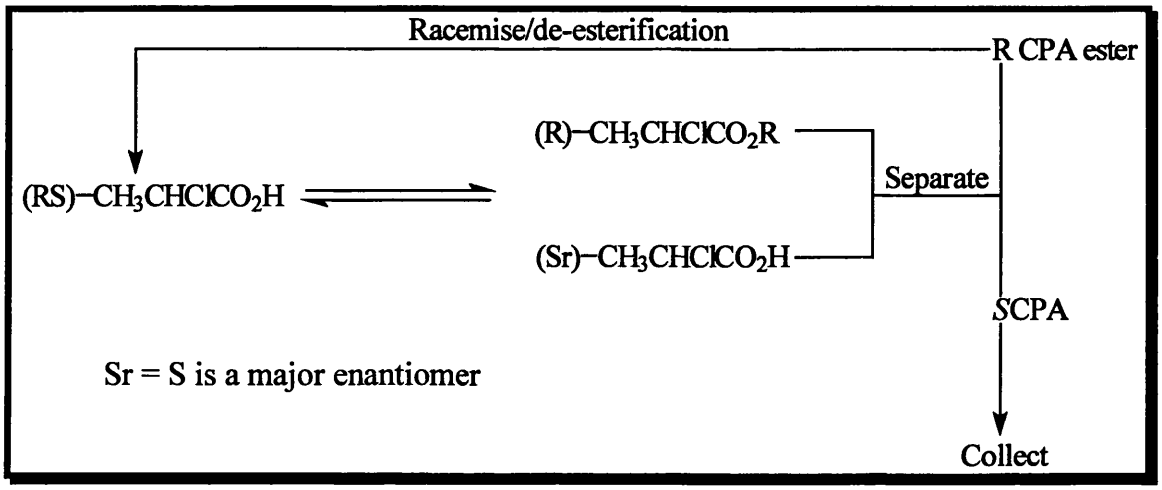
2.1.2 Enantioselective synthesis:

Scheme 12 would be operated as a esterification route, the same enzyme could be used as in **scheme 10**. A modest E_R value is required, particularly if the *R*-CPA is inverted. However the product is the (2*S*)-CPA ester, as it is in **scheme 11**, rather than (2*S*)-CPA itself.



Scheme 12: Esterification of racemic acid with the recycling of *R*-CPA

Scheme 13 is again an esterification method. The reaction needs driving close to 50% conversion to obtain high e.e. in the residual substrate. A further processing of the products would resemble the routes in **scheme 13**.



Scheme 13: Esterification of racemic acid with the recycling of *R*-CPA ester

In order to increase the efficiencies of the above schemes, the hydrolysis and the esterification reactions should be carried out in a continuous mode. This could be achieved by continuously feeding racemic CPA ester into a column packed with lipase immobilised on a suitable adsorbent. The ester should adsorb to the column while the CPA produced will be removed from the column and passed through a racemisation and an esterification unit prior to feeding back into the column. The remaining ester should bind to the resin which can be eluted once the column is fully saturated with adsorbed ester (**fig 25**).

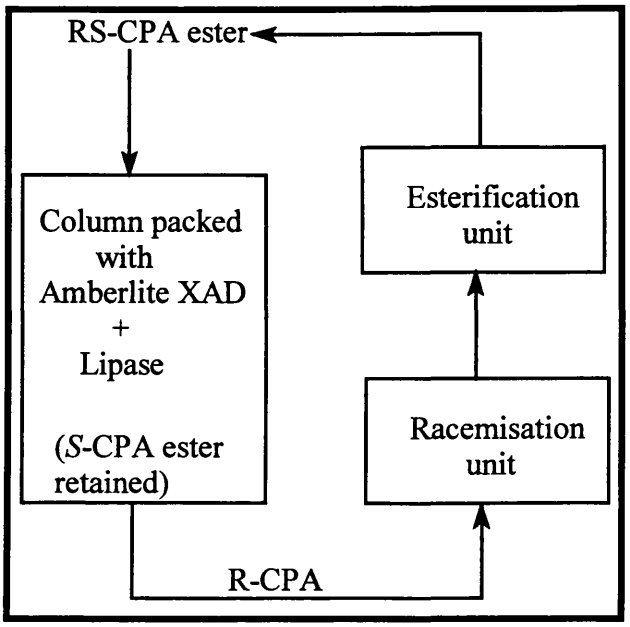


Figure 25: Schematic representation of a reactor for the continuous production of CPA and its esters

To establish a continuous column system for the production of optically pure (2*S*)-CPA according to the above schemes, we envisage that the following criteria which will have to be investigated:

- A chiral assay method to determine the optical purity of the CPA produced from the enzymatic hydrolysis of its esters. To accomplish this we plan to reduce the CPA produced to the 2-chloropropanol and the derivatisation of the alcohol to its Mosher ester (*Mosher, 1973*). Optical purity may also be determined on chiral GC.

- The synthesis of various esters of CPA, which will include aliphatic primary and secondary esters and cyclic primary and secondary esters. The purity of these substrates will be analysed on GC.

- Esters of CPA will be screened against commercially available lipases on a small scale. The screen will be followed qualitatively by thin layer chromatography (tlc)

and favourable lipase and substrate combinations will be shortlisted for large scale investigation.

- An analytical method is required to follow the rate of reaction on a large scale. For this GC internal standard method will be used (*Littlewood, 1970*).

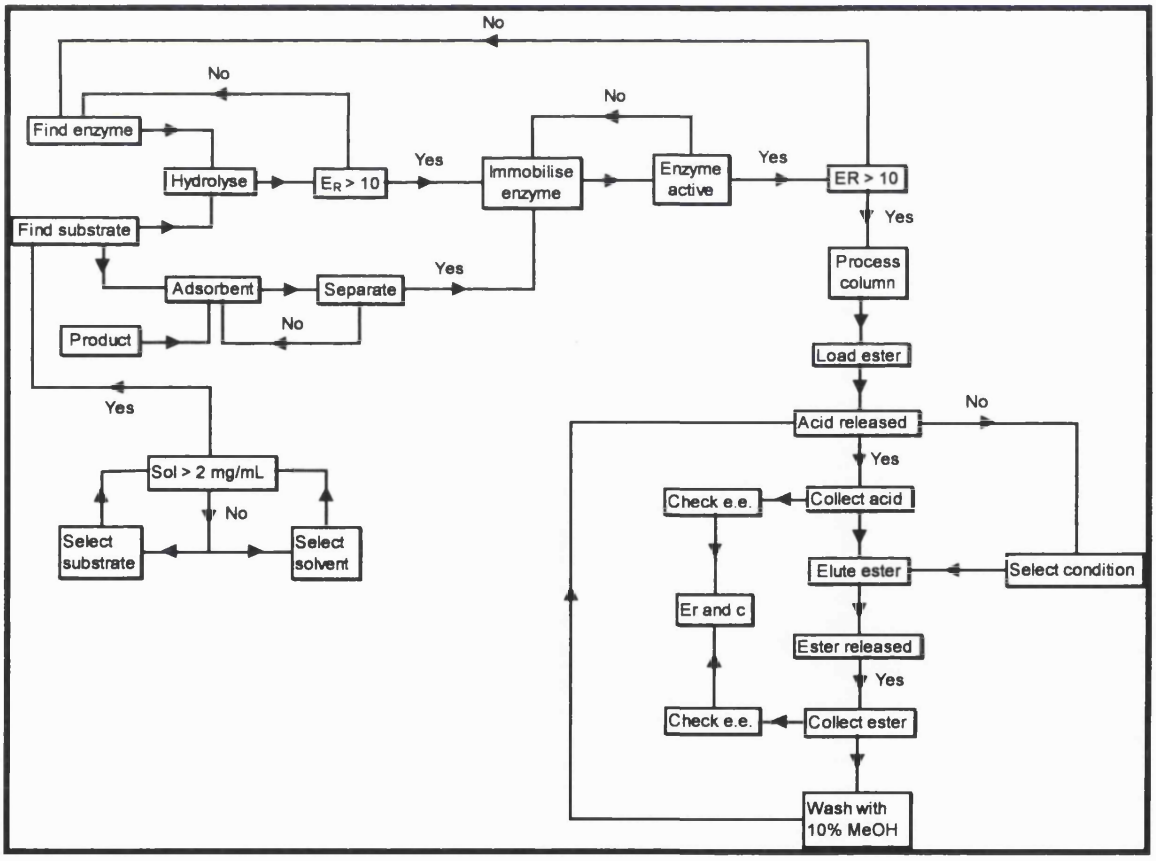
- To carry out the hydrolysis on the large scale and to calculate the optical purity of the product, the overall conversion of the reaction and E_R of the lipase. As mentioned above the E_R of the lipase must be higher than 10, to achieve this the substrate can be modified or completely changed and the lipase can be purified or activated.

- The selected lipase ($E_R > 10$) will be immobilised on to the macro reticular adsorbent (*Basri, 1990*). The immobilisation must be compatible with the lipase activity, the specificity of the lipase will be tested again and E_R must be greater than 10. The immobilised lipase will be cross-linked, once again cross-linking has to be compatible with the lipase activity and specificity. If the lipase losses its activity or specificity upon immobilisation or cross linking then the new support must be tried to obtain $E_R > 10$. A new lipase will have to be found if efforts have failed to find the right support. This is a major checkpoint in the system as all available substrates would have to be screened against the new lipase.

- The support used for immobilisation must also be able to adsorb the ester preferentially and release the acid. If the resin is not capable of separating the acid and the ester then a new support must be found. The adsorption will be carried out in a single phase system, but that the, solubility of the substrate must be higher than 2mg/mL in the adsorption solvents to achieve a reasonable process intensity. A substrate will have to be found to satisfy the solubility criteria. This step will have to be followed closely with the immobilisation of the lipase.

- The column will be set-up according to the above conditions. Ester dissolved in adsorption solvent is to be fed into the column at a fixed flow rate and released acid will be isolated from the effluent and its optical purity will be determined. At the end of the each run, adsorbed residual ester will be eluted and analysed for enantiomeric excess. Conversion and E_R of the column will be calculated from the enantiomeric excess data. Regeneration of the column is the crucial step as ester elution must be compatible with lipase activity, this is necessary for the repeated use of the column. If the lipase activity is destroyed during the elution then the new elution solvent which is compatible with lipase activity will have to be found

The above strategies are summarised and have been linked together in **scheme 14** below.



Scheme 14: Outline of the research necessary to establish a continuous reactor

The following thesis is divided into nine chapters, where chapters 3 and 4 describe the development of basic analytical methods required to quantify the optical purities of products, lipase activities and protein contents. Chapter 5 deals with the chemical methods employed to synthesise various esters of α-substituted propionic acid and chapter 6 analyses the screening process for an enantiospecific lipase. Chapter 7 describes the immobilisation of *Candida rugosa* lipase on macroreticular resins. Chapter 8 defines the separation conditions for the residual ester from the products of their hydrolysis, with a necessary reinvestigation of a suitable esters. Chapter 9 integrates the hydrolysis of esters with the separation of ester and acid into a single reactor which could form the basis for the continuous process described in chapter 1. Chapters 10 and 11 discuss the implications of these results and the necessary research for the successful development of a continuous reactor.

Materials and Methods.

The proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian VXR-400 (400 MHz) spectrometer, and are reported in δ ppm values. The spectra were recorded in deuterochloroform ($CDCl_3$). Gas chromatograms were recorded on Perkin Elmer 8700, using a capillary column, the retention times are reported in minutes. Absorbance of substrates were measured on UVIKON, absorbance is recorded in absorbance units (A.U.)

Commercially available precoated silica gel plates (250 M) with a fluorescent indicator (E. Merck) were used for analytical thin layer chromatography. They were initially visualised with ultra-violet light and permanently stained with potassium permanganate. Flash column chromatography was performed using 220-400 mesh silica

All chemical reagents used for ester synthesis were of analytical research grades. General purpose reagents grade solvents were used for flash column chromatography. A temperatures of -10 °C was achieved with a mixture of ice and sodium chloride.

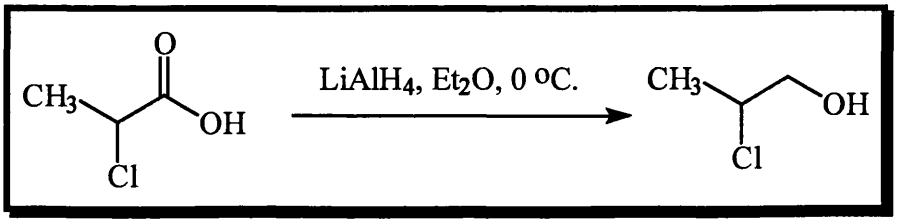
Commercially available lipases were bought from the following suppliers and were stored at 4 °C. All lipase catalysed hydrolyses were performed at room temperature unless otherwise stated. IPA treated *C. rugosa* lipase was stored at 4 °C in a presence of 0.02% W/V sodium azide and was used within three months.

Enzyme	Supplier
Pseudomonas fluorescens lipase	Biocatalysts. Fluka
Chromobacterium viscosum lipase	Biocatalysts. Fluka
Mucor miehei lipase (Lipozyme)	Novo. Fluka
Candida antarctica lipase	Novo. Fluka
Candida cylindracea lipase	Biocatalysts. Fluka
Porcine Pancreatic lipase	Biocatalysts. Sigma
Aspergillus niger lipase	Fluka
Mucor javanicus lipase	Fluka
Rhizopus arrhizus lipase	Fluka
Pseudomonas cepacia lipase	Fluka
Wheatgerm lipase	Fluka
Candida lipolytica lipase	Fluka
Penicillium roqueforti lipase	Fluka
Rhizopus niveus lipase	Fluka
Pig liver acetone powder	Sigma
Bakers yeast	Sainsbury's

3 Chiral Analysis

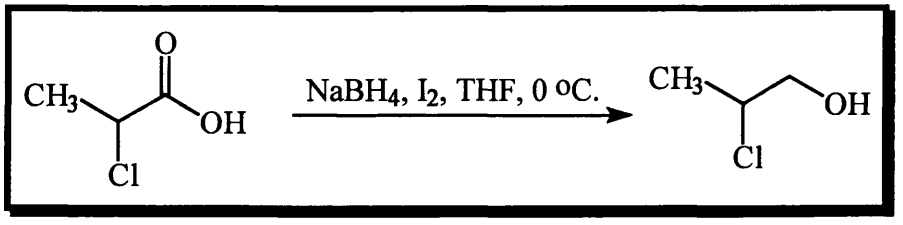
3.1 Experimental

3.1.1 Reduction of CPA with LiAlH (Harwood and Moody, *1996*)



Lithium aluminium hydride (13.3 mg, 0.35 mmol) was added cautiously to a stirred solution of racemic 2-CPA (01 mL, 1.17 mmol) in diethylether (2 mL) at 0 °C under a nitrogen atmosphere. After 25 min the reaction mixture was warmed to room temperature and stirred for a further 40 mins. The tlc showed multiple spots, therefore, the reaction was stopped and no attempts were made to isolate any of the products.

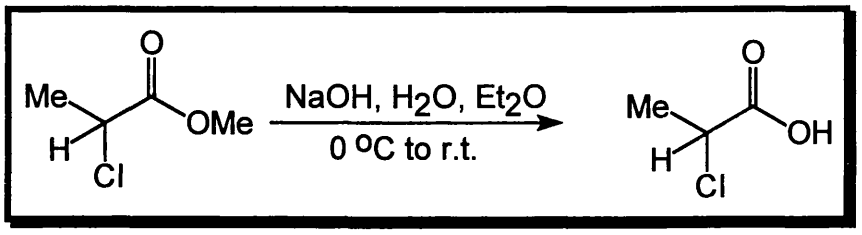
3.1.2 Reduction of CPA with $NaBH_4$ and Iodine (Kanth, *1991*)



Solution of CPA (0.2 mL, 2.34 mmol) in THF (2 mL) was added dropwise to a stirred suspension of $NaBH_4$ (106 mg, 2.81 mmol) in THF (2 mL) at 0 °C, under nitrogen atmosphere. After 5 min a solution of iodine (297 mg, 1.17 mmol) in THF (2mL) was added dropwise. The reaction mixture was warmed to room temperature and stirred

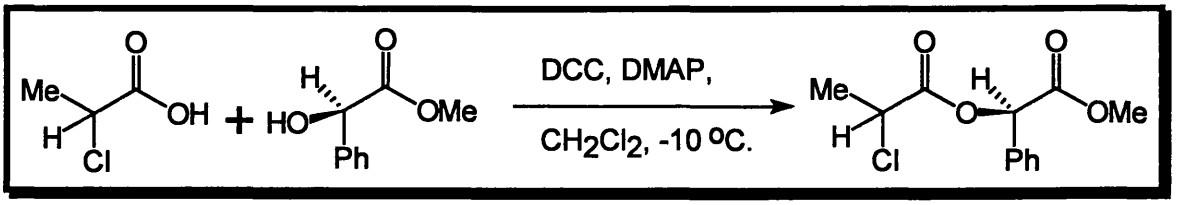
for a further 70 mins. At this stage tlc showed multiple spots and the reaction was stopped.

3.1.3 Hydrolysis of methyl-chloropropionate (Harwood and Moody, *1999*)



Sodium hydroxide (3.7 mg, 0.093 mmol) in water (1 mL) was added cautiously to a stirred cold (0 °C) solution of racemic methyl-2-chloropropionate (0.1 mL, 0.93 mmol) in Et_2O (1 mL). After 10 min the reaction mixture was warmed to room temperature and stirred for 2 h. At this stage a further 0.1 eq of NaOH was added to the reaction mixture. No reaction was observed after 2 h, and therefore the reaction was abandoned at this stage.

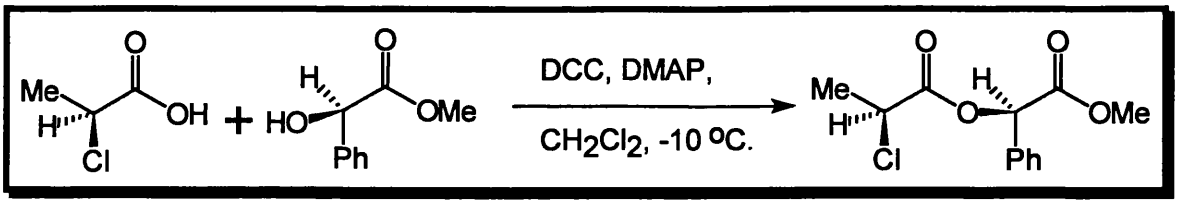
3.1.4 Mandelate ester of racemic CPA (Hassner, *1978*):



To a stirred solution of racemic 2-chloropropanoic acid (0.1 mL, 1.17 mmol) and 4,4-dimethylaminopyridine (14.3 mg, 0.117 mmol) in dichloromethane (10 mL), at -10 °C were added (+)-methyl-L-mandelate (194 mg, 1.17 mmol) and dicyclohexylcarbodiimide (241 mg, 1.17 mmol). After 3.5 h the reaction mixture was

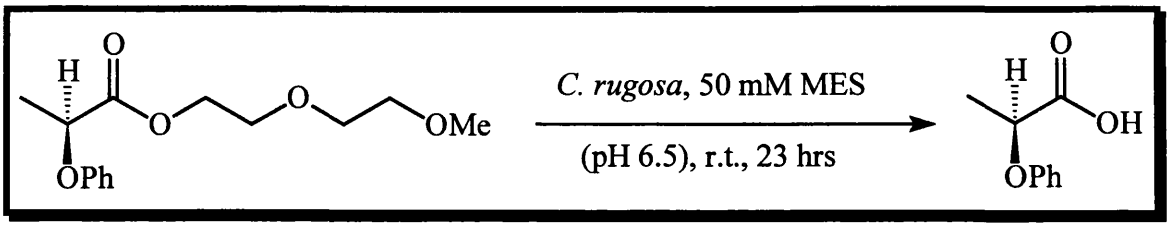
filtered and solvent was evaporated. The residue was taken up in petroleum spirit (40-60 °C), the precipitate formed was filtered, and the filtrate was concentrated in *vacuo*. The residue was purified by flash column chromatography on silica gel (petrol 40-60 °C:ethyl acetate 20:1) to give the product as a colourless oil (0.24 g, 79% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44-7.42 (4 H, m), 6.01 (1 H, s), 6.00 (1 H, s), 4.67 (2 H, m), 3.76 (3 H, s), 3.75 (3 H, s), 1.81 (3 H, d, J = 7 Hz,), 1.78 (3 H, d, J = 7 Hz).

3.1.5 Mandelate ester of (2*S*)-CPA (Hassner, *1978*)**:**



To a stirred mixture of (2*S*)-chloropropanoic acid (0.1 mL, 1.17 mmol) and 4,4-dimethylaminopyridine (0.0143 g, 0.117 mmol) in dichloromethane (10 mL), at -10 °C were added (+)-methyl-L-mandelate (0.1944 g, 1.17 mmol) and dicyclohexylcarbodiimide (0.2414 g, 1.17 mmol). After 3 h the reaction mixture was filtered and the solvent was evaporated. The resulting residue was taken up in petroleum spirit (40-60 °C), the precipitate was filtered, and the filtrate was concentrated. The residue was purified by flash column chromatography on silica gel (petrol:ethyl acetate 20:1) to furnish the product as a colourless oil (0.22 g, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47-7.40 (4 H, m), 5.99 (1 H, s), 4.57 (1 H, q, J = 7 Hz), 3.38 (3 H, s), 1.79 (3 H, d, J = 7 Hz).

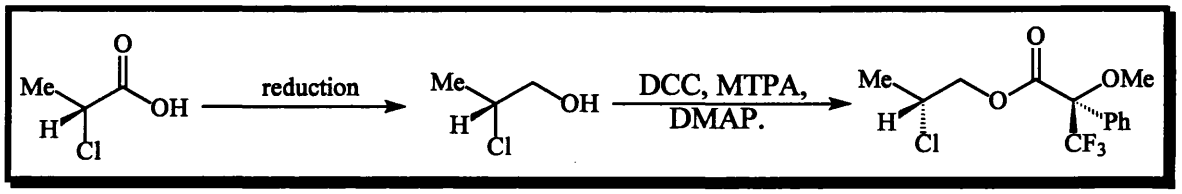
3.1.6 Hydrolysis of the residual DEGME-phenoxypropionate:



To a stirred mixture of the DEGME ester (0.211g, 0.786 mmol) and 50 mM MES buffer (75 mL, pH 6.5) was added crude *C. rugosa* (0.50 g). The reaction was stirred at room temperature for 23 h. The reaction was quenched with saturated sodium hydrogen carbonate. The organic phase was extracted with ether (3x 100 mL). The combined organic extracts were dried over anhydrous magnesium sulphate , filtered and concentrated to give *di*-ethyleneglycolmethyl ether. The aqueous phase was acidified to *p*H 1 with 3N HCl. The organic contents were extracted with ether (3 x 100 mL). The combined organic extracts were dried over anhydrous magnesium sulphate, filtered and concentrated in *vacuo* to yield 2-phenoxypropionic acid (0.1068 g, 82 % yield)

3.2 Results and Discussion

Our first objective was to establish a chiral assay method, to determine the enantiomeric excess (e.e.) of 2-chloropropanoic acid liberated from the enzymatic hydrolysis of its esters. To execute this strategy (**scheme 15**), it was decided to reduce the CPA to the corresponding primary alcohol and then derivatise it to the corresponding Mosher ester (*Mosher, 1973*).



Scheme 15: Synthesis of Mosher ester of CPA

In our first attempt to reduce the CPA, lithium aluminium hydride (LAH) was used (*Harwood and Moody[a], 1996*), but due to its strong reducing powers, it gave rise to a complex reaction mixture (as showed by the tlc). Different ratios of LAH to the CPA were unsuccessfully tried to minimise the side reactions. One of the side product is thought to be stable α, β-unsaturated propionic acid (**fig 26**), which is produced due to the elimination of chlorine at the α-carbon. This unsaturated acid can also undergo reduction to produce the corresponding alcohol.

As an alternative sodium borohydride was tried, this is a mild reducing agent, not capable of reducing carboxylic acids to the corresponding secondary alcohols. Therefore iodine and sodium borohydride mixture was used (*Kanth, 1991, Periasamy, 1992*). Iodine complexes with $NaBH_4$ and increases its reduction powers, but this method was also unsuccessful as it gave rise to another complex reaction mixture, no attempts were made to isolate any of the products of the reaction.

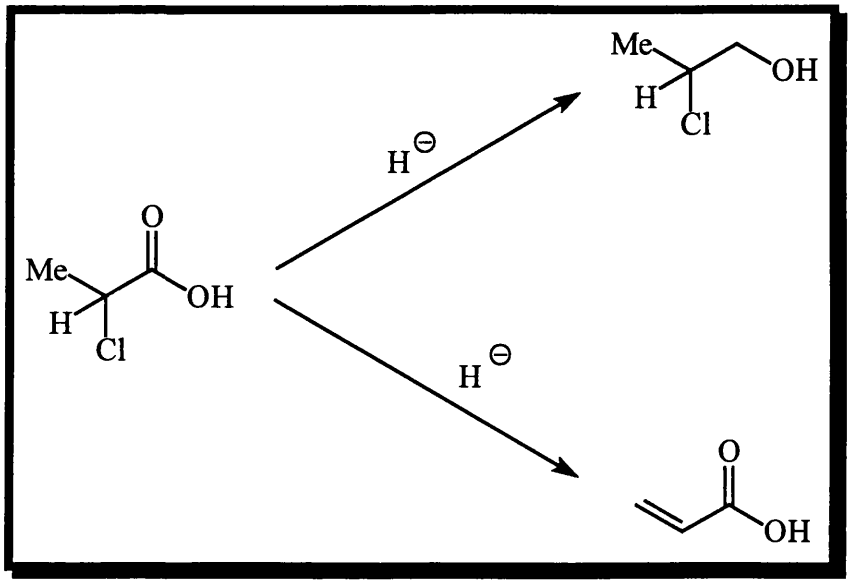


Figure 26: Possible reactions during the reduction of CPA.

At this stage attempts to make a Mosher ester were abandoned and an alternative coupling approach (**fig 27**) was adopted, where the CPA was coupled to (+)-methyl-L-mandelate (*Schwab, 1984*) using a standard DCC coupling procedure (*Hassner, 1978*). In this procedure DCC is initially used to deprotonate the carboxylic acid and then it is attacked by the anion formed to generate a good leaving group. The intermediate is then attacked by the mandelate to give the corresponding mandelate ester.

To assign an absolute configuration at the α-carbon, the mandelate ester of racemic CPA and (2*S*)-CPA were synthesised and analysed by NMR. A typical nmr spectrum of racemic mandelate ester of CPA contained a doublet of doublet at ~1.8 ppm for methyl group at α-carbon, where nmr spectra of mandelate ester of (2*S*)-CPA contained a high intensity doublet due to the *S*-enantiomer and a very low intensity doublet due to a trace amount of *R*-enantiomer present in the sample.

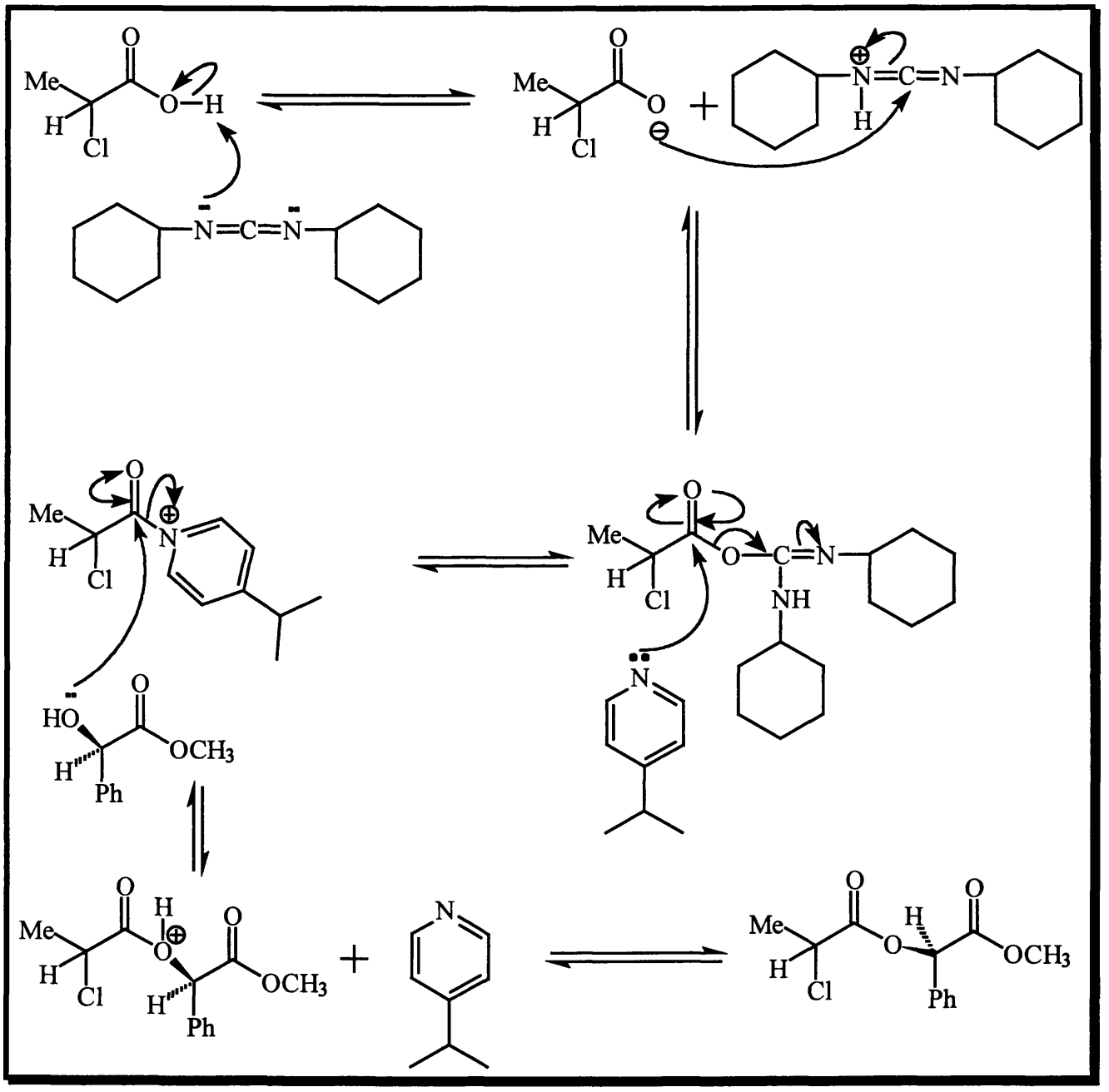


Figure 27: Reaction mechanism of the DCC coupling to form the mandelate ester of CPA

The NMR spectra of mandelate ester of (2*S*)-CPA showed no sign of any racemisation during the reaction (**fig 28**).

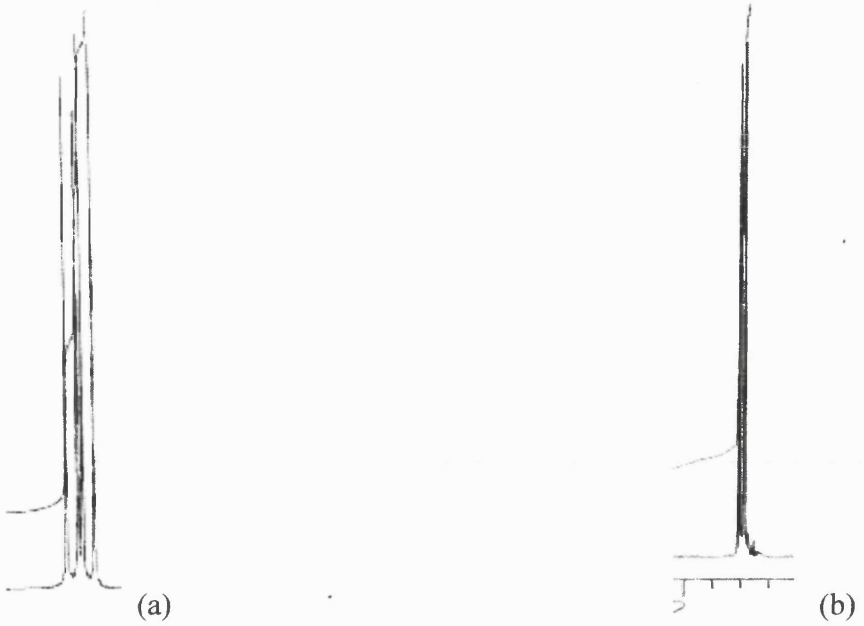


Figure 28: Partial NMR of the mandelate esters of racemic and (*S*)-CPA
a) Signal for methyl protons in NMR spectra of mandelate ester of racemic CPA (ppm ~1.8)
b) Signal for methyl protons in NMR spectra of mandelate ester of *S*-CPA (ppm ~1.8)

Enantiomeric excess of 2-phenoxypropionic acid was also determined by its derivatisation to the corresponding mandelate ester. The absolute configuration was assigned by forming mandelate ester of 2-*R*-chloro-4-methylphenoxypropionic acid (CMPP) and then comparing the nmr spectra of mandelate ester of (*R*)-CMPP with the spectra of mandelate ester of 2-phenoxypropionic acid released from the hydrolysis of its esters.

To determine the optical purity of the residual ester from the hydrolytic reaction, it was decided to use an excess amount of crude *C. rugosa* lipase. The crude lipase is

found to be non specific towards both enantiomers of CPA or 2-phenoxypropionic acid based esters ($E_R = 2$). In a typical reaction lipase and the residual ester in the presence of MES buffer (pH 6.5) were stirred at room temperature, the progress of the reaction was followed by tlc. The reaction was only stopped when it was confirmed by the tlc that the reaction is complete, this was necessary to accurately determine the optical purity.

The 2-phenoxypropionic acid produced was derivatised to the mandelate ester and analysed by NMR for its optical purity.

3.3 Conclusion

- Reduction of CPA with strong to mild reducing agents results in a complex reaction mixture, hence this strategy is non advisable

- Mandelate ester of CPA and 2-phenoxypropionic acid can be easily synthesised using DCC coupling method. The resulting diastereomer give rise to separate peaks for both enantiomers in the NMR spectrum.

- The residual ester can be hydrolysed using non specific crude *C. rugosa* lipase under very mild conditions to yield 2-phenoxypropionic acid without racemisation.

4 Analytical methods

4.1 Experimental

4.1.1 Bradford protein method:

Protein concentrations were determined by using the Coomassie Brilliant blue dye (G250) binding method of Bradford. Aliquots of diluted samples (100 μL) were added to Bradford reagent (Biorad, 2.9 mL) in 3 mL acrylic cuvettes. The cuvettes were inverted to ensure thorough mixing, and the colour was allowed to develop for 5 min. Samples were then read against a reagent blank (595 nm). Bovine Serum Albumin (BSA) was used to calibrate the reagent in the range 0-1 mg/mL. The calibration curve was linear in the range of 50 to 500 μg/mL (*Bradford, 1976*).

4.1.2 Lowry method:

5% Sodium potassium tartrate (2.5 mL) and 0.5% w/v copper sulphate pentahydrate (2.5 mL) were diluted to 100 mL with 0.1M sodium hydroxide in 2% w/v sodium bicarbonate. This working reagent was freshly prepared before use. Diluted protein samples to be assayed (0.5 mL) were first placed in a test tubes to which the working reagent (2 mL) was added. These were mixed and allowed to stand for 30 mins. Folin-Ciocalteu (F.C) phenol reagent was diluted with water in the ratio of 1:2. 0.5 mL. Diluted F.C reagent was then added to each sample and immediately mixed vigorously. The colour was allowed to develop for 30 mins at room temperature. The absorbance of the solutions was measured in a spectrophotometer at 660 nm. Solutions of know concentration of bovine serum albumin were used for preparation of a calibration curve (*Lowry, 1951*).

4.1.3 Spectrophotometric lipase activity assay

*p*NPA (100 μL of 125 mM in *i*-PrOH) was added directly to an acrylic cuvette (1.2 mL) to which MES buffer (50 mM, pH 6.5, 800 μL) was added. Enzyme solution

(100 μL) was added to the reaction mixture and the increase in absorbance at 410 nm at 37 °C by using an ATI Unicam UV/Vis spectrometer (model UV2). The enzyme was diluted appropriately to give rates between 0.4 and 0.6 ΔA/min (*Winkler, 1979*).

One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute.

4.1.4 Titrimetric lipase activity assay:

Lipase activity was measured using 20% *S*-ethyl lactate in 10 mM NaCl as the substrate. The assays were carried out in a *p*H stat (Radiometer ETS822 end-point titration system). Substrate was placed into a titration beaker such that the final volume of the enzyme and the substrate was 25 mL. The *p*H of the substrate was adjusted to *p*H 7 after which the enzyme solution was added to the beaker and the reaction was autotitrated at *p*H 7. The rate of addition of 0.1 M NaOH was recorded at one minute intervals for 10 min, the rate of addition was used to calculate the linear rate (*Lee, 2000*). The linear rates (mL/min) were converted to units/mL by taking into account the dilution of the enzyme in the substrate using the following equation.

The consumption of 1 mL of 0.1 M NaOH was equivalent to the liberation of 100 μmoles of fatty acid, thus:

$$\text{Volume activity} = \frac{\text{volume of NaOH consumed}}{\text{volume of reaction vessel}} \text{ X 100 X dilution factor (U / mL)}$$

5 % Triacetin, esters of CPA and 2-phenoxypropionic acid were also used as an alternative to the *S*-ethyl lactate.

4.1.5 Gas Chromatography analysis:

Gas chromatography analysis were carried out on Perkin Elmer 8700, with the following column specifications:

Column type = Capillary column, length = 30 m, phase = Supelcowax10, column I.D. = 0.32 mm, film thickness = 0.25 microns.

4.1.5.1 Retention times

The retention times were measured using the following G.C. conditions:

Oven temperature = 60 °C

***iso*-Time** = 2 min

Ramp rate = 15 °C/min

Final oven temperature = 250 °C

***iso*-Time (2)** = 1 min

He = 13.4 psi

Detector temperature = 300 °C

Injector temperature = 250 °C

Injection volume = 0.2 μL.

Compound	Retention times (min)
Dodecyl-2-CPA ester	13.7
1-Octyl-2-CPA ester	10.5
2-Octyl-2-CPA ester	11.8
3-Octyl-2-CPA ester	9.1
Hexyl-2-CPA ester	6.1
2-Pentyl-2-CPA ester	6.5
Butyl-2-CPA ester	6.7
1-Octene-3-yl-2-CPA ester	8.2
1-Phenethyl-2-CPA ester	10.9
Cyclohexyl-2-CPA ester	8.8
Octyl-2-phenoxypropionate	14
Hexyl-2-phenoxypropionate	12.9

Table 1: Retention times of esters of CPA and 2-phenoxypropionic acid

4.1.5.2 Response factors

1) Various concentration solutions of internal standard solutions were prepared in petroleum spirit 80-100 °C.

2) Alcohols, esters and internal standard solutions were accurately weighed and diluted in set volume of petroleum spirit.

Three solutions of different concentration of the alcohols and esters were prepared and analysed by the above method (sec 4.1.5).

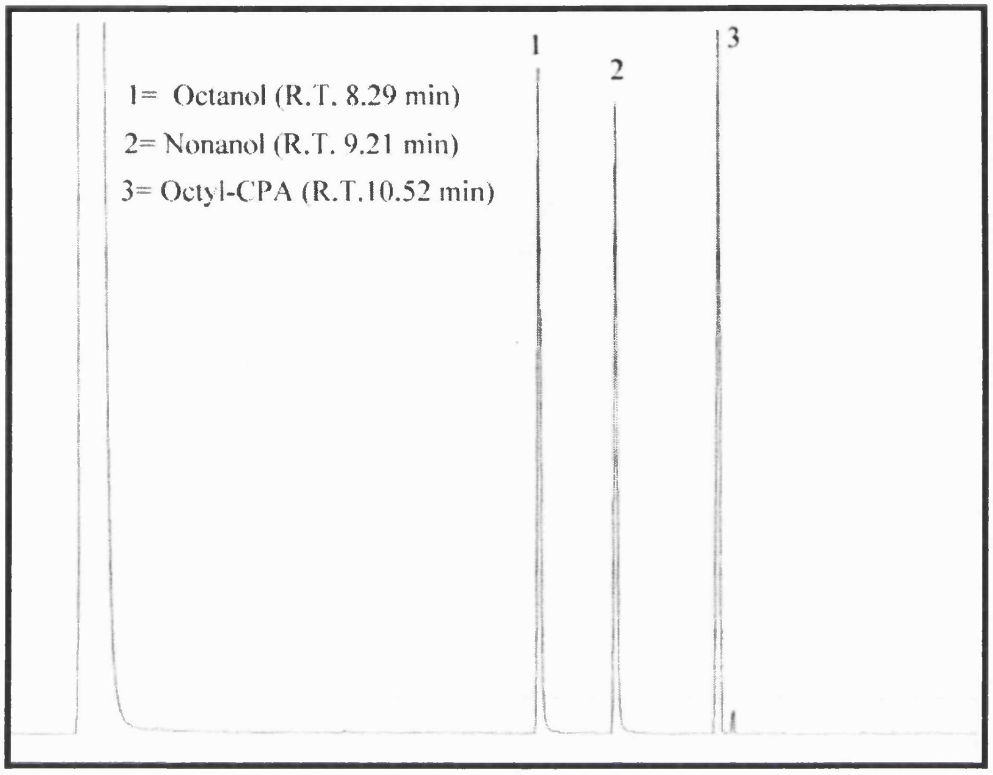


Figure 29: Typical gas chromatogram used for the calculation of response factors.

4.2 Results and discussion

In order to determine the degree of hydrolysis quantitatively on a large scale using gas chromatography, the response factors of esters and alcohols had to be calculated, using an internal standard method (*Littlewood, 1970*).

Relative response factors were calculated using the following equation

$$RRF_i = \frac{A_i \text{ x } C_S}{A_S \text{ x } C_i}$$

Equation 4: Equation to calculate the response factors

Where:

$\mathbf{RRF}_i$ = relative response factor of the injected sample.

A_i = peak area of the injected sample.

C_s = concentration of the internal standard solution.

A_s = peak area of the internal standard.

C_i = concentration of the injected sample.

The above equation (**eq 4**) was rearranged to **eq 5** and the resulting equation was used to calculate the unknown concentrations of ester and alcohol in a hydrolytic reaction.

$$C_i = \frac{A_i \text{ x } C_S}{A_S \text{ x } RRF_i}$$

Equation 5: Equation to calculate the unknown concentration.

During the reaction aliquots (2 mL) were withdrawn from the reaction. The crude mixture was diluted with saturated sodium hydrogen carbonate (to remove the carboxylic acid produced), organic contents were extracted with diethyl ether. Ether was removed under reduced pressure and the resulting residue was diluted with an internal standard solution (5 mL). The solution was analysed by GC and the concentrations of ester and alcohol were calculated using **equation5**. The degree of conversion (%) was calculated using **equation 6**

$$\frac{\text{Concentration of alcohol}}{\text{Concentration of ester}} \times 100$$

Equation 6: Equation to calculate the degree of conversion.

Relative response factors (**table 2, chapter 5**) of all esters synthesised were calculated according to **equation 4**

It was necessary to use dodecanol instead of nonanol as an internal standard for secondary octyl esters and alcohols, because of the very close retention times of secondary octyl alcohols and nonanol, which sometimes resulted in an overlap in the chromatogram.

Protein contents of the crude lipase and the IPA treated lipase were measured by Bradford and Lowry protein assays. The actual value determined depended on the assay used. According to the Lowry protein assay there is three times more protein present in a commercially available *C. rugosa* lipase than the protein contents calculated according to the Bradford assay. Although both methods gave different results, but the Lowry method was considered more reliable and accurate and hence was used where accurate results were required.

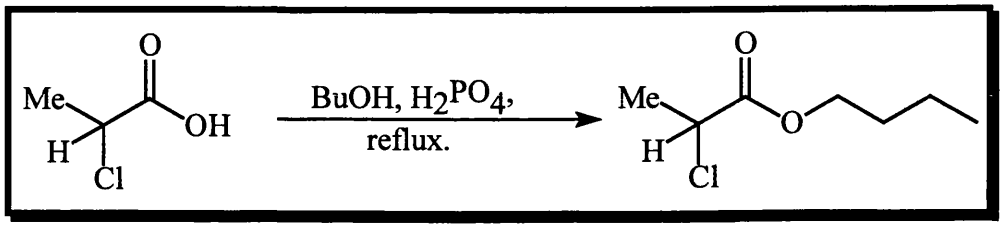
To calculate the mass balance for the IPA treatment of *C. rugosa*, the lipase activity was calculated using two activity assay methods. Firstly, using *p*-nitrophenylacetate

as a substrate, the rate of production of *p*-nitrophenol from the hydrolysis was measured at 410 nm. Secondly, with 20% *S*-ethyl lactate as a substrate, in which the pH of the reaction mixture was brought to 7 and the enzyme was added. The pH of the solution was maintained by the addition of known molarity sodium hydroxide. The rate of addition of NaOH was then used to calculate the activity of the lipase (U). Hexyl ester of CPA and 2-phenoxypropionic acid were also used as substrates. The latter esters are water insoluble hence the rate were determined in two phase system. Activity of the immobilised lipase was also determined using the above methods.

5 Ester Synthesis

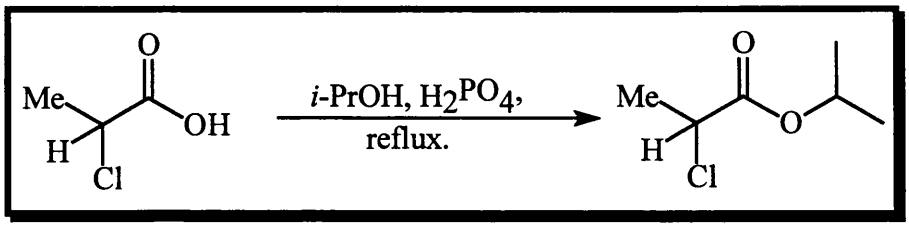
5.1 Experimental

5.1.1 Butyl ester (Harwood and Moody, *1996*):



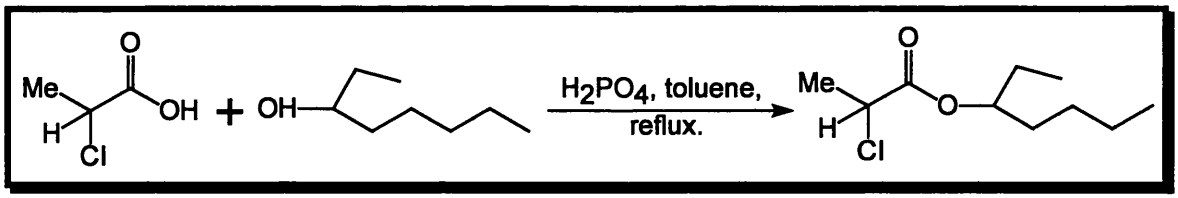
2-Chloropropanoic acid (33.8 mL, 0.37 mol), butanol (55 mL, 0.74 mol), and *o*-phosphoric acid (cat) in toluene (100 mL) were refluxed for 5 h. The water of esterification was azeotropically removed with toluene using Dean Stark apparatus. The reaction mixture was cooled to room temperature and washed with saturated aqueous sodium hydrogen carbonate solution (2 x 50 mL). The organic layer was washed with water (1 x 100 mL) and dried over sodium sulphate, and filtered. The filtrate was purified by distillation to give the butyl ester as colourless oil (44.6 g, 74% yield, b.p. 145-164 °C, G.C. retention time = 8.61 min) (for method see sec 4.1.5).

5.1.2 Iso-propyl ester:



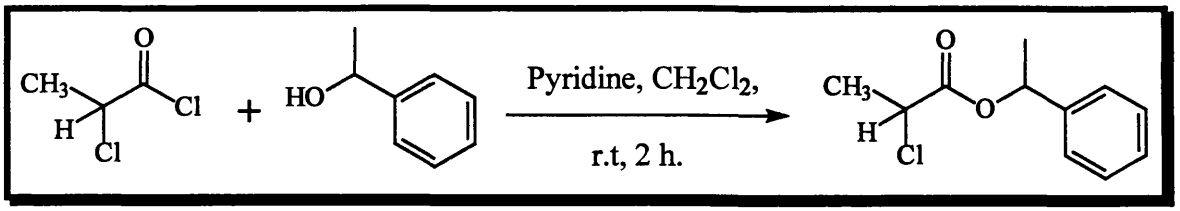
A mixture of racemic 2-chloropropanoic acid (33.8 mL, 0.37 mol), *i*-PrOH (28.3 mL, 0.37 mol) and *o*-phosphoric acids (0.23 g) was distilled and excess *i*-PrOH was removed. The flask was then charged with more *i*-PrOH (28.3 mL, 0.37 mol), and excess *i*-PrOH was removed by distillation. This procedure was repeated 10 times. The final residue was purified by distillation to give *i*-propyl ester as a colourless oil (16.13 g, 29% yield, b.p. 135-140 ^{o}C).

5.1.3 3-Octyl ester (Harwood and Moody, *1996*):



The mixture of 3-octanol (66.5 mL, 0.42 mol), racemic 2-chloropropanic acid (39.4 mL, 0.46 mol), and *o*-phosphoric acid (0.23 g) were refluxed in toluene (50 mL). The water of esterification was removed by the use of Dean Stark apparatus. After 6 h the reaction mixture was cooled to room temperature and carefully quenched with saturated sodium hydrogen carbonate solution. The organic phase was separated and dried over sodium sulphate, and filtered. The filtrate was purified by vacuum distillation to yield 3-octyl ester as a colourless oil (52.91 g, 57% yield, b.p. 171-173 ^{o}C, 600 mm, Hg. G.C. retention time = 11.18 min).

5.1.4 *sec*-Phenethyl-2-chloropropionate (Harwood and Moody, *1999* [b])



A mixture of *sec*-phenethyl alcohol (42.34 g, 0.35 moles) and pyridine (24.76 g, 0.31 moles) in dry dichloromethane (100 mL) was added dropwise over 50 min to a stirred solution of 2-chloropropionyl chloride (39.24 g, 0.31 moles) in dry dichloromethane (150 mL), under a nitrogen atmosphere. After 2 h the reaction was stopped by the addition of water. The organic phase was extracted with dichloromethane and washed with water. The organic extracts were dried over magnesium sulphate, filtered, and concentrated. Purification of the residue by flash column chromatography (petrol : EtOAc 25: 1) yielded the ester (57.7 g, 66% yield).

5.2 Results and Discussion

After establishing a chiral assay method, large scale synthesis of various esters of CPA in large quantities were carried out. For this purpose an acid catalysed esterification method was employed (**fig 30**), in which CPA, alcohol, and a catalytic amount of *O*-phosphoric acid, in toluene were refluxed, the water of esterification was removed (using Dean Stark apparatus). As water catalyses the hydrolysis of the esters, it was necessary to remove the water of esterification from the reaction media in order for the reactions to proceed in the direction of esterification. The esters formed were purified by vacuum distillation or flash column chromatography using petroleum spirit and ethyl acetate as a mobile phase. The purity of the esters was determined by gas chromatography. All straight chain esters (C4 and higher) were synthesised according to the Dean Stark method (*Harwood and Moody, 1996*).

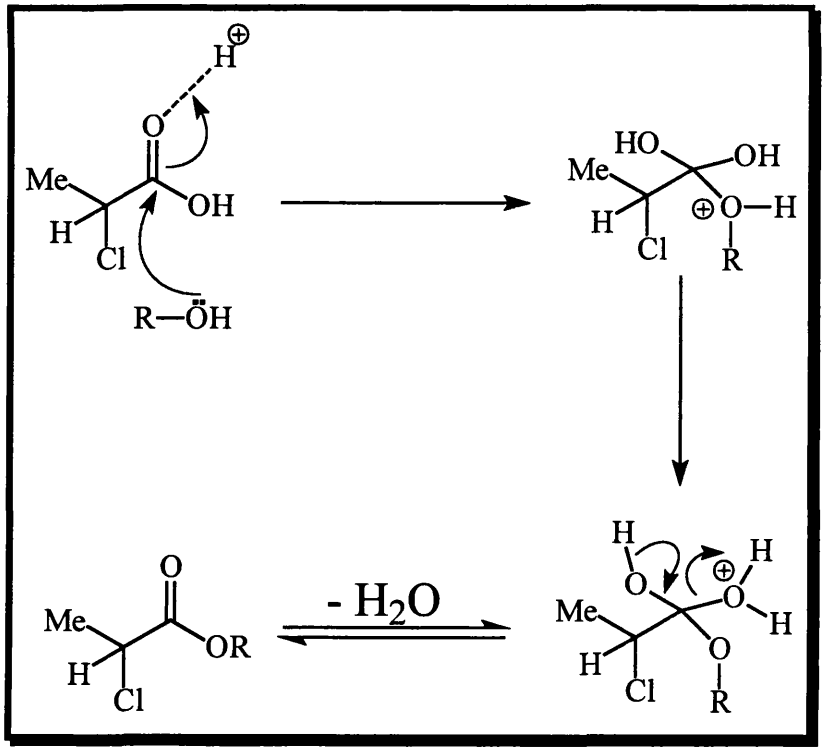


Figure 30: Reaction mechanism of acid catalysed ester synthesis

Synthesis of the *iso*-propyl ester was not possible by that method because of the lower boiling point of *iso*-propanol compared to toluene. For the *iso*-propyl ester, another acid catalysed method was employed, where two molar excess of alcohol, and acid were charged in the flask and the *iso*-propyl alcohol was removed bydistillation. A further two equivalence of alcohol was added and removed by distillation this procedure was repeated 10 times. This particular method was found to be very inefficient as ester was recovered in low yield, and large excess of alcohol was consumed, therefore this method was not used for any other ester synthesis.

Esters prepared using *sec*-phenethyl alcohol and 1-octene-3-ol were made by a different method than the two outlined above. Primary alcohol based esters are prone to a rearrangement under acidic conditions leading to a mixture of compounds. The rearrangement is outlined in **fig 31**.

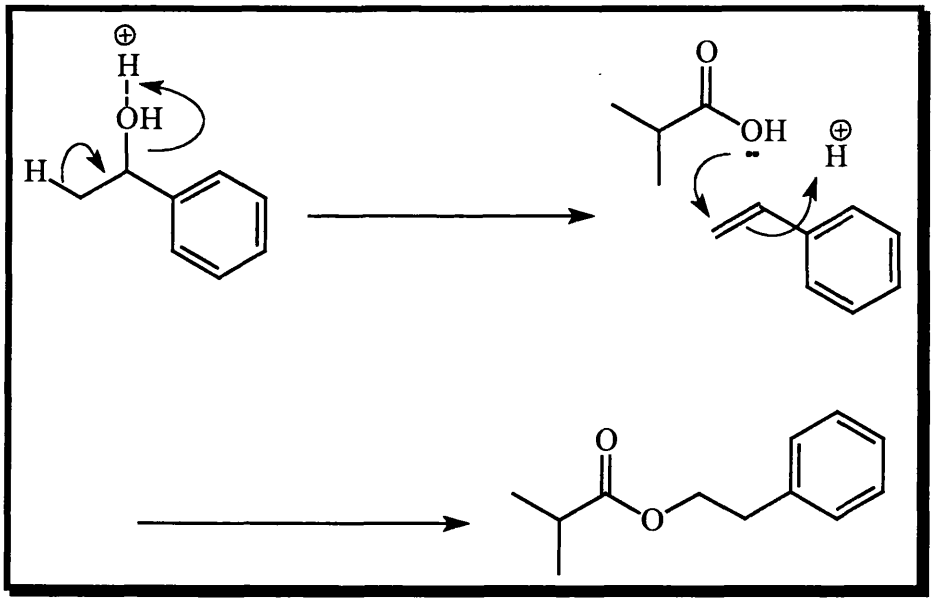


Figure 31: Possible rearrangement of secondary esters under acidic conditions

In acidic medium α-proton collapses to release water to give an alkene which is then attacked by the carboxylic acid at the least hindered carbon to give an undesired ester

as well as the desired ester. Keeping this situation in mind we used a mild esterification method using the acid chloride as a starting material (for mechanism see **fig 32**). To the acid chloride in dry dichloromethane under nitrogen atmosphere was added dropwise the mixture of an alcohol and pyridine in dry dichloromethane. The reaction was stirred at room temperature and monitored by tlc. The two roles of pyridine in this reaction were to activate the acid chloride by making a pyridinium complex, which then undergoes a nucleophilic attack by an alcohol to produce the ester. The second role was to remove the hydrochloric via the formation of pyridinium hydrogen chloride as a by-product, which was filtered at the end of the reaction. This reaction gave relatively high yields and purity compared to the normal acid catalysed esterification reaction.

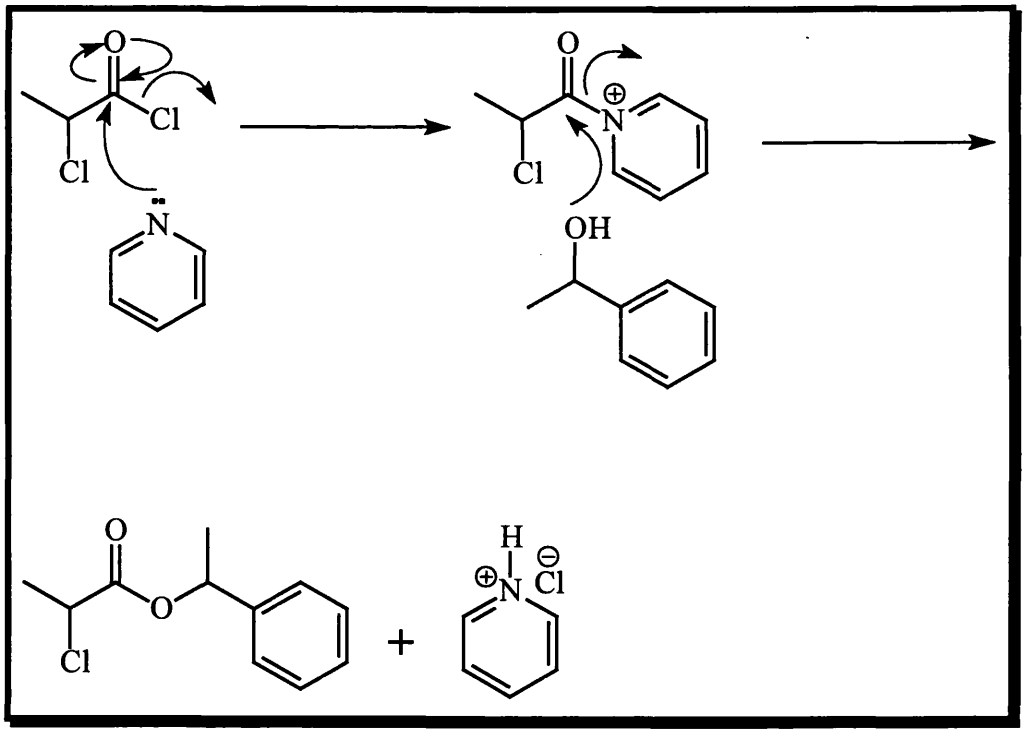


Figure 32: Reaction mechanism of ester synthesis using pyridine as a catalyst

All the esters prepared along with their analytical data are summarised in the following table (**table 2**).

Ester	RRF	% Yield	b.p (°C)	
Dodecyl-2-CPA	1.3	53	238 (Hg, 620 mm)	
Octyl-2-CPA	1.1	61	181 (Hg, 600 mm)	
2-Octyl-2-CPA	0.7	48	172 (Hg, 620 mm)	
3-Octyl-2-CPA	0.8	57	173 (Hg, 620 mm)	
1-Octene-3-yl-2-CPA	0.8	45	167 (Hg, 620 mm)	
Hexyl-2-CPA	0.9	77	158 (Hg, 620 mm)	
2-Pentyl-2-CPA	0.7	63	131 (Hg, 620 mm)	
Butyl-2-CPA	0.6	74	164	
2-phenylethyl-2-CPA	0.9	66		
Cyclohexyl-2-CPA	0.7	68		ε_{270} (mol^{-1} cm^{-1})
	-			
Hexyl-2-phenoxypropionate	1.4	82		1186
Octyl-2-phenoxypropionate	1.8	78		1464
di-ethylene glycol methyl ether ester of 2-phenoxypropionic acid	1.4	79		1166

Table 2: List of the esters prepared along with the analytical data

5.3 Conclusions

- Primary esters of CPA and 2-phenoxypropionic acid can be synthesised in good yield using acid catalysed Dean Stark apparatus. Products can be purified by distillation under reduced pressure or flash chromatography. Flash chromatography is advisable when purifying higher esters.

- Synthesis of secondary esters by acid catalysed esterification reaction results in considerable amount of the by-products being formed. Secondary esters can be readily made from the acid chloride in a presence of weak base (pyridine) at room temperature. This method can also be used for the synthesis of small esters.

- Small esters of CPA (C1 - C3) can also be made by the acid chloride method.

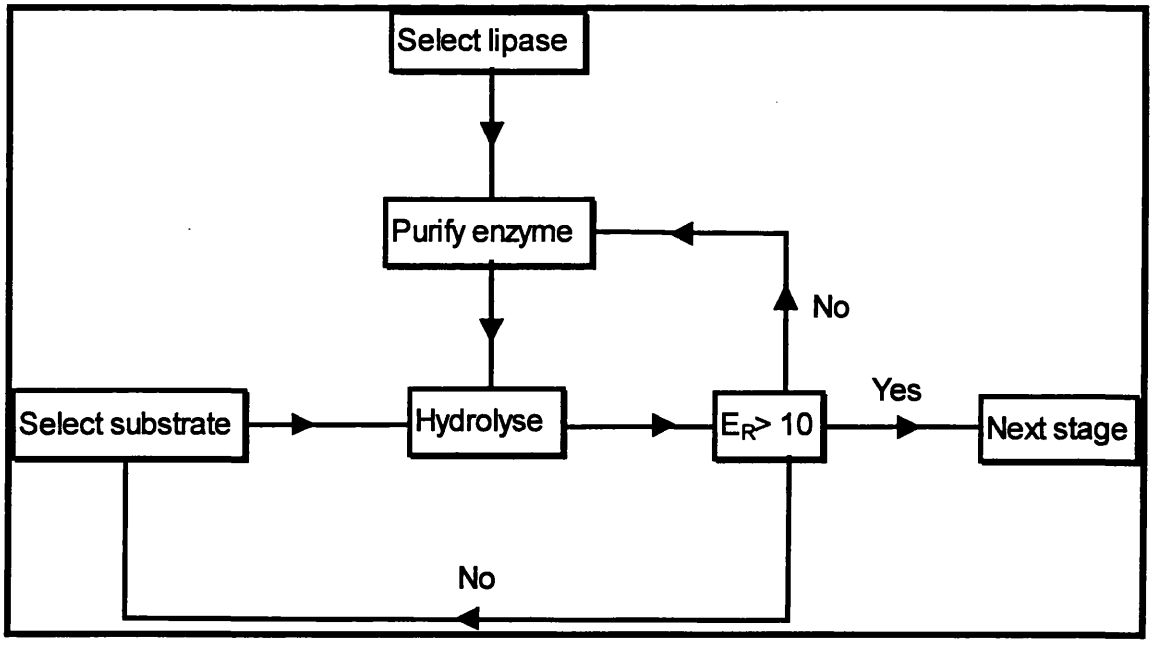
6 Lipase Screening

6.1 Aim

- To screen all commercially available lipases against esters of CPA on a small scale

- To use the successful lipase and substrate combinations on a large scale

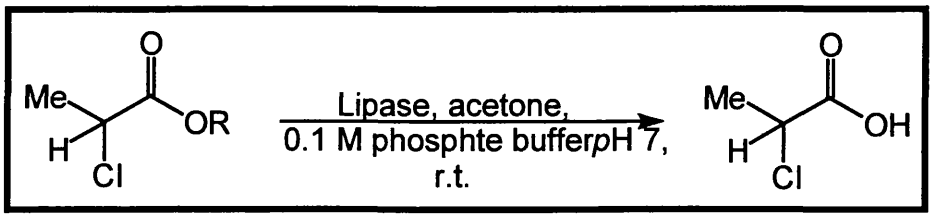
All commercially available lipases must be screened against custom made esters. Aterwards we will qualitatively analyse the screen and short list the promising combinations. The combinations have to be tried on a large scale where the product and residual substrate can be isolated and analysed for their optical purity. An enzyme with an E_R of 10 or above for CPA is required for the operation of a column reactor. If no lipase is found to have an E_R above 10, the crude lipase must be purified or activated in some way to increase its specificity. Sbstrates can also be modified to find the optimal combination.



Scheme 16: Guidelines for selecting lipase and substrate combination

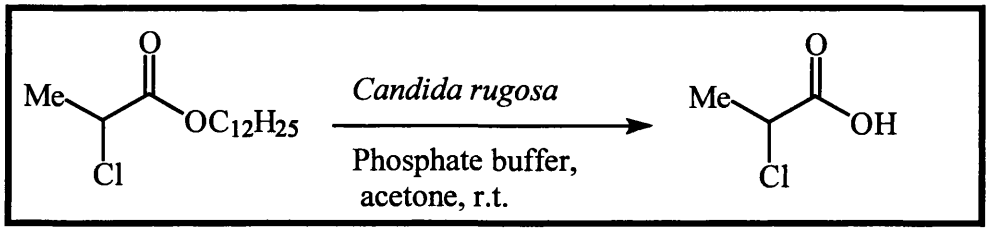
6.2 Experimental

6.2.1 Hydrolysis of esters using lipase screening kit:



Ester (20 mg), acetone (0.1 mL), and lipase (20 mg) in 0.1M phosphate buffer *p*H 7 (0.9 mL) were shaken on a vortex shaker for 20-24 h. The progress of the reaction was monitored by tlc (solvent system pet:EtOAc 4:1, staining agent $KMnO_4$).

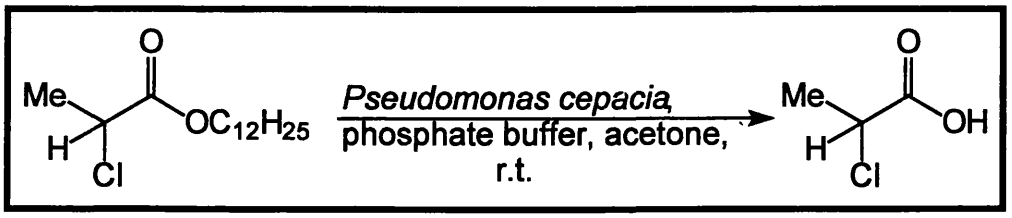
6.2.2 Hydrolysis of dodecyl ester with *Candida rugosa* lipase:



A mixture of the dodecyl ester (1.48 g, 6.1 mmoL), 0.1 M phosphate buffer *p*H 7 (15 mL), acetone (5 mL), and *Candida rugosa* lipase (0.71 g), were stirred vigorously at room temperature for 16 h. The *p*H of the reaction was acidified to *p*H 1 with 3N HCl. The aqueous phase was extracted with ether (5 x 50 mL). The combined organic phase was washed with saturated sodium bicarbonate solution (1 x50 mL). The organic phase was dried over sodium sulphate, filtered and concentrated to give a mixture of dodecyl ester, dodecanol and CPA. The mixture was purified by silica

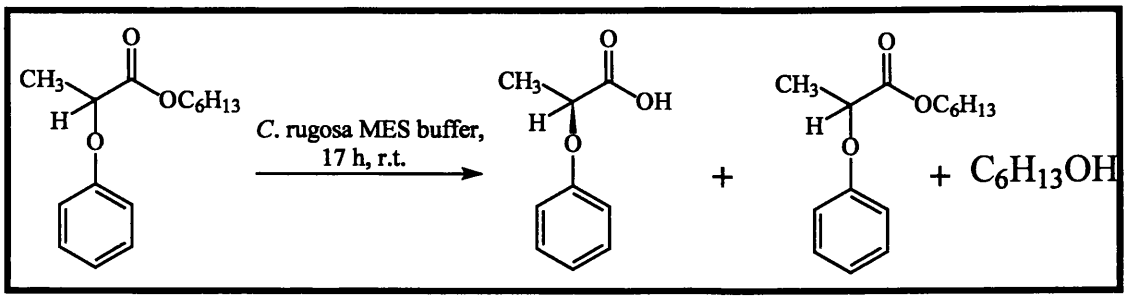
column chromatography (pet:EtOAc 25:1, 4:1) to give dodecyl ester (0.58 g, 89%). and CPA as a colourless oil (0.078 g, 24 %).

6.2.3 Hydrolysis of dodecyl ester with *Pseudomonas cepacia* lipase:



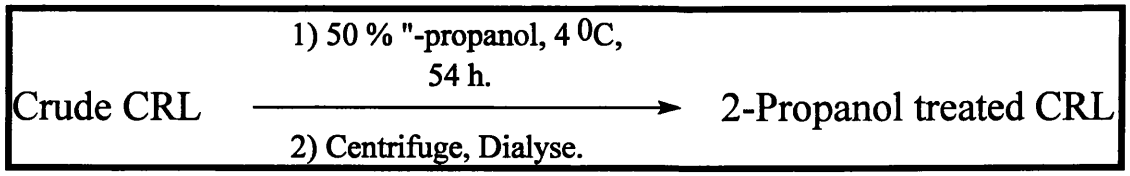
A mixture of dodecyl ester (1.52 g, 6.3 mmol), 0.1 M phosphate buffer *p*H 7 (17 mL), acetone (4 mL) and *Pseudomonas cepacia* lipase (0.50 g), were stirred vigorously at room temperature for 15 h. The reaction was quenched with saturated sodium bicarbonate (15 mL). The aqueous phase was extracted with ether (5 x 50 mL). The combined organic phase was dried over sodium sulphate, filtered and concentrated to give the mixture of dodecyl ester and dodecanol. The mixture was purified by silica column chromatography (petroleum spirit : EtOAc 25:1) to give dodecyl ester (0.84 g, 86 % yield). The aqueous phase was acidified to *p*H 1 with 3N HCl. The organic layer was extracted with ether (5 x 30 mL). The combined organic extracts were dried over sodium sulphate, filtered and concentrated in *vacuo*. Purification of the residue by silica column chromatography (pet:EtOAc 3:1) yielded CPA(0.04 g, 19 %).

6.2.4 Hydrolysis of hexyl-2-phenoxypropionate with *C-rugosa* pre-treated with IPA:



A mixture of hexyl-2-phenoxypropionate (2.58 g, 10.3 mmol), 50 mM MES buffer *p*H 6.5 (75 mL), *C. rugosa* lipase (5 mL), was stirred vigorously at room temperature for 17 h. The reaction was quenched with saturated sodium bicarbonate. The aqueous phase was extracted with ether (5 x 100 mL). The combined organic phase were dried over sodium sulphate, filtered and concentrated to give a mixture of hexyl-2-phenoxypropionate and hexanol. The mixture was purified by silica column chromatography (pet:EtOAc 25:1) to give (2.08 g). The aqueous phase was acidified to *p*H 1 with 3N HCl. The organic layer was extracted with ether (5 x 100 mL). The combined organic extracts were dried over sodium sulphate, filtered and concentrated in *vacuo*. Purification of the residue by silica column chromatography (pet:EtOAc 3:1) yielded 2-phenoxypropionic acid (0.33g).

6.2.5 IPA treatment of crude *C. rugosa* lipase:



Crude *C. rugosa* lipase (5.06 g) was dissolved in MES buffer (50 mL, 50 mM, *p*H 6.0, 4 °C) by stirring for 30 min. 2-Propanol (50 mL) was added dropwise over 20 min. After 54 hours the precipitate was removed by centrifuging at 3000 rpm for 30 mins at

4 °C. The pellet was resuspended in MES buffer (90 mL). The supernatant and resuspended pallet were dialysed separately against distilled water (3 x 4 L), and then against PEG 4000. The final volume of the supernatant fraction (90 mL) and resuspended pellet (25 mL) were stored at 4 °C with 0.02 wt/vol. % sodium azide.

6.3 Results and discussion

After synthesising esters of CPA, they were hydrolysed on a small scale, with commercially available lipases. The hydrolysis of the esters were qualitatively followed using thin layer chromatography, which was visualised under UV source and then stained with potassium permanganate. The degree of hydrolysis was estimated from tlc. Below is the qualitative assessment of the hydrolysis, divided into two sections based on the esters of primary (**table 3**) and secondary alcohols (**table 4**).

The degree of conversion was visually estimated by observing the appearance of a spot for alcohol and the disappearance of the spot for ester on a tlc plate. The results were divided into four categories:

1. – where conversion was estimated to be between 0-20 %

2. ± where conversion was estimated to be between 20-40% conversion

3. + where conversion was estimated to be between 40-60% conversion

4. + + where conversion was estimated to be between 60-100% conversion

An ideal lipase and substrate combination is where conversion is around 50% after 2 h and remains at 50% even after 24 h. The combination of lipase and substrate which falls in category 2 and 3 was chosen for further studies.

Enantiospecific lipase will only hydrolyse one enantiomer of the ester, therefore, the hydrolysis should stop once the degree of conversion has reached 50%. If the conversion is carried on beyond 50% then it is clear that the lipase does not distinguish between two enantiomers, hence the product isolated will have low enantiomeric excess. However, if the degree of conversion after 24 h is below 25% then it is not safe to assume that the lipase is non specific, as the lipase may be

specific but shows low activity towards the substrate. In this case the reaction time will be long and hence uneconomical. The results have been characterised according to the above information.

6.3.1 Hydrolysis of hexyl-chloropropionate.

Lipases L1-L3, L5, L10 and L14-L16 catalysed non specific hydrolysis of the hexyl ester as the degree of conversion was approximately 100%, while lipases L11-L13 were inactive. Approximately a 50% degree of hydrolysis was obtained with the lipases L8, L17 and L19 and this suggests some enantiospecificity and therefore, these enzyme and substrate combinations were chosen for further studies.

6.3.2 Hydrolysis of octyl-chloropropionate.

Lipases L3, L4, L11, L12, L13, and L18 were found to be inactive towards the octyl ester. Lipases L1, L2, L6, L8-L10, L14, L16-L17 catalysed the hydrolyses to the desired degree of conversion (50%) and were chosen for the large scale hydrolysis.

6.3.3 Hydrolysis of dodecyl-chloropropionate.

Lipases L6, L14, L17 were found to be the desired lipases. Lipases L11-L12 were also found to be inactive towards the dodecyl ester.

Most of the lipases were found to be inactive towards the secondary alcohol esters. Only lipases L5, L10, L17 and L19 possessed preferred activity towards the secondary esters (**table 4**).

Lipases	Visually estimated hydrolysis					
	Dodecyl		Octyl		Hexyl	
	3 h	22 h	4 h	23 h	3 h	22 h
L1 *Pseudomonas fluorescens*	+	++	+	+	++	++
L 2 *Chromobacterium viscosum*	±	++	±	++	++	++
L 3 *Mucor miehei*	-	-	-	-	-	++
L 4 *Candida antartica*	-	-	-	-	-	-
L 5 *Candida rugosa*	+	++	+	+	-	++
L 6 *Porcine Pancreatic*	-	+	-	±	-	-
L 7 *Aspergillus niger*	-	-	-	-	-	-
L 8 *Mucor Javanicus*	±	++	-	++	±	±
L 9 *Rhizopus arrhizus*	-	±	±	+	-	-
L 10 *Pseudomonas cepacia*	+	++	-	+	+ +	++
L 11 Wheatgerm	-	-	-	-	-	-
L 12 *Candida lipolytica*	-	-	-	-	-	-
L 13 *Penicillium roqueforti*	-	±	-	-	-	-
L 14 *Rhizopous niveus*	-	+	±	++	-	++
L 15 Pig liver acetone	-	+	-	±	++	++
L 16 Bakers yeast	streak	streak	-	++	+	++
L 17Bovine liver acetone powder	±	+	+	+	-	+
L 18 *Aspergillus oryzae*	-	-	-	-	-	-
L 19 Horse liver acetone powder	±	++	-	+	+	+

Table 3: Screening of primary alcohol based esters of CPA against commercially available lipases

Lipases	2-octyl		3-octyl		1-octene-3-ol	
	2.5 h	20 h	3 h	20 h	3 h	20 h
L 1 *Pseudomonas fluorescens*	-	-	-	-	-	-
L 2 *Chromobacterium viscosum*	-	-	-	-	-	-
L 3 *Mucor miehei*	-	-	-	-	-	-
L 4 *Candida antartica*	-	-	-	-	-	-
L 5 *Candida rugosa*	±	+	-	+	-	-
L 6 *Porcine Pancreatic*	-	-	-	-	-	±
L 7 *Aspergillus niger*	-	-	-	-	-	-
L 8 *Mucor Javanicus*	-	-	-	-	-	-
L 9 *Rhizopus arrhizus*	-	-	-	-	-	-
L 10 *Pseudomonas cepacia*	-	+	-	+	±	±
L 11 Wheatgerm	-	-	-	-	-	-
L 12 *Candida lipolytica*	-	-	-	-	-	-
L 13 *Penicillium roqueforti*	-	-	-	-	-	-
L 14 *Rhizopous niveus*	-	-	-	-	-	-
L 15 Pig liver acetone	-	-	-	±	±	+
L 16 Bakers yeast	-	-	-	-	-	-
L 17 Bovine liver acetone powder	±	+	-	+	±	+
L 18 *Aspergillus oryzae*	-	-	-	-	-	-
L 19 Horse liver acetone powder	+	+	-	+	+	+

Table 4: Screening of secondary alcohol based esters of CPA against commercially available lipases

The lipases and the substrate combination originally thought to be promising were short listed and are summarised in **table 5**.

Lipases	Dodecyl	1-Octyl	2-Octyl	3-Octyl	1-Octene	Hexyl
L 1 *Pseudomonas fluorescens*	✔	✔				
L 2 *Chromobacterium viscosum*	✔					
L 5 *Candida rugosa*	✔	✔	✔	✔		
L 8 *Mucor Javanicus*	✔					
L 9 *Rhizopus arrhizus*	✔	✔				
L 10 *Pseudomonas cepacia*	✔	✔	✔	✔	✔	✔
L 14 *Rhizopous niveus*	✔	✔	✔			
L 17 Bovine liver acetone powder	✔	✔	✔	✔	✔	✔
L 19 Horse liver acetone powder	✔	✔	✔	✔	✔	✔

Table 5: List of lipase and substrate combinations selected for large scale studies

Pseudomonas cepacia, bovine and horse liver acetone powder were found to be active against all the esters screened while *Candida rugosa* lipase was thought to be an ideal enzyme for most medium to large sized primary and secondary esters of CPA screened. The above ester and lipase combination were carried out on large scale. **Table 6** contains the data from large scale hydrolysis.

All hydrolysis were carried out at room temperature, unless otherwise stated. In a typical hydrolytic reaction 2.5 g substrate, 0.5 g lipase and buffer (pH 6.0) were stirred in a round bottom flask for 24 h. The conversion was determined by GC (internal standard method). The reaction was stopped by the addition of saturated sodium bicarbonate. The CPA produced and the residual ester was purified by flash column chromatography. The purified CPA was derivatised to the corresponding mandelate ester and the optical purity was determined by NMR.

Substrate	Lipase	Conversion (%)	e.e. of CPA (%)
Hexyl ester	Bovine	51	30
Hexyl ester	*P. cepacia*	59	5
Octyl ester	*C. rugosa*	41	25
Octyl ester	*C. rugosa*	29	37
Octyl ester	*C. rugosa*	24	39
Octyl ester	*P. fluorescense*	34	20
Octyl ester	*P. cepacia*	47	22
2-Octyl ester	Bovine	29	23
2-Octyl ester	*P. cepacia*	37	16
Dodecyl ester	*P. fluorescence*	43	22
Dodecyl ester	Chromobacterium	31	27
Dodecyl ester	*C. rugosa*	41	25
Dodecyl ester	*P.cepacia*	36	25
Dodecyl ester	*R. niveus*	73	38

Table 6: e.e. values of CPA produced from the hydrolysis of its esters using various lipases.

The highest enantiomeric excess of the CPA produced was 39% at 24% conversion, this was achieved by hydrolysing the octyl ester with *C. rugosa* at 37 °C. Despite the data recorded in the literature (Klibanov, ***84***, Dahod, ***87***) regarding the production of optically pure (2*S*)-CPA, we were unsuccessful in isolating the (2*S*)-CPA in a high enantiomeric excess. It is assumed that the low enantiomeric excess could be due to the fact that the acid moiety is very small and does not fit very well in the active site of the lipase, or the size of chlorine atom and the methyl group at the chiral centre is very similar and the lipase can not distinguish between the two enantiomers hence hydrolysing both enantiomers at the same rate.

The alcohol moiety was changed from the straight chain to the cyclic structure to make the molecule more bulky and rigid. *Sec*-phenethy and cyclohexyl esters of CPA were synthesised and were screened against commercially available hydrolase enzymes.

Lipases	*sec*-phenethyl		cyclohexyl	
	3 h	33 h	2 h	24 h
L 1 *Pseudomonas fluorescens*	++	++	-	++
L 2 *Chromobacterium viscosum*	++	++	-	++
L 3 *Mucor miehei*	+	++	-	++
L 4 *Candida antartica*	++	++	-	++
L 5 *Candida rugosa*	++	++	-	++
L 6 *Porcine Pancreatic*	+	+	-	-
L 7 *Aspergillus niger*	-	+	-	-
L 8 *Mucor Javanicus*	++	++	±	+
L 9 *Rhizopus arrhizus*	±	++	-	+
L 10 *Pseudomonas cepacia*	++	++	++	++
L 11 Wheatgerm	-	-	-	-
L 12 *Candida lipolytica*	-	±	-	-
L 13 *Penicillium roqueforti*	-	-	-	-
L 14 *Rhizopous niveus*	++	++	±	++
L 15 Pig liver acetone	+	++	±	++
L 16 Bakers yeast	++	++	-	±
L 17 Bovine liver acetone powder	++	++	++	++
L 18 *Aspergillus oryzae*	++	++	±	++
L 19 Horse liver acetone powder	++	++	++	++

Table 7: Screening of cyclic esters of CPA against commercially available lipases

The results obtained from the initial screening (**table 7**) were quite disappointing as not many lipases were found to be active with these substrates. Only a combination of *Porcine pancreatic* lipase (PPL) and *sec*-phenethyl ester and *M. javanicus* lipase and cyclohexyl ester were considered to be promising. The large scale hydrolysis revealed that both lipases were not highly specific towards the two substrate (**table 8**).

Substrate	Lipase	% Conversion	e. e. CPA (%)
2-PhEt ester	PPL	21	38
Cyclohexyl ester	*M. javanicus*	7	20

Table 8:e.e. of CPA produced from the enzymatic hydrolysis of its cyclic esters

As changing the alcohol moiety of the ester made no considerable difference to the enantiomeric excess of the CPA produced, it was then decided to manipulate the substituents at the α-carbon one obvious choice was to replace the chlorine with phenoxy group. The resulting racemic 2-phenoxypropionic acid is commercially available and optically pure 2-phenoxypropionic acid is in fact synthesised from enantio pure CPA. Therefore at this stage attempts to resolve CPA were temporarily stopped, and efforts were made to resolve 2-phenoxypropionic acid by means of *C. rugosa* catalysed hydrolysis of its hexyl and octyl esters (**table 9**). The lipase was obtained from Biocats and Sigma, the lipase from both suppliers was nonspecific, but the lipase from Biocats was discovered to be twice as specific towards the (*R*) enantiomer of the hexyl ester of 2-phenoxypropionic acid as the lipase purchased from Sigma.

Supplier	Ester	Conversion (%)	e.e. (%)	E_R
Biocats	hexyl	38	56	5
Sigma	hexyl	18	37	2

Table 9: E_R of crude *Candida rugosa* lipase (from two different sources) towards hexyl ester of 2-phenoxypropionic acid

The yeast *C. rugosa* synthesises and secrets a mixture of five lipase isozymes (*Alberghina, 1994*). There is a strong evidence that at least one of the *C. rugosa* enzymes has a somewhat different substrate specificity, which can result in a low enantiomeric excess of the product. There is also evidence that *C.rugosa* lipase preparations from various suppliers show variations in their catalytic efficiency, and stereospecificity (*Shaw, 1994*). This is due to the presence of different forms of lipases (*Gago, 1999, Pandey, 1998, Valero, 1998*) present in different preparations (*Valero, 1999*).

Researchers have carried out several approaches to enhance the specificities of the lipases, such as adding additives to reduce the hydrolysis of the slow-reacting enantiomer.

A rather controversial strategy for increasing the specificity of *C. rugosa* is to change the conformations of isozymes present to one conformation using an organic solvent treatment (*Colton, 1995, Cipiciani, 1999, Chamorro, 1998*). Colton *et. al.* have considered three possible mechanism by which the organic solvent can effect the enantiospecificity.

i) Removal of contaminating hydrolases with lower, or opposite, enantioselectivity.

ii) Removal of non-protein contaminants that inhibit the lipase and lower its specificity.

iii) Change of conformation of the lipase, thereby activating the lipase and changing its specificity.

They concluded from their studies that conformation change does take place when crude *C. rugosa* is treated with organic solvent. However, the other two possibilities cannot be ruled out.

Despite the controversy the organic solvent treatment remains the easiest way to enhance the specificity of the *C. rugosa.* We have carried out extensive study into the lipase activity and specificity behaviour when treated with different concentrations of *i*-PrOH over a period of time. From our study we concluded the following observations.

6.3.5 Protein content

The protein content of the commercial lipase derived from *C. rugosa* is low. The actual value depends on the assay used, the Bradford protein assay suggests that it contains less than 5% protein, the Lowry assay shows that the 14 % of commercial sample consists of protein (**table 10**). The actual values do not affect the mass balance of the fractionation, but due to the large discrepancies it was necessary to present the data on the basis of the quantity of enzyme recovered per gram of crude enzyme, rather than in terms of the input of protein

Assay method	Protein content (mg/g sample)
Lowry	141 ± 15
Bradford	46 ± 12

Table 10: Protein content of commercially available crude *C. rugosa* lipase (carried out by J.Vaghjiani)

6.3.6 Lipase activities:

According to the large range of intrinsic lipase activities shown in **table 11**, substrates can be arbitrarily divided into two groups, those whose hydrolysis is catalysed with rates above 10 mmol/g (HCPA and pNPA) and those with rates below 0.5 mmol/g. (SEL, triacetin and HPPA). The rates of hydrolysis of HCPA is found to be about 1000-times faster than it is against HPPA.

Although only one sample was assayed a sufficient number of times to record both a mean and a standard error, it does appear the variability of the assays for any one sample is about 10%, except for the assays with SEL. In this instance the results are much more variable (**table 11**). This was particularly true for a sample supplied without the usual stabilising additives (batch 4), in which the activity fell progressively from 1.3 mMole/g to about 0.45 mmol/g over a period of about a month.

	mmol/min/g		μmol/min/g		
Batch #	HCPA	pNPA	SEL	Triacetin	HPPA
1	8.0				
2	15.5		710 ± 350 (4)		
3	9.3 ± 0.7 (5)		340 ± 50 (5)		
4	23.8	14.4	460	54	28.5

Table 11: Lipase activity of crude *C. rugosa* lipase towards various esters (pNPA assays were carried out by Mr. J. Vaghjiani)

6.3.7 Fractionation with 20% IPA:

Fractionation with 20% IPA is necessary before the crude *C. rugosa* can be induced to crystallise which is essential for the manufacture of CLECs®.

6.3.7.1 High activity enzyme:

The bulk of the activity resides in the 20% supernatant (**table 12**). There is an over recovery of the activity against HCPA in the presence of IPA which is lost when samples are dialysed to remove IPA. This indicates that the enzyme is activated by the presence of organic solvent.

6.3.7.2 Low activity enzyme:

The recovery of the low activity enzymes is more equally divided between the two fractions (**table 13**). The activation of the activity in the 20% IPA pellet is more noticeable than it is for the high activity enzymes particularly because it increases with the length of the IPA treatment. Some of the increased activity remains even after the dialysis (when the dialysed samples from the 2 h and 60 h treatment are compared) and it is particularly marked with the activity measured against HPPA. In this case there is a significant over-recovery of the activity even after the dialysis (total activity recovered is 40 μmol/min/g compared to an input of only 28 μmol/min)

	mmol /min/g			
Substrate	HCPA		pNPA	
Crude	24		14.4	
Fractionation time (h)	2	60	2	60
Supernatant	40	48	11.6	10.6
Pellet	11.8	11.4	2.4	3.0
Dialysed supernatant	22	26	9.8	9.0
Dialysed Pellet	10.1	4.3	1.6	1.3

Table 12: Activity of crude *C. rugosa lipase* and lipase treated with 20% IPA against hexyl ester of CPA and pNPA. (pNPA assays were carried out by J. Vaghjiani

Substrate	μmol/min/g SEL		Triacetin		HPPA	
crude solid	460		54		29	
Fractionation Time (h)	2	60	2	60	2	60
Supernatant	263	236	31	32	22	22
Pellet	190	259	25	48	20	35
Dialysed supernatant	112	166	21	34	14	23
Dialysed Pellet	128	142	17	25	11	16

Table 13: Activity of crude *C. rugosa lipase* and lipase treated with 20% IPA towards hexyl ester of 2-phenoxypropionic acid, triacetin and *S*-ethyl lactate

6.3.7.3 Enantiospecificity:

Neither the supernatant, nor the pellet from the 2 h and 60 h treatment had any significant effect on the specificity of the lipase for either enantiomer of HPPA (**table 14**). The E_R is 1 for the dialysed pellet and 2 for the supernatant and crude lipase.

Treatment time (h)	E_R Supernatant	Pellet
2	2	1
60	2	1

Table 14: Specificity of different fractions of *C. rugosa* lipase treated with 20% IPA towards the hexyl ester of 2-phenoxypropionic acid

The supernatant from 20% IPA treatment (2 h) was treated with 48% 2-methyl-2,4-pentanediol (MPD) for approx. 60 h. This treatment resulted in the crystallisation of the protein. The crystals were separated and cross-linked with glutaraldehyde. The

crystals, crystallisation supernatant and CLECs® prepared in the lab by J. Vaghjiani and from Altus were tested for their specificity for HPPA (**table 15**).

Lipase fraction	E_R
Crystals	8
Crystallisation supernatant	5
CLECs ® (Altus)	2
Ours	8

Table 15: Specificity of CLECs ® and crystals obtained from *C. rugosa* lipase (treated with 20% IPA) towards hexyl ester of 2-phenoxypropionic acid

As mentioned before, the supernatant from 20% IPA treatment of the lipase for 2 h was crystallised. Upon treatment with MPD over 60 h the enzyme was crystallised and the E_R value of the lipase increased four fold (2 to 8). The crystals were centrifuged and the crystallisation supernatant isolated exhibited E_R value of 5 towards the hexyl ester. The cross linked lipase prepared in our laboratory showed same E_R value as the crystals i.e. there was no change in the E_R upon cross-linking, however, the CLECs ® bought from Altus showed considerably lower E_R (2) when compared to our CLECs ®.

In short, the 20% IPA treatment did not enhance the specificity of the lipase, but the MPD treatment for crystallisation significantly enhanced the specificity.

6.3.8 Fractionation with 50% IPA:

This fractionation is considered essential for the successful resolution of HPPA.

6.3.8.1 High activity enzyme:

The bulk of the activity resides in the pellet. Although there is a considerable increase in the activity against HCPA which is lost upon dialysis, there is a similar loss of activity against pNPA even though there is no activation in the presence of IPA (**table 16**). This suggests that at least some of the enzymes are unstable in the presence of high concentrations of IPA even if others are actually activated. Note the large increase in activity against HCPA with the increase of duration of treatment with IPA.

6.3.8.2 Low activity enzymes:

The pellet mainly consists of low activity enzymes, but the bias is less extreme than for the high activity enzymes. In this regard the distribution resembles that observed after the 20% IPA fractionation where again the low activity enzymes were the more equally distributed.

	mmol/min/g			
Substrate	HCPA		pNPA	
Crude	24		14.4	
Fractionation time (h)	2	60	2	60
Supernatant	4.5	4.0	0.4	0.4
Pellet	46	64	8.4	7.9
Dialysed supernatant	3.3	1.2	0.4	0.2
Dialysed Pellet	23	26	3.3	2.8

Table 16: Activity of crude *C. rugosa lipase* and lipase treated with 50% IPA against hexyl ester of CPA and pNPA. (pNPA assays were carried out by J. Vaghjiani)

There is considerable activation of all of the activities (**table 17**) in the precipitates, but this does not generally survive the dialysis which removes the IPA. After the

dialysis, the total recoveries of the activity against triacetin are close to 100% both after 2 and 60 hours of treatment. The recovery in the pellet does increase with time at the expense of the supernatant, and this might seem to be a progressive precipitation were it not for the effect on the activity against HPPA. Here the precipitate does increase its activity with time, and there is no compensating decrease in the supernatant.

Much of the supernatant activity against SEL does not survive the 50% IPA treatment. The activity falls from about 200 to 30 μMole/min/g crude solid and is further reduced on dialysis. These results were confirmed in a separate experiment (**table 18**) where the activity even after 2h treatment was rather lower. Therefore it can be concluded that this substrate is measuring an unstable activity in the supernatant, and this instability is reflected in the loss of activity against the substrate which is observed in an unstabilised batch of the crude solid (batch 2 in **table 11**).

	μmol/min/g					
Sample	SEL		Triacetin		HPPA	
Crude	460		54		29	
Fractionation time (hr)	2	60	2	60	2	60
Supernatant	199	30	32	32	8.8	9.3
Pellet	190	210	48	70	18.5	32
Dialysed supernatant	10	13	25	14	8.4	7.1
Dialysed Pellet	106	129	26	36	6.4	16

Table 17: Activity of crude *C. rugosa lipase* and lipase treated with 50% IPA towards hexyl ester of 2-phenoxypropionic acid, triacetin and *S*-ethyl lactate

	μmole/min/g	
Crude	460	
Fractionation time (h)	2	60
Supernatant	73	24
Dialysed supernatant	32	16

Table 18: Lipase activity of crude *C. rugosa* lipase and lipase treated with 50% IPA over 2 and 60 h towards *S*-ethyl lactate

6.3.8.3 Enantiospecificity:

As before all the fractions were analysed for their specificity towards the hexyl ester.

	E_R	
Treatment time	Supernatant	Pellet
2	9	2
60	16	8

Table 19 Specificity of different fractions of *C. rugosa* lipase treated with 50% IPA towards the hexyl ester of 2-phenoxypropionic acid

The 50% IPA treatment over 2 h increased the specificity of the lipase in supernatant from 2 to 9, however there was no increase in specificity observed in the pellet fraction (**table 19**).

The IPA treatment over 60 hours increased the E_R value of the lipase from 2 to 16. This eight fold increase is the largest E_R increase observed in our investigation of IPA treatment of the *C.* rugosa. The pellet from 60 h treatment also showed same specificity as the CLECs ® prepared from the 20% IPA supernatant towards the

hydrolysis of the hexyl ester. Note that all fractions show preference for the (*R*)-enantiomer.

Supernatant and pellet from the 50% IPA (60 h) were also analysed for its specificity towards the octyl ester of 2-phenoxypropionic acid

The supernatant showed over three times more specificity towards the (*R*)-enantiomer than the crude lipase. This increase is much less than the increase observed for the hexyl ester. The E_R of the pellet for octyl ester has increased slightly from 2 to 5 (**table 20**).

Fraction	E_R
Crude	2
Supernatant	7
Pellet	5

Table 20 Specificity of different fractions of *C. rugosa* lipase treated with 50% IPA (60 h) towards the octyl ester of 2-phenoxypropionic acid

In light of the increased specificity with solvent treatment, hydrolysis of the octyl CPA were attempted again with fractions from 50% IPA treatment and CLECs ® prepared.

It is clear from the table (**table 21**) that IPA treatment did not considerably enhance the specificity of the lipase towards the octyl esters of CPA. Note the higher E_R value of the pellet fraction compared to supernatant, contrary to the results for HPPA, where the supernatant fraction exhibited higher E_R value than the pellet fraction

Fraction	E_R
Supernatant	2
Pellet	4
CLECs ®	2

Table 21: Specificity of different fractions of *C rugosa* lipase treated with 50% IPA (60 h) and CLECs ® towards the octyl ester of CPA

6.4 Conclusion

- CPA cannot be resolved using straight chain esters and commercially available crude lipases

- Crude *C. rugosa* lipase from different suppliers exhibit different specificities towards 2-phenoxypropionic acid

- IPA treatment enhances the specificity of the *C. rugosa* lipase. The increase depends on the concentration of IPA and the treatment time

- *C. rugosa* treated with 50% IPA 60 h shows maximum increase in its specificity.

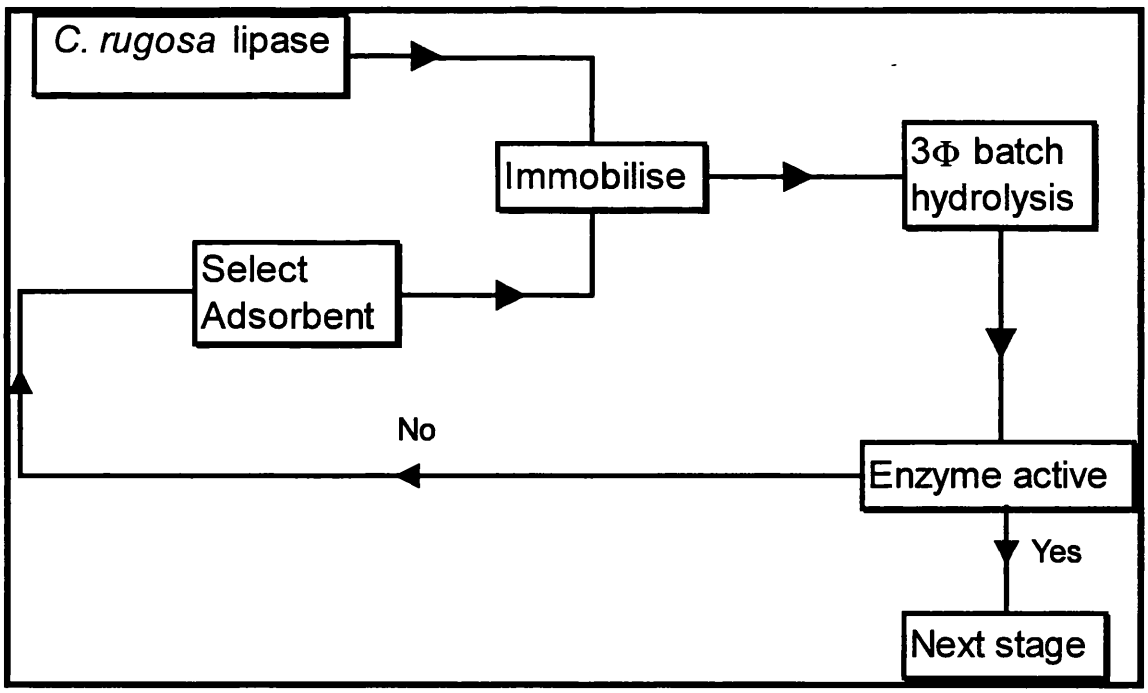
- Crystals and CLECs ® of *C. rugosa* also show enhanced specificity compared to their source

7 Lipase Immobilisation

7.1 Aim:

To immobilise the lipase onto a macro reticular adsorbent.

The activity of the immobilised lipase should be measured before and after the immobilisation, because the lipase must retain its activity during the immobilisation process. Lipase must also retain its E_R value for a particular substrate. The immobilisation conditions can be modified to obtain an active lipase. If the lipase is found to be inactive under optimum conditions, then a new adsorbent must be selected (resin must be able to separate the substrate and products).



Scheme 17: Guidelines for the successful immobilisation of *C. rugosa* lipase

7.2 Results and Discussion

The hydrophobicity or hydrophilicity of the matrix surface has a critical effect on the adsorption capacity and both characteristics confer advantages for lipase immobilisation. Amberlite XAD resins are classified as macro porous polymers from which XAD-7 has an intermediate polarity and a high pore size, while XAD-2 and 4 are less polar polymer with higher surface area and a smaller pore size than XAD-7. Higher pore size of XAD-7 could be the reason why enzymes are more efficient while immobilised on XAD-7 than it is on XAD-2 and XAD-4 (*Yunus, 1992*). The immobilisation results (**table 22**) obtained are consistent with the above given explanation.

Lipase from 50% IPA treatment over 60 hours was used for all immobilisation studies unless otherwise stated.

In a typical immobilisation experiment Amberlite XAD type (2g), 0.2 M MES buffer pH6 (0.3 mL), *C. rugosa* lipase (3 mL, 25 U), and 1M NaCl (30 μL) were shaken (125 rpm) at 27 °C for 16 hours. The resin was filtered and washed with immobilisation buffer (0.2 M MES, 100 mL). The immobilised lipase was stored at 4 °C.

The resin and the supernatant were assayed for lipase activity using a *p*H stat method (sec 4.1.4) with 20% *S*-ethyl lactate in 10 MmoL sodium chloride as the substrate.

The *S*-ethyl lactate is dissolved in water, no steric hindrance should occur for the lipase powder particle to reach the substrate. The assay is an esterase assay (the substrate is dissolved in water, no interface is present), which is a drawback when one is interested in lipase activities, since the activity at an interface (*Geluk, 1992*). Furthermore, the crude lipase preparation used contains several lipases, esterases and inhibitors (chapter 6). Preferential immobilisation can take place under the immobilisation conditions and inhibitors may be lost; therefore the direct comparison between the two fractions could be misleading.

The best results of immobilisation were achieved with Amberlite XAD7, where 192% of the lipase activity was retained (**table 22**). Amberlite XAD2 had less affinity for the lipase then XAD7, while XAD4 had least affinity for the lipase, only adsorbing 12% lipase activity.

Several examples of an increase in lipase activity upon immobilisation have been reported (*Celibi, 1981, Norin, 1988, Basri, 1996*). This increase in activity is presumably due to the increased hydrophobicity of lipase and its hydrophobic milieu. The local concentration of hydrophobic substrates around the enzyme may be higher and more accessible to the active site (*Basri, 1995*). Also, as mentioned earlier that immobilisation may have acted as a purification step, where some or all of the inhibitors may have been removed from the medium, resulting in higher lipase activity.

	Activity (U)		
XAD type	Input	Output	% Immobilised
2	25	23	92
4	25	3	12
7	25	48	192

Table 22: Activity of free and immobilised *C. rugosa* lipase against 20% *S*-ethyllactate (immobilisation pH = 6.5)

The activity of the immobilised enzyme was also checked against the hexyl and di-ethyleneglycolmethyl ether based (DEGME) esters of 2-phenoxypropionic acid (**table 23**) as these substrates will be used to establish the column reactor.

XAD-7 adsorbed 64% of the activity input (DEGME ester as a substrate) while XAD-4 took up the least (9%) amount of activity input. This trend is consistent with the results obtained when the activity was determined using *S*-ethyl lactate as a

substrate (**table 22**), although there is no over-recovery of the catalytic activity measured with either of these substrates.

XAD	Activity (U)					
	DEGME			Hexyl		
	Input	Output	%Immobilised	Input	Output	%Immobilised
2	107	14	13	35	4	11
4	107	10	9	35	3	9
7	73	47	64	46	13	28

Table 23: Activity of free and immobilised *C. rugosa* lipase against racemic DEGME and hexyl esters of 2-phenoxypropionate

The free enzyme hydrolysed the DEGME ester 1.6 times faster than the hexyl ester, while the immobilised lipase hydrolysed the DEGME ester 2.3 times faster than the hexyl ester. This increase in the activity upon immobilisation could be due to the diffusion limitations imposed by immobilisation (*Braun, 1996*) or the preferential immobilisation of a lipase whose preferred substrate is the DEGME ester.

By their nature, lipases are most effective at organic/aqueous interfaces. The linking of lipase to the solid support presents a problem of efficiently bringing reactants located in two separate liquid phases to the active site of the tethered enzyme (*Braun, 1996*). Water and Hexyl/DEGME esters of 2-phenoxypropionic acid are virtually insoluble in one another. If the lipase is immobilised on the hydrophobic support then delivering water to the active site of the enzyme becomes a rate determining step.

As all activity assays were carried out with wet immobilised lipase resin, this restricted the diffusion of Hexyl/DEGME esters of 2-phenoxypropionic acid through the matrix, resulting in a relatively low hydrolytic activity of immobilised *Candida rugosa* lipase compared to the activity towards *S*-ethyl lactate.

In the light of the above immobilisation results XAD-7 was chosen for further immobilisation and adsorption studies.

To determine the optimum pH of the immobilisation, the immobilisation was carried out at pH 5, 6, 7 and 8.5 on Amberlite XAD7. Activity was determined on pH stat against 20% *S*-ethyl lactate.

pH	Activity input (U)	Activity output (U)	% U immobilised
5	8.6	3.5	41
6	8.6	8.6	100
7	8.6	3.3	38
8.5	8.6	2.5	29

Table 24: Activities of immobilised *C. rugosa* lipase on XAD-7 against *S*-ethyllactate at various pH values

The results (**table 24**) show that maximum activity is immobilised on XAD7 at pH 6. The supernatant were also analysed and were found to contain no protein or lipase activity. Note that the lipase gained 92% activity when immobilised at pH 6.5 (**table 22**), while there was no increase in activity at any other pH. This strange behaviour of the *Candida rugosa* lipase was not further explored, however, to avoid discrepancies in future work it was decided to carryout further immobilisation at pH 6.0

The above immobilisation method is based on an enzyme being physically adsorbed or ionically attached to the resin and in both cases the binding forces between the protein and the resin are weak. During the operational conditions the enzyme may leak from the resin. To avoid the loss of enzyme it was decided to cross-link the enzyme with glutaraldehyde. This process involves the covalent linkage of lysine. This method produces dimer or polymer of adsorbed protein, hence reducing its solubility in water and reducing the probability of leakage of immobilised enzyme into

the liquid phase during usage. The activity resulting from the cross-linking are shown in **table 25.**

% Glutaraldehyde	Activity input (U)	Activity output (U)	% Activity lost (U)
2	29.3	21.3	27
5	28.3	18	36
11	28.4	16.6	42

Table 25: Activity loss upon cross-linking of *C. rugosa* lipase immobilised on XAD-7 against *S*-ethyl lactate

The cross-linking was carried out by shaking immobilised enzyme in the presence of glutaraldehyde and MES buffer (0.2 M, pH 6) for 2.5 h at 27 °C. As expected the glutaraldehyde had deactivated nearly a third of activity at 2% concentration but the increase of glutaraldehyde concentration from 5% to 11% only resulted in a relatively small further deactivation.

Activities of cross-linked enzyme were also measured against hexyl and DEGME esters of 2-phenoxypropionic acid and are summarised in **table 26.**

	DEGME	Hexyl
Activity input	23.8	6.9
Activity output (U)	19.3	5.5
% Activity lost	19	20

Table 26: Activities of cross-linked *C. rugosa* lipase on XAD-7 against DEGME and hexyl esters of 2-phenoxypropionic acid

As expected there was 20% activity loss upon cross-linking. Surprisingly the cross-linked enzyme was now 3.5 times more active towards the DEGME ester than the hexyl ester, compared to 2.3 times, before the cross-linking (**table 23**)

The activity of immobilised *C. rugosa* lipase during multiple runs was also tested against *S*-ethyl lactate (**table 27**), the hexyl ester of 2-phenoxypropionic acid (**table 28**), triacetin (**table 29**) and the DEGME ester of 2-phenoxypropionic acid (**table 30**)

Run	Activity (U/g)	% Activity lost
1	19	0
2	11.3	41
3	8.7	54

Table 27: Activity loss of immobilised *C. rugosa* lipase (on XAD7) after three successive runs against *S*-ethyllactate

Immobilised *C. rugosa* lipase lost 40% of its hydrolytic activity after just one run, the lipase lost further 13% of activity after second run (**table 27**). This loss in activity could be due to enzyme inhibition by ethanol produced during the reaction or enzyme deactivation under operating conditions.

Run	Activity (U/g)	% Activity gained
1	0.26	0
2	0.49	88
3	0.66	154

Table 28: Activity gained by immobilised *C. rugosa* lipase (on XAD7) after four successive runs against hexyl ester of 2-phenoxypropionic acid.

Immobilised *C. rugosa* lipase exhibited completely opposite activity behaviour against the hexyl ester of 2-phenoxypropionic acid when compared with *S*-ethyllactate. The immobilised lipase gained almost 90% hydrolytic activity after

one run, lipase gained further 66% activity after the second run (**table 28**). Remember that the lipase lost almost half of its hydrolytic activity after two runs when analysed against *S*-ethyllactate (**table 27**).

Run	Activity (U/g)	%Activity gained/lost
1	2.4	0
2	2.3	(4)
3	2.5	4
4	2.3	(4)

Table 29: Activity of immobilised *C. rugosa* lipase (on XAD7) after four successive runs against triacetin.

Immobilised *C. rugosa* lipase showed no significant activity loss or gain when tested against triacetin after multiple runs (**table 29**). This trend in activity contradicts the activity behaviours observed in **table 27** and **table 28**.

Run	Activity	% Activity loss
1	2.40	0
2	2.39	0.4
3	2.31	4

Table 30: Activity of immobilised *C. rugosa* lipase (on XAD7) after three successive runs against DEGME ester of 2-phenoxypropionic acid

The activity of immobilised *C. rugosa* lipase after three runs against the DEGME ester of 2-phenoxypropionic acid showed no considerable change (**table 30**). This trend is consistent with the results obtained against triacetin (**table 29**), however, these results are opposite to the trends outlined in **table 27** and **table 28**.

This peculiar behaviour of the immobilised *C. rugosa* lipase was not fully investigated as it was outside the scope of this thesis. However, these results indicate the presence of multiple lipases in the fractions used for immobilisation, hence the variable activities observed against different substrates. The increase and decrease in activities after multiple runs could be due to the simple activation and deactivation phenomenon respectively, but any other explanation could not be ruled out until further analysis.

Due to the nature of the column, it was essential that the mobile phase consists of a single phase, hence the activity of the free and immobilised lipase was tested in the presence of organic solvents. The activity of the free lipase was measured against 20% *S*-ethyl lactate in the presence of 30% aqueous acetonitrile (**table 31**). Free lipase was found to be incompatible with 30% acetonitrile, the lipase lost 85% of its hydrolytic activity against 20% *S*-ethyl lactate when the hydrolysis was carried out in the presence of 30% acetonitrile.

% Acetonitrile	Activity (U/mL)
0	9.6
30%	1.48
% Activity lost	85%

Table 31: Activities of cross-linked *C. rugosa* lipase on XAD-7 in aqueous and 30% acetonitrile against *S*-ethyl lactate

The activity of the free and immobilised lipase were measured against the DEGME ester in a single phase system (DEGME ester (4mg/mL), 10% methanol in 10 mM NaCl (25 mL) and immobilised lipase) and two phase system (DEGME ester (0.5 mL), *C. rugosa* (1 mL) and 10 mM NaCl (23.5 mL)). As before (**table 31**) free lipase lost most of its hydrolytic activity in the presence of organic solvent (10% aqueous methanol), indicating that the hydrolytic activity of the free lipase is highly sensitive

to the organic solvents (**table 32**). The immobilised lipase also lost significant amount of its hydrolytic activity (71%) in presence of 10% methanol, however, the immobilised lipase was twice as active as the free lipase, indicating an effect of diffusional limitation on the activity of the immobilised enzyme upon immobilisation.

Lipase	2 Phase	Single phase	Activity lost (%)
Free	1.62(U/mL)	0.2 (U/mL)	88
Immobilised	1.73 (U/g)	0.51 (U/g)	71

Table 32: Activities of free and immobilised and cross-linked *C. rugosa* lipase in aqueous and 10% MeOH against DEGME ester of 2-phenoxypropionic acid

Activity of the immobilised lipase was determined after successive runs in the presence of 10% aqueous methanol, this was necessary to establish the long term stability of the immobilised lipase in 10% methanol. Hydrolytic activity of the immobilised lipase was measured against DEGME ester, this time the same fraction of the immobilised lipase was used in three successive runs, after each run the substrate was drained off and the immobilised lipase was washed three times with 10 mM MES buffer (pH6.0) and analysed again for its hydrolytic activity.

The results (**table 33**) were quite encouraging as the immobilised lipase did not lose activity during the three runs, indicating that the immobilised lipase can withstand multiple runs or long term exposure to the aqueous organic solvents without losing noticeable activity.

Run	Activity (U/g)	% Activity lost
1	0.51	0
2	0.49	(4)
3	0.55	8

Table 33: Activity of immobilised and cross-linked *C. rugosa* lipase against DEGME ester during three successive runs

Although the immobilised lipase lost almost 70% of its hydrolytic activity when used in aqueous organic systems, its consistent activity during the multiple runs, suggested that this system could be used to establish the column for continuous production of 2-phenoxypropionic acid or its esters.

It was important that the lipase does not lose its enantiospecificity upon immobilisation, therefore, the E_R values of the immobilised and cross-linked lipase were determined against the hexyl ester.

Fraction	E_R
50% IPA supernatant (60 h)	16
50% IPA supernatant (60 h) cross-linked on XAD7	25

Table 34: E_R of free and cross-linked *C. rugosa* lipase for hexyl 2-phenoxypropionate

The free enzyme exhibited an E_R of 16 towards the hexyl ester but surprisingly the enzyme immobilised and cross-linked on XAD-7 showed a 40% increase in its E_R value upon cross-linking with 2% glutaraldehyde (**table 34**). For a comparison the DEGME ester was also hydrolysed using immobilised and cross-linked enzyme.

The immobilised and cross-linked lipase was discovered to be less enantiospecific towards the DEGME ester then the free enzyme. The lipase lost 30% of its E_R value upon immobilisation (**table 35**). As discussed earlier (**table 23**) upon cross-linking the enzyme hydrolyses the DEGME ester 3.5 times faster than the hexyl ester. This increase in activity is at the expense of specificity, even after 30 % loss of specificity upon cross-linking, the enzyme is still more specific towards DEGME ester than the hexyl ester.

Fraction	E_R
50% IPA supernatant (60 h)	30
50% IPA supernatant (60 h) cross-linked on XAD7	20

Table 35: E_R of free and cross-linked *C. rugosa* lipase for DEGME ester of 2-phenoxypropionic acid

Note that the E_R of free enzyme towards DEGME ester is 47 % higher than for the hexyl ester. Interestingly the enzyme losses 33% of its specificity towards DEGME ester when treated with glutaraldehyde for cross-linking this trend is completely opposite to the one observed for the hexyl ester where the enzyme has 40% increased specificity towards the *R*- enantiomer upon cross-linking.

We believe this is due to the presence of a contaminating non-specific lipase. This lipase is considered to be more active towards the DEGME ester than the hexyl ester and more specific towards the hexyl ester than the DEGME ester. It is assumed that this lipase is preferentially immobilised and retains its activity upon cross-linking. The lipase specific towards the DEGME ester loses its activity upon immobilisation and cross-linking, however, a more detailed study of immobilisation and specificity is required to fully understand the trends observed in this section.

7.3 Conclusions

- Best immobilisation results are achieved when immobilisation is carried out over a long period of time (16 h) and at pH 6

- Maximum lipase activity is when immobilised on XAD-7 followed by XAD-2 while the least amount of activity was when immobilised on XAD-4

- Approximately 25% of immobilised lipase activity was lost upon cross-linking with 2% glutaraldehyde

- Immobilised lipase is less active in the presence of organic solvents than the free lipase in aqueous system

- Immobilised lipase shows increased specificity towards the hexyl ester than the free lipase, however, this trend was reversed when immobilised lipase was tested against the DEGME ester

8 Separation of Acid and Ester

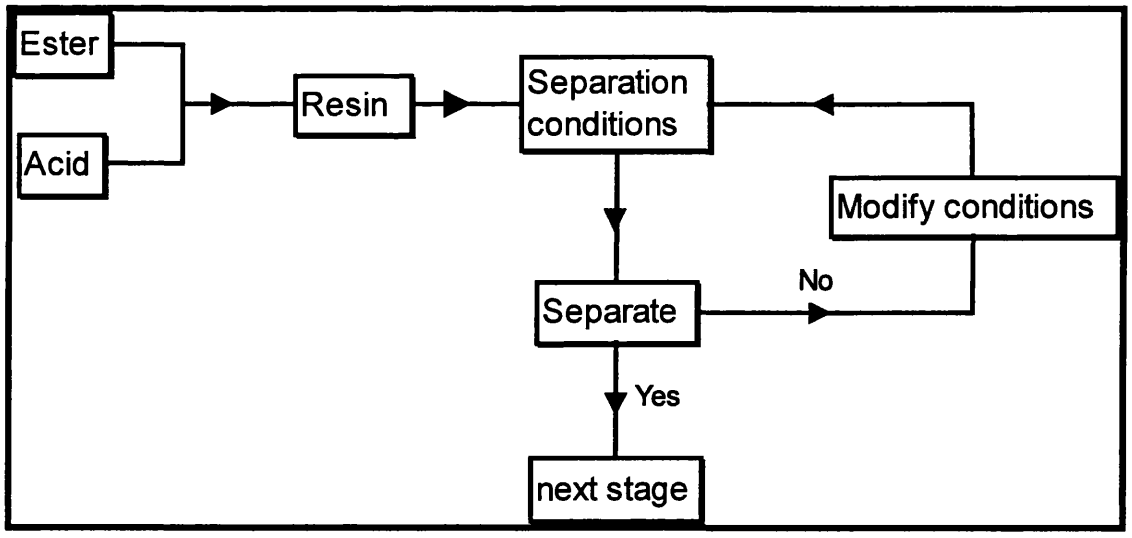
8.1 Aim:

This chapter deals with two criteria

1. Conditions for the separation of the acid and ester
2. The solubility of esters

8.1.1 Separation criteria:

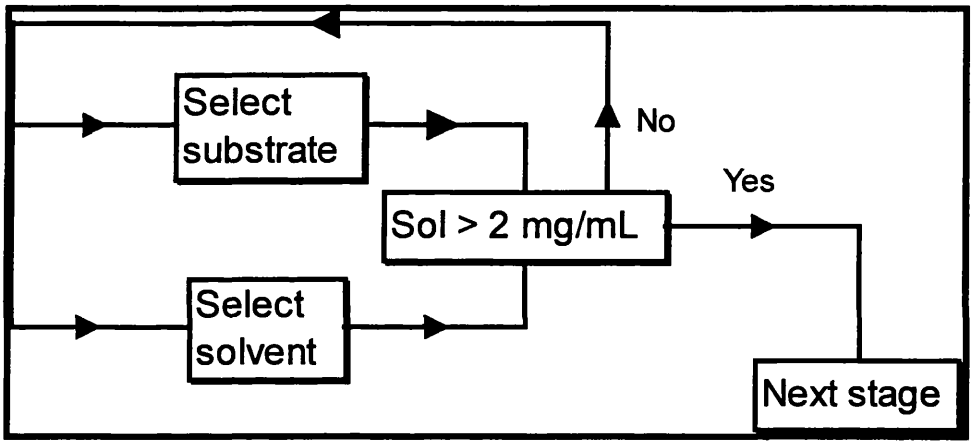
The resin must be able to separate the substrate (ester) and the product (2-phenoxypropionic acid). The separation is to be carried out by adsorbing the ester and releasing the 2-phenoxypropionic acid produced. If separation is not achieved in the first instance the separation conditions (solvent concentration) must be modified. The resin and the separation conditions must be compatible with lipase activity.



Scheme 18: Conditions for the separation of acid and ester

8.1.2 Solubility of esters:

To follow the reaction quantitatively by UV and for the trouble free isolation of products and residual substrate for the optical purity analysis, it was decided that the solubility of the ester must be 2mg/mL or higher, during the loading stage. The set limit of solubility must be achieved in a low concentration of organic solvent in a aqueous system to avoid enzyme activity loss during the run. The substrate can be changed to achieve the target solubility but the solvent and substrates must be compatible with lipase activity and specificity.



Scheme 19: Selection of substrate and solvents for the column

8.2 Experimental:

8.2.1 Solubility of DEGME ester in aqueous methanol :

DEGME ester (0.7624 g) was dissolved in methanol (9.9 mL). A 2 mL aliquot was withdrawn and water (0.2 mL) was added to the solution, the mixture was shaken vigorously at room temperature to assure complete mixing. Further aliquots (9 x 0.2 mL) of water were added until the solution turned cloudy at which point the concentration of methanol was 50% and solubility of the DEGME ester was 38.5 mg/mL

8.2.2 Adsorption of DEGME ester on XAD-7

Amberlite XAD-7 (7.84 g, wet weight) was packed in a Pharmacia column (height = 5.5 cm, i.d = 1.6 cm). The mobile phase (10% methanol : MES buffer (50 mM, pH 6.5) was passed through the column (from bottom to top) at a fixed flow rate (1.1 mL/min) for 60 min. The mobile phase was changed to 10% methanol containing DEGME ester (4 mg/mL), the solution was pumped at a same flow rate. Effluent was collected and analysed by UV every 20 mins for the presence of DEGME ester (0.59 g DEGME ester was loaded).

8.2.3 Elution of DEGME ester

After adsorption, the top end of column was opened and carefully 60% methanol was added (at no time the bed surface was disturbed or column was run dry). Effluent was collected and analysed by UV. Methanol (60%, 324 mL) was added until no more DEGME ester was detected (0.58g, 98.5% DEGME ester was recovered).

8.3 Result and Discussion

In order to carry out the adsorption studies, solubilities of the esters of 2-phenoxypropionic acid had to be calculated in aqueous organic solvents. The solubilities were calculated by making a known concentration solution of esters in an organic solvent and then adding small aliquots of water, after each addition the mixture was shaken vigorously to ensure complete mixing. Aliquots of water were added until the solution turned cloudy, then water added and the organic solvent ratio was calculated along with the final concentration. The solubility of octyl phenoxypropionate in aqueous methanol is shown in **table 36**.

% Methanol	Solubility (mg/mL)
42	0.09
48	0.26
50	0.40
59	1.60
62	2.63
67	3.40
71	7.54
83	21.80

Table 36: Solubility of octyl ester of 2-phenoxypropionic acid in aqueous methanol.

The octyl ester of 2-phenoxypropionate is poorly soluble in below 50% MeOH concentration, but the solubility of the ester increases sharply as the concentration of MeOH reaches beyond 60%. There is a huge increase in the octyl ester solubility when MeOH concentration reaches beyond 67% (**fig 37**). The desired ester concentration of 2 mg/mL falls in a region of unfavourable MeOH concentration (62%). High MeOH concentration increase the chances of transesterification, esterification, poor adsorption and perhaps most importantly the loss of lipase activity

through deactivation or inhibition (**Dahod, *87***), therefore, methanol concentration have to be kept at its minimum. As the desired octyl ester concentration requires large quantities of methanol, this combination was considered to be not feasible.

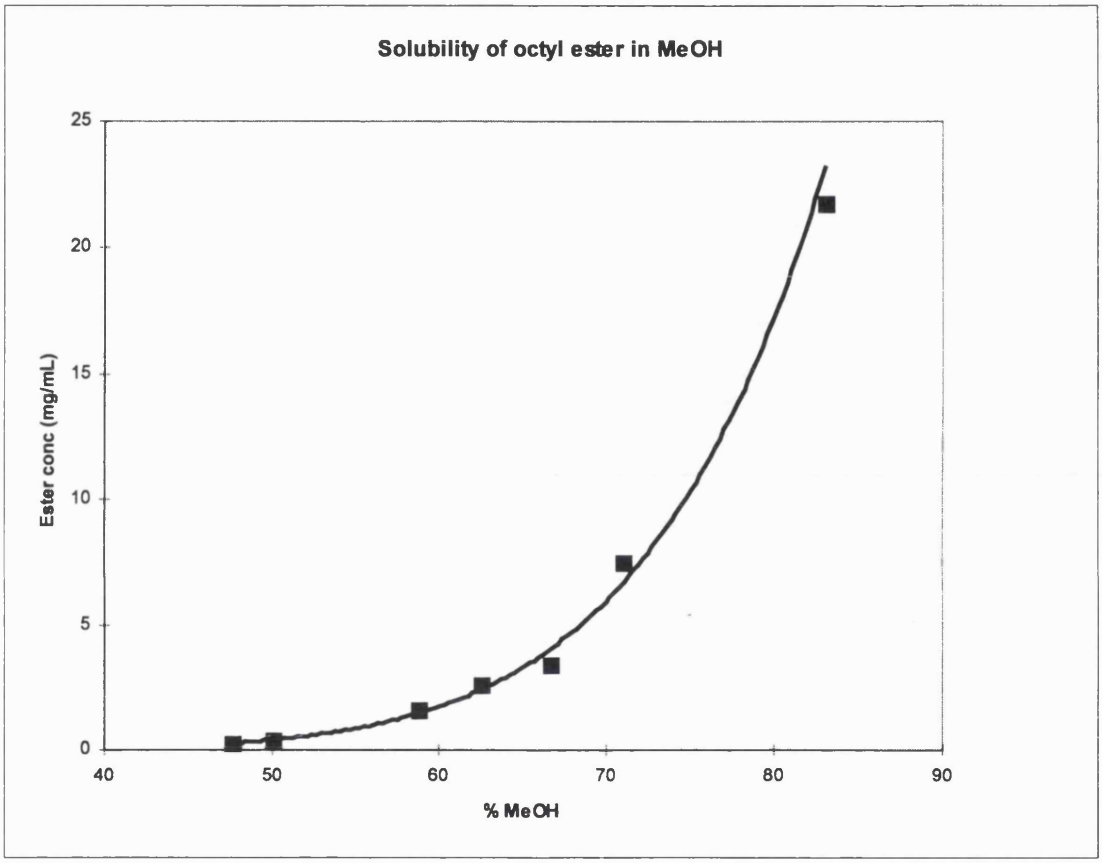


Figure 33: Graphical representation of the solubility of the octyl ester of 2-phenoxypropionic acid in aqueous methanol

Acetonitrile was chosen as an alternative solvent and the octyl ester was replaced by a shorter alkyl chain hexyl ester. The solubility of the hexyl ester (**table 37**) was determined in a similar manner as outlined above.

%MeCN	Solubility (mg/mL)
27	0.178
35	0.56
39	0.96
40	1.376
42	1.71
43	2.1
50	4.21

Table 37: Solubility of hexyl ester of 2-phenoxypropionic acid in aqueous acetonitrile.

The hexyl ester was found to be much more soluble in acetonitrile than octyl ester in methanol. The solubility of the ester starts increasing once the concentration of acetonitrile reaches 40% and above (**fig 38**). It was decided to use 50% aqueous acetonitrile, where the solubility of the hexyl ester is 4.1 mg/mL, to study the adsorption of hexyl phenoxypropionate on XAD-7.

8.3.1 Absorbance Coefficient

It was necessary to develop a quantitative method to measure the adsorption of the ester on the resin. For this purpose we decided to use the Beer Lambert law (**eq 6**). To use the law we had to calculate the molar absorbance coefficient (ε) of the esters and the free acid. The absorbance coefficient for 2-phenoxypropionic acid was calculated over a wide *p*H range, while the absorbance coefficient for the hexyl ester was only calculated in neat acetonitrile. The absorbance maxima for the acid and the ester lies near 220 nm, as this region is usually contaminated due to the absorbance from the solvents, therefore, the second maxima at 270 nm was chosen for the calculations.

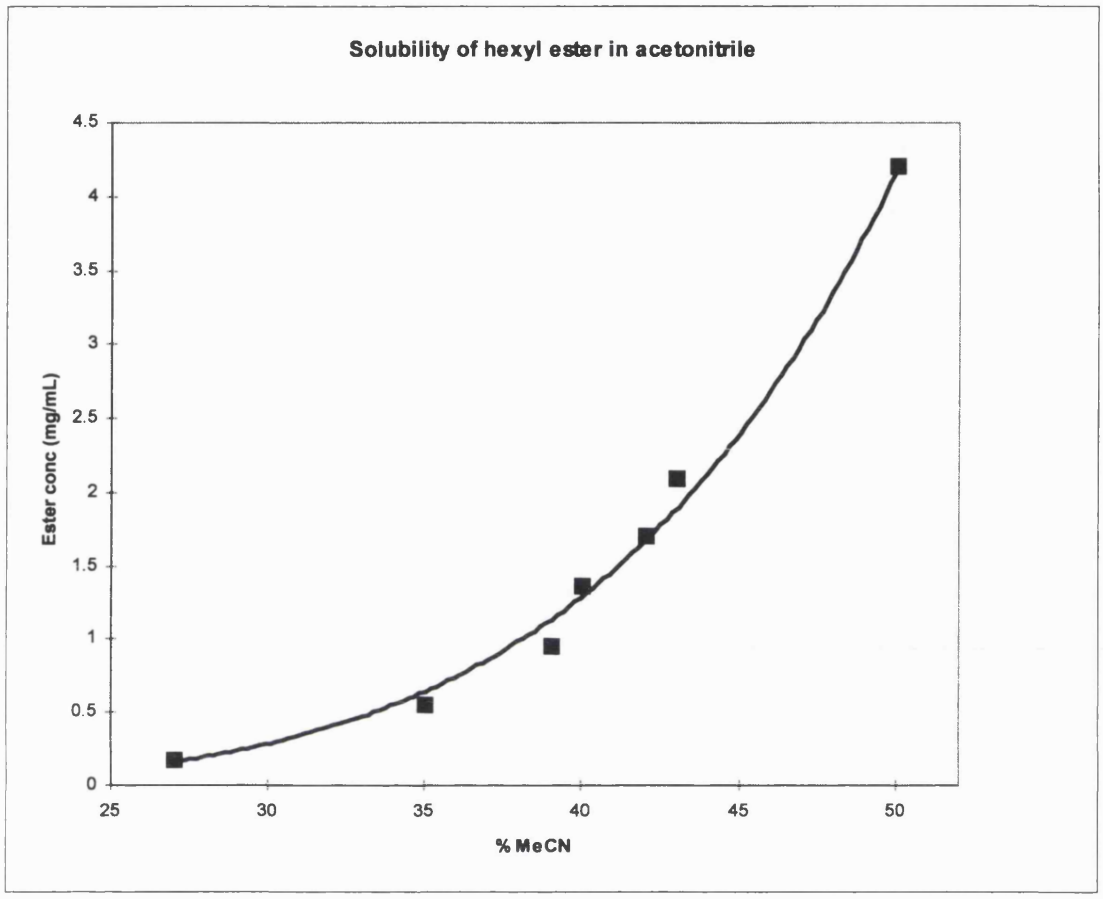


Figure 34: Graphical representation of the solubility of the hexyl ester of 2-phenoxypropionic acid in aqueous acetonitrile

$$A = c \times \varepsilon \times l$$

Equation 7: Beer Lambert law equation

where A = absorbance, C = concentration, ε = molar absorbance coefficient, l = cell path length (1 in this case).

Different concentration of solutions of 2-phenoxypropionic acid were prepared and absorbance of the solutions were recorded (**table 38**) and the ε was calculated by rearranging the **eq 6**.

Molar absorbance coefficient of 2-phenoxypropionic acid is fairly constant in aqueous solutions at different *p*H values but the molecular absorbance coefficient decreases by 19% in neat methanol. The molar absorbance coefficient (ε_{270}) of hexyl ester in methanol was calculated to be 1.18×10^3 mol^{-1} cm^{-1}, which is slightly lower than the ε_{270} of 2-phenoxypropionic acid in neat methanol.

After establishing a method for the calculation of unknown concentrations of the 2-phenoxypropionic acid and its ester, the adsorption of the hexyl ester on Amberlite XAD-7 was attempted. Adsorption was carried out in an a Pharmacia (**fig 39**) column packed with XAD-7 (8 mL, 5.7 g) The mobile phase was run from the bottom of the column to the top of the column. The column was equilibrated with 50% acetonitrile over 1-2 h at a set flow rate. After equilibration the mobile phase was changed to 50% acetonitrile containing hexyl ester (4 mg/mL) and set volume fractions were collected and analysed by U.V to determine the break-through point. However, each fraction contained a small amount of the ester but there was 99% retention of hexyl ester from the first three fractions. After the third fraction there was a large drop in the degree of retention, dropping from 99% to 36 % in 5 fractions (**table 39**). The retained ester was eluted with 100% MeOH running this time from top to bottom.

pH	λ (nm)	Abs (A.U)	Conc M/L ($X10^{-4}$)	M.C (ε)
5	269	1.01	5.8	1723
5	269	0.75	4.4	1713
5	269	0.50	2.9	1710
5	269	0.26	1.5	1756
8	269	0.91	5.6	1617
8	269	0.68	4.2	1617
8	269	0.46	2.8	1639
8	269	0.23	1.4	1666
10.2	269	1.20	7.0	1699
10.2	269	0.90	5.3	1704
10.2	269	0.60	3.5	1701
10.2	269	0.31	1.8	1767
MeOH	270	0.96	6.9	1395
MeOH	270	0.73	5.1	1417
MeOH	270	0.48	3.4	1409
MeOH	270	0.24	1.7	1388

Table 38: Molar absorbance coefficient of 2-phenoxypropionic acid at different pH values.

Units and Abbreviations:

A.U = Absorbance units, M/L = moles/ dm^3, Conc = concentration,

M.C = molar absorbance coefficient mol^{-1} cm^{-1}

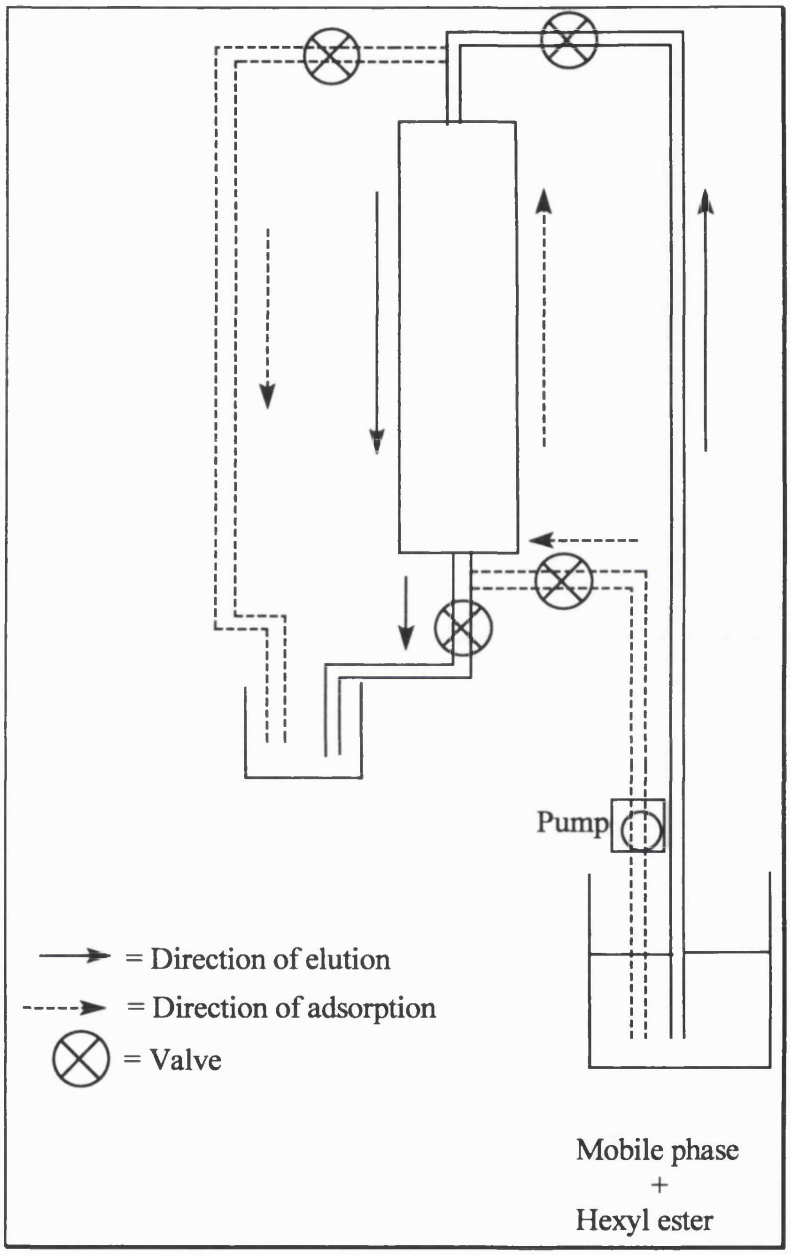


Figure 35: Set-up of the column used for the adsorption studies

Vol (mL)	A_{270}	Weight retained (mg)	%Retention
17	0.01	67.6	99.4
17	0.13	67.5	99.3
17	0.11	67.6	99.4
17	1.30	63.3	93.1
17	4.10	53.3	78.4
17	8.02	39.3	57.7
17	10.34	31.0	45.6
17	11.93	25.2	37.1

Table 39: Adsorption of hexyl ester of 2-phenoxypropionic acid on XAD-7, using 50% aqueous acetonitrile as a mobile phase

We believed that the large decrease in the degree of retention (fraction 4-8) was due to the high concentration of the acetonitrile, but the initial 1% release indicates that the rate of adsorption at low concentration is slow. This problem can be overcome by installing a small size column containing XAD-2 resin at the end of the main column.

To minimise the large drop in retention, the concentration of the aqueous acetonitrile was reduced to 40%. The decrease in the polarity of the mobile phase indeed increased the degree of retention of the ester.

The flow rate of the mobile phase was slightly lower than before, and the concentration of the hexyl ester was 1.4 mg/mL (note this concentration is lower than the limit set earlier). The initial retention of the ester on XAD7 (11 mL, 7.80g) was slightly lower with 40% acetonitrile when compared to 50% aqueous acetonitrile, but overall retention was 97% and there was no large decrease in the retention after few fractions (**table 40**).

Vol (mL)	A_{270}	Weight retained (mg)	% Retention
14	0.019	18.98	97.85
14	0.028	18.96	97.72
14	0.052	18.89	97.35
14	0.044	18.91	97.47
14	0.030	18.95	97.69
14	0.027	18.96	97.74
14	0.026	18.96	97.75
14	0.041	18.92	97.53
14	0.117	18.69	96.36

Table 40: Adsorption of hexyl ester of 2-phenoxypropionic acid on XAD-7, using 40% aqueous acetonitrile as a mobile phase

The adsorption capacity of XAD-2 resin was also studied with 40% acetonitrile. A total of 206 mL of mobile phase containing 1.4 mg/mL of hexyl ester was passed through the column. The result showed 100% retention of the ester and no sign of a break through.

It was found that the immobilised enzyme is totally inactive in 40% acetonitrile, therefore 40% acetonitrile was considered to be unfavourable (**table 31**).

At this stage attentions were directed towards the synthesis of a fairly water soluble substrate. The criteria for the new substrate were minimum solubility of 2mg/mL in 10% aqueous organic solvents and E_R greater than 10.

di-Ethylenediglycolmethylether based ester of 2-phenoxypropionic acid was synthesised which was found to be much more soluble in aqueous methanol (**table 41**) than the hexyl and octyl ester.

% MeOH	Solubility (mg/mL) Hexyl ester	DEGME ester
10		4
45	0.46	63
53	1	150
56	2	214

Table 41: Solubility of *di*-ethylenediglycolmethylether based ester of 2-phenoxypropionic acid in aqueous methanol

The solubility of DEGME ester in 10% MeOH was found to be twice as much as the set limit of 2 mg/mL. The solubilities of hexyl and DEGME esters were also determined in a aqueous acetone (**table 42**).

% Acetone	Solubility (mg/mL) Hexyl ester	DEGME ester
8		6
38	0.45	41
42	0.95	54
50	2.95	86

Table 42: Solubility of hexyl and *di*-ethylenediglycolmethylether based ester of 2-phenoxypropionic acid in aqueous acetone.

The solubility of the hexyl ester in aqueous acetone is measured to be approximately three times more than the solubility in aqueous methanol but the desired solubility range (2 mg/mL) still falls in a unfavourable solvent concentration (50%).

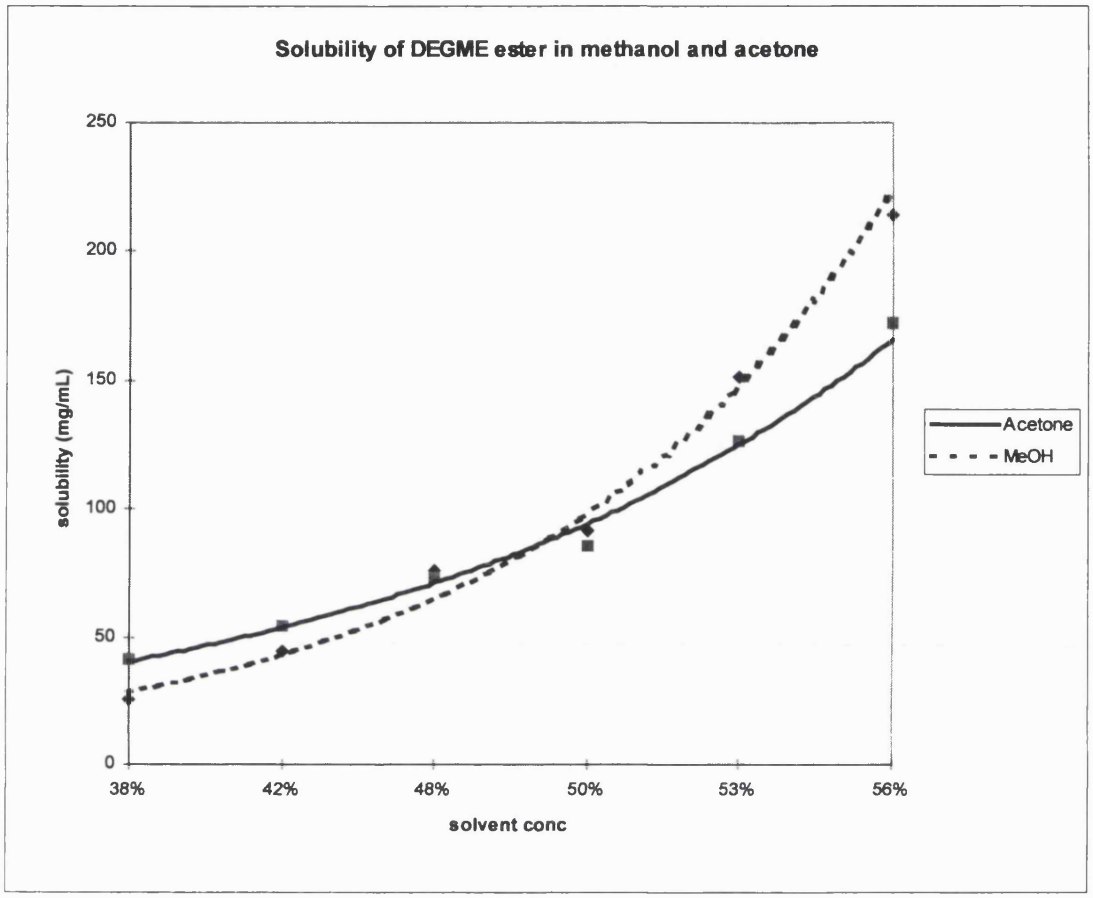


Figure 36: Graphical representation of the solubility of *di*-ethylenediglycolmethylether based ester of 2-phenoxypropionic acid in aqueous methanol and acetone

DEGME ester is found to be more soluble in 8% acetone than 10% methanol, but as the solvent concentration increases, the DEGME ester becomes less soluble in acetone than methanol (**fig 36**).

Even the solubility of DEGME ester in aqueous acetone is higher than in methanol at low solvent concentrations but acetone could not be used as a solvent during loading of the substrate on the column, due to its high UV absorbance in the UV range under investigation.

Vol (mL)	A_{270}	Weight retained (mg)	% Retention
20	0.07	80.77	99.96
22	0.12	88.94	99.93
22	0.12	89.94	99.93
22	0.12	87.90	99.89
22	0.36	85.82	99.79
22	0.37	87.81	99.78
17	0.40	65.85	99.77

Table 43: Adsorption of the DEGME ester on XAD-7 in 10% methanol

The DEGME ester was loaded on the column using 10% MeOH as a solvent in this adsorption experiment, 587.8 mg of DEGME ester was loaded onto XAD7 (8.1 g, 5.68 cm) packed in the column (**table 43**). During the adsorption 0.13% (0.8 mg) was released from the column compared to the 3% discharge when hexyl ester in 40% acetonitrile is adsorbed on to the XAD7.

In conclusion DEGME ester proved to be better substrate than the hexyl or octyl esters in terms of solubility and adsorption affinity towards XAD-7.

The desorption profiles (**fig 37**) of DEGME ester with various concentrations of aqueous methanol were investigated. In a typical experiment DEGME ester (88mg) was adsorbed on XAD7. The adsorbed ester was eluted with different concentrations of methanol.

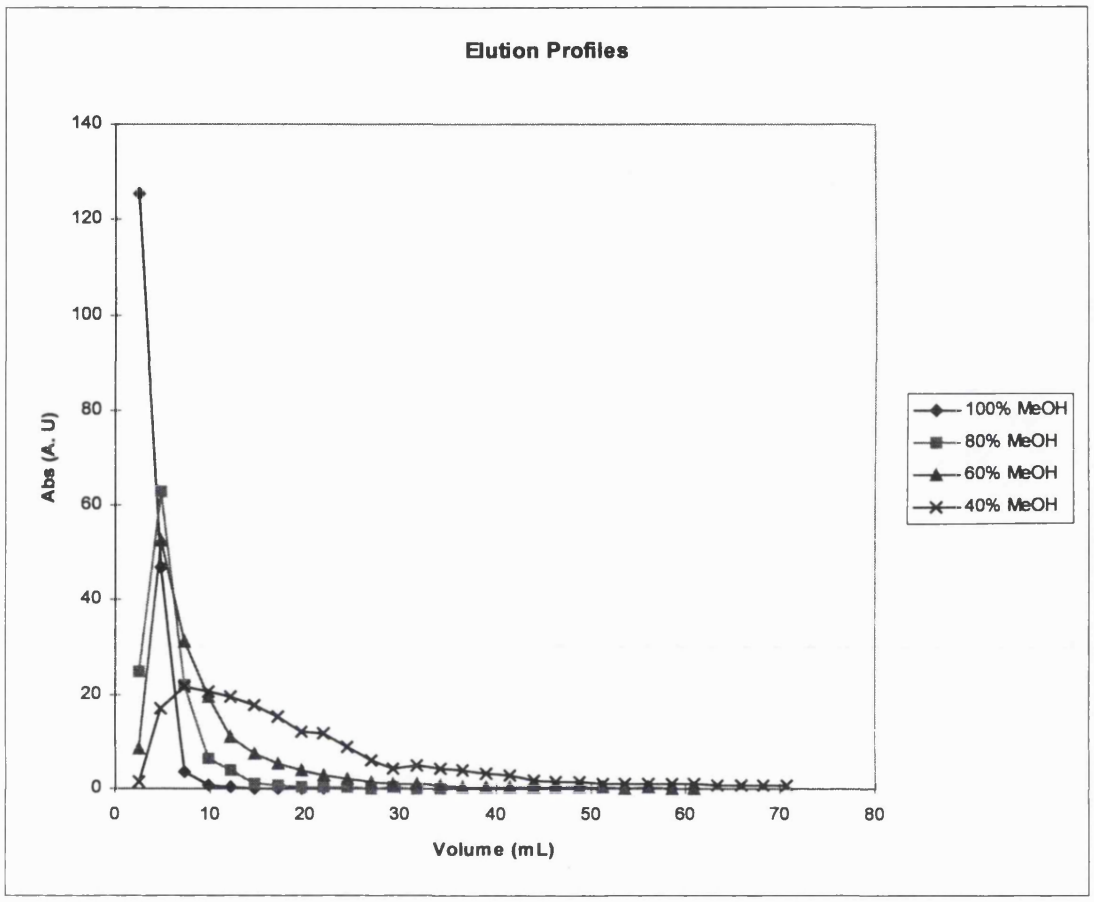


Figure 37: Elution profiles of DEGME ester with various concentrations of aqueous methanol

From the profile it appears that approximately 80% of the methanol concentration is required to elute the adsorbed ester efficiently but this concentration was found to deactivate the enzyme (**fig 41, sec 9.2.2**) hence methanol was considered to be unfavourable solvent for elution.

Elution with acetone was also investigated (**table 44**), due to its high UV absorbance, the elution was analysed by tlc instead of UV absorbance.

Solvent concentration (%)	Elution volume (mL)
20 % Acetone	42
40 % Acetone	22
40 % Methanol	80
80 % Methanol	36

Table 44: Elution of DEGME ester of 2-phenoxypropionic acid with aqueous acetone

The elution volumes suggest that 40% acetone is a much better elution solvent than 80% methanol and lipase is also found to be active and retains all its activity after elution with acetone.

8.4 Conclusions:

- XAD-7 and XAD-2 are capable of separating the acid and ester

- Hexyl and octyl ester are poorly soluble in methanol and acetonitrile, therefore, they can not be used as a substrate in a column reactor.

- DEGME ester is much more soluble in methanol than the hexyl and octyl ester and DEGME ester can also be adsorbed on to the XAD-7, hence, it can replace hexyl ester.

- 10% methanol is chosen as an adsorption solvent, while 40% acetone is selected as an elution solvent.

9 Optimisation of the Column Reactor

9.1 Aim

- The optimisation of lipase activity
- The optimisation of elution
- The optimisation of E_R

9.1.1 Optimisation of lipase activity

As lipase looses its activity considerably in aqueous organic mixtures it is necessary to determine the critical amount of activity necessary to operate the column effectively.

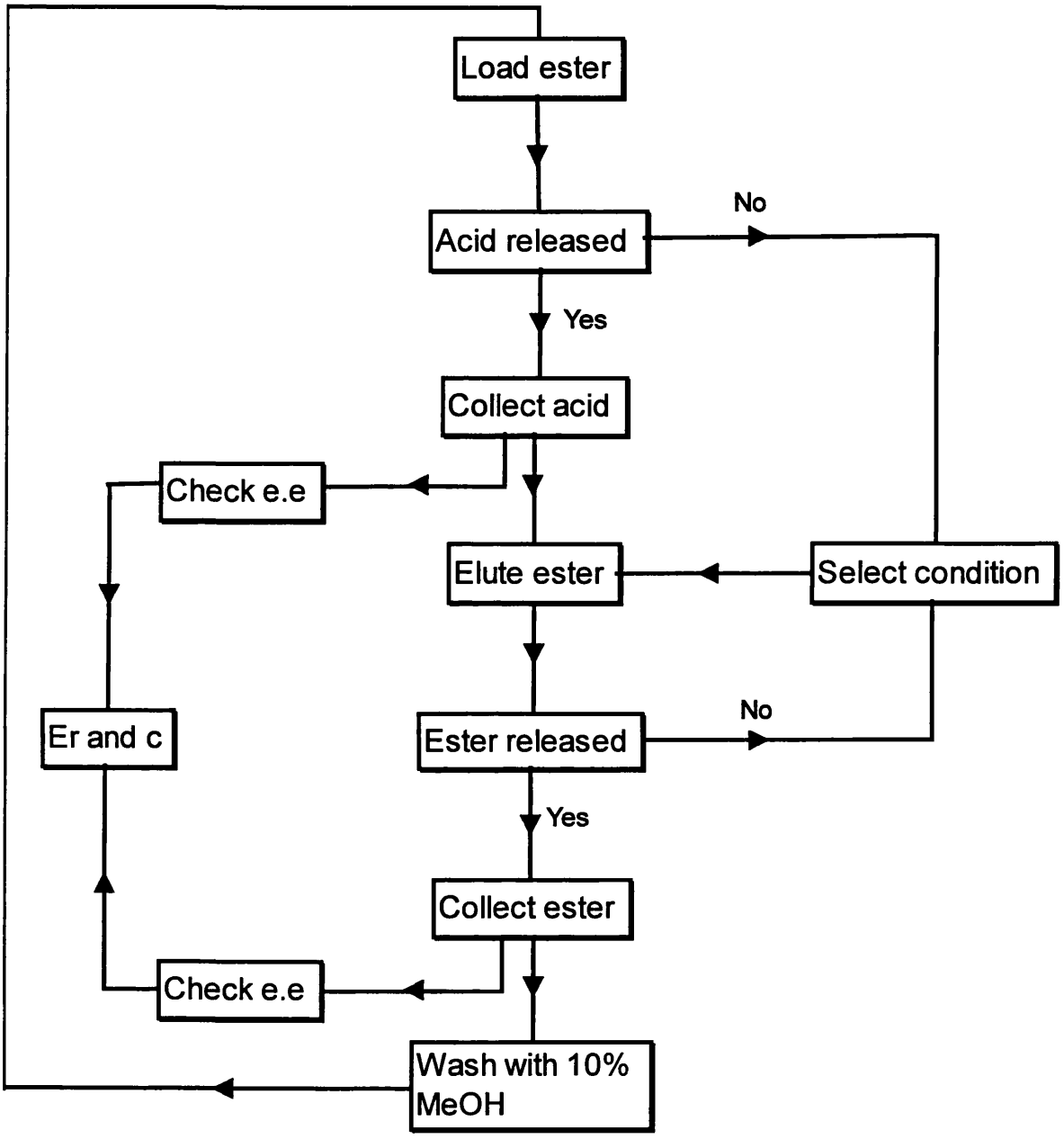
9.1.2 Optimisation of elution

For the column to be economical it is essential that the lipase does not loose its activity during the run and especially during the elution of adsorbed ester with polar solvents. The elution solvent must be volatile so the products can be easily recovered.

9.1.3 Optical purity

To analyse the productivity of the column in terms of its capability of producing optically pure esters of and 2-phenoxypropionic acid itself, the run must be split into different stages. Product from each stage must be collected and analysed for its optical purity. At the end of each run adsorbed ester is to be eluted and analysed for the optical purity, this step is necessary to calculate the E_R of the enzyme and the conversion.

The column is to be equilibrated with the adsorption solvent (10% methanol) prior to the second run.



Scheme 20: Guidelines for establishing a column reactor

9.2 Results and Discussion

9.2 1Optimisation of lipase activity:

The activity of the immobilised *C. rugosa* in 10% MeOH is found to be much lower than the activity in pure aqueous system therefore it is essential that the column must have sufficient enzyme activity for the hydrolysis in 10% MeOH. Different activity (U/g) lipase was immobilised on XAD7. The immobilised lipase was packed in the column and the 10% MeOH solution containing DEGME ester was pumped through the column. The 2-phenoxypropanoic acid released due to the hydrolysis was analysed by U.V (**fig 38**).

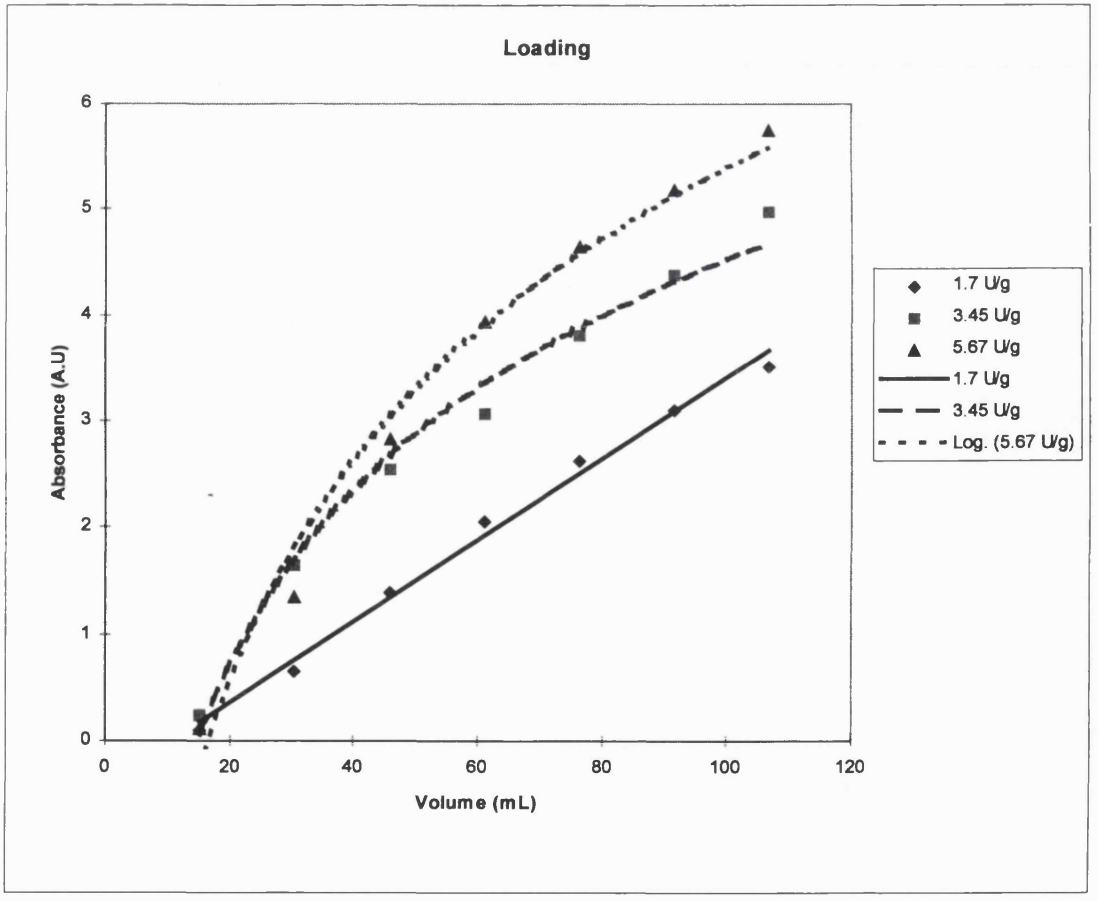


Figure 38: Production of 2-phenoxypropionic acid, released from column reactors with different *C. rugosa* lipase activity during the loading stage

The activity of the immobilised lipase was investigated in two parts. Firstly the loading stage, where the release of the 2-phenoxypropionic acid was monitored (**fig 38**), while the substrate was being loaded onto the column. During the initial stages of the loading, there was approximately twice as much acid produced from the column containing 3.45 U/g lipase activity than the column containing half the lipase activity (1.7 U/g). The column lost 25% of its activity during 3.5 hours of loading. While the column containing 5.7 U/g lipase activity produced approximately the same amount of the free acid during the initial stages of the loading than the column containing 3.45 U/g lipases activity. At the end of the loading stage the difference in activity between the two higher activity columns was approximately 1.1. In summary the column containing 40% extra activity only produced approximately 13% extra 2-phenoxypropanoic acid.

After loading the substrate, 10% methanol was circulated through the column in a recycling manner. During this stage the circulating solution was analysed every 30 mins for the presence of the 2-phenoxypropanoic acid. The results are plotted in the **fig39**.

The rate of hydrolysis during this stage for the three different lipase activity fraction is very similar, this could be due to the decreased concentration of the fast reacting enantiomer (*R*), which lowers the overall rate of hydrolysis, therefore the increased activity may not increase the rate of hydrolysis.

In light of the above results it was decided to achieve approximately 3.5 U of lipase activity per gram to XAD7 resin during the immobilisation for the process to be economical in-terms of lipase activity.

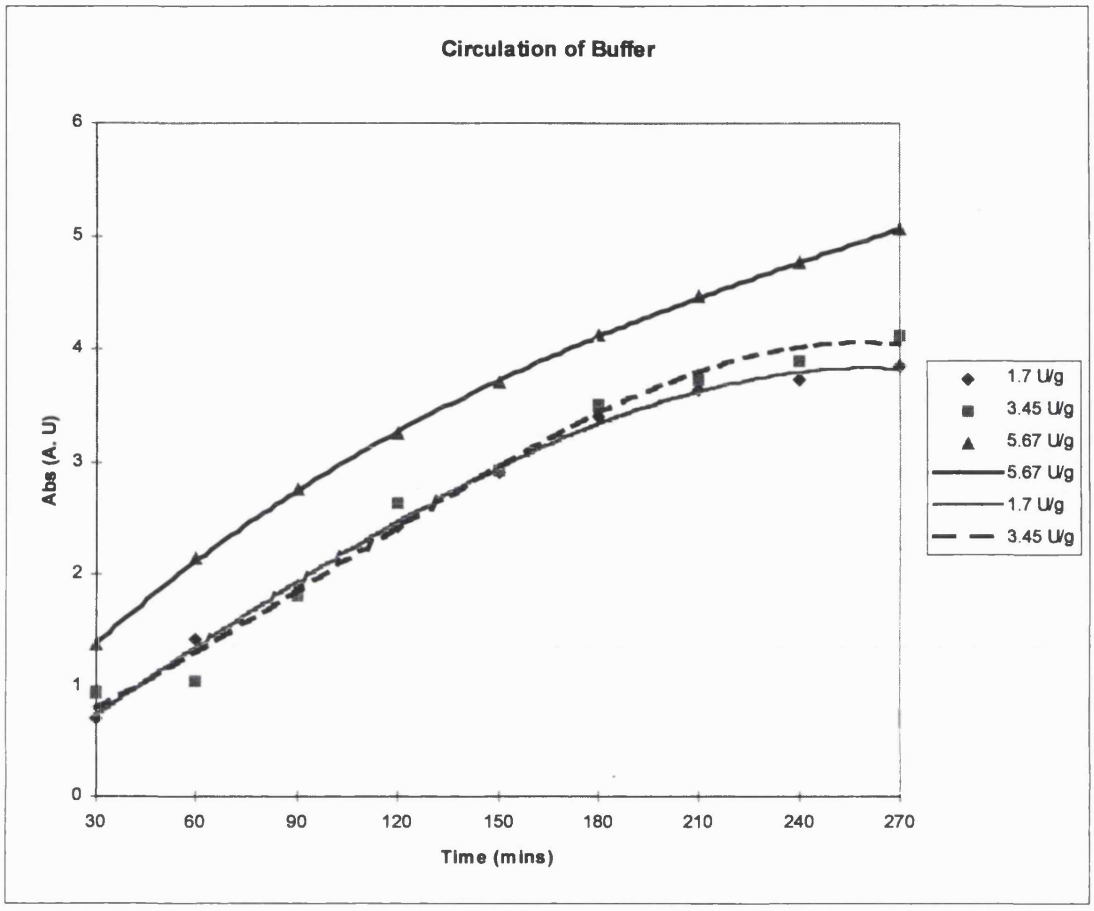


Figure 39: Release of 2-phenoxypropionic acid from column reactors with different *C. rugosa* lipase activity during the circulation of buffer stage

9.2.2 Optimisation of elution:

For column to be efficient and economical it was essential to reuse the column without any loss of lipase activity. From the elution profiles (**chapter 8**) the two preferred elution solvents were thought to be 60% aqueous methanol and 40% aqueous acetone.

Fraction	Absorbance (A. U) Run 1	Run 2
1	0.05	0.094
2	0.62	0.12
3	1.58	0.25
4	2.42	0.39
5	3.13	0.41

Table 45: Release of 2-phenoxypropionic acid from the column, run 2 was carried out after the regeneration of the column with 60% aqueous methanol after run 1

The DEGME ester was loaded onto the column and six fractions of approx. 16 mL were collected during the process. After which the MES buffer (25 mL) was re-circulated through the column for 20 hours (15.49 A.U). The adsorbed ester was eluted with 60% aqueous methanol (185 mL). DEGME ester was loaded onto the washed column and as before, eight fractions were collected during the loading process and were analysed by U.V to detect the presence of free acid (**table 45**). The fractions had very low U.V absorbance which could be due to the loss of the DEGME ester from the column rather than the production of 2-phenoxypropanoic acid. The data is graphically presented in **fig 40**. Activity of the washed resin was also analysed by the pH stat (using DEGME ester as a substrate) to detect any residual lipase activity, the result were negative. It was then concluded that 60% aqueous methanol deactivates the lipase.

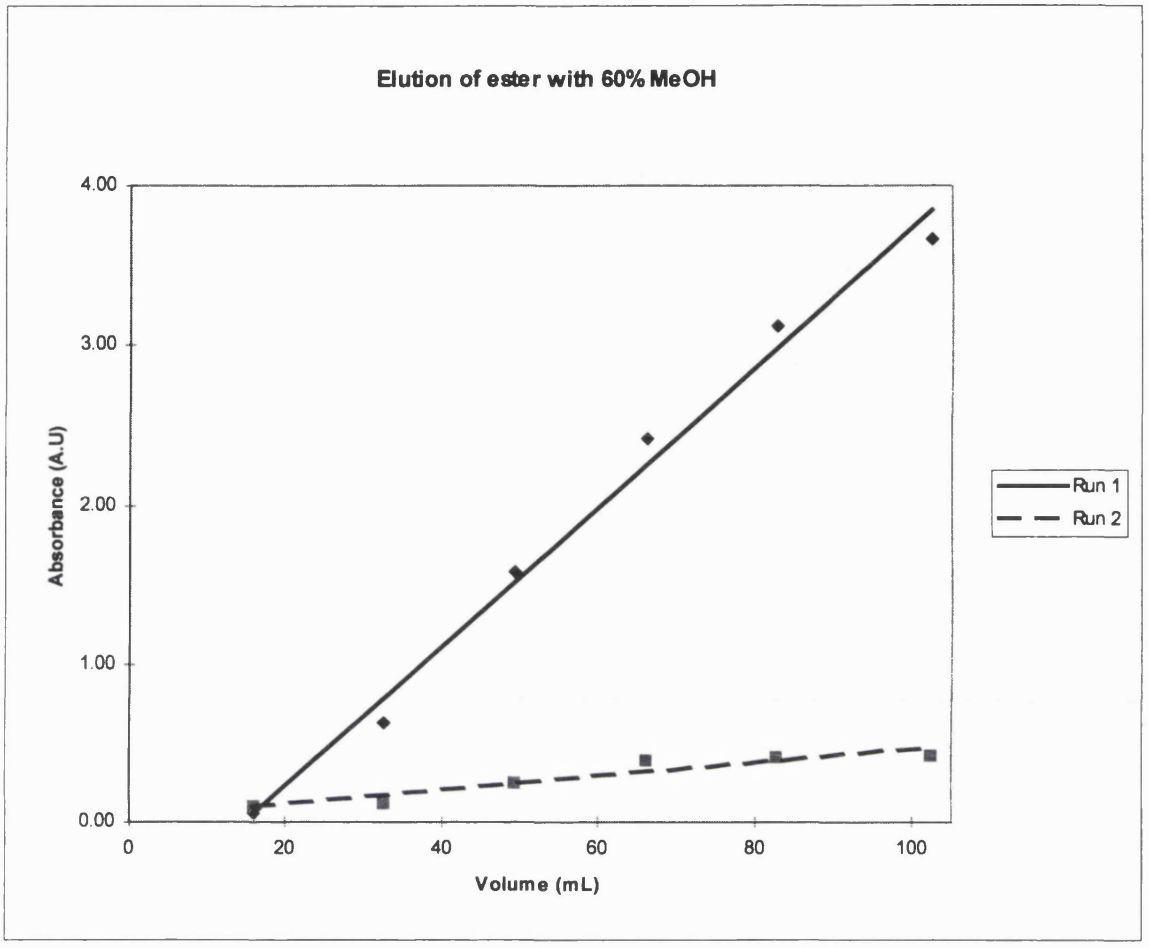


Figure 40: Graphical representation of the loss of *C. rugosa* lipase activity after the regeneration of the column with 60% aqueous methanol

Elution of the adsorbed DEGME ester with 40% acetone was also investigated in an identical way. Six fractions of 15 mL effluent were collected during the loading. As before the presence of acid was determined by the U. V of the solution. The absorbance data is listed in **table 46**. The DEGME ester was eluted with 40% aqueous acetone (125 mL) immediately after the adsorption stage. The column was equilibrated with 10% methanol before the second run. The second run was carried out under the identical conditions. The acid produced and the residual ester from both runs were isolated and analysed for the optical purity.

	Absorbance (A. U)	
Vol. (mL)	Run 1	Run 2
15	0.0530	0.0466
15	0.9195	0.9067
15	2.045	1.894
15	2.598	2.9445
15	3.6195	3.687
15	4.4147	4.285

Table 46: Release of 2-phenoxypropionic acid from the column, run 2 was carried out after the regeneration of the column with 40% aqueous acetone after run 1

The pictorial representation of the above data is shown in **fig 41**. It is clear from the data that the column retained its activity, during the acetone treatment to elute the residual ester. From these results, the suitability of 40% acetone as an eluent becomes apparent and hence it was decided to use 40% acetone as an eluent for the future work.

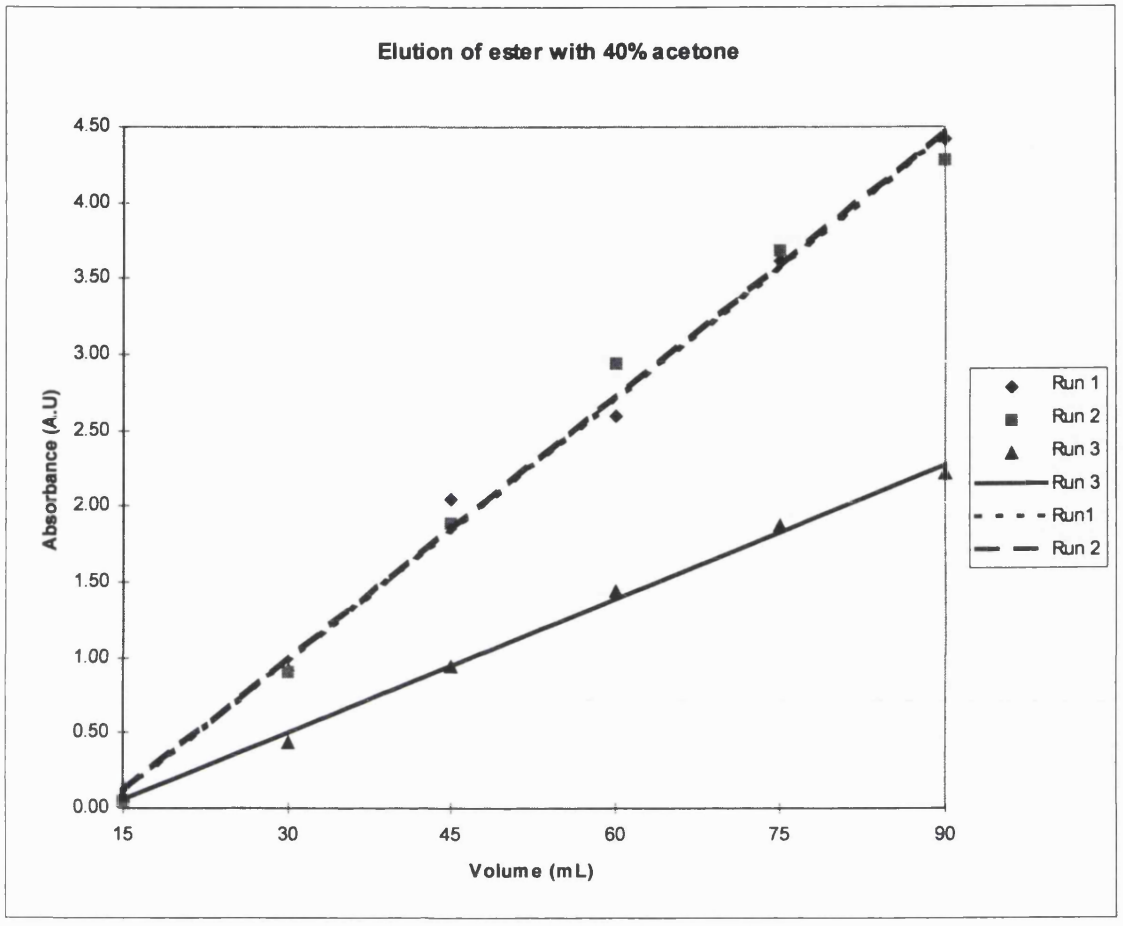


Figure 41: Graphical representation of the retention of *C. rugosa* lipase activity after the regeneration of the column with 40% aqueous acetone

9.2.3 Optimisation of E_R:

As the hydrolysis in the column was carried out in a single phase system, it was essential to determine the E_R of the *C. rugosa* for the DEGME ester in 10% methanol. The results (**table 47**) were quite disappointing as the lipase was found to loose considerable E_R towards DEGME ester when the hydrolysis was carried out in a single phase system. Further 33% loss in E_R was observed upon immobilisation. In summary the E_R of the free enzyme in two phase system was reduced from 30 to 6, when the hydrolysis was carried out using immobilised enzyme in a single phase system.

Fraction	E_R
50% IPA (60 h)	30
50% IPA (60 h) on XAD7 c-lin'd	20
50%IPA (60 h) in 10% MeOH	13
50%IPA (60 h) on XAD7 c-lin'd 10%MeOH	6

Table 47: E_R of the free and immobilised *C. rugosa* lipase for the DEGME ester of 2-phenoxypropionic acid in the presence of aqueous methanol.

The above results discourage the use of a single phase system for the column, however the column was further investigated in terms of the enantiomeric excess of the 2-phenoxypropionic acid produced at different stages of the run.

The run was split in three stages, initial stage loading followed by circulation of 10% MeOH (in a continuous mode) for approx. 3 hours and finally the circulation of buffer at pH 6.5 (in a recycling mode) was carried out for approx. 16 hours. As shown in **table 48** the enantiomeric excess of the acid produced, decreased with time. Initially the acid was produced in high enantio purity, but the optical purity decreased sharply in fraction three, where the acid was only recovered in 8% enantiomeric excess.

Fraction	Weight (mg)	% e.e.
1	22.4	86
2	23.4	70
3	78.8	8
Total	**124.6**	**34**

Table 48: % e.e. of the 2-phenoxypropionic acid produced during different stages of the column

The average enantiomeric excess of the 2-phenoxypropionic acid was calculated to be only 34%, however, the residual ester was isolated in high enantiomeric excess (86%), as high as the acid produced in the first fraction. From the enantiomeric excess of the product and the residual substrate, the conversion was calculated to be 72%. The 2-phenoxypropionic acid was isolated in 68% yield and the residual DEGME ester was recovered in 75% yield. The high conversion is considered to be the reason for the low enantio purity of the product and high enantiomeric excess of the residual ester.

The column was run again in an identical way except the stage three, where 10% MeOH was re-circulated through the column instead of the buffer. The results in **table 49** for stage 1 and stage 2 are identical to the results in **table 48**, but in stage three the acid was recovered in 32% enantiomeric excess, this led to the overall higher enantiomeric excess (54%) of the 2-phenoxypropionic acid when compared with the results in **table 48**. The 2-phenoxypropionic acid was isolated in 67% yield and the residual DEGME ester was also recovered in 68% yield. The conversion was calculated to be 48%. The enantiomeric excess of the residual ester was relatively low (49%), hence, neither the product or the residual substrate were produced in high enantiomeric excess. E_R of the enzyme for both run was calculated to be 5.

Fraction	Weight (mg)	% e.e.
1	19.3	84
2	21.2	70
3	41	32
Total	**81.5**	**54**

Table 49: % e.e. of the 2-phenoxypropionic acid produced during different stages of the column

Due to the overall low E_R of the run, it was decided to determine the E_R of the enzyme for the loading stage. This involved loading 400 mg of DEGME ester (over 3 h) on

the column, stage two and three were omitted from this run. The 2-phenoxypropionic acid released was collected and the residual adsorbed ester was eluted with 40% acetone.

Compound	% e.e.
2-phenoxypropionic acid	82
Residual DEGME ester	20

Table 50: % e.e. of the product and the residual substrate isolated from the column after stage 1.

As expected, the 2-phenoxypropionic acid was isolated in high enantiomeric excess and the residual DEGME ester was recovered in poor enantio purity (**table 50**). The conversion was calculated to be 20% and the E_R of the run was found to be 12.

This increase in the E_R value could be due to the fact that the DEGME ester was pumped continuously through the column, hence the concentration of the fast reacting enantiomer (*R*) was kept high during the run, this led to the preferential hydrolysis of the (*R*) enantiomer and increased E_R of the enzyme. This was not the case in the earlier runs because during the stage two and three, no substrate was pumped through the column, this led to the decreased concentration of the (*R*) enantiomer and increased rate of hydrolysis of the (*S*) enantiomer, hence the low E_R of the enzyme.

The low E_R of the earlier runs could also be due to the presence of the low activity contaminating lipase with the opposite specificity. This enzyme slowly hydrolyses the opposite (*S*) enantiomer and over a long period of time destroys the E_R of the reaction.

To fully understand the reason for different E_R values further experimental work has to be carried out.

One of the main concerns for this type of the column system is the regeneration of the column once it has reached its loading capacity. We have demonstrated that the column can be regenerated with 40% aqueous acetone and product and the residual starting material can be easily recovered in moderate to good yields and optical purity.

9.3 Conclusions

- Immobilised *C. rugosa* must have activity of about 3.5 U/g for the column to be economical in-terms of lipase consumption.

- Methanol deactivates the immobilised enzyme.

- 40% Acetone is a good solvent for the elution of adsorbed residual DEGME ester.

- To obtain optically pure 2-phenoxypropionic acid the column must be stopped immediately after the loading stage.

- To obtain the residual ester (opposite stereo chemistry than the carboxylic acid) in enantio pure form, the conversion must be beyond 70%.

- This column is suitable for the recovery of the optically pure residual ester rather than the production of carboxylic acid.

10 Discussion

This chapter deals with the overall review of the work carried out to establish a column reactor, for the production of optically pure carboxylic esters and carboxylic acids.

In order to determine the optical purity of the products, a chiral analytical method had to be established. To accomplish this, it was decided to reduce the CPA to the corresponding primary alcohol, 2-chloropropanol. Various reducing agents were employed to carryout this transformation, but due to the good leaving group at the α-carbon, the attempts to carry out this transformation were unsuccessful. However, an alternative method of synthesising the mandelate esters was successfully established, which involved mild coupling conditions. The second objective was to hydrolyse the residual ester from the hydrolyses reaction. Base catalysed hydrolysis were thought to racemise the chiral centre, due to the very labile chlorine at the α-carbon. It was decided to use an excess of non specific lipase (crude *C. rugosa* lipase) to carry out the hydrolysis.

Various straight chain esters of racemic 2-chloropropionic acid (CPA) were synthesised and were screened against commercially available lipases. From the screen several combinations of ester and lipase were selected and were analysed on a larger scale. Each hydrolysis reaction was followed analytically using gas chromatography, the products of hydrolysis were isolated and purified. The CPA produced was derivatised to its mandelate ester and the optical purity was determined by NMR. The shortlist of straight chain esters and lipase combination only produced CPA in very low enantiomeric excess. Cyclic esters were also synthesised and hydrolysed on large scale, but once again CPA was isolated in low enantiomeric excess.

Attempts to resolve CPA were stopped and a new substrate, 2-phenoxypropionic acid was chosen for the resolution studies while *C. rugosa* lipase was selected as a catalyst for their hydrolysis. Initially hexyl and octyl esters were prepared and hydrolysed against *C. rugosa* purchased from Sigma and Fluka. It was discovered that the lipase

provided by both manufacturers showed a preference for (*R*)-enantiomer but exhibited different E_R values. Both lipase produced 2-phenoxypropionic acid in poor enantiomeric excess.

At this stage *C. rugosa* fractionation was carried out by treating the lipase with IPA. During this investigation *C. rugosa* lipase was treated with 20% and 50% IPA over 2 and 60 h. Activity of the treated lipase was analysed extensively against various substrates. The fractionated lipase from each treatment was also tested for its specificity against various esters of 2-phenoxypropionic acid. It was discovered that to increase the specificity of the lipase the IPA concentration must be around 50% and the treatment should be carried out over a long period of time. Mr J. Vaghjiani crystallised the lipase from the fraction treated with 20% IPA over 2 h and used the crystals to produce CLECs ®. The specificity of crystals and CLECs ® for hexyl ester was then tested. The crystals and CLECs ® showed similar specificity ($E_R = 8$) towards the ester, a 4-fold specificity increase upon crystallisation. We believe this increase in specificity is due to the extended treatment of the lipase with MPD. CLECs ® obtained from Altus showed much lower specificity ($E_R = 2$), the same as the lipase in the supernatant obtained from the 20% IPA treatment over 2 h ($E_R = 2$).

The fractionated lipase (50% IPA, 60 h) was immobilised on Amberlite XAD2, 4 and 7. Contrary to the information in the literature, we obtained maximum lipase activity when immobilisation was carried over 16-20 h, no lipase activity was detected when immobilisation was carried out over two hours. It was also found that the maximum lipase activity is immobilised at pH 6.0, suggesting that the protein immobilised has iso-electric point of approximately 6. The immobilised lipase was cross-linked with 3% glutaraldehyde, lipase lost 20% of its activity upon cross-linking. The cross-linked lipase showed increased specificity (16 to 25) towards the hexyl ester, while it exhibited decreased specificity (30 to 20) towards the DEGME ester. The cross-linked lipase also showed increased activity towards the DEGME ester and decreased activity towards hexyl ester upon cross-linking. We believe this is due to the preferential immobilisation and cross-linking of an enzyme which is less active but highly specific towards the

hexyl ester and this lipase is highly active towards the DEGME ester but only shows moderate specificity.

It was necessary that the adsorption of esters on XAD7 is carried out in a single phase, hence the solubilities of hexyl and octyl esters of 2-phenoxypropionic acid were determined in aqueous methanol and acetonitrile. Solubilities of the esters in low concentration aqueous solvents were found to be inadequate for the adsorption studies. Very high solvent concentrations were required to achieve satisfactory solubility. High solvent concentrations were found to be incompatible with lipase activity, hence it was necessary to synthesise an ester which is fairly soluble in low concentrations of organic solvents. DEGME ester was synthesised and was found to be sufficiently soluble in low concentration organic solvents (4 mg/mL in 10% methanol, 6 mg/mL in 8 % acetone).

According to the immobilisation results it was clear that the maximum lipase activity is immobilised on Amberlite XAD7, hence XAD7 was chosen for the adsorption studies. In initial adsorption experiments, attempts were made to adsorb hexyl ester (4 mg/mL) in 50% acetonitrile. It was discovered that the acetonitrile concentration was too high for the adsorption to take place and it was deemed necessary to reduce the acetonitrile concentration. Adsorption of hexyl ester (1.4 mg/mL) in 40% acetonitrile was carried out and was found to be successful. However at latter stages it was discovered that the lipase loses the majority of its activity in 40% aqueous acetonitrile. Adsorption of DEGME ester (4 mg/mL) in 10% methanol on XAD7 was highly successful as approximately 99.7% DEGME ester was adsorbed on to the XAD7 compared to 97% hexyl ester adsorption in 40% acetonitrile.

Next stage was to elute the adsorbed ester. For this purpose small column was packed with Amberlite XAD7 and approximately 88 mg DEGME ester was adsorbed onto the resin. The elution was carried out with 40%, 60% 80% and 100% methanol, volume of eluent required to elute the adsorbed ester were recorded. According to the elution profiles 60-80% methanol was required to elute the adsorbed ester efficiently. Acetone was chosen as a second eluent and elution of the adsorbed ester was investigated with

20%, 40% and 60% aqueous acetone. According to the data collected, 40% acetone was as effective as 100% methanol. Regeneration of the adsorbed ester on a XAD7 in a presence of lipase was attempted with 60% methanol, 60% aqueous methanol was discovered to be incompatible with the lipase activity. Regeneration of the column with 40% aqueous acetone was successful and lipase retained all its activity during the elution process. Surprisingly this trend was only observed for one elution, column regeneration with 40% aqueous acetone for the third run resulted in approximately 50% lipase activity loss. Further investigation is essential to determine the cause for activity decrease.

Final stage was to set up the column according to the above conditions and monitor the optical purity of the products produced. The column was packed with *C. rugosa* lipase immobilised on Amberlite XAD7 and the DEGME ester (4mg/mL) in 10% aqueous methanol was fed into the column at the fixed rate. The run was split in to 3 parts, initially the loading stage, circulation of the mobile phase in a continuous fashion and finally the circulation of the mobile phase in a recycling manner. 2-Phenoxypropionic acid produced in each stage was isolated and analysed for its optical purity. After final stage the adsorbed residual ester was eluted with 40% aqueous acetone and hydrolysed with crude *C. rugosa* lipase and the resulting acid was tested for the optical purity. As expected the optical purity of the 2-phenoxypropionic acid was at its highest in first stage and at its lowest in stage three. Optical purity of the residual ester was as high as the acid produced in the first stage. E_R of the reaction was calculated to be 5. The Column was run again and this time stage 2 and 3 were omitted from the run. The Column was regenerated immediately after the loading and the acid produced and the residual ester released were tested for their optical purity. The optical purity of the 2-phenoxypropionic acid was same as in the previous runs and as expected the residual ester was recovered in very low enantiomeric excess, however, the E_R of the reaction increased from 5 to 12. The difference in the E_R could be due to the presence of a low activity contaminating lipase which shows preference towards the slow reacting enantiomer and over a long period of time destroys the optical purity of the 2-phenoxypropionic acid produced. The increase in E_R could also be due to the fact that

during the loading stage, substrate concentration of both enantiomers (due to the continuous feed of the substrate) was kept same and this led to the preferential hydrolysis of the fast reacting enantiomer. The acid produced and residual ester were recovered in 65-75% yield.

This column is currently at its initial stage and requires a lot of work for continuous operation. However, from the work carried out so far it is evident that this column shows a huge potential for the production of optically pure residual esters. This column will be setup as a simulated moving bed system, where number of small columns will be connected in series to aid isolation of the products (for the set-up see chapter11). Incorporation of a racemisation unit to the simulated moving bed technology will provide a highly economical method for the production of carboxylic esters or carboxylic acids depending on the individuals needs.

11 Future Work

- To investigate the reason for the activity loss after the second run. This may involve search for an alternative solvent for elution.

- At the moment the ester is loaded onto the column with aqueous methanol and eluted with aqueous acetone. In future we plan to use one solvent for adsorption as well as the elution stage. To achieve this the column must be connected to HPLC. The online HPLC analysis will enable us to run the column unattended for longer period of time and UV analysis will not be necessary.

- Investigate the conditions required to racemise the 2-phenoxypropionic acid produced or the residual ester. Thio esters of 2-phenoxypropionic acid have been racemised using triethylamine under mild conditions, however, racemisation of 2-phenoxypropionic acid will require much stronger conditions.

- Link the racemisation step with the column, this will enable us to obtain 2-phenoxypropionic acid or its esters in high yields.

11.1 Continuous separation

The separation column (packed with immobilised *C. rugosa* lipase and Amberlite XAD7) can be set-up according to the following configuration to continuously resolve racemic esters of 2-phenoxypropionic acid. Remember, the outlets will be connected to the on-line HPLC machine to follow the progress of the column.

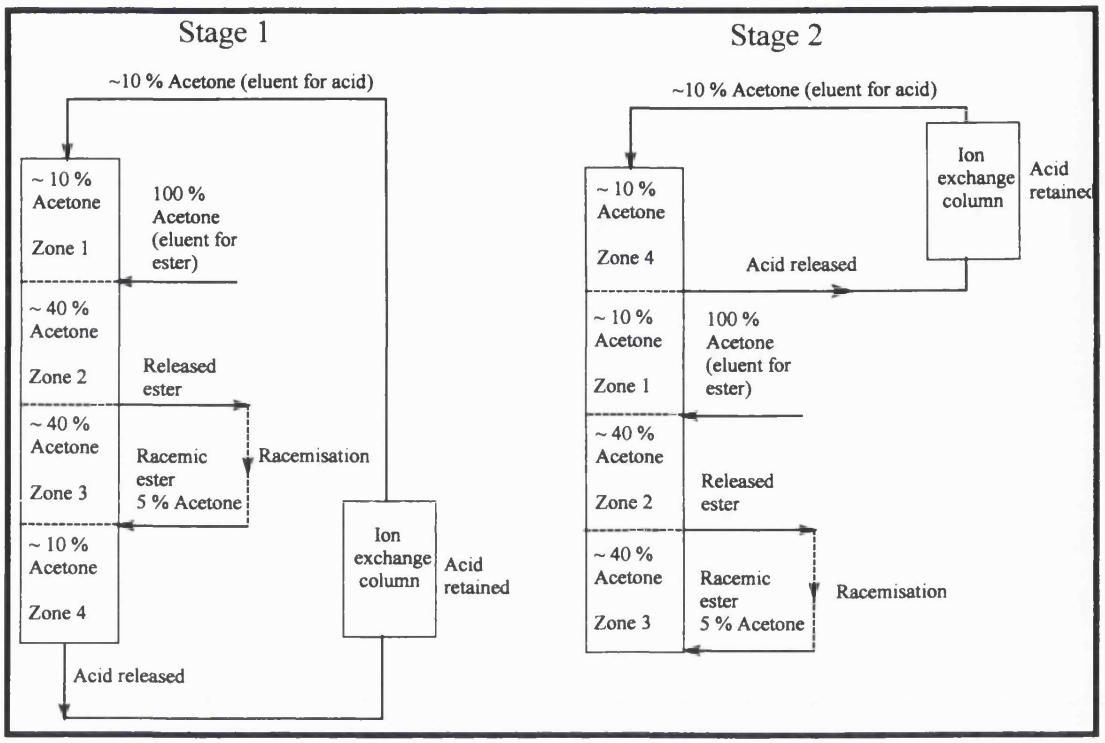


Figure 42: Column reactor for the continuos production of carboxylic esters

The eluent (10% aqueous acetone) will be introduced to the column at a higher level as the eluent moves down the column, it will mix with the second stream of eluent (100% acetone, introduced to the bed at the start of zone 2). Flow rates of both streams will have to be carefully monitored to produce 40% aqueous acetone, this concentration will be retained in zone 3 as well. The racemic ester will be introduced to the column in zone 4 for separation purposes.

At the start there are two main zones in the column **zone 4** and **zone 3**, the functions of both zones are summarised bellow :

Zone 4:

The primary function of this zone is to hydrolyse only one enantiomer of the racemic ester introduced to the column. The fluid/solution entering the zone 3 will only carry the residual ester. The liquid stream which will flow downwards, countercurrent to

the movement of the injection ports will only contain optically enriched 2-phenoxypropionic acid produced.

The optically enriched 2-phenoxypropionic acid will be allowed to leave the column. The feed containing the acid will be passed through the second column (packed with ion exchange resin) where the acid will be removed from the feed, the mobile phase leaving the ion exchange column will be introduced to the first column. The acid can be eluted from the ion exchange column by a polar solvent when required.

Zone 3:

The main purpose of the zone is to remove the adsorbed ester from the resin entering from zone 4. The desorbed ester will be collected at the top of zone3. The residual ester removed from the column in zone 3 will have to be racemised and esterified (depending on the racemisation conditions) prior to the introduction to the column.

The feed and the extraction points will be systematically moved around the column to simulate the moving bed environment. Racemic ester will be introduced to the column in zone 1 (stage 2), residual ester will be collected in zone 3 (stage 2) and the released acid will be collected from the start of zone 1 (stage 2).

11.2 Racemisation of 2-phenoxypropionic acid and its esters

Drueckhammer (1998) has successfully carried out in-situ racemisation of thioesters of α--substituted propionic acids using, triethylamine as a base under mild conditions. However, their work with *C. rugosa* lipase has shown that the high activity and enantiospecificity in oxoester hydrolysis does not guarantee high activity and enantiospecificity in hydrolysis of a thioester of the same acid.

We believe that the 2-phenoxypropionic acid or its esters can be racemised under acidic or basic conditions, however, high temperatures maybe required. High

temperatures should not effect the running of the column as racemisation will be carried out in a separate unit.

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