THE UNIVERSITY OF HULL

THE DEVELOPMENT AND IMPROVEMENT OF BIOCATALYTIC REACTIONS

Being a Thesis submitted in accordance with the requirements for the degree of Doctor of Philosophy in the Department of Chemistry at The University of Hull

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September 2008

ABSTRACT

Conventional chemical processes often require a series of complex synthesis steps that utilise a great deal of energy and raw materials. There is a huge commitment by the chemical industry to search for "green", environmentally friendly processes. Biocatalysis, using enzymes to catalyse a chemical reaction, is emerging as an economical and ecological production route for a wide range of fine chemicals, pharmaceuticals and food ingredients. In addition to biocatalysis, miniaturised chemistry has also become of interest and significance to the chemical industry over the last decade. Microreactors allow for the synthesis of small volumes of compounds quickly and in high yield, with fast optimisation of conditions. Additionally, when used with immobilised biocatalysts, these miniaturised devices can enable the rapid throughput screening of a wide range of enzymes and their substrates. Despite this however, developments of applications in the field of miniaturised enzymatic processes are still in the initial stages.

Using lipase catalysed, enzymatic esterification as a model reaction, **Chapter 2** demonstrates the use of miniaturised technology for biocatalytic reactions. The work described within this chapter explored the feasibility of combining biocatalysis with miniaturisation and enabled us to gain familiarity with both the biocatalytic processes and miniaturised technology. Benchmarked again batch reactions, nine alkyl esters were successfully synthesised enzymatically, using Novozyme 435, in a pressure driven, packed-bed, miniaturised, continuous flow reactor. In some cases close to 100% ester conversions were achieved. **Chapter 2** also demonstrates the ability to screen the enzyme for substrate specificity.

In nature, many molecules exist as racemic mixtures. As discussed in **Chapter 3**, enantiomers of racemic compounds can often exhibit diverse pharmacological and therapeutic properties. In fact, different drugs usually have their biological activity based mainly on one enantiomer. Consequently, research into the preparation of enantiomerically pure chiral compounds is increasing in interest. Dynamic kinetic resolution is emerging as a potentially efficient process, whereby standard enzymatic

kinetic resolution is coupled with *in situ* racemisation of the slow reacting starting substrate, using a suitable racemising catalyst. However, compatibility issues between the biocatalyst and racemising agent limit the number of applications to which this dynamic kinetic resolution set up can be applied. With this in mind, **Chapter 3** reports the development of a dynamic kinetic resolution process. A thorough study into the kinetic resolution, racemisation and dynamic kinetic resolution batch reactions of a range of substrates, of interest to the pharmaceutical industry, are detailed in **Chapter 3**. Efficient methods for the enantioselective hydrolysis of a series of esters, using a range of lipases, were primarily developed. The D-forms of the racemic ester and thioester substrates were found to be substrates for lipase catalysed enantioselective hydrolysis to the corresponding chiral acids, in excellent optical purity (>99%ee_D). Base catalysed racemisation studies showed that the ester substrates could not be racemised to any extent, under the reaction conditions studied. However, three organic bases were found to be suitable racemising catalysts for the L-enantiomers of the two thioester substrates under investigation. The combined reactions allowed for a dynamic kinetic resolution process to be established for the two thioesters with excellent to good conversions and enantioselectivies being achieved. The successful dynamic kinetic resolution processes were subsequently investigated with the aid of miniaturised reactor chemistry as a means of improving the technique further. **Chapter 4** therefore, details a combination of the miniaturised reactor technology described in **Chapter 2** and the biocatalytic resolution reactions presented in **Chapter 3**. The purpose here was to determine if the inherent advantages associated with miniaturisation, discussed in **Chapter 2**, could be observed for enzymatic resolution set ups. Additionally, in an attempt to overcome compatibility issues, with regards to the enzyme and racemising agent in the dynamic kinetic resolution process, as discussed in **Chapter 3**, **Chapter 4** discusses the development of a separated dynamic kinetic resolution reaction.

Unfortunately, issues surrounding the activity of the immobilised bases, used as racemising agents, did not allow for the full dynamic kinetic resolution reaction to be achieved separately. However, kinetic resolution reactions utilising an immobilised lipase were successfully conducted in the miniaturised system with inherent advantages over the equivalent batch process being observed.

CONTENTS

ACKNOWLEDGEMENTS

Firstly, I must thank my academic supervisors, Professor Gillian Greenway and Dr Paul Watts for providing me with the opportunity to carry out research within the group and for giving me continued support over the past four years. Additionally, my industrial supervisors Andrew Wells and Steve Eyley are thanked for their guidance over the past 36 months, in particularly during my stay at AstraZeneca, Charnwood in 2006, which was most productive and very enjoyable.

Importantly, I would like to thank my colleague Dr Charlotte Wiles for her advice, interest and enthusiasm with regards to my work. Jools Hooper, Leanne Marle, Benji Wahab and Gareth Wild are also thanked for helping to create a pleasant working environment. Colleagues from my six month stay at AstraZeneca, Charnwood, must be recognised. In particular, Phil O'Keefe, who was a brilliant landlord, Mark Dow for being a great mate in a town where I did not know anyone and John Singleton for being an unassigned mentor and taking me under his wing in the department.

On a more personal note, I acknowledge my family and friends. Most importantly my Dad, Mick who has been there for me every step of the way since the day I was born. My dear Grandma, Paddy, my not so little anymore brother, Ashley and my best friend in the whole world, Sam must also be thanked for their constant support, encouragement and hugs over the past four very difficult years.

Finally, the funding of the EPSRC and AstraZeneca was gratefully received.

DECLARATION

The work described in this thesis was carried out in the Department of Chemistry, The University of Hull, under the supervision of Prof. G.M. Greenway and Dr. P. Watts between September 2004 and December 2007. Except where indicated by reference, this work is original and has not been submitted for any other degree.

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September 2008

ABBREVIATIONS

γ	Gamma
α	Alpha
λ	Wavelength
%	Percentage
^{o}C	Degree centigrade
D-	Dextrorotary
L-	Levorotary
k_D	Rate of reaction with D- enantiomer
k_L	Rate of reaction with L- enantiomer
K_{rac}	Rate of racemisation
3D	Three dimensional
6-APA	6-aminopenicillanic acid
μTAS	Micro total analytical system
Å	Angstrom
ACN	Acetonitrile
ADP	Adenosine diphosphate
AJM	Abrasive jet machining
APTS	3-aminopropyltrithoxysilane
ATP	Adenosine triphosphate
BMR	Batch membrane reactor
CAD	Computer aided design
CALB	*Candida antarctica* lipase B
CLEA	Cross linked enzyme aggregates
CLEC	Cross linked enzyme crystals
CLPC	Cross linked protein crystals
CPA	Controlled pore alumina
CPG	Controlled pore glass
CPT	Controlled pore titania
CPZ	Controlled pore zirconia
CRL	*Candida rugosa* lipase
CSTR	Continuous stirred tank reactor

DABCO	Diazabicyclo[2.2.2] octane
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCHA	Dicyclohexylamine
DCM	Dichloromethane
D_f	Thickness of stationary phase film in HPLC column
DIPEA	*N,N* diisopropylethylenediamine
DKR	Dynamic kinetic resolution
DMEA	Dimethylethanolamine
DNA	Deoxyribonucleic aicd
DVB	Divinyl benzene
EC	Enzyme commission
ee	Enantiomeric excess
EOF	Electroosmotic flow
EPSRC	Engineering and Physical Sciences Research Council
FBR	Fluidised bed reactor
FPLC	Fast protein lipid chromatography
GC	Gas chromatography
GC-FID	Gas chromatography flame ionisation detection
GC-MS	Gas chromatography mass spectrometry
HPLC	High performance liquid chromatography
Hz	Hertz
ID	Internal diameter
IUBMB	International Union of Biochemistry and Molecular Biology
kDa	kilo Dalton
KR	Kinetic resolution
LC	Liquid chromatography
MALDO TOF MS	Matrix assisted laser desorption time of flight mass spectrometry
MW	Molecular weight
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
ODS	Octadecylsilane
PBR	Packed bed reactor
PFR	Plug flow reactor

PS	Proton sponge
PY	Pyridine
R&D	Research & Development
RT	Room temperature
STR	Stirred tank reactor
TCT	Thermal conductivity detector
TEBA	*Tert*-butylamine
TEOS	Silicon tetraethoxide
TFA	Trifluoroacetic acid
THF	Tetrahydrofluorane
TLC	Thin layer chromatography
TMA	Trimethylamine
TMS	Tetramethyl silane
TOA	Trioctylamine
TRBA	Tributylamine
UV	Ultra violet
UV Vis	Ultraviolet visible
wt/wt	Weight for weight

1 INTRODUCTION

1.1 BIOCATALYSIS

Conventional chemical processes often require a series of complex synthetic steps that utilise a great deal of energy and raw materials. However, millions of years of evolution have created millions of microorganisms containing enzymes known to catalyse almost many chemical reactions used to make xenobiotic chemicals. It is the use of such enzymes to catalyse a specific chemical reaction that is known as biocatalysis. Biocatalysis uses living cells or isolated enzymes to replace the multi-level processes of classical chemistry. With the correct research and development, this area can yield new products such as fine chemicals, pharmaceuticals and food ingredients. In fact, for a high proportion of chemical processes, biocatalysis is the most economically and ecologically feasible production route. Ultimately, the use of enzymes in chemical synthesis can give rise to new chiral building blocks such as amines, alcohols and carboxylic acids, where access has been previously limited (Patel, 2008). Such biocatalytic processes are highly selective and efficient whilst providing an environmentally benign, economically feasible and sustainable production process for complex chemical conversions. Biocatalysts also have the advantages of allowing reactions to be carried out under milder reaction conditions by removing the need for elevated temperatures and perhaps the use of noxious chemicals. Likewise, biocatalytic reactions tend to incorporate less reaction steps than traditional chemical synthesis due to the high selectivity of the biocatalyst eliminating the need for protection and deprotection procedures which may otherwise be necessary.

Challenges exist however in coercing nature to work on an industrial scale and, with few exceptions, most applications have been for low volume, high value products, such as drugs and fine chemicals. Essential drawbacks of biocatalysis include instability of the enzyme in the desired media, a limited supply and range of biocatalysts which often have high costs due to isolation and purification, and the prolonged development cycles associated with biocatalytic reactions. Enzymes may deactivate under a range of conditions such as extremes of temperature or pH value, when subjected to physical forces, upon removal from natural environment and/or

when exposed to a variety of solvents. Another important factor to consider is product inhibition. Product inhibition, should it arise, can cause the productivity of enzymatic processes to become low.

In biocatalysis, a distinction is made between fermentative and enzymatic processes. In fermentation, whole cells are generally used (Kiss *et al*., 2004) and in enzymatic synthesis, a single enzyme usually isolated from a microorganism, is used to catalyse just one specific reaction step. In fermentation, a process that typically uses whole cells with the ability to carry out several transformations, the metabolic functions of living microorganisms are used. The organisms are supplied with a raw material and the finished product is then obtained following separation from the reaction batches. One of the largest volume chemicals produced biocatalytically is bioethanol from the fermentation of sugars (Balat *et al*, 2008), which are often generated by breaking down starches from corn, potatoes, beets, sugar cane or wheat. Isolated enzymes, utilised in enzymatic synthesis can often be treated more like traditional heterogeneous chemical catalysts. That is, isolated enzymes can be purified, immobilised, and stabilised to improve performance. Isolated enzymes can work in continuous processes and are generally used for a specific and highly selective process.

1.2 ENZYMES

The concept of enzyme catalysis was first recognised by Kirchoff in 1811. However, the actual word 'catalysis' was not coined until 1838 by Berzelius and it wasn't for another 40 years that the word 'enzymes', which were later found to be proteins, was introduced by Kuhne. Modern enzyme chemistry was heralded by the proposed hypothesis for enzyme reactions authored by Michaelis and Menten (1913).

Enzymes are highly selective (*i.e*. they possess an active site which specifically binds to a substrate with a precise size and shape that only fits into this active site), efficient, readily available, environmentally benign biocatalysts. In general, enzymes have a high catalytic activity for virtually all synthetic and degradative reactions in biological systems. Enzymes function by increasing the rate of an otherwise slow reaction, without being consumed by the overall reaction. Most enzymes do however, undergo a conformational change on turnover of the enzymatic process. These biocatalysts can often be used under ambient conditions, neutral pH and atmospheric pressure whilst producing minimal waste and consuming a low amount of energy, which reduces capital costs. Factors that affect the enzyme activity are pH, temperature, stability of the enzyme and the enzyme's kinetic parameters, all of which need to be optimised. Although not all enzymes can do so, many enzymes have enantioselective properties which have the ability to distinguish between the two enantiomers of a racemic or prochiral substrate. Whereby, the 3D cavity of the active site will only usually be matched to one enantiomer which will be bound tightly whilst the other will not. Consequently, the use of such biocatalysts for organic transformations, especially in the resolution / synthesis of enantiomers, has become an increasingly attractive alternative to conventional chemical methods (Kelly & Waldmann, 1999).

Biocatalysts may be obtained from living microorganisms, selected by screening and harvested by fermentation procedures utilising reactors or flasks to yield the biocatalysts of commercial interest. Of the hundred or so enzymes currently being used industrially over half are from fungi and yeast, over a third are from bacteria and the remainder are derived from plant sources such as spinach or animals,

including snake venom. Purification usually involves a chromatographic technique with the aid of fast protein lipid chromatography (FPLC) apparatus. Other concentration techniques include salting, co-solvent precipitation, vacuum centrifugation, ultra filtration and dialysis or lyophilisation. Once purified, the enzyme can be characterised by techniques such as electrophoresis, analytical centrifugation, matrix assisted laser desorption - time of flight mass spectrometry (MALDI-TOF-MS), ultraviolet visible (UV Vis) spectroscopy, circular dichroism and fluorescence spectroscopy and structurally characterized by NMR spectroscopy or x-ray crystallography. Functional characterization is carried out by determining the enzyme activity and stability at varying temperatures and pH values.

Structurally, enzymes are proteins composed of long, linear chains of amino acids that fold to produce a globular three dimensional (3D) enzymatic product. There are four distinct aspects of a protein's structure; primary, secondary, tertiary and quaternary. The primary structure is the amino acid sequence of the peptide chains, the secondary protein structure (the α-helix or the β-pleated sheet) occurs when the sequence of amino acids are linked by hydrogen bonds, the tertiary arrangement is the 3D structure of a single protein molecule and occurs when additional attractions are present between the α-helix or the β-pleated sheets and ultimately the quaternary structure of proteins would be described as being a complex of several protein molecules consisting of more than one amino acid chain. The activities of enzymes are determined by their 3D structure. Only a small portion of the enzyme, known as the active site, is involved in catalysis. In some cases, the 'backbone' which exists to maintain structure and ensure the active site amino acids are in close proximity is the only component in the structure. However, there are usually additional non-protein groups present which may or may not participate in the catalytic activity of the enzyme, but affect its stability and solubility. Other factors often present are cofactors, such as metal ions and low molecular weight organic molecules known as coenzymes. These cofactors are loosely or tightly bound *via* covalent or non covalent forces and not only contribute to the enzymes stability but also to their activity. In fact, in some cases, these cofactors and coenzymes are a requirement for the enzyme to be active. If so this must be recognised in order for the enzyme to be used efficiently, particularly if the reaction is continuous where there may be a

tendency for the cofactors or coenzymes to become separated from an enzymes moiety and hence decrease the activity.

For classification purposes, the report of the '*Nomenclature Committee*' appointed by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1984 is often used. This enzyme commission assigned each enzyme a recommended name with defined subclasses and sub-subclasses, identified by what is known as an enzyme commission (EC) number. This is a 4-part distinguishing number where the first digit indicates the general class of the enzyme, the second indicates the sub-class of the enzyme and will vary according to the first, the third further divides the enzymes into sub-subclasses and the fourth and final digit indicates the specific enzyme and is unique for each known enzyme reaction. There are six main classes of enzymes from which an enzyme can be chosen for a biocatalytic reaction; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.

Oxidoreductases are an extensive class of enzymes that catalyse a variety of oxidation-reduction (redox) reactions, where hydrogen or oxygen atoms (or electrons) are transferred between molecules, frequently using NAD(P)H and adenosine triphosphate (ATP) as co-factors. Common names of such enzymes include; dehydrogenases, oxidases *e.g.* glucose oxidase (EC 1.1.3.4), oxygenases, peroxidases, reductase and catalase. Transferases catalyse the transfer of an atom or group of atoms between two molecules. Common names include acetyltransferase, aminotransferase, *e.g.* aspartate aminotransferase (EC 2.6.1.1), methylase, protein kinase and polymerase. The third group of enzymes are classified as hydrolases and involve hydrolytic reactions, whereby a molecule is split into two or more smaller molecules by the addition of water. The subsequent reversal reactions, *i.e.* condensation can also be catalysed by this same group of enzymes. At present, this is the most commonly encountered class of enzymes within the field of enzyme technology. Common names of hydrolases include; esterases, nucleases, phosphatases, lipases and proteases *e.g.* chymosin (rennin), EC 3.4.23.4. Lyases, such as histidine ammonia lyase (EC 4.3.1.3) are distinct from the hydrolases as they do not cleave by the addition of water. This type of enzyme is generally involved in elimination reactions, during which a group of atoms is removed from the substrate, leaving double bonds. The group includes aldolases, decarboxylases and

dehydratases. The isomerases such as xylose isomerase (EC 5.3.1.5) are responsible for the catalysis of molecular isomerations *via* atomic rearrangement within a molecule and include epimerases, racemases, rotamase and intramolecular transferases. The final group of enzymes to be classified by the IUBMB are the ligases, also know as synthetases. The ligases form a relatively small group of enzymes which cause the formation of a covalent bond joining two molecules together. Glutathione synthase (EC 6.3.2.3) is a common example involved in adenosine diphosphate (ADP) forming from ATP.

Lipases, also known as triacylglycerol ester hydrolases (EC 3.1.1.3) are the focus of the work described herein. Lipases are extra cellular, soluble enzymes that in their natural environment catalyse the hydrolysis of fats and oils. Their high commercial availability, low costs, broad substrate specificity, selectivity, lack of need for co-factors or co-enzymes and catalytic versatility under mild operating conditions within a large range of temperatures have made lipases important biocatalysts in many applications, with considerable attention from chemists in fundamental and applied research (Villeneuve *et al*., 2000). In the appropriate reaction media lipases have been shown to be very active in the synthetic biocatalysis of reactions such as the esterification of fatty acids (Cramer *et al*., 2007) and transesterifications (Yasmin *et al*., 2006). Abundant in nature, these enzymes can occur in several organisms. Animals including cattle, sheep and pigs (pancreatic lipases) are an important source of lipases. As are plants, fungi, many types of yeast such as *Candida antarctica* (*C. antarctica*) and *Candida rugosa* (*C. rugosa*), moulds such as *Aspergillus niger* (*A. niger*) and bacteria such as *Chromobacterium viscosum* (*Ch. Viscosum)*. Lipases have many industrial and pharmaceutical applications, these include the synthesis of surfactants and soaps, use in leather treatment, the controlled hydrolysis of milk fat for the acceleration of cheese ripening, hydrolysis, glycerolysis and alcoholysis of bulk fats and oils, and in the production of optically pure compounds for flavourings, such as esters.

Synthetic reactions catalysed by enzymes such as lipases may be performed in aqueous media, organic solvents (Klibanov, 2001), in supercritical fluids (Karmee *et al*., 2007), in ionic liquids (Sheldon *et al*., 2002) or in solvent free systems (Hobbs & Thomas, 2007). However, the advantages associated with the use of enzymes in

organic media, such as improved reagent solubility, an increase in chemical stability of the organic substrates by avoiding denaturation or deactivation, facile product recovery and no bacterial contaminations has led to much research focusing on the application of enzyme reactions in organic solvents. The choice of solvent in which biocatalysis is carried out is very important, not only with regards to enzyme stability and the immobilisation technique (**Section 1.2.1**) but also in terms of the nature of the chemical reaction taking place. Although a proportion of biocatalytic reactions, including hydrolysis reactions, need a certain amount of polarity in order for the reaction to proceed successfully, it is well known that high polarity solvents can denature an enzyme and thus decrease its catalytic activity (Catoni *et al*., 1996; Carrea *et al*., 1995). Non polar organic solvents such as hexane (Zaidi *et al*., 2000), heptanes (Langrand *et al*., 1990), isooctane (Blattner *et al*., 2006; Bezbradica *et al*., 2006) and cyclohexane (Norin *et al*., 1988) are the most effective choice of reaction media for use in biocatalysis (Laane *et al*., 1987; Wehtje *et al*., 1997) when reactions such as esterification, are being conducted (Ghamgui *et al*., 2004). Difficulty in adjusting the pH of an organic solvent solution in large scale processes and poor conformational flexibility of some enzymes in organic media are some points to be considered with this choice of reaction media. However, a lack of conformational flexibility can lead to stabilization of the protein in organic media and can also lead to an increase in enantioselectivity since access to the active site can shrink, making this an advantage with a chiral reaction.

Whilst a diverse array of genetically distinct lipase enzymes are found in nature, which represent several types of protein folds and catalytic mechanisms, most are built on an alpha/beta hydrolase fold and employ a hydrolysis mechanism involving a catalytic triad, that is buried inside the protein, composed of a serine nucleophile, an acid residue (usually aspartic acid) and a histidine. Mechanistically, the serine residue is activated by the histidine and aspartate residues and a tetrahedral acyl enzyme intermediate is formed between the reactant and active site of the enzyme. Stabilisation is maintained by the presence of an oxyanion hole which can adopt an '*open*' and '*closed*' formation giving rise to the high level of specificity of the lipase enzymes. X-ray crystallographic techniques can often be applied to characterise the sequence and crystal structure of lipase enzymes to develop further understandings of their specific substrate specificity.

1.2.1 ENZYME STABILISATION

In general, free enzymes are soluble in aqueous reaction medium. Soluble enzymes may exhibit a slow reaction rate as a result of the addition of organic solvents or reagents, can be unstable under practical operating conditions such as elevated temperatures and can have a low storage stability. For pharmaceuticals and fine chemicals, the use of a free enzyme can result in complex downstream processing where the product may be contaminated with potentially toxic protein. The removal of this can involve extra purification costs as a result of the need for ultra filtration. This remaining residual activity cannot usually be commercially recovered for reuse and is therefore wasted, which is not desirable if, as is often the case, the initial cost of the enzyme was high. It is for these reasons that soluble enzymes are not of much interest to industry and, whenever possible enzymes should be protected against denaturation which can lead to a loss in activity, and utilised in an efficient manner. Consequently, stabilisation of enzymes either *via* protein engineering or protein immobilisation is often important.

Protein engineering, which can be used in order to enhance both stability and activity, is laborious and currently, only one property of one protein can be altered at any one time. Therefore, immobilisation of the enzyme to a suitable support is the most attractive method of enzyme stabilisation. Enzyme immobilisation (Sheldon, 2007) is achieved by literally attaching an enzyme to or within some other material thus making the enzyme insoluble. Immobilisation separates the enzyme and product during the reaction using a two phase system; one phase containing the enzyme and the other phase containing the product. The enzyme is imprisoned within its phase, allowing its continuous use or subsequent reuse, but preventing it from contaminating the product. Other molecules however, including the reactants are able to move freely between the two phases.

Immobilisation increases enzyme stability thereby allowing applications even under harsh environmental conditions of pH, temperature and organic solvents. In addition, immobilisation of a biocatalyst can alter selectivity, provides greater control over the bioreactor system, eliminates waste and gives improved productivity as the enzyme

molecule can be easily separated from the products of the reaction and recycled and reused, with a considerable saving in enzyme, labour and over head costs. The availability of a product in greater purity is very crucial in the food and pharmaceutical industry since contamination could cause serious toxicological, sensory, or immunological problems.

On a more negative note, the use of an inert solid support in the immobilisation procedure may lead to a lower overall catalytic activity, where in the immobilised state the enzyme may have a more restrained conformation. This could however, be advantageous if the reaction investigated is chiral. Likewise, insoluble immobilised enzymes are of little use when any of the reactants are also insoluble. With respect to cost, there may be a high initial investment for the immobilisation compared to the free enzyme. Therefore, in so far as large companies are concerned, if there is a cheap source of soluble enzyme on the market or other successful processes are well established, it is doubtful whether the newly developed immobilised enzyme process would survive. These cost considerations of immobilisation must take into account such factors as cell production, matrix type and form, immobilisation conditions, reactor design and product purification. Although economic and advantageous processes can be devised using immobilised enzymes, their general industrial adoption will need time and encouragement and if a process will work well in water with a cheap enzyme then the cost of immobilisation may be prohibitive. However, most enzymes for use in organic solvent would have to be support prior to processing since free proteins are often inactivated in such reaction media.

One of the earliest reports of immobilised enzymes was that of Nelson and Griffin in 1916, who reported the adsorption of the enzyme invertase on charcoal and alumina and demonstrated that these immobilised enzymes retained their catalytic activity. Despite their success it was over 30 years before immobilisation was further developed, when in 1949, Michael and Ewers used the azide derivative of carboxymethylcellulose to immobilise a variety of proteins. However it was not until Schleith, who in 1954 used a diazo derivative of poly-ρ-amino styrene to immobilise pepsin, amylase and carboxypeptidase that any large scale experimentation was carried out in this field. One of the problems at this time was the scarcity of commercially available supports which could be used to successfully immobilise the

enzymes. Although a number of scientists showed an interest in the early 1950's, including chemists McLaren (1954) and Zittle (1953) who studied the adsorption of enzymes onto inorganic carriers, such as kaolinite, their work went unnoticed as they were considered to be ahead of their time. Work followed by Katchalski (1960) and Mitz and Summaria (1961) who concentrated on the covalent binding of enzymes to organic copolymers and the coupling of trypsin and chymotrypsin to diazotised ρ-aminobenzoyl cellulose and the hydrazide derivative of carboxymethylcellulose, respectively. It was not until the work of Manecke in 1961 and his observation that, in general, the level of activity of an immobilised enzyme depends on the degree of hydration of the polymer matrix, that polymers other than cellulose were investigated as supports to which enzymes could be covalently bonded. Later, in 1963, Rimmon used a copolymer of L-leucine-ρ-amino-DL-phenylalanine to immobilise chymotrypsin, papain, pepsin and streptokinase. The use of supports other than polymers was investigated in the late 1960's and early 1970's, when Weetal (1969) used glass and, Inman and Hornby (1972) used nylon.

Immobilisation can be carried out using either the whole cell or an isolated enzyme. Whilst in practice there is little to choose between the immobilised cell and the immobilised purified enzyme in so far as stability is concerned, if the enzyme requires the control of the whole cell to remain stable, then whole cells would tend to be used. Whole cell immobilisation avoids the lengthy and expensive operations for enzyme purification whilst preserving the enzyme in its natural environment thus preventing it from inactivation during immobilisation or in its use in a continuous system. Whole cell immobilisation may also provide a multi-purpose catalyst, especially when the process requires the participation of several enzymes in sequence, whilst any cofactors or coenzymes that may be needed when immobilising the isolated enzyme should already be present. Although this method of immobilisation has gained popularity in recent years, with uses in analytical and biochemistry, the major limitations that need to be addressed whilst using such cells are diffusion of substrate and product through the cell wall, unwanted side reactions due to the presence of other enzymes in the cell and cost. One theoretical advantage in the use of the immobilised purified enzyme as opposed to the immobilised cell is that it is possible to obtain higher loadings of catalyst.

A large number of techniques (Yahia *et al*., 1998; Maury *et al*., 2005) are available for the immobilisation of enzymes on a variety of natural and synthetic supports, many of which have been reviewed by Kolot (1981). The choice of support used in the immobilisation depends upon the nature of the enzyme, substrate and ultimate application. Care has to be taken when selecting the support materials as well as the reagents used for immobilisation, particularly when their ultimate applications are in the food and pharmaceutical industries. An important consideration when undertaking the immobilisation of enzymes is the physical properties of the support, such as their flow properties, form, shape, density, porosity, pore size distribution, operational stability, particle size distribution and maximum enzyme loading. Other factors including cost, toxicity and availability are discussed by Gekas and Lopeiz-Leiva (1985). In particular, the average pore diameter and surface area can have a significant effect on the final properties of the immobilised enzyme. Therefore, it is essential to determine the optimum characteristics for the enzyme and application under investigation. Overall, matrices must provide facile, secure immobilisation with good interaction with substrates that conform in shape, size and density to the use for which they are intended. The manufacture of high quality products on a low scale may allow the use of relatively expensive supports and immobilisation techniques, whereas these would not be economical in the large scale production of low added value materials. A substantial saving in costs occurs where the carrier may be regenerated after the useful lifetime of the immobilised enzyme. The ideal support is cheap, inert, physically strong and stable. Clearly most supports possess only some of these features but a thorough understanding of the properties of the immobilised enzyme does allow suitable engineering of the system to approach the optimal qualities. The carriers utilised for immobilisation may be broadly classified into two categories; inorganic and organic. Inorganic carriers include kaolinite, colloidal silica (Talon *et al*., 1996), glass particles, controlled pore glass (CPG), alumina, controlled pore alumina (CPA), controlled pore titania (CPT), nickel oxide, controlled pore zirconia (CPZ), zirconia coated CPG, carbon, hydroxyapatite, and iron oxide. Some organic supports are cellulose, agarose, starch, polyacrylamides, dextran, nylon, collagen and organic co-polymers (Queiroz & Nascimento, 2002).

The choice of immobilisation method is based upon factors such as substrate molecular weight and size, stability of the enzyme molecule and the requirement for

a specific physical form of the immobilised enzyme or support material. Although to some extent the choice of immobilisation method is governed by such factors, some will not be apparent until the technique is tried, therefore there is little alternative to the screening principle. Following immobilisation, the enzyme must be stable under the required conditions and the active site should be protected in some way. Techniques for immobilisation have been broadly classified into four categories, depending upon the physical relationship of the enzyme catalyst to the matrix; adsorption and covalent binding to a prefabricated support, cross-linking of enzyme molecules and entrapment in organic or inorganic polymer matrices. However, it is usually the case that which ever immobilisation technique is chosen the result is likely to be a combination of the methods. For example, during the process of cross-linking within a carrier it is rather obvious that some adsorption to the surface of the carrier occurs and likewise for the covalent attachment of an enzyme to a carrier it is probable that the enzyme is also adsorbed onto the carrier surface. Therefore, when a particular bonding technique is described in this review, it merely implies that the proposed attachment method is the predominant type of bonding.

1.2.1.1 ADSORPTION

Physical adsorption of an enzyme onto an insoluble, prefabricated carrier, without covalent binding, is the oldest commercial application for the immobilisation of an enzyme. The process is a very simple, easy to perform, economical procedure for the immobilisation of enzymes, with wide applicability and capabilities of high enzyme loading, whilst not grossly altering the biocatalysts activity under the correct conditions. In practice, the enzyme is simply mixed with a suitable adsorbent under appropriate conditions of pH and ionic strength for a sufficient incubation period. After which, the loosely bound and unbound enzyme is washed off to produce the immobilised enzyme in a directly usable form. The particular choice of adsorbent, a variety of which are available, depends principally upon minimising leakage of the enzyme during use. Although the physical links between the enzyme molecules and support are often very strong, they may be reduced by many factors including the introduction of the substrate. Additional care must be taken so that the binding forces are not weakened during use by inappropriate changes in pH or ionic strength.

Suitable adsorbents include ion exchange matrices, porous carbon, clays, hydrous metal oxides, glasses and polymeric aromatic resins. Stability of the adsorbed enzyme derivative will depend on the strength of the non-covalent bond formed between the support and the amino acid residues on the surface of the protein. Therefore adsorption is not permanent like covalent bonding. The driving force causing this bonding is usually a combination of hydrophobic effects and the formation of several salt links per enzyme molecule. The binding forces may be ionic, hydrogen bonds or van der Waals' interactions. The number and nature of bonds formed are controlled by the iso-electric point of the protein and the pH at which immobilisation occurs, as both factors determine the respective surface charges. In addition to simplicity, wide applicability for both whole cells and isolated enzymes and low cost, adsorption has the advantages of mild operating conditions, the availability of a ready prepared polymer material suitable for use in column reactors and the potential for regeneration of the immobilised enzyme or support as the binding is reversible. Additionally, there are minimum limitations for access of the substrate to the bound enzyme whilst causing no harm to the protein itself as chemical attachment might do. There is however, one principal disadvantage associated with adsorption as a method of immobilisation. That is, the reversible nature of the interactions between support and enzyme which are much weaker than in the case of the covalent binding. This can result in the adsorbed enzymes easily being desorbed from the support surface if there are changes in temperature, pH, ionic strength or even in the presence of substrate, maybe at a critical time. Also, the amount of enzyme bound per unit of adsorbent is often low so the enzyme is often partially or totally inactivated. A further disadvantage to mention is non-specific further adsorption of other proteins or other substances that may take place and subsequently alter the properties of the immobilised enzyme or, if the substance adsorbed is a substrate for the enzyme, then the rate will probably decrease depending on the surface mobility of the enzyme and substrate. Although this method is usually only used for scientific studies or for so called 'disposable' enzymes, adsorbed enzymes have been very useful in several areas of biotechnology and are still considerably more stable than an enzyme in solution (Bickerstaff, Methods in Biotechnology).

1.2.1.2 COVALENT BINDING

Covalent binding is the covalent chemical attachment of an enzyme to a water insoluble supporting material through the activated groups of the support and some external functional groups of the protein. Attachment of an enzyme to a carrier through covalent binding is probably the most extensively studied and used method of immobilisation. Immobilisation of an enzyme *via* covalent binding generally involves two steps; activation of the support and coupling of the enzyme to the activated support. In order to make the functional groups on the chosen support strongly electrophilic, which is necessary for the second step to take place, the functional group of the support must be activated with a specific reagent. As the activated supports have unstable and reactive functional groups, immobilisation of the enzyme should follow immediately after activation, unless steps are taken to preserve the activated support. The activation process can be carried out using a number of reagents. Trichlorotriazine (TCT) gives excellent results in the immobilisation of monomeric proteins and glycoproteins. Glycidol is recommended if the enzyme is very sensitive to hydrochloric acid, liberated during immobilisation using TCT, but not if the enzyme has catalytic functional groups that are sensitive to the redox reagents used in this methodology ($NaIO_4$ and $NaBH_4$). Trialkyloxysilanes such as the most popular activator, 3-aminopropyltriethoxysilane (APTS) allow even apparently inert materials such as glass to be coupled to enzymes. Highly activated commercial supports such as epoxy-activated Eupergit® or Sepabeads may also be used.

The second step is the coupling reaction where the external functional nucleophilic groups of the enzyme which are not essential for catalytic activity are covalently attached to the activated support material *via* a chemical reaction. Usually, this step is carried out using a bi-functional agent as a bridge between the enzyme and support. The range of chemical coupling procedures used is enormous, however the selection of methodology is dependant on the nature of the support and enzyme and it is important to study the active sites of the enzyme prior to choosing a coupling method as the immobilisation method must not involve any group at that point and bonding must take place through other groups in the enzyme, such as hydroxyl, phenolic,

thiol, carboxyl or amino groups. For example, lysines have been found to be the most generally useful groups for covalent bonding of enzymes to insoluble supports due to their widespread surface exposure, high reactivity, especially in slightly alkaline solutions and the fact that they appear to be only very rarely involved in the active sites of enzymes. Alternatively, these active sites can be protected during attachment as long as the protecting groups can be removed without loss of enzyme activity. Coupling reactions involved in covalent immobilisation fall into the following categories (Cabral & Kennedy, 1991); diazotisation, peptide bond formations, alkylation or arylation, Schiff base formations, Ugi reactions, amidation reactions, thiol-disulphide interchange reactions, mercury-enzyme interactions and γ-irradiation induced coupling. Covalent binding is very strong with minimal leakage of enzyme from the support. Theoretically, this method of immobilisation should not alter the enzymes conformational flexibility and yield stable, insolubilised enzyme derivatives that maintain activity for several cycles without enzyme desorption. Disadvantages however, are that the covalent coupling of an enzyme can produce a considerable loss of activity due to the influence of the coupling conditions and to conformational changes in the enzyme structure. Also, irreversible binding of the enzyme to the carrier during covalent coupling does not allow for the recovery of the carrier from the carrier-enzyme complex and extensive, difficult and tedious preparations are often involved with high associated costs. Covalent binding is only applicable to a handful of enzymes with only small amounts of enzyme being immobilised by this technique. In addition, immobilisation through covalent binding does not offer microbial protection and it is not a good technique for the immobilisation of whole cells. Covalent binding to inorganic supports has been extensively investigated by, amongst others (Husain & Jafri, 1995), Moreno and Sinisterra (1994), who studied the covalent immobilisation of *C. rugosa*.

1.2.1.3 CROSS-LINKING

Intermolecular cross-linking of an enzyme is essentially an extension of covalent bonding techniques. To achieve immobilisation, the enzyme forms links to an insoluble, solid support matrix or itself, using homo- and hetero-bifunctional cross linking agents such as glutaraldehyde. There are two main types of cross-linked

enzymes available; cross linked enzyme crystals (CLEC) and cross-linked enzyme aggregates (CLEA). CLECs arise when the enzyme has been crystallised prior to the cross-linking process, thereby stabilising the crystalline lattice and its constituent enzyme molecules, resulting in highly concentrated immobilised enzyme particles with improved resilience to organic solvents that can be lyophilised and stored indefinitely at room temperature. Chemical cross-linking of enzyme crystals was first developed in the 1960's by Quiocho and Richards (1964), to stabilise enzyme crystals in order to facilitate the manipulation and the collection of crystallographic data by X-ray crystallography. Soon after however, it was recognised by the same authors that cross-linked enzyme crystals were catalytically active (1966). In practice, the enzyme is crystallised before being chemically cross-linked using a bifunctional reagent such as dialdehydes like glutaraldehyde or diamines activated with carbodiimide to 'lock' the crystalline state, obtaining an insoluble active protein matrix in the form of homogenous enzyme crystals. CLECs are an active, insoluble, mechanically robust and microporous protein matrix that retain a very high catalytic activity and stability even under harsh conditions of temperature and pH and exposure to organic or aqueous solvents (Goncalves *et al*., 1996). In fact, the level of activity of CLECs is far greater than that of conventionally immobilised proteins as the final preparation is basically pure protein with a high concentration of enzyme per unit volume. Immobilisation of enzymes *via* cross-linking is a particularly attractive, relatively cheap immobilisation alternative. However, the actual development of an adequate crystallisation protocol is expensive and time consuming and often yields very little bulk of immobilised enzyme with a very high intrinsic activity. Therefore, the development of CLECs or more generally, cross-linked protein crystals (CLPCs) is still very much under way and the number of commercial suppliers of this type of enzyme is limited. The cross-linking of enzyme aggregates developed by López-Serrano *et al.* (2002) is basically the covalent cross-linking of a precipitated enzyme without the need for crystallisation, based on the amply demonstrated notion that enzymes and proteins in general can be precipitated by agents such as inorganic salts or organic solvents without undergoing denaturation. The resulting CLEAs tend to have high enzymatic activity but lower stability than the CLECs.

1.2.1.4 ENTRAPMENT

The immobilisation of a biocatalyst *via* entrapment within gels, membranes or fibres (1981) to generate hydro-gels or water-soluble polymers, is in principle, an easy to perform process. The enzyme or whole cell becomes confined within a matrix by dissolving the biocatalyst in a solution of the chemicals required for the synthesis of the enzyme phase and then treating this so that a distinct phase is formed. The process may be purely physical caging or involve covalent binding, resulting in a system where in theory the enzyme cannot escape, whilst substrates and products have the ability to diffuse in and out. The costs associated with entrapment are usually quite low, the method is applicable to a wide variety of enzymes, is simple and requires mild conditions whilst providing microbial protection. The method is desirable as a high enzyme loading is usually achieved with high activity, whereas *via* chemical immobilisation, activity of the enzyme is often lost during the immobilisation procedure. Despite these advantages, entrapment, although used extensively for the immobilisation of whole cells, is not often used for the insolubilisation of isolated enzymes due to the major limitation of possible slow leakage during continuous use, in view of the small molecular size compared to the cells. This problem would result in activity losses as well as contamination of the product with the enzymes. The entrapment of enzymes is a convenient method for use in processes involving low molecular weight substrates and products. However, the difficulty which large molecules have in approaching the catalytic sites of the entrapped enzymes precludes the use of entrapped enzymes with high molecular weight substrates. Additionally, entrapment can involve difficult preparation, results in a weak binding force and is thus associated with high running problems, such as cost.

There are a number of materials that can be utilised in the entrapment procedure, the choice of which is usually determined by the size of the enzyme. Polymer entrapment is the inclusion of a biocatalyst in the lattices of a polymerised gel in order to achieve immobilisation, allowing the free diffusion of low molecular weight substrates and reaction products. In practice, the biocatalyst is dissolved in a solution of the polymer precursors, after which polymerisation is initiated. The polymeric

mass is then broken up to the desired particle size, whilst the enzyme remains 'trapped' in the gel, 'net-like' matrix, which in theory, has pores that are large enough to permit entry of low molecular weight substrates but too small to allow the escape of the enzyme. A wide variety of natural polymers like agar, agarose and gelatine have been used for polymer entrapment, as well as a number of synthetic polymers, such as the photo-crosslinkable resins, polyurethane prepolymers and acrylic polymers like polyacrylamide (d'Souza & Nadkarni, 1980). However, the broad distribution in pore size of synthetic gels of the polyacrylamide type inevitably results in leakage of the entrapped enzyme, even after prolonged washing. This may be overcome by cross-linking the entrapped protein with glutaraldehyde (**Section 1.2.1.3**). Sol-gel matrices, a colloidal suspension of solid particles (inorganic silica glass in a liquid), are also often used for the entrapment process. In the sol-gel system, metal alkoxide precursors are used for the preparation of a colloid containing a metal or metalloid element surrounded by various ligands. The sol-gel is formed by a hydrolysis or condensation reaction, where if the enzymes or cells are introduced whilst the condensations are taking place, they become entrapped in the matrix, as it is polymerising. The most widely used example is silicon tetraethoxide (TEOS). Amorphous gels are cast from the silicate solution produced by the hydrolysis of the TEOS, then after the gel has dried over a sufficiently long period of time an optically transparent glassy material is produced. Characteristics making this substrate attractive are the possibility of room temperature or even lower processing conditions, chemical inertness, no swelling and tenable porosity. This immobilisation procedure in which enzymes are incorporated in hydrophobic organic or inorganic hybrid materials with the aid of a sol-gel process is extending rapidly. Over the past few years, these materials have been investigated for the immobilisation of biomolecules by a variety of procedures such as physiosorption, micro encapsulation and sandwich configurations. Narang *et al*. (1994) have described one protocol for sol-gel preparation. Membrane confinement of enzymes may be achieved by a number of quite different methods, all of which depend on the semi permeable nature of the membrane. The simplest of these methods is achieved by placing the enzyme on one side of the semi permeable membrane whilst the reactant and product stream is present on the other side. Hollow fibre membrane units are available commercially with large surface areas relative to their contained volumes and permeable only to substances of molecular weight substantially less

than the enzymes. Although costly, these are very easy to use for a wide variety of enzymes without the additional research and development costs associated with other immobilisation methods. Compared to other entrapment techniques, this method has minimal enzyme leakage, very wide applicability, no matrix effects, large diffusional barriers and microbial protection. He *et al.* (2005) used a lipase immobilised fabric membrane to synthesise 2-ethylhexyl palmitate and found the membrane reactor allowed for reuse of up to twenty one batches with high percentage ester conversion.

1.2.2 ENZYME REACTORS

An enzyme reactor usually consists of a vessel or series of vessels lined with a support. The purpose of the enzyme reactor is to allow the enzyme and substrate to come into contact for a sufficient period of time to perform the desired conversion *via* enzymatic means. After which, one needs to be able to easily separate the product and enzyme. Several configurations of enzyme reactors exist, such as the packed bed, stirred, tubular and membrane reactor systems that can be sub-divided further into batch and continuous set ups.

Prior to the choice of reactor to be used for a particular process being decided, several important factors must be thought through. One of the most important considerations is of course costs, inclusive of labour, processing, overheads, process development, fabrication and operation of the reactor. The enzyme type to be used (*i.e.* free or immobilised), physical and chemical properties of the immobilised support such as density, compressibility, robustness, particle size and regenerability must also be considered. Other factors including the scale of operation, the need for pH and temperature control, the requirement for the supply and removal of gases and or enzyme and substrate and product stability must also be taken into account before choosing a reactor set up.

1.2.2.1 BATCH REACTORS

Batch reactors, which can be of the stirred tank or batch membrane variety, can be used with either a free or immobilised enzyme and are generally large stirred tanks

consisting of enzyme and substrate. The reaction is allowed to proceed to completion before the reactor is drained and the product separated from the enzyme. If a soluble enzyme has been used it is usually removed by denaturation, which is economically feasible so long as the enzyme is cheap. However, if an expensive enzyme is required then they are first immobilised (**Section 1.2.1**) and following the reaction, the immobilised enzyme is recovered by centrifugation or filtration. However, as the immobilised enzyme may be destroyed by the recovery process batch reactors are often only used with cheap, soluble enzymes and their use with immobilised enzymes has limited potential. Also, as the batch reactor is emptied and refilled regularly this arrangement accumulates higher operating costs than a continuous process making the reactor unproductive with uneven demands on labour and services. In addition, the reactor suffers from batch to batch variations as the conditions are constantly changing with time. Despite these disadvantages, the batch reactor is cheap in comparison to other reactor types and maintains ease and simplicity with respect to usage and process development. Likewise, the reactor has good control and as the reactions carried out are slow, the compositions of products can be accurately monitored, whilst conditions can be varied throughout the reaction.

1.2.2.2 CONTINUOUS FLOW REACTORS

The principle of a continuous flow reactor is the constant addition of substrate to the reactor in conjunction with continual removal of the product. Continuous reactors are easy to make, low cost, effective mixers that have ease of access which allows parameters such as pH to be altered quickly when required, whilst maintaining environmental control. Likewise, enzyme replacement is relatively simple making this type of reactor particularly attractive when the enzyme (usually immobilised) has a short half life. The continuous enzyme reactor has a wide applicability and can therefore be adapted to many different applications. The advantages of immobilised enzymes as processing catalysts are most markedly appreciated in continuous flow reactors where the average residence time of the substrate molecules within the reactor is far shorter than that of the immobilised enzyme catalyst. This results in far greater productivity from a fixed amount of enzyme than is achieved in standard batch processes.

Whilst other forms of continuous reactor do exist, such as the continuous stirred tank reactor (CSTR), it was the packed bed continuous flow reactor (PBR) that was used primarily for the work reported herein and is therefore discussed in further detail. By adopting the continuous reactor into a packed bed, flow design, the mechanical stability of the support used in the enzyme immobilisation is not as important as it will not be subjected to movement throughout the reaction time and the surface to volume ratio is increased. Consisting of a settled bed of immobilised enzyme particles and if required, exterior heating or cooling coils, the packed bed, continuous flow reactor is often used in a 'recycle' mode, where the substrate is passed through the column, over the enzyme and the product is obtained from the outlet. It is one extreme of process kinetics in relation to continuous flow reactors where upon mixing with a turbulent flow regime the enzyme comes into contact with high substrate and low product concentrations, whilst the entire substrate stream flows with good heat transfer, at the same velocity, parallel to the reactor axis with, ideally, no back mixing. All of the material present at any given reactor cross section has had an identical residence time. Hence all substrate molecules have equal opportunity for reaction. In practice, the substrate stream may enter from either the top or the bottom of the column, which may be constructed as a tall column or a flat bed. As an alternative to using columns packed with immobilised enzyme particles, the enzyme may be immobilised to a membrane or sheet of material and confined in a filter press arrangement. Whilst PBRs are the preferred reactors for processes involving product inhibition, substrate activation and reaction reversibility, the enzyme reactor is not compatible with free enzymes. Instead, PBRs are generally used with fairly rigid immobilised enzyme catalysts as an excessive increase in flow rate often experienced in PBRs may distort compressible or physically weak particles. Such particle deformation can result in reduced catalytic surface area of the particles contacting the substrate containing solution, poor external mass transfer characteristics and a restriction to the flow. A vicious circle of increased back pressure, particle deformation and restricted flow may eventually result in no flow at all through the PBR. Other disadvantages include expense in fabrication, possible clogging and fouling, difficulty in commissioning on plant scale, possible bacterial contamination, no pH control by the addition of acids or bases and poor temperature control. Access to the reactor is limited so other operational problems also exist, for example, this type of reactor is relatively difficult to recharge with enzyme so is not

ideally suited for use with enzymes with a short operational half life. Despite this bundle of disadvantages, the PBR is capable of high substrate conversions, even in the presence of product inhibition, using less immobilised enzyme than a tank reactor. The reactor also has relative ease of automation, which is a major cost benefit, and once operational the reactor will virtually look after itself. It is many of these disadvantages that this work, utilising a miniaturised version of this reactor type, hopes to overcome.

1.3 MICROREACTORS

Microreactors (Ehrfeld *et al*., 2000) are generally defined as miniaturised reaction systems that have internal structure dimensions in the millimetre to sub millimetre range and are fabricated by using, at least partially, methods of precision technology. The term "microreactor" is the proposed name for a wide range of devices that simply have small dimensions and it is not the norm to further categorise such systems, into nano or minireactors for example.

The cleanliness and efficiency of synthetic processes have become of particular interest and significance to the chemical industry over the past decade as environmental issues have gained importance. In particular, pharmaceutical companies often have to synthesise and screen novel chemical entities representative of the universe of drug like compounds which may be in the order of 10^{200} compounds (Watts & Haswell, 2005). Additionally, as this is an industry where costs for starting materials are extremely high there is a pressure to shorten optimisation cycle times, handling times to assay and reagent costs. Miniaturised reactor chemistry is showing great promise as an efficient method to achieve these aims allowing for a faster transfer of results of development work into production, earlier start of production at lower costs, lower costs with respect to materials, energy and transportation, and a more flexible response to market demands with high throughput. Similarly, miniaturisation provides scope for new chemical processes in which the reactions generally produce the desired product in higher yield over shorter periods of time due to an increase in surface to volume ratio, whilst increasing our knowledge of complex chemical processes (Watts & Haswell, 2005). Importantly, sufficient quantities can still be generated to perform all structural characterisation (Watts & Haswell, 2005) whilst producing minimal waste. There is also improved process safety associated with the use of microreactor systems as any hazardous material can be synthesised as required and at the point of use in precisely defined quantities. Shorter residence times result in there being localised control over reagents and products compared to traditional batch scale reactions. Finally, when larger amounts of a product are needed, miniaturised reaction systems can be 'numbered-up' as oppose to 'scaled-up' like on a plant. This was shown by Fletcher

and Haswell (1999) who proposed that 1000 microreactors operating continuously could produce 1 kg of material in a day. This is somewhat cheaper and easier than the complex, time consuming and cost intensive increase in a plant size.

Consequently, the technique of miniaturisation (Wiles & Watts, 2007) has drawn considerable attention in synthetic chemistry over the past decade with many groups having begun the task of transferring synthetic methodology from batch to miniaturised reactors (Watts & Wiles, 2007). Microfluidic devices were first developed within analytical chemistry to enable the rapid analysis of chemical and biological samples (Manz *et al*., 1991). More recently however, microreactors have been used for fine chemical synthesis (Schwalbe *et al*., 2002) and it is now well established that chemical reactions conducted in such devices produce products in higher yield, purity and selectivity when compared to batch reactors (Wiles & Watts, 2008). Miniaturised reactor set ups do however, suffer from a number of disadvantages. For example, they are prone to fouling and blocking, whilst other problems include the transport of solids, film formation, and surface effects.

For synthetic chemistry carried out in a miniaturised flow system, micro or nanolitre volumes of reagents are brought together, mixed and allowed to react for a specified time in a controlled region of the miniaturised reactor. One of two main pumping techniques are usually used to provide movement of materials within the device; hydrodynamic or electroosmotic flow (Spence & Crouch, 1998). In hydrodynamic or pressure driven flow, the fluid is pumped through the device using syringe or peristaltic pumps. Hydrodynamic flow does not interfere with enzyme actions, is usually sufficient and provides fast optimisation of results if only two solutions are being reacted. It does however, become far more complex to accurately control the fluidics when introducing more than three reagents into the device (Watts & Haswell, 2005). Also, in very small channels the required pressure drop in the channel could be large. Therefore hydrodynamic pumping as a choice of movement through the reactor may become impractical in very small devices (Souders *et al*., 2003). As an alternative, electrokinetic forces, such as EOF can be used. EOF produces a flat velocity profile across the channel. EOF refers to the bulk movement of an aqueous solution past a stationary solid surface due to an externally applied electric field, requiring the existence of a charged double layer at the solid / liquid interface, *via* a

pair of electrodes. When feasible, this method is preferential over hydrodynamic pumping techniques as there are no moving parts involved and the chance of back pressure is dramatically reduced. However, due to its ease of use, definite lack of interference with enzyme activity and the fact that no more than two reagents are necessary for the work presented herein, hydrodynamic pumping was the chosen method of movement of material through the packed bed reactor for the miniaturised flow reactions investigated.

There are two main types of so called microreactors reactor; miniaturised continuous flow system set ups and lab on a chip, discussed in **Sections 1.3.1** and **1.3.2**. For completeness however, it is important to mention that others do exist such as biocapsules (Desai *et al*. 1998) and electrochemical micro systems (Suzuki, 2000).

1.3.1 MINIATURISED CONTINUOUS FLOW REACTORS

Miniaturised continuous flow reactor set ups are, in principal, a miniaturised version of the continuous flow reactor (**Section 1.2.2.2**). Structurally, the reactor portion is typically a small glass capillary in varying length (*ca.* 30mm) and diameter (~0.5-1.5mm). The capillary is usually packed with the immobilised enzyme or chemical, connected to some form of mixing device (hydrodynamic or EOF) and a product collection point is situated at the opposite end (Wiles *et al*. 2006). To date, the majority of work carried out in miniaturised flow reactors is associated with synthesis using immobilised organic catalysts as opposed to enzyme catalysed reactions. Most commonly the immobilised reagent is packed into the reaction channel. Chemical reagents may be supported by attachment to beads (Seong *et al*., 2003), incorporating membranes into chips, creating supports using micro fabrication or fabricating gel, polymer and silica (Kawakami *et al*., 2005) monoliths within micro fluidic channels (Peterson, 2005). Wiles *et al.* (2004) investigated the use of silica and polymer supported bases within a flow reactor and successfully synthesised eight condensation products with high yields (>99%) and product purity (>99%).

1.3.2 LAB ON A CHIP

When in the form of a chip, the microreactor device still operates on the continuous reactor principle and is fabricated by etching of the channels which are typically in a 'T' shape with three reservoirs; A, B and C. Reservoirs A and B would normally hold the substrates and reservoir C is where the product would be collected. In the case of using immobilised catalysts, the channel in the middle of the chip would be wider and the immobilised enzyme or chemical would be 'loaded' into this if possible. The design of microreactor chips can be altered accordingly depending upon the reaction to be carried out. For example, more or less reservoirs can be added and the reactor portion can be made longer, to provide longer reaction times, by implementing a 'serpentine' type channel. The substrate used for constructing a lab on a chip set up must allow for the mobilisation of organic reagents and aqueous solutions using pumping mechanisms such as hydrodynamic and EOF (Spence & Crouch, 1998). A number of materials such as silicon, quartz, glass, metals and polymers have been used to construct so called microreactors using a range of fabrication methods (Ehrfeld *et al*., 2000) such as hot embossing, wet etching (James *et al*., 1993), dry etching (Bhardwaj & Ashraf, 1995), photolithography (Backhouse *et al*., 1999), powder blasting, injection micro moulding (Weber & Ehrfeld, 1998), bulk micro machining (James *et al*., 1993) and laser micro forming (Zissi *et al*., 1996). Photolithography, using glass because of its physical properties, such as being mechanically strong and optically transparent and its chemical inertness is the most common microreactor fabrication technique used today. Despite the many techniques available, the final choice is not simple and certain factors such as process costs and time, accuracy, reliability, material choice and accessibility have to be considered when choosing a fabrication technique for lab on a chip, which may in some cases, be a combination of two or more (Arnold *et al*., 1995).

1.3.3 APPLICATIONS OF ENZYMES IN MICROREACTORS

There are two main disadvantages associated with biocatalysis employing immobilised enzymes in a traditional batch reactor. Firstly, is the enzyme being subjected to mechanical forces which may result in denaturation of the biocatalyst

due to poor operational stability and secondly, is the high cost of many enzymes, which are often only available in small amounts. These pitfalls can be overcome to some extent by using a miniaturised set up. The use of an enzymatic microreactor is advantageous for biocatalytic reactions using expensive enzymes, allowing high throughput and 'on site' production of high value products. As a result, in recent years enzymatic microreactors have been developed to have applications in biochemical analysis, kinetic studies and biocatalysis. A review by Urban *et al*. (2006) summarises recent work in the field of enzymatic microreactors, including the use of a homemade microreactor incorporating trypsin immobilised onto controlled pore glass, for the analysis of proteins (Bonneil & Waldron, 2005).

For its successful use within a microreactor, the enzyme first needs to be immobilised (**Section 1.2.1**). However, a low expenditure of enzyme is often a result of its immobilisation and the range of immobilised enzymes available with satisfactory characteristics is still limited (Buchholz *et al*., 2005). There have however, been many useful approaches for incorporating immobilised enzymes within miniaturised devices (Miyazaki & Maeda, 2006). The initial method was to immobilise the enzyme onto silica or polymer microparticles which are then packed into the appropriate reaction channel of the microreactor (Seong & Crooks, 2002; Seong *et al*., 2003). Andersson *et al*. (2000) and Wang *et al*. (2000) packed beads (40-50μm) with immobilised trypsin into a chamber on a chip for on chip protein digestion. However, the packing of particles into the channel of a microchip is not facile and capacity is often low. Grinding of the enzyme to decrease the particle size may affect the enzymes activity, slurry packing is often very laborious and time consuming and, as they cannot be exposed to the high temperatures necessary for fabrication, packing prior to the bonding of the top plate at extreme temperatures would more than likely to deactivate the enzyme before any reactions have even begun. The small dimensions within a microreactor may also cause several other problems. For example, favoured supports for enzyme immobilisation such as Sepharose can swell and cause large back pressures meaning that silica based substrates are preferred. Immobilising enzymes onto the inner wall of the miniaturised reactor has been investigated as an alternative method (Kaneno *et al*., 2003). Whilst this approach overcomes the issues associated with high back pressures, enzyme loading is often found to be lower (Hisamoto *et al*., 2003; Holden *et al*., 2004). Incorporating membranes into micro chips has also been studied

with nylon membranes formed at liquid/liquid interfaces. However this system is difficult to achieve and is not suitable if organic solvents are utilised. Similarly, Honda *et al*. (Honda et al., 2007) attempted to form the membrane at the micro channel wall in a modified process of forming cross linked enzyme aggregates. Monoliths are well-ordered macroporous materials with low flow resistance, high reaction efficiency and good flow-through properties. Monoliths also have the ability to be easily formed by *in situ* polymerisation, making them ideal for use as components in micro reaction systems (Peterson, 2005; Kawakami *et al*., 2005). The application of monoliths as a form of enzyme immobilisation within microreactors has proved to be successful for glucose oxidase (Wilson *et al*., 2000) and a protease (Kawakami *et al*., 2005). Whilst monoliths appear to be the most feasible and successful method of incorporating an immobilised enzyme into a microreactor, further development to achieve a monolith with the required properties is required to eliminate disadvantages such as back pressure. It is for these reasons, in addition to a higher obtainable throughput with a continuous flow reactor, that the miniaturised packed bed, continuous flow reactor, incorporating the immobilised enzyme required (up to 630μm) into a glass capillary (30 x 1.65mm) was used for the work presented in this report.

1.4 THESIS AIMS

Conventional chemical processes often require a series of complex chemical synthetic steps that utilise a great deal of energy and raw materials. There is a huge commitment by the chemical industry to search for "green", environmentally friendly processes. Biocatalysis, using enzymes to catalyse a chemical reaction, is emerging as an economical and ecological production route for a wide range of fine chemicals, pharmaceuticals and food ingredients. Lipases have high commercial availability, low costs, broad substrate specificity, selectivity and catalytic versatility under a range of operating conditions. Consequently, lipases have received considerable attention in the field.

In addition to biocatalysis, miniaturised chemistry has also become of interest and significance to the chemical industry over the last decade. Microreactors allow for the synthesis of small volumes of compounds quickly and in high yield with fast optimisation of conditions. Additionally, when used with immobilised biocatalysts, such miniaturised devices would enable the rapid throughput screening of a wide range of enzymes and their substrates. Despite this however, developments of applications in the field of miniaturised enzymatic processes are still in the initial stage. In fact, very few enzymes have been applied within microreactors and there are few published patents describing the construction of enzymatic microreactors (Urban *et al*., 2006). There is a range of immobilised lipases available on the current market and an expanding commercialisation of microsystem components. With this in mind, further development of microreactors and biocatalytic methodologies, with enzymes that are frequently used, is achievable. The work described herein aims to investigate prospective fields for the application of enzymatic microreactors to improve screening, synthetic and combinatorial chemistry.

2 LIPASE CATALYSED ESTERIFICATION REACTIONS

2.1 AIMS OF CHAPTER 2

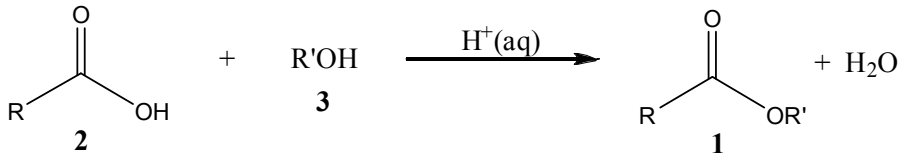
The biocatalytic synthesis of esters has been studied extensively and is well reported in the literature using various lipases in batch reactions (Bezbradica *et al*., 2006). However, disadvantages associated with batch reactions, such as long reaction times (da Silva *et al*., 2005), a high amount of enzyme required, operational instability of the enzyme and water production have resulted in an increasing interest in methodologies to overcome these problems. Miniaturised reactor chemistry allows for small volumes of compounds to be generated quickly and in high yield, with fast optimisation of conditions. Additionally, in terms of biocatalysis, only small amounts of enzyme are necessary in the reactor capillary. Traditional acid catalysed esterification has been previously investigated in a glass microreactor by Brivio *et al* (2003). However, as discussed in **Chapter 1**, studies on enzyme catalysed organic synthesis in a miniaturised reactor are limited.

Chapter 2 therefore, aims to investigate the already well reported area of batch enzymatic esterification, within a miniaturised continuous flow reactor. Whereby, ideally, the disadvantages associated with enzymatic batch syntheses will be overcome whilst achieving good, reproducible percentage ester conversion.

2.2 ESTERIFICATION

Short chain esters are common in organic chemistry and these materials often have a characteristic pleasant, fruity odour. Consequently, esters have notable commercial significance in the fragrance, cosmetics, food (Kumar *et al*., 2005) and pharmaceutical industries. Esterifications are among the simplest and most often performed organic transformations, traditionally produced *via* one of two main routes; by heating to reflux a free acid and hydroxy group in the presence of a catalyst with water as a byproduct (Fischer Esterification), or by transesterification.

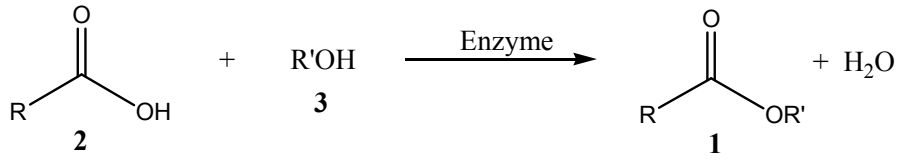
The current commercial manufacture of esters **1** is realised by reacting a carboxylic acid **2** with an alcohol **3** at a high temperature in the presence of an acid catalyst (**Scheme 1**). However, this high temperature acylation process can lead to degradation of the ester, the formation of undesired side products and the energy costs are high. Also, these methods often require complex separation processes which result in lower yields, whilst generating large amounts of acidic aqueous waste.



Scheme 1: Fischer Esterification.

In an attempt to overcome the disadvantages associated with the production of esters by chemical means and with an increasing orientation towards natural production, many companies are looking to switch to biocatalytic methods that offer the advantages of mild operating conditions, high specificity and reduced side reactions (Villeneuve *et al*., 2000). In addition, biocatalytic synthesis should remove the need for expensive separation techniques whilst yielding valuable products, often with a much higher selling price. Consequently, development of the research area of biocatalytic esterification, that is environmentally benign (Aran *et al*., 2000), economically feasible and provides a sustainable production process for complex chemical conversions, has been rapid over the past twenty years (Yahia *et al*., 1998).

There are three major biotechnological methods available to generate such compounds; the use of isolated enzymes, the use of microorganisms and the use of plant cells and culture of tissues (Gatfield *et al*., 1995), however the application of enzymes (**Scheme 2**) is the most common technique.



Scheme 2: Enzyme catalysed esterification.

Lipases, which are considered to be 'natural' by the food legislation agencies (Welsh *et al*., 1990) have been widely investigated for ester synthesis, mainly in organic solvents (Langrand *et al*., 1990), due to their applicability at a wide range of temperatures and pH. There are various sources from which the lipase can be taken for enzymatic synthesis, including *C. antarctica* (Blattner *et al*., 2006), *Mucor miehei* (*M.miehei*) (Zhao *et al*., 2005), *C. rugosa* (Bezbradica *et al*., 2006; Hilal *et al*., 2006), *Rhizopus oryzae* (*R. oryzae*) (Ghamgui *et al*., 2004), *Rhizopus sp*. (Melo *et al*., 2005), *Candida sp*. (Zhao *et al*., 2005), *Pseudomonas sp*. (Crespo *et al*., 2005), porcine pancreas (Zhao *et al*., 2005), *Candida lipolytica* (*C.lipolytica*) (Zhao *et al*., 2005), and *Bacillus sp.* (Dosanjh & Kaur, 2002). However, it is the yeast lipases, specifically *C. antarctica* lipase B (CALB), a serine hydrolase, which are of particular importance for ester synthesis (Bezbradica *et al*., 2006). Many authors have used free lipases in enzymatic esterification (Leszczak *et al*., 1998) but it is the use of immobilised lipases that is gaining importance. As discussed in **Chapter 1**, immobilisation can be carried out by a variety of methods on a wide range of support materials (Warmuth *et al*., 1995) and results in a biocatalyst that in general is more stable than soluble enzymes, is simpler to use in operation and can be recovered and reused, hence reducing associated costs.

Novozyme 435, which can be used for the synthesis of esters irrespective of chain length (Kumar *et al*., 2005), is a non-specific, commercially available, immobilised form of lipase B from *C. antarctica* stabilised onto a macro porous acrylic resin. This interesting lipase has potential for application in a number of industrial

processes, such as the synthesis of esters in the flavour industry (Yadav & Lathi, 2003) and is commonly used due to its wide availability, applicability and heat tolerance to extreme temperatures (Vitro *et al*., 2000).

2.3 RESULTS & DISCUSSION

2.3.1 PREPARATION OF ESTER STANDARDS

Most of the esters studied (**Figure 1**) were commercially available and so represented synthetic targets for the enzyme catalysed reactions in batch and in the miniaturised continuous flow reactor. However, methyl octanoate **4** and butyl octanoate **5** were not, therefore batch, conventional chemical reactions were carried out to obtain these esters.

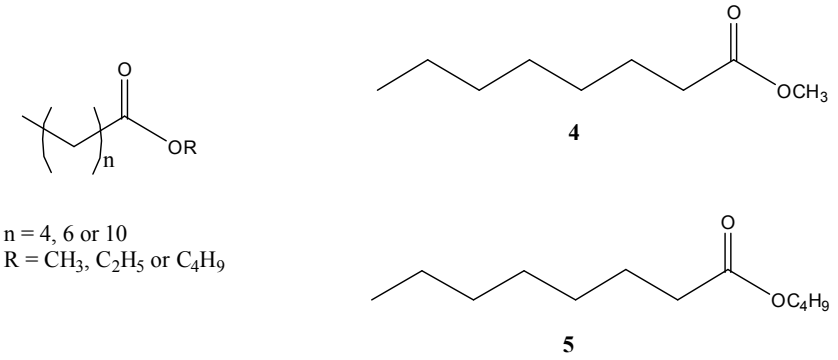


Figure 1: Ester standards, methyl octanoate **4** and butyl octanoate **5**.

GC-MS was used as a method of analysis for the ester standards, the results of which can be seen in **Table 1**.

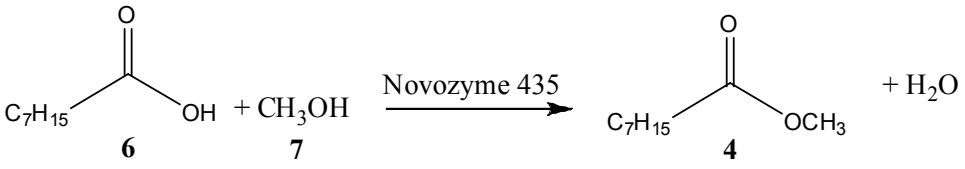
Compound	Approximate Retention Time / Min	M^++1 Value
Methyl octanote	8.0	159
Butyl octanote	9.4	201

Table 1: GC-MS retention times and M^+ +1 values for in-house prepared ester standards.

2.3.2 BATCH ENZYMATIC ESTERIFICATION REACTIONS

In order to benchmark the efficiency of the flow reactors, a series of batch reactions were carried out, studying the ability of Novozyme 435 to catalyse the synthesis of methyl octanoate **4** from commercially available octanoic acid **6** and methanol **7**

according to **Scheme 3**. Parameters considered, based upon previous studies to optimise enzymatic synthesis were temperature (Bezbradica *et al*., 2006), reaction media, acid: alcohol molar ratio (Melo *et al*., 2005) and enzyme concentration.



6 **7** **4**

Scheme 3: Enzymatic preparation of methyl octanoate **4** using Novozyme 435.

The impact of temperature on the reaction was studied at 22, 37, and 70 ^{o}C. Room temperature because this is the ideal from an environmental and ease of reactor set up perspective, 37 ^{o}C because this is the optimum operating temperature of free *C. antarctica* lipase, and 70 ^{o}C as this is suggested by the commercial provider of Novozyme 435 (an immobilised form of *C. antarctica* lipase) to be the operating temperature (Romero *et al*., 2005). There was no significant difference in the percentage ester conversion of octanoic acid **6** and methanol **7** to methyl octanoate **4** over a 24 hr period, at the three temperatures studied. Percentage ester conversions of 92, 93 and 92% were seen at 22, 37, and 70 ^{o}C respectively. Consequently, all subsequent experiments were conducted at room temperature for simplicity and in order to minimise the energy requirements. The lack of differences in the results confirms what is discussed in the literature, that Novozyme 435 is tolerant and remains active at a wide range of temperatures.

Synthetic reactions catalysed by lipases may be performed in aqueous media, organic solvents, in supercritical fluids (Lozano *et al*., 2004), in ionic liquids (Lozano *et al*., 2001) or in an organic media. However, the choice of solvent used, if any, is highly dependant upon the nature of the reaction being carried out. Esterification reactions, using hydrolytic enzymes are commonly performed in a suitable non-polar organic solvent (Zhao *et al*., 2005), such as hexane, heptane, isooctane and cyclohexane. However, when choosing a solvent for the lipase catalysed esterification reactions, the selection becomes more limited. A suitable organic solvent should dissolve the substrates for lipase catalysed esterification whilst not affecting the lipase activity and stability (Zhao *et al*., 2005). Esters and alcohols cannot be used as they act as

substrates and ethers are undesirable because of peroxide formation (Wehtje *et al*., 1997). Solvent free systems (Bucala *et al*., 2005; Foresti & Ferreira, 2005) are highly concentrated media which are economically and operationally interesting for industrial processes because of considerable simplification of downstream processing and a reduced environmental hazard (Ghamgui *et al*., 2004). However, a solvent was needed in this study in order to dissolve any solid starting material. Although hexanoic and octanoic **6** acids were liquids, a solvent was used for all experiments conducted to maintain continuity. The effect of the choice of reaction media was studied for the enzymatic esterification of octanoic acid **6** and methanol **7** at room temperature, using Novozyme 435 with the solvents hexane, toluene, dichloromethane and acetonitrile, to represent a range of polarities.

As the polarity of the solvent decreases, the percentage ester conversion of methyl octanoate **4** after 24 hr at room temperature was seen to increase. In ACN, only a 77% conversion of acid to ester was seen after 24 hr compared to 92% in the non-polar hexane. These results are consistent with others in the literature (Voutsas *et al*., 2002) including those by da Silva *et al.* (2005) who found that for the enzymatic batch esterification of pentyl laurate, hexane was the best solvent giving almost 100% ester conversion after 192 hr and 0% in the extremely polar acetone. Likewise, using immobilised *R. oryzae* lipase, Ghamgui *et al.* (2004) obtained their highest percentage ester conversion of 73% for 1-butyl oleate in the presence of hexane with substrate concentrations of 0.1M and Whetje *et al*. (1997) found that the product yield is dependant on the solvent used when they carried out a lipase catalysed esterification and achieved 95% yield in hexane and 64% yield in 5-methyl-2-hexanone.

It is well known that acid: alcohol ratio is one of the most important parameters in enzymatic esterification. Since the reaction is reversible an increase in the amount of one of the reactants will result in higher ester yields and as expected, this will shift the chemical equilibrium to the product. One way of shifting the reaction towards synthesis is to increase the alcohol concentration (Dormo *et al*., 2004). However 1:1 acid: alcohol is ideal, especially in the miniaturised continuous flow reactor, as a work up step, involving the removal of excess alcohol, is then avoided. Kinetic studies have shown that ester synthesis catalysed by *C. antarctica* B lipase is

inhibited by an excess of alcohol (Blattner *et al*., 2006), therefore the ideal ratio of 1:1 for acid: alcohol and 1:40 (used as a model excess amount) were studied to determine if this is the case. Various papers have investigated the effect of the molar ratio in more detail (Ghamgui *et al*., 2004; Dormo *et al*., 2004). In the batch lipase catalysed esterification reaction presented here, where Novozyme 435 catalysed the esterifcation of octanoic acid **6** with methanol **7** to yield methyl octanoate **4**, an excess of alcohol resulted in a maximum percentage esterification of only 15% compared to 92% when stoichiometric quantities of substrates were used. These results were in agreement with those in the literature where when the molar substrate ratio is considered with respect to overall conversion, a high excess of alcohol drastically reduces the yield of esters (Divakar *et al*., 2001). For example, Bezbradica *et al.* (2006) found that in the synthesis of amyl isobutyrate by the lipase from *C. rugosa* the nucleophilic substrate amyl alcohol acted as an inhibitor and Ghamgui *et al.* (2004) found an acid: alcohol ratio of 1:1 was the optimum for 1-butyl oleate synthesis with immobilised lipase from *R. oryzae*. The reason for this is thought to be due to an inhibitory effect of the alcohol where the mechanism of inhibition includes the formation of a non-reactive, dead end complex between the enzyme and alcohol, thereby decreasing the esterification rates (Yadav *et al*., 2004). Alternatively, it may be that an excess of a polar alcohol, in this case methanol, may have a dehydrating effect on the enzyme and thus reduced its activity. Consequently an acid: alcohol ratio of 1:1 was used for all subsequent experiments. Although this study does not identify if 1:1 is better than say 1:2 or 1:3, it does confirm that an excess of alcohol in the esterification reaction is problematic and that high conversions of acid to ester can be achieved with an acid: alcohol ratio of 1:1. Various papers have investigated the effect of the molar ratio in more detail (Dormo *et al*., 2004; Ghamgui *et al*., 2004).

From an economical point of view, achieving high esterification utilising low enzyme concentration is important and a number of authors have studied the effect of enzyme concentration on enzyme catalysed esterifications. Melo *et al.* (2005) found that the optimum value for enzyme concentration (wt/wt acid) was *ca.* 6% when using free and immobilised lipase from *Rhizopus sp*. Similarly, Garcia *et al*. (1999) studied catalyst concentrations between *ca.* 2 and 7% whilst optimising the lipase catalysed synthesis of 3-hexen-1-yl acetate by direct esterification in hexane and a

solvent free medium. The impact of enzyme/substrate ratio on the initial reaction rate for the biocatalytic synthesis of methyl octanoate **4** was studied, with concentrations of Novozyme 435 from 0.25–7% (wt/wt acid), at room temperature with an acid: alcohol ratio of 1:1.

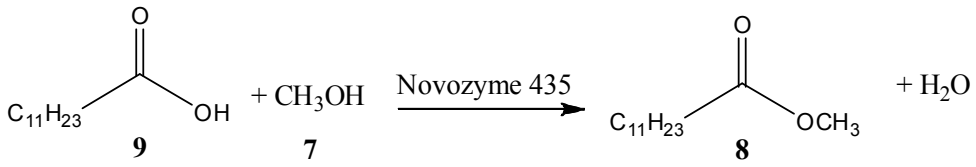
The results indicated that there is a definite correlation between the concentration of biocatalyst used and the conversion of acid to ester after 24 hr, where the amount of enzyme is likely to be the rate determining factor up to 1% (wt/wt acid). A percentage ester conversion of 70% was seen with 0.25% (wt/wt acid) of enzyme, which increased to 91% when 1% (wt/wt acid) of Novozyme 435 was used in the batch reaction. However, with an amount of enzyme between 1 and 7% the differences became insignificant (<3%) and any further increase in the amount of enzyme did not appear to result in a significant increase in the percentage ester conversion. This is likely to be because the substrate concentration has become a limiting factor in the face of an excess of active enzyme sites, as found by Mutua and Akoh (1993). According to these results, Novozyme 435 was used in all further experiments at a concentration of 1% (wt/wt acid). Although this aspect of work is not really applicable to the miniaturised continuous flow reactor system that was ultimately used, the optimum enzyme concentration was carried out so that when the amount of enzyme in the flow reactor was determined the figure could be compared to the batch reaction optimum in order to ensure that a lower concentration of enzyme was not being used and hence jeopardising the success of the reaction.

2.3.3 MINIATURISED FLOW REACTIONS

The results obtained for the batch experiments in this study were comparable to and in some cases better than similar studies (Garcia *et al*., 1999). Silva and Jesus (2003), achieved yields of between 41 and 97% after 48 hr in hexane, with 1:1 substrate ratios, for the esterification of a series of similar esters using a range of lipases immobilised onto chrysolite. Similarly Guvenc *et al*. (2002) investigated isoamyl acetate production using several lipases in batch and obtained 80% ester conversion after 6 hr under their optimised conditions. The investigation was therefore

transferred to the miniaturised continuous flow reactor. The conditions used were based on the batch lipase catalysed esterification reaction results, where all experiments were carried out using Novozyme 435 as the biocatalyst, hexane as the solvent with 1:1 acid: alcohol ratio and at ambient temperature.

Various parameters (flow rate and reaction mixture concentration) relating to the miniaturised reactor were investigated using the enzymatic synthesis (**Scheme 4**) of methyl laurate **8** from lauric acid **9** and methanol **7** as a model reaction. This ester was chosen because lauric acid is a solid and so this was thought to be the so-called 'worse case' scenario in terms of solubility in the flow system.



Scheme 4: Enzymatic preparation of methyl laurate **8**, using Novozyme 435.

It can be seen from the results shown in **Table 2** that as the flow rate through the miniaturised, continuous flow reactor is decreased an increase in percentage ester conversion is observed. At 25 and 1μLmin^{-1}, conversions of 46.5 and 95.9% were seen respectively, after collection of product for 10 min.

Flow Rate / μLmin-1	% Conversion to Ester (after 10 minute collection)
25.0	46.5
10.0	51.7
4.0	67.5
2.0	81.1
1.0	95.9

Table 2: Enzymatic preparation of methyl laurate, **8** using Novozyme 435 in a miniaturised, packed bed, continuous flow reactor, at varying flow rates. Reaction conditions; 0.2M reaction mixture in hexane, 1: 1 acid/alcohol ratio, *ca.* 10mg Novozyme 435, at room temperature, with a flow rate ranging from 25 to 1μLmin^{-1}.

It would seem that at 1μLmin^{-1} the optimum percentage conversion has been reached and further decreases in the flow rate do not appear to increase the conversion any

further (at a flow rate of 0.5μLmin^{-1} a conversion of 95% was seen). The decrease in percentage ester conversion observed at the higher flow rates is due to a reduced residence time of the reactants in the capillary and hence lower conversion of starting materials to product. Whereas, as the flow rate is decreased, the increased residence time of the reactants over the catalyst bed provides more opportunity for reaction, resulting in higher conversions of acid and alcohol to the ester. At 1μLmin^{-1} the maximum percentage ester conversion appears to have been reached. Therefore, flow rates of 1μLmin^{-1} were maintained for the remainder of the work.

Also for optimisation purposes with respect to the miniaturised flow reactor, a series of experiments were conducted using different reactant concentrations for the preparation of methyl laurate **8** from lauric acid **9** and methanol **7**. It can be seen from the results shown in **Figure 2** that at the lowest reaction mixture concentration (0.05M) only a 52.1% ester conversion was observed after 10 min of collection. However, after the same period of time a reaction mixture concentration of 0.2M resulted in the highest percentage ester conversion (> 99%).

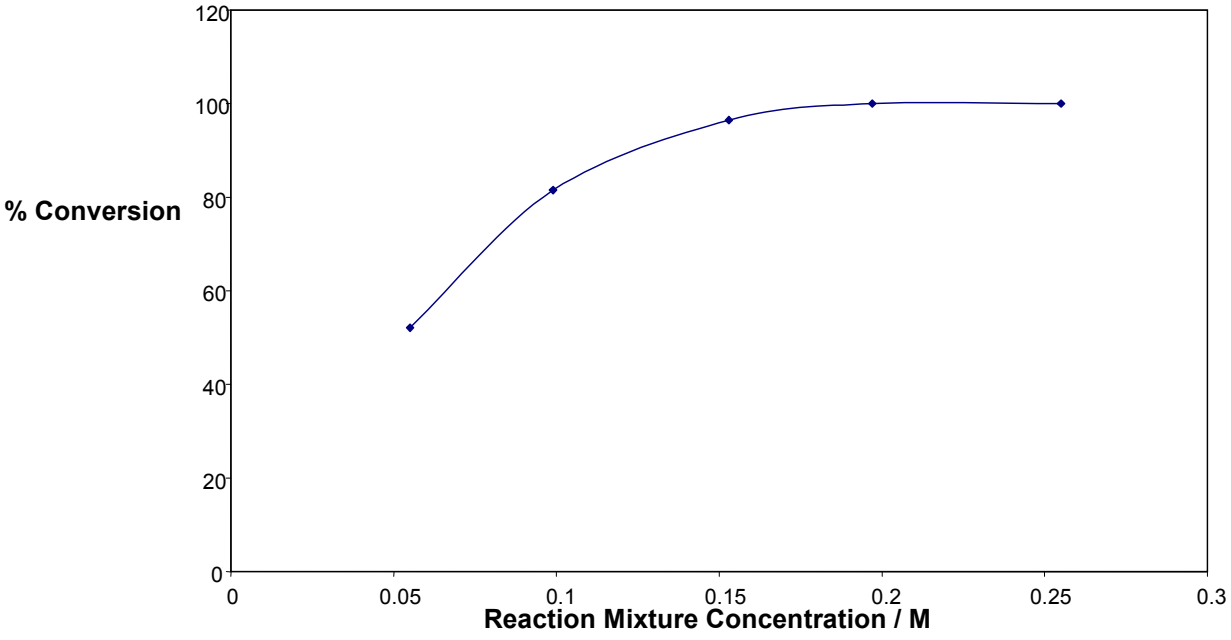
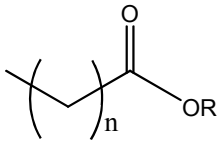


Figure 2: Enzymatic preparation of methyl laurate, **8** using Novozyme 435 in a miniaturised, packed bed, continuous flow reactor, at varying reaction mixture concentrations. Reaction conditions; 0.05M-0.2M reaction mixture in hexane, 1:1 acid / alcohol ratio, *ca.* 10mg Novozyme 435, at room temperature, with a flow rate of 1μLmin^{-1}.

As 0.2M appeared to be the optimum value for reaction mixture concentration under the conditions studied, this was the concentration used for all subsequent reactions.

Following the optimisation of the flow rate of substrates through the reactor and reaction mixture concentration, for the preparation of methyl laurate **8**, where up to >99% ester conversion was achieved, it was decided to investigate the use of Novozyme 435 in the same system to synthesis a series of esters. Based on previous batch studies carried out (Blattner *et al*., 2006; Kumar *et al*., 2005), including those by Macedo *et al*. (2003) who obtained results which suggested the size of an aliphatic chain from an acyl donor is important to the conversion rate, different chain length acids (hexanoic, octanoic and lauric) were investigated with the addition of various alcohols (methanol, ethanol and butanol). The range of alkyl esters (Macedo *et al*., 2003) (**Figure 3**) were synthesised enzymatically under the following conditions; 0.2M reaction mixture in hexane, 1:1 acid: alcohol ratio at an optimised flow rate of $1\mu Lmin^{-1}$ over a packed bed of Novozyme 435. The conversion percentages were determined every 10 min over a period of 2 hours.



n = 4, 6 or 10
R = CH_3, C_2H_5 or C_4H_9

Figure 3: Series of alkyl esters prepared in the miniaturised, packed bed, continuous flow reactor.

For the synthesis of the methyl esters; methyl hexanoate **10**, methyl octanoate **4** and methyl laurate **8** (**Figure 4**), apart from experimental error, there was no obvious difference in the average percentage biocatalytic conversions of acid and alcohol to the corresponding ester in the miniaturised continuous flow reactor.

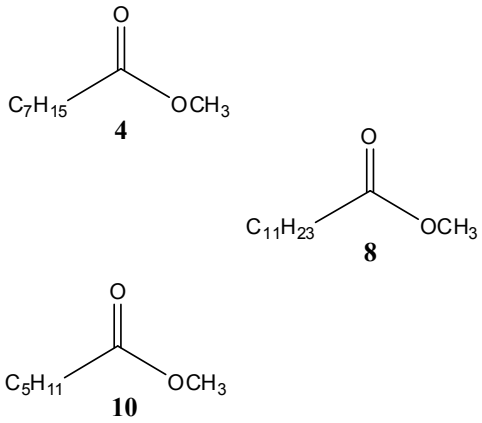


Figure 4: Methyl hexanoate **10**, methyl octanote **4** and methyl laurate **8**.

Over the 2 hr period, average results of 92, 92, and 91% were observed for the preparation of methyl hexanote **10**, methyl octanoate **4** and methyl laurate **8** respectively (**Figure 5**).

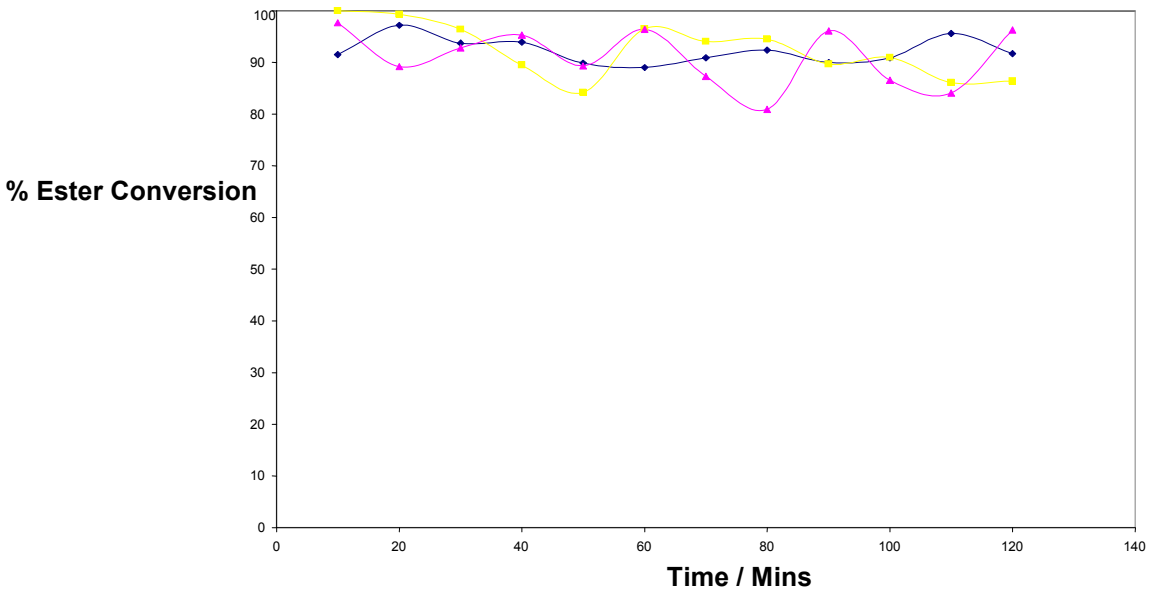


Figure 5: Enzymatic preparation of methyl hexanoate (♦), methyl octanoate (■) and methyl laurate (▲) in a miniaturised, packed bed, continuous flow reactor at 1μLmin^{-1} over a period of 120 minutes. Reaction conditions; 0.2M reaction mixture in hexane, 1:1 acid : alcohol ratio, *ca.* 10mg Novozyme 435, at room temperature, with a flow rate of 1μLmin^{-1}.

Having achieved success with the methyl ester preparations, further investigations were carried out with longer chain alcohols. For the ethyl esters; ethyl hexanote **11**,

ethyl octanoate **12** and ethyl laurate **13 (Figure 6)**, only differences that could be accounted for by experimental error were noticed over time, with consistent results over the 120 min period for the three esters individually.

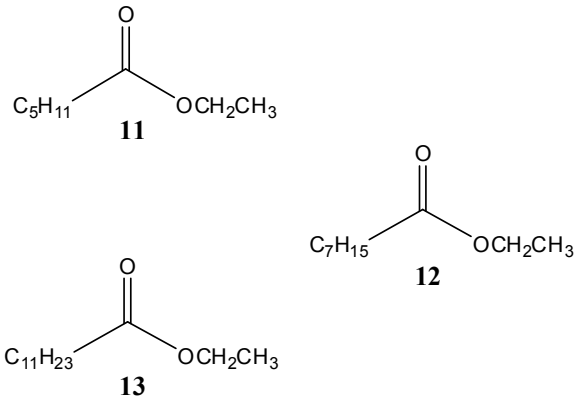


Figure 6: Ethyl hexanoate **11**, ethyl octanoate **12**, ethyl laurate **13**.

In so far as the individual ethyl esters were concerned, the percentage conversions of acid and alcohol to ester were consistent over the 2 hr period (**Figure 7**). However, it was found that Novozyme 435 does not catalyse the synthesis of ethyl laurate **13** to the same extent as it does ethyl hexanoate **11** and ethyl octanoate **12**. In fact, the average conversion was only 80% for ethyl laurate compared to 92 and 95% for ethyl hexanoate and ethyl octanoate respectively. This probably reflects lower enzyme specificity for the longer chain length lauric acid with ethanol.

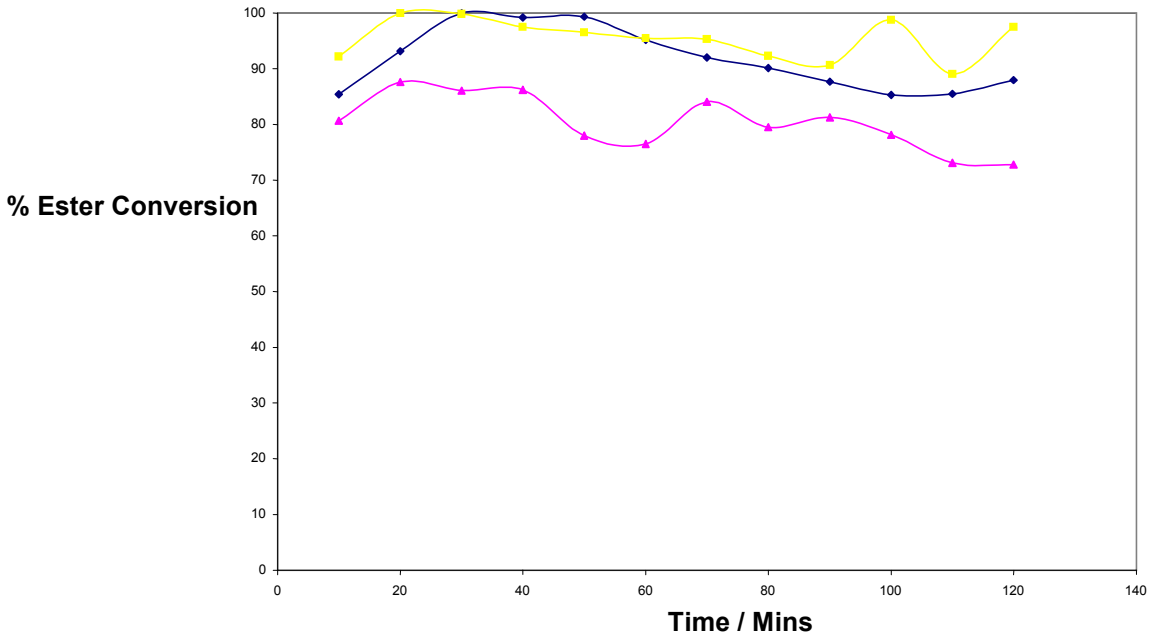


Figure 7: Enzymatic preparation of ethyl hexanoate (♦), ethyl octanoate (■) and ethyl laurate (▲) in a miniaturised, packed bed, continuous flow reactor at 1μLmin^{-1} over a period of 120 minutes. Reaction conditions; 0.2M reaction mixture in hexane, 1:1 acid : alcohol ratio, *ca.* 10mg Novozyme 435, at room temperature, with a flow rate of 1μLmin^{-1}.

It was the synthesis of the butyl esters; butyl hexanoate **14**, butyl octanoate **5** and butyl laurate **15** (**Figure 8**) that illustrated the most obvious difference between the production of the three esters.

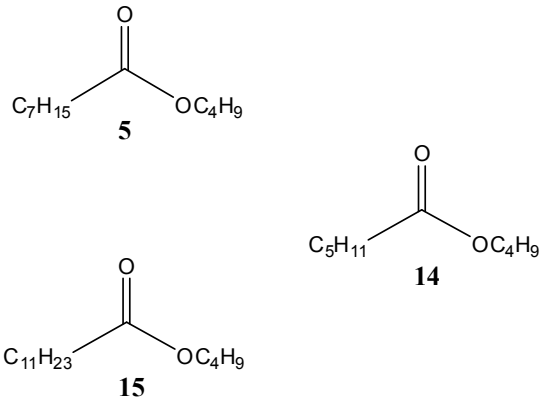


Figure 8: Butyl hexanoate **14**, butyl octanoate **5** and butyl laurate **15**.

Butyl hexanoate **14** had excellent percentage conversions (up to >99%) of acid and alcohol to ester, consistently over the 2 hr period, when using Novozyme 435 in the

miniaturised, continuous flow reactor set up. However, butyl octanoate **5** and butyl laurate **15**, despite having high initial conversions, >99 % and 90% respectively, showed a large decrease in percentage ester conversions over time (**Figure 9**). In fact, in the case of butyl laurate, the conversion of acid to ester had decreased to 67% after the 2 hr period. Thus, the larger product molecules appear to be problematic in the enzymatic esterification reaction, possibly as a result of competition between the product and the substrate in the active site, resulting in lower percentage ester conversions overall. The suggestion that the enzyme specificity for the higher molecular weight acid is insufficient to achieve similar conversions as with hexanoic and octanoic equivalents was supported by the lower initial conversion observed after 10 min. However, gradual adsorption of the product to the enzyme support matrix, leading to product inhibition from product binding to the enzyme active site and therefore, limited access of the substrate to the enzyme, must also be considered as a reason for the decrease in conversion of acid to ester over time.

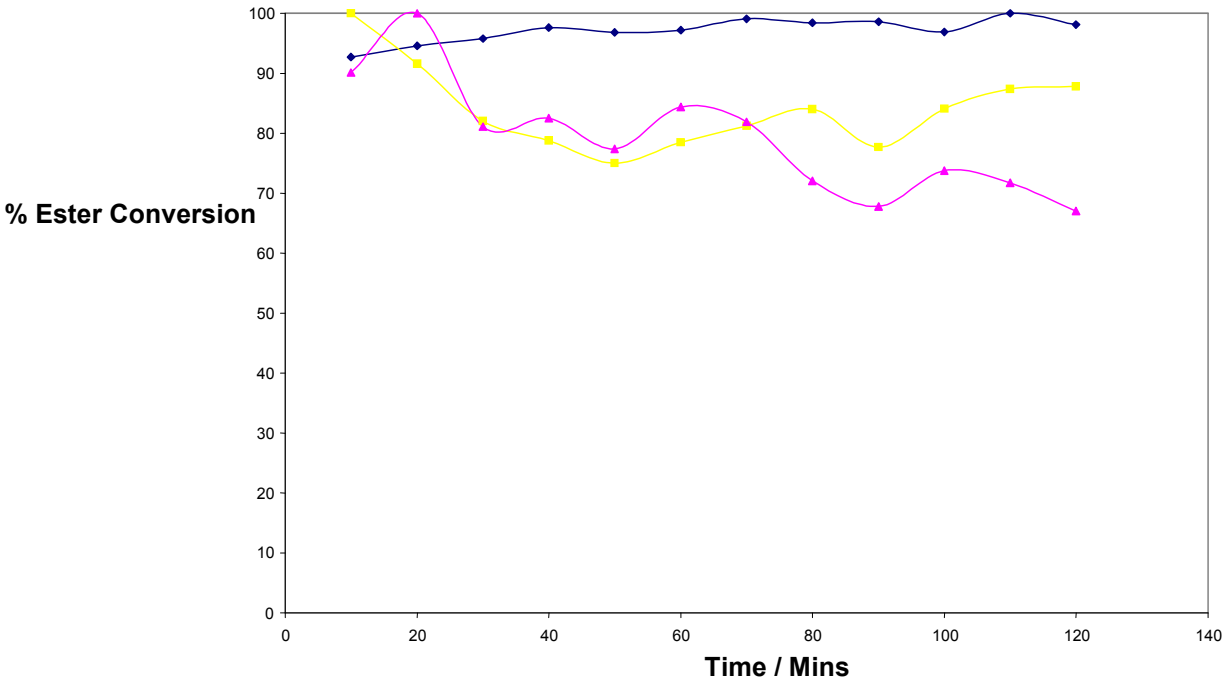


Figure 9: Enzymatic preparation of butyl hexanoate (♦), butyl octanoate (■) and butyl laurate (▲) in a miniaturised, packed bed, continuous flow reactor at 1μLmin^{-1} over a period of 120 minutes. Reaction conditions; 0.2M reaction mixture in hexane, 1:1 acid : alcohol ratio, *ca.* 10mg Novozyme 435, at room temperature, with a flow rate of 1μLmin^{-1}.

It is well reported that water content has a massive influence on the kinetics of enzymatic esterification (Bloomer *et al*., 1992; Halling, 1994). Not only does water drive the equilibrium in the opposite direction in the case of esterifications, it also plays an important role in enzyme structure and function (Klibanov, 1997). As water is being produced in the discussed esterification reactions, the production of water in the reaction is an alternative factor that should be considered when discussing the decrease in conversion of acid to ester over time. This would theoretically allow enzymatic hydrolysis to compete with esterification, resulting in a decreasing conversion. In a standard batch reactor, water production and the consequent enzymatic hydrolysis of the ester would probably pose problems at the concentrations studied, so water removal techniques would have to be investigated. In fact, there is extensive literature relating to the problems of water in batch enzymatic esterification reactions (Almeida *et al*., 1998; Condoret *et al*., 1997) and ways in which this issue can be controlled (Wehtje *et al*, 1997). However, with the continuous flow reactors, the product and any generated water are continuously removed from the enzyme environment and hence the equilibrium of the reaction within the reactor capillary is controlled without the need for additional water removal techniques.

Out of the nine alkyl esters studied, butyl hexanoate **14** showed the most promising results (**Figure 9**) with the lowest deviation in results over time (7.3%) and what would appear to be no decrease in the percentage ester conversion over the 2 hr period. This would suggest that in the case of Novozyme 435, a longer chain alcohol (butanol) and shorter chain acid (hexanoic) is preferable with an increased affinity to reach 100% ester conversion due to the restricted size pocket of the acyl donor in the active site. However, it must be noted that different enzymes have different specificities. For example, previous work carried out by Ghamgui *et al* (2004) found that shorter chain alcohols and oleic acid were more suited to enzymatic esterification when using lipase from *R. oryzae*.

One of the disadvantages associated with the use of packed bed, continuous flow reactors is that access to the reactor is limited. As a result, this type of reactor is difficult to recharge with the enzyme making it not ideally suited for use with enzymes with a short operational half life. To confirm that the decrease in

percentage ester conversion over time observed for some of the esters, in particular butyl laurate, where a 23.1% decrease was seen after two hours, was most likely due to one of the points previously raised, such as enzyme inactivation by some product inhibition of the enzyme by the production of some of the esters within the miniaturised flow reactor, and not a general decrease in enzyme activity (operational half life), a further study was carried out. The reaction of butanol and hexanoic acid to yield butyl hexanoate was studied further as it showed the most consistent results with no significant decrease in ester conversion over the 2 hr period.

It was found that the conversion of acid to alcohol, within the limits of experimental error, did not vary from 97% over a period of 7.5 hr for the synthesis of butyl hexanoate in the miniaturised, continuous flow reactor with Novozyme 435. This suggests that it is likely to be either enzyme inactivation or limited access of the substrate to the enzyme caused by product inhibition, that is an important factor for the preparation of some esters, but not others, and that the actual efficiency of the enzyme does not appear to diminish for up to 7.5 hr at least, with the most favoured substrates.

Following optimisation of the miniaturised, continuous flow reactor experiments, with respect to flow rate and reaction mixture concentration, and the successful preparation of a series of alkyl esters in the system, using Novozyme 435, a comparison of a standard bench top, one pot, batch reaction and the miniaturised reaction was carried out. This would determine if the inherent advantages of miniaturised reaction systems such as faster reactions due to increased surface to volume area ratios can be realised. Again, butyl hexanoate **14** was chosen for the study because of the consistent results this ester achieved in the miniaturised continuous flow reactor set up. The reaction conditions were identical in batch (0.2M reaction mixture, 1:1 acid: alcohol ratio and room temperature) and samples were taken every 10 min to mimic the miniaturised reaction and analysed using the same analytical methodology.

It is evident from the results shown in **Figure 10** that miniaturisation of the enzymatic esterification reaction has several advantages with respect to the rate of the reaction and perhaps water control. After 10 min in the batch reaction only a

percentage ester conversion of 3.3% was achieved compared to 92.7% in the miniaturised reaction set up. Although the percentage ester conversion in the batch reaction increased with time, it was not until 70 min that a result comparable to the miniaturised set up (94.2%) was reached. This improved efficiency in the miniaturised reactor set up reflects the smaller reaction environment in the miniaturised capillary. This means that, compared with batch reactions, the effective concentration of enzyme in the miniaturised flow reactor is higher due to a high surface to volume ratio, resulting in a greater percentage ester conversion in a shorter amount of time. It can also be seen from the results that, after reaching a maximum, the percentage ester conversion for butyl hexanoate begins to decrease in the batch reaction, a trend not observed when synthesising butyl hexanoate **14** using the miniaturised continuous flow reactor for up to 7.5 hr. This supports the theory that, as water is produced from the reaction over time, the Novozyme 435 catalyses both the forward and backward reaction in the batch set up. This problem does not appear to occur in the miniaturised reactor where the water is continually being removed from the system with the product, keeping contact time with the biocatalyst to a minimum.

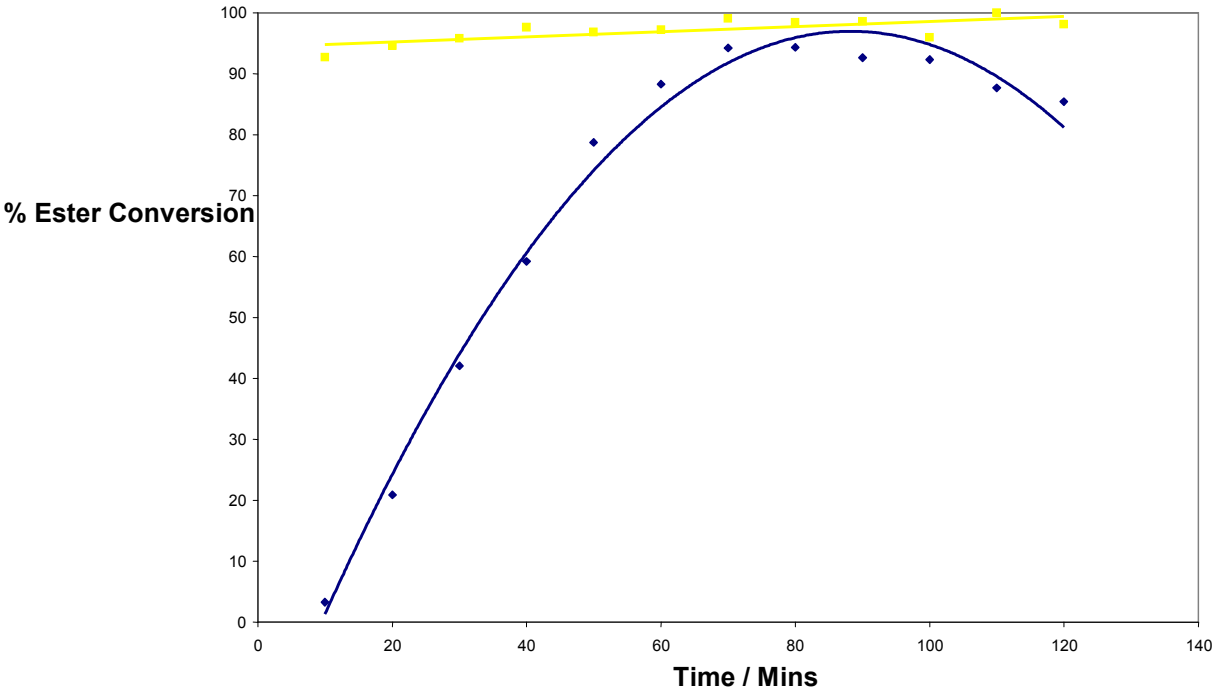


Figure 10: Enzymatic preparation of butyl hexanoate in a traditional batch reactor (♦) and in a miniaturised, packed bed, continuous flow reactor (■). Reaction conditions; 0.2M reaction mixture in hexane, 1:1 acid : alcohol ratio, 1% (wt/wt acid) or *ca.* 10mg Novozyme 435, at room temperature, with a flow rate of 1μLmin^{-1} in the miniaturised set up and 800rpm in the batch reactor.

Within the flow reactor described, which contained *ca.* 10mg of Novozyme 435, 2.04mg of product was formed per hour (in a volume of 60μL) at the set flow rate of 1μLmin^{-1}. While comparable productivity was seen with the batch reactor, it is clear that the timing of the reaction is critical for achieving the optimum percentage conversion, while this is maintained continuously and even appears to increase during the operation of the flow reactor. It is well established within flow reactor technology that larger quantities of product may be prepared using the technique of scale-out, where many reactors are replicated in parallel. The advantage of this is that optimisation can be conducted on the small scale, while larger quantities of product can be prepared without having to re-optimise the reaction on a pilot-plant. Hence, if 1000 reactors were run in parallel, over 2g of product could be prepared in an hour.

2.4 EXPERIMENTAL

2.4.1 MATERIALS

Hexanoic (99%) and octanoic acid (>98%) were purchased from Lancaster. Lauric (>98%) and benzoic (>98%) acid and seven of the nine ester standards (>98%) were from Sigma Chemie, Steinheim, Germany. The two commercially unavailable ester standards, methyl and butyl octanoate were prepared in house (**Section 2.3.1**). All solvents used were analytical grade and the water used was deionised. Dried (max 0.005% water), analytical reagent grade methanol was obtained from Reidel-de-Haen, Sigma Aldrich Laborchemikalien, Seelze, whilst Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK supplied the same grade ethanol and butanol. Novozyme 435 beads, a commercial form of lipase from *C. antarctica* B immobilised onto a macro porous resin, diameter 320-630μm with 1-2% water content was purchased from Sigma Aldrich Chemie, Steinheim, Germany and the glass capillary tubes (1.65x100mm) were supplied by Bibby Sterilin Ltd, Staffordshire, UK.

2.4.2 INSTRUMENTATION

2.4.2.1 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Gas chromatography mass spectrometry (GC-MS) data was obtained using a Varian gas chromatograph (CP 3800) coupled to a Varian mass spectrometer (Saturn 2000) with a CP Sil 8 (30m) column manufactured by Phenomenex (Zebron ZB-5), using ultra high purity helium (99.999% Energas) as the carrier gas at a flow rate of 1mL min^{-1}. After injection of the samples (200 ^{o}C), the temperature of the column oven was kept constant at 50 ^{o}C for 4 min then linearly increased to 250 ^{o}C at a rate of 30 $^{o}C\ min^{-1}$. There was a filament delay of 3.0 min.

Under these conditions, GC-MS was used as a method of analysis for the ester standards. Likewise, as only a comparative study was needed for the benchmarking of the reaction conditions, analysis of the product from the batch reactions, after 24

hr was also carried out using the same GC-MS method as described above, where the calculated conversions were based on the ester and acid peak areas, as illustrated in **Equation 1**.

$$\% \text{ Conversion} = \frac{\text{Peak Area Ester}}{\text{Peak Area Ester} + \text{Peak Area Acid}}$$

Equation 1: Calculation of percentage ester conversion on GC-MS.

When the gas chromatography-flame ionisation detection (GC-FID) indicated a 100% conversion, GC-MS was used as confirmation by noting the absence of any acid peak.

2.4.2.2 GAS CHROMATOGRAPHY-FLAME IONISATION DETECTION

For a more accurate method of analysis, as carboxylic acids tend to retain on the GC column and hence give a percentage ester conversion greater than is perhaps true, the internal standard calibration method (eight point, (0-0.2M)) was applied to analysis of the miniaturised continuous flow reactor streams, using toluene as an internal standard and pure ester standards either purchased from Sigma or prepared in house.

Analysis was carried out using a gas chromatograph equipped with a CP Sil 8 (30m) column from Shimadzu and a flame ionisation detector (GC-FID), which gave an error in peak area of 15%. Ultra high purity helium (99.999%) was used as a carrier gas at a flow rate of $1 mL min^{-1}$. After injection of the samples, the temperature of the column oven was kept constant at 50 ^{o}C for 4 min then linearly increased to 270 ^{o}C at a rate of 25 $^{o}C\ min^{-1}$. This temperature was maintained for the remaining time of analysis. Injector and detector temperatures were both 250 ^{o}C.

Successful calibrations of all nine esters gave the equations (**Table 3**) from which to calculate the percentage ester conversion achieved, based on the ester and toluene peak areas.

Ester standard	Equation of line of best fit / y=[1]	R^2 value
Methyl hexanoate	6.824x	0.9948
Methyl octanote	10.287x	0.9964
Methyl laurate	13.542x	0.9996
Ethyl hexanote	9.724x	0.9901
Ethyl octanoate	11.697x	0.9965
Ethyl laurate	15.716x	0.9996
Butyl hexanoate	11.698x	0.9966
Butyl octanoate	14.488x	0.9924
Butyl laurate	14.093x	0.9966

Table 3: GC-FID calibrations for the nine ester standards.

Under the same conditions, standard mixtures were analysed to generate retention times that, once the peaks were identified, could be used as markers, according to **Table 4.**

Compound	R_T / Min
Toluene (internal standard)	3.7
Hexanoic acid	7.6
Octanoic acid	8.9
Lauric acid	10.8
Methyl hexanoate	6.2
Methyl octanote	8.1
Methyl laurate	10.2
Ethyl hexanote	6.9
Ethyl octanoate	8.6
Ethyl laurate	10.7
Butyl hexanoate	8.5
Butyl octanoate	9.7
Butyl laurate	11.7

Table 4: GC-FID retention times for the ester standards, starting materials and calibration internal standard.

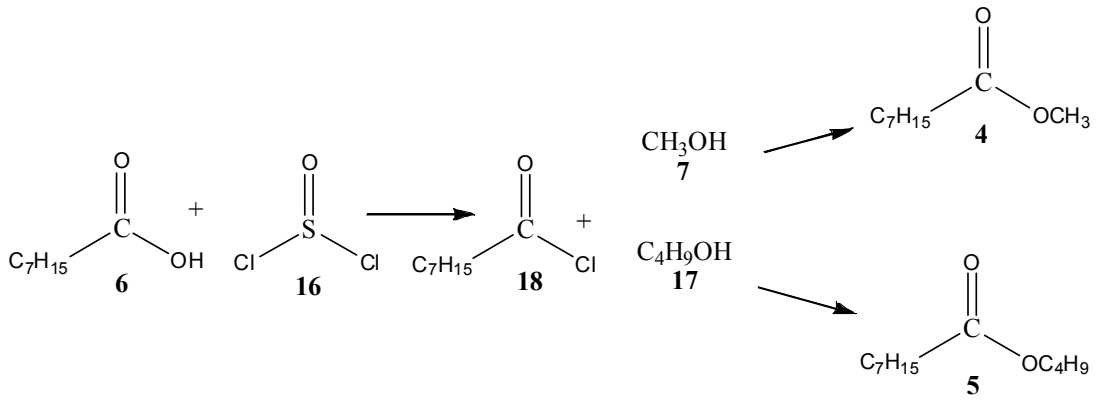
[1] Where y = Product / toluene ratio and x = Concentration of product

Analysis of the miniaturised reactor product stream was carried out at 10 min intervals for 2 hr, using the same GC-FID method. An error up to 15% (for peak area) was observed and in cases where over 100% was obtained, >99% was recorded as the value, confirmed by running the sample on GC-MS and noting the absence of an acid peak. All subsequent results were altered accordingly.

2.4.3 METHODS

2.4.3.1 PREPARATION OF ESTER STANDARDS

Octanoic acid **6** (3.5mmol, 0.175M) was dissolved in tetrahydrofuran (THF) (20mL) with stirring at room temperature. Thionyl chloride **16** (3eq, 1.5g) was added and the mixture was allowed to stir at room temperature for a further 1 hr. After which, without isolation of the chloride intermediate **18**, methanol **7** or butanol **17** (excess, 10mL) was added and the reaction mixture was allowed to stir for a further 2 hr prior to work up. The THF and alcohol were removed *in vacuo* and the resulting dark orange liquid was dissolved in dichloromethane (DCM). Following washing with water (3x50mL) and drying over magnesium sulphate, the product was concentrated *in vacuo* yielding (64 and 68% for methyl octanoate **4** and butyl octanoate **5** respectively) the esters as orange liquids with a distinctive sweet ester smell (**Scheme 5**). The products were analysed by GC-MS.

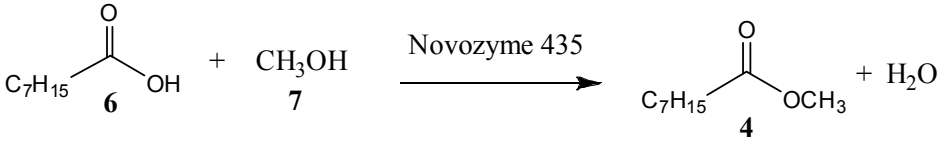


Scheme 5: In-house preparation of ester standards.

2.4.3.2 BATCH ENZYMATIC ESTERIFICATION REACTIONS

Standard reaction (Scheme 6)

A 1: 1 mixture of octanoic acid **6** (30mmol, 0.2M) and methanol **7** (30mmol) in hexane (150mL) was stirred (800rpm) continuously for 24 hr at atmospheric pressure in a stoppered round bottom flask (250mL), containing Novozyme 435 (5% (wt/wt acid)). After which, the Novozyme 435 was filtered off and the reaction mixture extracted into DCM (250mL). Following washing with water (3x50mL), drying over magnesium sulphate and evaporation of the solvent *in vacuo* the resulting product, a pale yellow oil of methyl octanoate **4** was analysed using GC-MS.



Scheme 6: Enzymatic preparation of methyl octanoate using Novozyme 435

Optimisation of batch reaction

Following the same protocol as described above, the reaction was repeated changing one parameter at a time. Parameters investigated in batch, with the aim of optimising the conditions for methyl octanoate and hence other alkyl esters, were temperature (22, 37, and 70 ^{o}C), solvent (hexane, toluene, DCM and acetonitrile (ACN)), ratio of acid to alcohol (1: 1 and 1: 40) and enzyme concentration (0.25-7% (wt/wt acid)). In all cases, control reactions without enzyme were also conducted.

Comparison of batch and miniaturised reactions

A 1: 1 mixture of hexanoic acid (30mmol, 0.2M) and butanol (30mmol) in hexane (150mL) was stirred (800rpm) continuously for 2 hr at atmospheric pressure in a stoppered round bottom flask (250mL) containing Novozyme 435 (5% (wt/wt acid)). Samples were removed every 10 min over a period of 2 hr and analysed by GC-FID. Conversion of the starting material to product was determined according to the calibrations.

2.4.3.3 MINIATURISED FLOW REACTIONS

A miniaturised continuous flow reactor set up (**Figure 11**) was used where the Novozyme 435 (an immobilised form of *C. antarctica* lipase) beads (*ca.* 10mg) were incorporated into a small glass capillary (1.65mm diameter x 30mm length) resistant to the solvents and reagents required. In a similar way to work carried out by Peterson (2005), the Novozyme 435 beads were trapped within the channel to ensure they were not flushed out with operation, using a micro porous silica frit fabricated (Christensen *et al*., 1998) at the end of the capillary. This ensured that the immobilised enzyme was held in place whilst the reaction solution was pumped over the biocatalyst. A pressure driven, hydrodynamic syringe pump set up which could operate at flow rate ranges between 0.5 and 100µLmin-1 was used. The glass capillary reactor portion of the set up was connected to a glass luer lock syringe using combined standard HPLC finger tight fittings (1/16″) with PEEK tubing (360µm outer and 150µm internal diameters) from Upchurch Scientific and female-female luers interfacing the tubing and syringes, supplied by Supelco.

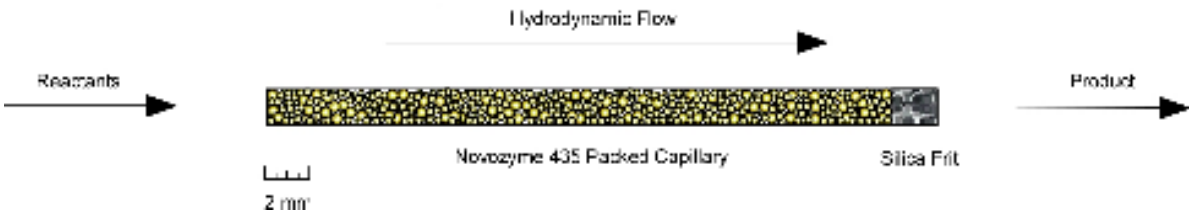
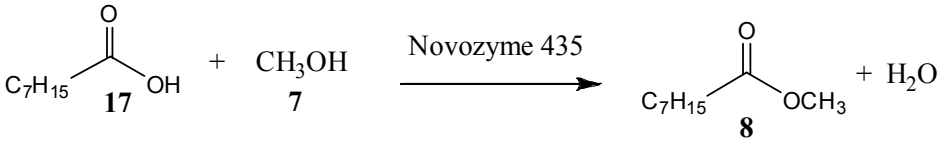


Figure 11: Set up of a miniaturised, packed bed, continuous flow reactor.

Standard reaction

A 1: 1 mixture of lauric acid **17** and methanol **7** in hexane (500µL, 0.05M-0.25M) was passed over (25-1µLmin^{-1}) the packed bed, continuous flow reactor containing Novozyme 435 (*ca.* 10mg) to yield methyl laurate **8** (**Scheme 7**). Following the reaction the product mixture, collected in a small glass vial outlet, was removed every 10 min over a period of 2 hr and analysed by GC-FID. Conversion of the starting material to product was determined, according to the calibrations.



Scheme 7: Enzymatic preparation of methyl laurate **8** using Novozyme 435.

Preparation of a series of alkyl esters

1: 1 mixtures of acid (hexanoic, octanoic or lauric) and alcohol (methanol, ethanol or butanol) in hexane (500μL, 0.2M) were passed over ($1\mu L min^{-1}$) the packed bed reactor containing Novozyme 435 (*ca.* 10mg) to yield the nine esters under investigation, individually. Following the reaction the product mixture, collected in a small glass vial outlet, was removed every 10 min over a period of 2 hr and analysed by GC-FID. Conversion of the starting material to product was determined, according to the calibrations.

Longevity of Novozyme 435

A 1: 1 mixture of hexanoic acid butanol in hexane (500μL, 0.2M) was passed over ($1\mu L min^{-1}$) the packed bed reactor containing Novozyme 435 (*ca.* 10mg) to yield butyl hexanoate. The product mixture, collected in a small glass vial outlet, was removed every 10 min over a period of 7.5 hr and analysed by GC-FID. Conversion of the starting material to product was determined, according to the calibrations.

2.5 CONCLUSIONS

Chapter 2 has explored the feasibility of combining biocatalysis with miniaturisation, using the enzymatic synthesis of methyl, ethyl and butyl esters in a packed bed, miniaturised continuous flow reactor. Initially benchmarking of the reaction conditions in batch was carried out. Parameters including temperature, enzyme concentration, reaction media and enzyme concentration were investigated. Under the optimised conditions, it took up to 24 hr to achieve reasonable percentage conversion of methanol and octanoic acid to methyl octanoate. The process of enzymatic esterification, utilising a cheap, commercial, readily available enzyme (Novozyme 435), was then transferred to a miniaturised continuous flow reactor. The successful synthesis of methyl laurate in the miniaturised reactor system allowed the study to be extended to the synthesis of nine methyl, ethyl and butyl esters. It would seem however, that the extent of the reaction using this lipase, in miniaturised conditions, is affected by the chain lengths of the acid and alcohol. Biocatalyst specificity problems with respect to the substrates, enzyme inhibition by the larger product molecules or adsorption of some of the products onto the enzyme support matrix, resulting in lower percentage ester conversions, are possible reasons for the differences observed between conversions of the esters. Despite this, the conversion of acid and alcohol to ester, at ten min intervals, and even after two hours, was in all cases greater than 65%. In fact, in the case of butyl hexanoate, excellent (>90%), consistent percentage ester conversions were achieved. The work detailed in **Chapter 2** successfully demonstrated that miniaturisation can be advantageous over bench top, one pot batch reactions for biocatalytic synthesis. Advantages include; an increased throughput, where high percentage ester conversions are obtained more rapidly than in a batch reaction, due to a high surface to volume ratio in the capillary; lower associated costs, as less reagents are required; increased environmental control, so there is less chance of the enzyme catalysing the reverse reaction; and a more benign process as smaller volumes of reagents are required. Additionally, the work described in **Chapter 2** demonstrated the ability to screen the enzyme for substrate specificity. Consequently, this concept can now be applied to other more complicated processes that involve enantioselective reactions or utilise expensive enzymes, where miniaturisation is of high importance.

3 LIPASE CATALYSED ENANTIOSELECTIVE RESOLUTION REACTIONS

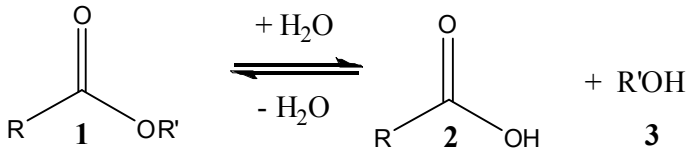
3.1 AIMS OF CHAPTER 3

Individual enantiomers of racemic compounds can often exhibit diverse pharmacological and therapeutic properties. In fact, drugs usually have their biological activity based mainly on one enantiomer. Consequently, research into the preparation of enantiomerically pure chiral compounds is increasing in interest. Dynamic kinetic resolution (DKR) is emerging as a potentially efficient process, whereby, standard enzymatic kinetic resolution is coupled with *in situ* racemisation of the slower reacting enantiomer, using a suitable racemising catalyst.

Chapter 3 investigates the kinetic resolution, racemisation and DKR reactions of a range of substrates of potential interest to the pharmaceutical industry. The main emphasis of the work will be based on the development of a process for the enantioselective hydrolysis of racemic esters into a single enantiomeric acid product in high yield, by making use of enzymatic kinetic resolution in conjunction with *in situ* chemical racemisation.

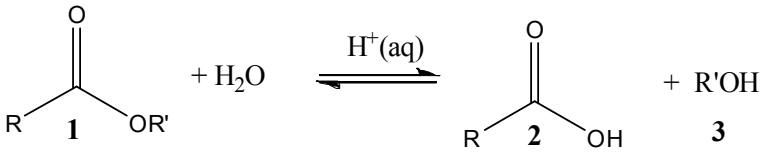
3.2 HYDROLYSIS

Hydrolysis is the chemical reaction or process in which a chemical compound is transformed by a reaction with water. In organic chemistry, hydrolysis can be considered as the reverse of a condensation reaction, such as esterification, whereby two molecular fragments; an acid **2** and an alcohol **3** are joined for each water molecule produced (**Scheme 8**). It is the hydrolysis of esters **1** thereby splitting them into one hydrolysis product containing a hydroxyl functional group (alcohol) and another containing a carboxylic acid functional group, as illustrated in **Scheme 8**, which is the focus of the work described herein.



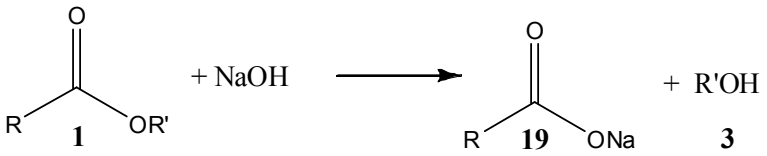
Scheme 8: Hydrolysis reaction.

Esters **1** can be hydrolysed to produce the carboxylic acid **2** and corresponding alcohol **3** using water in the presence of dilute acids or bases. However, the reaction is so slow that it is often never used and it is the heating of the ester **1** under reflux with a dilute acid such as hydrochloric acid or sulfuric acid that is still a common reaction in many laboratories (**Scheme 9**).



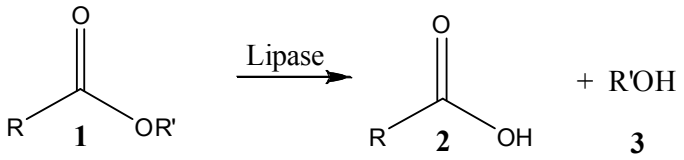
Scheme 9: Acid catalysed hydrolysis.

Alternatively, esters may also be hydrolysed into a carboxylate salt **19** rather than the carboxylic acid **2** and an alcohol **3** by heating the ester **1** under reflux with a dilute alkali such as sodium or potassium hydroxide solution. This approach has the advantages that the reactions are not reversible (**Scheme 10**) and the products are somewhat easier to separate.



Scheme 10: Base catalysed hydrolysis, using sodium hydroxide.

As is the case with most conventional chemical processes, these routes of hydrolysis can require complex separation processes which often result in lower yields whilst generating large amounts of aqueous and organic waste. In addition, as the reactions are generally carried out under reflux they utilise a considerable amount of energy which increases production costs. Consequently, biocatalytic hydrolysis (**Scheme 11**), using enzymes, in particular lipases, has gathered interest in recent years. This approach may allow for a reduction in the number of steps required in comparison to conventional chemical synthesis, has an increasing orientation towards natural production, maintains mild operating conditions, has high specificity with reduced side reactions and does not require any expensive separation techniques.



Scheme 11: Lipase catalysed esterification reaction.

3.3 CHIRALITY

Isomers are compounds with the same molecular formula but different structural formulae. There are two main forms of isomerism; structural isomerism and stereoisomerism. In structural isomers, the atoms and functional groups are joined together in different ways, whether it is variable amounts of branching on the hydrocarbon chains or the position and structure of a functional group. In stereoisomers however, the bond structure is the same but the geometrical positioning of the atoms and functional groups in space differs. This class includes enantiomers, derived from the Greek word for opposite forms, *enantiomorphs*. Enantiomers are different isomers that are non-super imposable mirror-images of each other and one enantiomer will not turn into the opposite unless you break and make some bonds. Molecules like this are said to be chiral and exhibit optical activity. Such optical isomers can occur when there is an asymmetric, tetrahedral bonded carbon atom, *i.e.* one which is bonded to four different groups that are able to form two spatially different compounds. For optical isomers to occur there must be four groups bonded to a carbon atom and all four must be different (**Figure 12**).

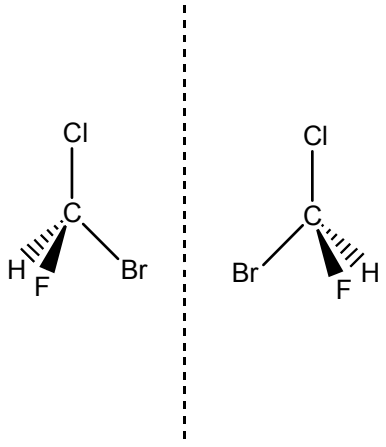


Figure 12: The two enantiomers of bromochlorofluoromethane.

It is the ability of the optical isomers to rotate the plane of polarisation of a beam of plane polarised monochromatic light that is passed through it, in opposite directions, that determines which enantiomer is which. The enantiomer that rotates the polarised light clockwise (to the right) is the dextrorotary (d-) or +ve enantiomer,

whereas the other, which rotates the polarised light anticlockwise (to the left) is called the levorotary (l-) or -ve enantiomer. The naming of enantiomers can be done *via* one of three methods; using the optical activity, (+)- and (-)- which must be determined experimentally, as discussed above, or by stereochemical rotation, using either the *R*- and *S*- or D- and L- configurations. The *R*/*S* system is the most commonly used nomenclature system for denoting enantiomers. It labels each chiral centre *R* or *S* according to a system by which its substituents are assigned a priority, according to the Cahn Ingold Prelog priority rules based on atomic number. If the centre is orientated so that the lowest priority of the four substituents is pointed away from the viewer and the priority of the remaining three substituents decreases in the clockwise direction, it is labelled *R*- (for *Rectus*). However, if the opposite is true then it is labelled *S*- (for *Sinister*). The *R*/*S* system has no fixed relation to the D/L or the (+)/(-) systems.

Using the D- and L- configuration, an optical isomer can be named by relating the molecule to glyceraldehydes **20**. In this system, compounds are named by similarity to glyceraldehyde, which is chiral itself and can undergo certain chemical manipulations without affecting its configuration. This has resulted in its use for nomenclature, which, in general, produces definite designations. It is however, easiest to use the D/L configurations in small biomolecules that are similar to glyceraldehyde. Again, the D/L labelling is unrelated to (+)/(-) and it does not indicate which enantiomer is dextrorotary and which is levorotary. Rather, it says whether the compounds stereochemistry is related to that of the dextrorotary **20a** or levorotary **20b** enantiomer of glyceraldehyde (**Figure 13**). Usually, the "CORN" rule can be applied to the naming of enantiomers according to the D/L nomenclature, where if the CO and the R groups are arranged adjacently, clockwise around the carbon atom it is the D- form and *vice versa*.

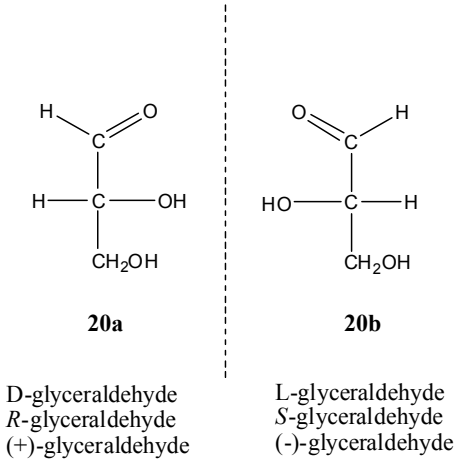


Figure 13: The two enantiomers of glyceraldehyde, named according to the (+)/(-), *R*/*S* and D/L nomenclature.

In chemistry, many molecules exhibit enantiomeric pairs that exist as mixtures in which both enantiomers are present in equal concentrations. The mixture is known as a racemic mixture or racemate where the percentage of each enantiomer is 50% which results in the racemate being optically inactive. The first known racemic mixture was 'racemic acid' which Louis Pasteur found to be a mixture of the two enantiomeric isomers of tartaric acid. Enantiomers of racemic compounds can often exhibit diverse pharmacological and therapeutic properties and different drugs usually have their biological activity based mainly on one enantiomer (Sheldon, 1993), *e.g.* 2-arylpropionic acids (profens) are an important class of non-steroidal anti inflammatory drug that work *via* the *S*-enantiomer (Hutt & Caldwell, 1984). Despite this, as a racemate can usually be easily obtained through chemical synthesis, for many years the pharmaceutical industry has simply used the racemic form of drugs. In some cases the chirality of a compound does not affect its biological properties and ability in obtaining the desired outcome meaning that the racemic compound is safe for consumption. However, this is not the case for many drugs, where in fact, the undesired enantiomer can elicit an undesired biological response e.g. toxicity. For example, in the case of the racemic mixture of thalidomide, one enantiomer (*R*-) has sedative properties and can be used as medication for pregnant women to treat morning sickness, whilst the *S*-enantiomer is teratogenic and can cause serious malformations to the foetus. Therefore, it would be advantageous and safer to administer only the enantiomer with desirable properties. Consequently, as the need for enantiomerically pure chiral drugs in the pharmaceutical industry is

increasing, the chiral preparation of such compounds, through synthesis or resolution, has become more important.

3.3.1 CHIRAL SYNTHESIS

Chiral synthesis is an approach that introduces one or more new and desired elements of chirality. There are three main chemical approaches to chiral synthesis; chiral pool synthesis (Ulrich Klar *et al*., 2005), chiral auxiliaries and asymmetric catalysis. However, in practice, a mixture of all three methods is often used to maximise the advantages associated with each process. This approach has many associated disadvantages, such as the number of possible reactions the molecule can undergo may be restricted whilst maintaining the chiral integrity of the substance, long synthetic routes may be required involving many steps with abundant losses in yield and enantiopure starting materials may be expensive if they do not occur in nature.

3.3.2 CHIRAL RESOLUTION

The most dominant production method to obtain a single enantiomer in industrial synthesis consists of the resolution of racemates (Taschner *et al*., 1993) by separating racemic starting compounds into their corresponding enantiomers (Porter, 1991). Chiral resolution can be divided into three main categories; crystallisation, chromatography and kinetic resolution.

3.3.2.1 CRYSTALLISATION

Around 5-10% of all racemates are known to crystallise as a mixture of enantiopure crystals, so called conglomerates. This type of resolution, also known as spontaneous resolution, has been demonstrated with racemic methadone (Zaugg, 1955). Another form of direct crystallisation is preferential crystallisation or resolution by entrainment. In this approach, an added seed of an enantiomerically pure compound to a racemic mixture should, in theory, have the corresponding enantiomer crystallising out relatively quickly and in high optical purity. This technique is widely used on the industrial scale, for example in the manufacture of the antimicrobial agent, eye drop ingredient, chloramphenicol (Amiard, 1959).

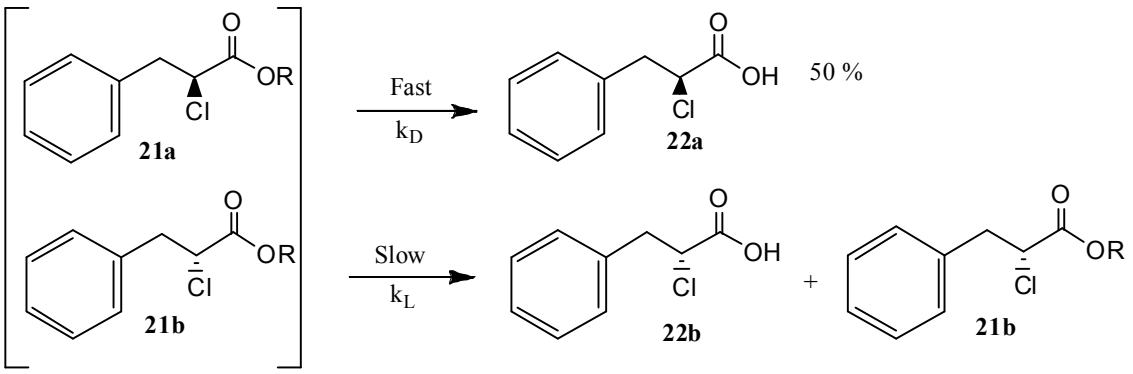
Chiral resolution through crystallisation has the disadvantage that it is technically only feasible with racemates that form conglomerates. Unfortunately however, less than 10% of all racemates are believed to be conglomerates with the rest comprising true racemic compounds that cannot be separated by this method.

3.3.2.2 CHROMATOGRAPHY

Chiral column chromatography is a variant of column chromatography, where the stationary phase is chiral. The enantiomers of the same compound then differ in affinity to the stationary phase, thus they have different retention times. The chiral stationary phase can be prepared by attaching a suitable chiral compound to the surface of an achiral support such as silica gel, which creates a chiral stationary phase.

3.3.2.3 KINETIC RESOLUTION

Kinetic resolution is a very old concept in organic chemistry for the synthesis of chiral molecules. Kinetic resolution was first observed by Marckwald and McKenzie in 1899 and works by the principal that the two enantiomers, for example D- and L-, of a racemic mixture show different reaction rates ($k_D \neq k_L$) with a chiral entity in a chemical reaction, thereby creating an excess of the less reactive enantiomer. The chiral entity should be present in catalytic amounts and may be a biocatalyst (enzyme or microorganism) or a chemocatalyst (chiral acid or base or a chiral metal complex). Usually, one enantiomer reacts much faster than the other ($k_D >> k_L$) but ideally one enantiomer would be the only reacting molecule, for example $k_L=0$. This concept can be illustrated using the enantioselective hydrolysis of a racemic ester **21**, where it is the D-ester **21a** which is preferentially hydrolysed to yield the corresponding enantiomeric acid **22a**, with perhaps a small amount of the opposite enantiomeric acid **22b** (depending upon the selectivity of the enzyme) and some residual enantiomeric ester **21b** (**Scheme 12**).



Scheme 12: Enzymatic kinetic resolution (KR) of a racemic α-chloro ester.

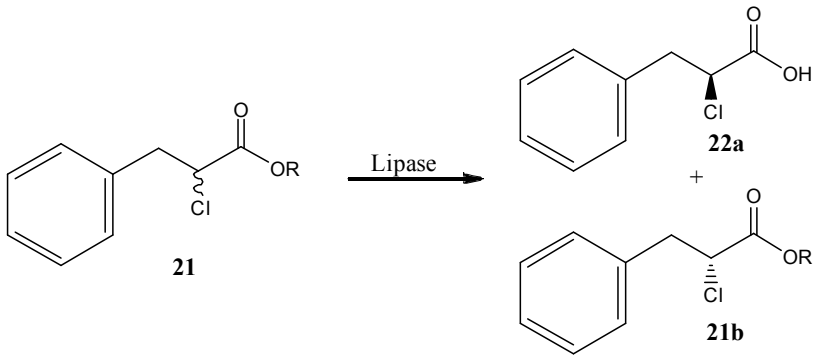
It is the use of enzymes to catalyse the kinetic resolution of racemic mixtures that is investigated in the work presented here, therefore any reference to kinetic resolution from here on in refers to that which is enzyme catalysed. Enzymes show chiral selectivity. That is, they have the ability to distinguish between the two enantiomers of a racemic mixture with good to excellent selectivity. This is because all enzymes are composed of only L-amino acids and the 3D cavities in the enzyme active site which bind with the substrates usually allow only one enantiomer to fit inside and be bound tightly while the other will not, due to the different spatial arrangement of the atoms. It is this characteristic which makes enzymes ideal catalysts for the kinetic resolution of racemic compounds and is why synthetic chemists have become increasingly interested in biocatalysis to synthesise enantiopure compounds as chiral building blocks for drugs and agrochemicals.

Enzymatic kinetic resolution was being used as far back as the 1980's when Gist Brocades, in a patent, disclosed the preparation of the anti inflammatory drug, *S*-naproxen, through the enantioselective hydrolysis of alkyl esters, using a biocatalyst from *Bacillus thai* (*B. thai*)and other microorganisms (Brocades, 1988). However, the reaction took as long as six days to complete and it is only in recent years that the use of enzymes for organic transformations to yield a wide range of enantiomerically enriched molecules including alcohols, acids and esters has become an increasingly attractive alternative to conventional chemical methods. In fact, using enzymatic kinetic resolution in organic media (Klibanov, 1990) to prepare optically pure enantiomers simply and with minimised environmental impact, from their racemates,

is one of the most commonly used resolution methodologies and is probably the most widespread application of enzymes as synthetic catalysts.

Members of the lipase family often display high applicability and efficiency in resolution processes in organic solvents and have been found to be particularly suitable for such applications, with improved enantioselectivity. For example, lipases from *C. rugosa*, *C. antarctica* and porcine pancreas are being used to resolve enantiomers of the anti inflammatory drugs naproxen (Tsai & Wei, 1994; Koul et al., 2003), ibuprofen (Tsai, 1997) and suprofen (Mertoli, 1996).

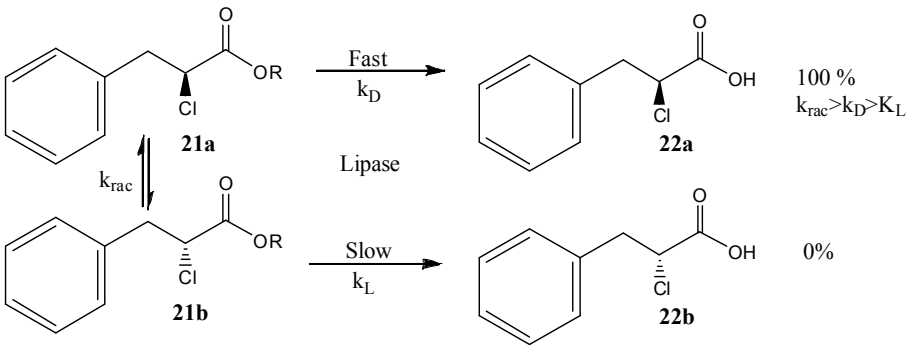
Enzymatic kinetic resolution (KR), illustrated in **Scheme 13**, has the advantage of easily separating both enantiomers using a single enzyme. However, the traditional KR process has the disadvantages that only a maximum 50% yield of the desired enantiomer is obtained (**Scheme 13**), with the unwanted enantiomer having to be discarded or recycled in a tedious manner; additionally, enzymes may be considerably high in cost.



Scheme 13: Enzymatic kinetic resolution (KR) of a racemic α-chloro ester.

In order to increase this yield to 100% and therefore avoiding having to discard any materials, classic kinetic resolution requires some additional steps whereby the unwanted enantiomer of the racemic starting material can be re-racemised and once again subjected to resolution (**Scheme 14**). Dynamic kinetic resolution (Williams *et al*., 2002) has been proven as a potentially efficient process in which standard KR is coupled with *in situ* racemisation of the starting substrate using a suitable racemising catalyst (Ebbers *et al*., 1997). DKR has attracted an increasing amount of interest over the past decade and is an important extension of KR. This approach tackles the

obvious drawbacks of the KR system, where the maximum conversion in the reaction is only 50% and the product then has to be separated from the reactants. If the rate of racemisation of the starting material, for example a chloro ester **21**, is fast relative to the rate of the biocatalytic transformation ($k_{rac}>>k_D$) then as the substrate is continuously racemised during the resolution process, the D- and L- substrates are in equilibrium which allows for the possibility of converting 100 % of the achiral reactant into the enantiomeric product (chloro acid **22a**).



Scheme 14: Dynamic kinetic resolution (DKR) of a racemic α-chloro ester.

Whilst some enzyme-enzyme processes are known, DKR often involves coupling an enzymatic reaction with the *in situ* chemo racemisation of the unreactive or slow reacting enantiomer. In some instances this has proved very successful, however the technique is not without its drawbacks. Persson *et al*. (1999) used a combination of enzyme and transition metal complexes to perform DKR of a set of secondary alcohols. Likewise, Kim *et al*. (2002) have studied DKRs and asymmetric transformation by enzymes coupled with metal catalysis, in particular lipase-ruthenium and lipase-palladium for the DKR of alcohols and amines respectively. One disadvantage however of this 'one-pot' approach is that the enzyme must be compatible with the choice of racemising agent in order for DKR to be successful. Consequently, improved techniques are required which separate the processes such that the methodology can be more widely applied, it is this that is the focus of the investigation discussed herein.

3.3.3 RACEMISATION

Racemisation of the unreactive or slow reacting enantiomeric substrate in order to achieve a successful DKR process can be performed chemically, catalytically or even

spontaneously. However, the conditions must be right, such that it does not result in the racemisation of the enantioenriched product, affect the kinetic resolution of the substrate or cause any adverse effects to the production of the desired compound. Under the correct conditions the DKR reaction should be able to produce 100% of the optically pure compounds out of the racemic starting substrate.

The racemisation step in the whole asymmetric transformation process is very important and there are a number of organic reactions whereby an optically active substance can be transformed into the corresponding racemic modification. However, only those reactions taking place in a single step and under mild conditions are suitable for use with DKR and the ease with which an optically active compound can be racemised varies greatly.

Methods of racemisation include base catalysed, Schiff base mediated, enzyme catalysed, thermal racemisation, oxidation and reduction and substitution / elimination reactions. Base catalysed racemisation is an important, well known and frequently used method for the racemisation of optically pure organic substrates. The process usually involves the removal of a proton from the chiral centre and equilibrates enantiomers *via* an enolate compound, if the substrate is an ester or a ketone. This method can be applied to most compounds with a stereogenic centre bearing an acidic proton adjacent to an electron withdrawing group, at the chiral centre. Schiff base mediated racemisation is feasible for compounds bearing a free amino (NH_2) group at the chiral centre. Although this approach is very extensively studied and is important in industry due to its application to the racemisation of amino acids, it has been less utilised in DKR reactions due to an abundance of compatibility issues. Similarly, enzyme catalysed racemisation, which uses the Schiff base method, is mainly restricted to amino acids and their derivatives and the scope is therefore limited. Thermal racemisation is widely applicable, simple and cheap, making it an attractive alternative. However, there is the inherent problem of decomposition of the substrate when high temperatures have to be applied. Oxidation or reduction is used primarily for chiral amines and alcohols. If the substrate is a chiral halide or nitrile, nucleophilic substitution or elimination with addition reactions can be considered for racemisation.

It is with these factors in mind that the DKR of a series of α-chloroesters was evaluated, with the aim of developing continuous flow methodology capable of affording a rapid screening or synthesising technique.

3.4 RESULTS & DISCUSSION

3.4.1 PREPARATION OF MATERIALS

As the racemic α-chloro esters (**23** & **24**) and α-chloro thioesters (**25** & **26**) of potential interest to the pharmaceutical industry (**Figure 14**) were not commercially available and, to our knowledge, had not been synthesised by any other groups previously, in the first instance these novel compounds were synthesised.

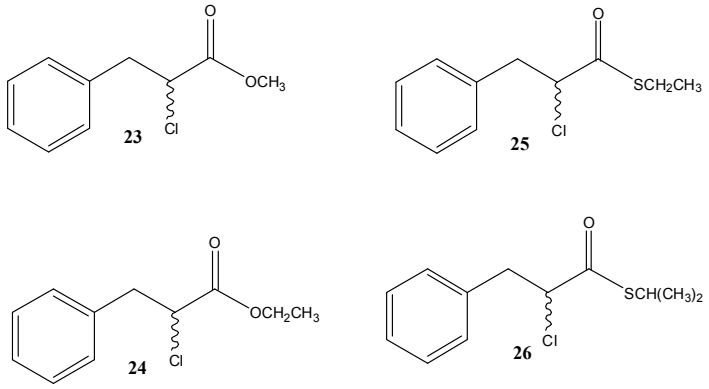


Figure 14: DL-α-chloro methyl ester **23**, DL-α-chloro ethyl ester **24**, DL-α-chloro ethyl thioester **25** and DL-α-chloro i-propyl thio ester **26** substrates of interest.

In order to represent a synthetic target for the enzyme catalysed hydrolysis reactions, a racemic standard of the α-chloro acid product **22** (**Figure 15**) was synthesised and four lipases, one immobilsied; Novozyme 435 and three free in solution; Lipozyme RM (*Rhizomucor miehei* (*Rh. miehei)*), Lipozyme TL (*Thermomyces langinisa* (*Th. langinisa*) and *C. rugosa* lipase were screened in an attempt to hydrolyse the racemic α-chloro methyl ester **23** to the α-chloro acid racemate **22**, as illustrated in **Scheme 15**.

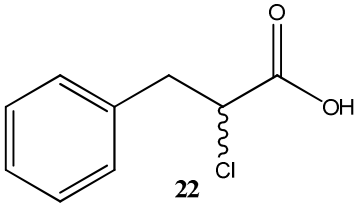
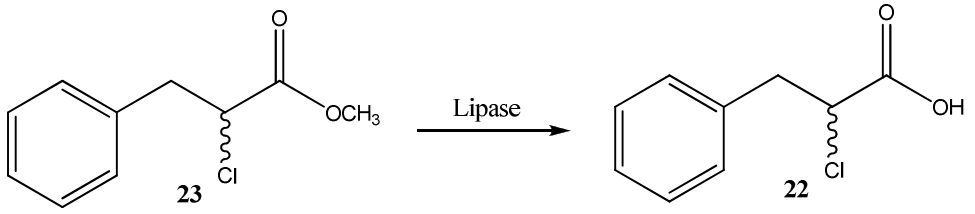


Figure 15: DL-α-chloro acid **22**.



Scheme 15: Enzymatic hydrolysis of DL-α-chloro methyl ester **23** to yield DL-α-chloro acid **22**.

It can be seen from the results shown in **Table 5** that Novozyme 435 and lipase from *C. rugosa* gave the greatest conversions of the DL-α-chloro methyl ester **23** to the DL-α-chloro acid **22**. Therefore in order to generate a useable quantity of the racemic acid **22** for subsequent investigations, the reaction was performed in batch using the immobilised lipase, Novozyme 435.

Lipase	Conversion / %
Novozyme 435	85.8
Lipozyme RM	62.6
Lipozyme TL	50.0
C. rugosa lipase	87.8

Table 5: Lipase catalysed hydrolysis of racemic α-chloro methyl ester **23** to yield racemic α-chloro acid, **22**. Reaction conditions; 40 ^{o}C, *ca*. 100mg lipase, 1mL substrate (0.02M in t-BuOH/Water (90: 10)) for 24 hr in batch.

3.4.2 ENZYMATIC KINETIC RESOLUTIONS

In order to evaluate the suitability of a range of lipases for enzymatic KR, screening reactions were carried out using the racemic esters depicted in **Figure 14**. This enabled the identification of those enzymes which possessed some enantioselectivity and may be suitable for the DKR, along with those that could be eliminated.

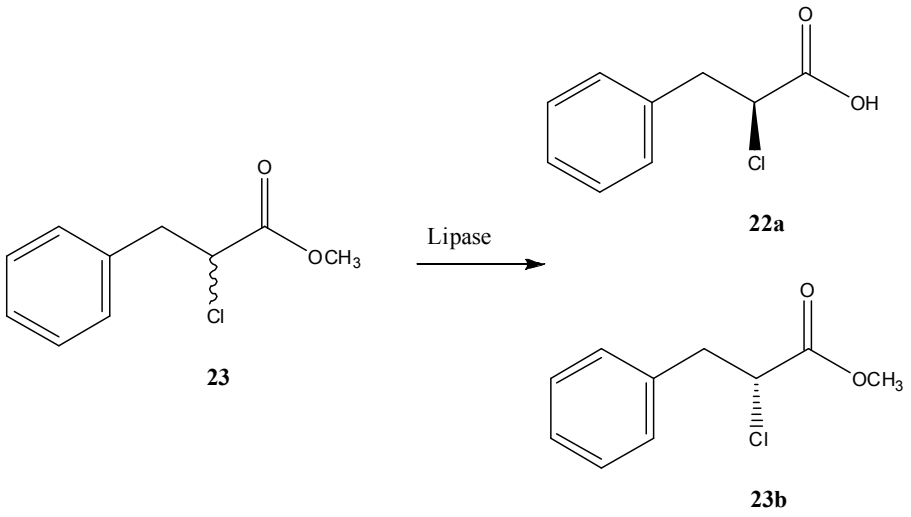
Using this approach, a range of free lipase enzymes from the following sources; *Achromobacter spp*.(AE04), *Pseudomonas stutzeri* (*Ps. stutzeri*) (AE07), *Rhizopus spp*.(AE08), *Alcaligines spp*. (AE011), *Pseudomonas cepacia* (*Ps. cepacia*) (Amano PS), *A. niger* (Amano AS) and *R. oryzae* (Amano D) were screened for the

enzymatic kinetic resolution of the racemic starting substrates **23**, **24**, **25**, and **26**. For clarity, the results obtained for each ester follow and are presented separately.

It is important to clarify at this stage that whilst 100mg (an excess) of each lipase enzyme was used in the free form in order to identify a suitable enantioselective enzyme, the comparative specific activity for each enzyme is not known. Therefore, whilst for the purpose of this investigation, this screening technique is sufficient and more than suitable to identify an appropriate enantioselective biocatalyst for each of the ester substrates under investigation, measurements of specific activities of each lipase enzyme would be necessary for a true comparative study.

3.4.2.1 RACEMIC DL-α-CHLORO METHYL ESTER

The lipase catalysed enantioselective resolution of DL-α-chloro methyl ester **23** to yield the desired enantiomeric D-acid product **22a** (compared to an enantiopure synthetic standard of L-α-chloro acid prepared in-house) and residual L-enantiomer, **23b** (**Scheme 16**) was investigated using the aforementioned lipases.



Scheme 16: Lipase catalysed enantioselective hydrolysis of the racemic ester **23** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **23b**.

It can be seen from the results shown in **Table 6** that of the lipases investigated, only AE04 and AE08 showed any sign of enantioselectivity towards the hydrolysis of the D-enantiomer **23a** of the DL-α-chloro methyl ester **23** and that lipase AE04 appears,

at this early stage be the most selective lipase of those studied. Enzymes AE07, Amano AS, AE011 and Amano D all had similar conversions of the ester to the acid with an average of 68% but these conversions, of over 50%, in conjunction with the low $\%ee_D$ values, illustrate that these lipases have very little, or no enantioselectivity towards either enantiomer. Although the rate of hydrolysis of the ester to acid appeared to be the fastest for lipase Amano PS, it can be seen from the $\%ee_D$ value (3%) that with this lipase there is again little or no enantioselectivity, hence this enzyme could also be discounted.

Lipase	Conversion to Acid / %	Acid / $\%ee_D$
AE04	52	47
AE07	71	-1
AE08	36	34
AE011	67	12
Amano PS	> 99	3
Amano AS	67	10
Amano D	66	-2

Table 6: Lipase catalysed enantioselective hydrolysis of the racemic ester **23** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **23b**. Reaction conditions; 40 ^oC, *ca.* 100mg lipase, 1mL substrate (0.02M in t-BuOH/water (90: 10)) for 24 hr in batch.

Having identified the most enantioselective lipases, AE04 and AE08, the kinetic resolution reaction was then repeated with the addition of trioctylamine (TOA) (1 equivalent), with the aim of maintaining a neutral pH to promote the reaction. The reaction was also analysed more frequently (2, 4 and 24 hr) using chiral HPLC and a summary of the results obtained can be seen in **Table 7**.

Lipase	Conversion to Acid / %			Acid / $\%ee_D$		
	2	**4**	**24**	**2**	**4**	**24**
AE04	42	52	61	72	48	41
AE08	29	37	42	63	34	30

Table 7: Lipase catalysed kinetic resolution screening reactions of the DL-α-chloro methyl ester racemate **23** with the most 'promising' lipase enzymes (AE04 and AE08), monitored at 2, 4 and 24 hr intervals. Reaction conditions; 40 ^oC, *ca.* 100mg lipase, 1mL substrate (0.02 M in t-BuOH/water (90: 10)) for 24 hr in batch.

It can be seen from the results that the conversion of the racemic DL-α-chloro methyl ester **23** to the desired enantiomeric acid product **22a** proceeds at a faster rate and with a greater degree of enantioselectivity for lipase AE04 *cf.* lipase AE08, where the conversion after 2 hr was 42 and 29% respectively (%ee_D = 71.9 and 63.0). After 2 hr, although the conversion of ester to acid does not massively increase with either enzyme (only an average 16% increase after 24 hr for both lipases), the enantiomeric excess of the D-acid, at 2 and 24 hr, decreases with an average of 32%. An observation that is attributed to the lipases catalysing the hydrolysis of the opposite L-enantiomer also, thus compromising the overall purity of the enantioenriched acid. Therefore, these results indicate that although lipase AE04 appears to be the most selective with a reasonable rate of reaction towards the D-enantiomer of the racemic ester substrate **23**, the rate of lipase catalysed hydrolysis of the L-enantiomer is not significantly slower ($k_D>k_L$, whereas $k_D>>k_L$ would be the ideal scenario). Consequently, unless a sufficient racemisation method can be established, in order to maintain a regular supply of the D-ester ($k_{rac}>k_D>k_L$), the batch enantioselective lipase catalysed reaction of this racemic α-chloro methyl ester **23** needs to be closely monitored in order to achieve D-acid **22a** in high enantiomeric purity. Following the screening experiments, the most selective enzyme, AE04, was used on a large scale and closely monitored to generate a useable quantity of the enantioenriched L-ester **23b** for racemisation studies conducted later.

From the results shown in **Table 8** it would seem that compared to the results obtained in the screening section, the rate at which lipase AE04 catalysed ester hydrolysis is somewhat slower (only 18 and 24% conversion of ester to acid after 2 and 4 hr) yet the selectivity is greater with only 1 and 3% of the unwanted enantiomer being consumed after 2 and 4 hr respectively. This observation is attributed to the use of a pH monitor and auto titrator on the larger scale as a means of maintaining the pH at 7, thus obtaining increased reaction control and selectivity, which consequently decreases the reaction rate.

It can be seen in **Table 8** that after 10 hr, the consumption of L-ester in the reaction becomes significant (6% L-ester consumed) despite there being some D-ester remaining in the reaction pot (18%). Therefore, the reaction was stopped after 10 hr and worked up accordingly. Chiral analysis followed the work up, affording the D-

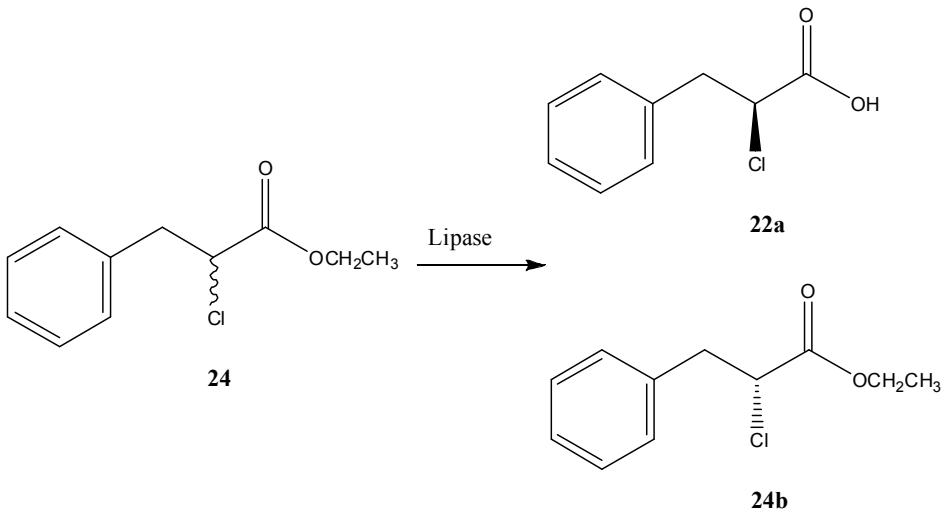
acid with an excellent 93%ee_D and enantioenriched α-chloro methyl ester with a respectable 60%ee_L. It is believed that the enantiomeric excess of the L-ester could have been improved had the reaction been allowed to proceed for longer, however this would have been at the cost of the purity of the enantiomeric acid and since the enantioenriched ester is only needed for racemisation purposes, a 60 %ee_L value (79% L-ester and 21% D-ester) was acceptable for purpose.

Time / hr	L-Ester / %	Acid / %
0.5	51	10
1	50	14
2	49	18
3	49	20
4	47	24
5	47	27
6	47	29
7	47	31
8	46	36
9	46	38
10	44	38

Table 8: Large scale, lipase catalysed, enantioselective hydrolysis of racemic α-chloro methyl ester **23** to yield the D-α-chloro acid **22a**. Reaction conditions; 40 ^oC, 1g lipase AE04 g $ester^{-1}$, 0.02M ester in t-BuOH/water (90: 10) for 24 hr in batch, with a constant pH of 7.

3.4.2.2 RACEMIC DL-α-CHLORO ETHYL ESTER

Having identified a suitable lipase for the kinetic resolution of DL-α-chloromethyl ester **23**, attention was turned to the identification of a suitable enzyme for the resolution of DL-α-chloroethyl ester **24**.



Scheme 17: Lipase catalysed enantioselective hydrolysis of the racemic ester **24** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **24b**.

Under the conditions described in **Section 3.4.2.1**, the resolution of ester **24** was evaluated and as **Table 9** illustrates, lipases AE04 and AE08 were again found to be the most selective, with conversions of 43 and 33% obtained respectively and high $\%ee_D$ values for the acid (51 and 49% respectively) after 24 hr. Lipases AE07 and Amano AS again resulted in conversions of greater than 50% (69 and 52% respectively) and exhibited low enantioselectivity (27 and 16% respectively). However, as illustrated in **Table 8** for the methyl ester, these two lipases may still show promise when monitored more closely (**Table 10**). In comparison, AE011, Amano PS and Amano D showed very little or no enantioselectivity, with high, conversions of ester to acid (76% average) and very low % ee_D of acid (2% average).

Enzyme	Conversion to Acid / %	Acid / % ee_D
AE04	43	52
AE07	69	27
AE08	33	49
AE011	79	0
Amano PS	81	3
Amano AS	52	16
Amano D	69	3

Table 9: Lipase catalysed enantioselective hydrolysis of the racemic ester **24** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **24b**. Reaction conditions; 40 ^{o}C, *ca.* 100mg lipase, 1mL substrate (0.02M in t-BuOH/water (90: 10)) for 24 hr in batch.

With this in mind, lipases AE04, AE07, AE08 and Amano AS were utilised in further kinetic resolution experiments of the ester substrate, employing TOA (1 eq.) to maintain a neutral pH and the reactions were analysed at intervals of 2,4 and 24 hr using chiral HPLC.

Lipase	Conversion to Acid / %			Acid / %ee_D		
	2	**4**	**24**	**2**	**4**	**24**
AE04	46	57	66	60	52	34
AE07	83	> 99	> 99	6	5	1
AE08	38	47	61	41	36	22
Amano PS	56	64	72	10	13	3

Table 10: Lipase catalysed kinetic resolution screening reactions of the DL-α-chloro ethyl ester racemate **24** with the most 'promising' lipase enzymes (AE04, AE07, AE08 and Amano PS), monitored at 2, 4 and 24 hr intervals. Reaction conditions; 40 ^{o}C, *ca.* 100mg lipase, 1mL substrate (0.02M in t-BuOH/water (90: 10)) for 24 hr in batch.

As **Table 10** illustrates, the additional screening confirms that AE07 and Amano AS are not selective and are not enantioselective for the reaction under investigation, owing to the fast reaction rates observed when using these two enzymes as the biocatalyst. Furthermore, the poor %ee_D values observed after 2 hr (6 and 10% respectively) discount lipases AE07 and Amano AS as enantioselective biocatalysts for the lipase catalysed resolution of DL-α-chloro ethyl ester **24**. Lipase AE04 in particular appeared to display enantioselectivity towards the D-substrate, compared to the resolution of racemic α-chloro methyl ester **23** (**Section 3.4.2.1**) and is the most suitable biocatalyst for the ethyl ester, exhibiting a good degree of selectivity and reaction rate.

In order to generate a sufficient quantity of the enantioenriched L-ester **24b** for further racemisation studies, lipase AE04 was employed in a large batch reaction.

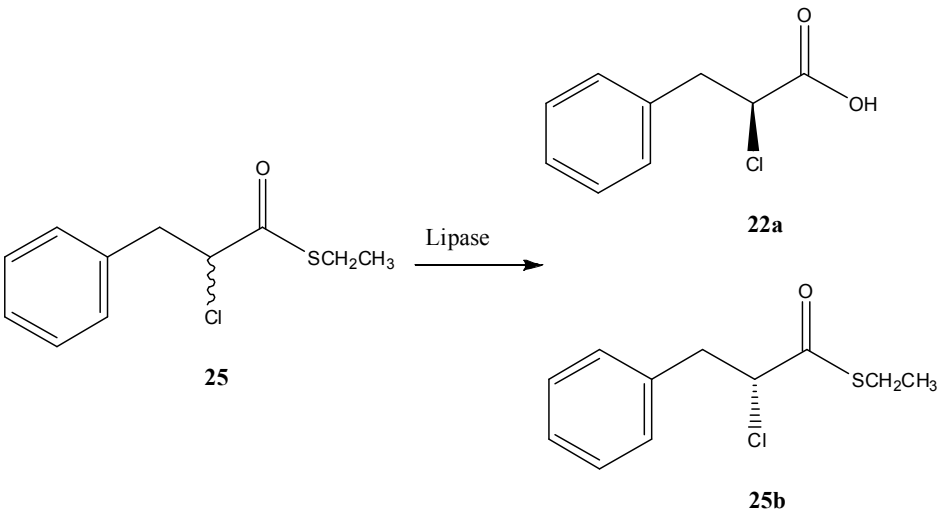
Time / hr	L-ester / %	Acid / %
0.5	50	3
1	51	7
2	50	14
3	49	16
4	49	16
5	48	16
6	47	17
7	47	17
8	45	18
24	43	35

Table 11: Large scale, lipase catalysed, enantioselective hydrolysis of racemic α-chloro ethyl ester **24** to yield the D-α-chloro acid **22a**. Reaction conditions; 40 ^{o}C, 1g lipase AE04 g ester^{-1}, 0.02M ester in t-BuOH/water (90: 10) for 24 hr in batch, with a constant pH of 7.

It can be seen from the results shown in **Table 11** that the enantioselective hydrolysis of the racemic α-chloro ethyl ester **24** is considerably slower than for the DL-α-chloro methyl ester **23** (**Table 8**); in fact, after 8 hr, there was only an 18% conversion of ester to acid. It was therefore decided to allow the reaction to proceed overnight in an attempt to synthesise L-α-chloro ethyl ester for the racemisation studies. After 24 hr the reaction was stopped and a work up performed to afford the D-acid with an excellent 94%ee$_D$ and the enantioenriched α-chloro ethyl ester with a poor 31%ee$_L$. It is believed that the enantiomeric excess of the L-ester could have been improved had the reaction been allowed to proceed for longer, however this would have again been at the cost of the enantiomeric purity of the acid and since the enantioenriched ester is needed for racemisation study, a 30 %ee$_L$ value (65% L-ester and 35% D-ester) was deemed acceptable for purpose.

3.4.2.3 RACEMIC DL-α-CHLORO ETHYL THIOESTER

Building on the successful screening of nine lipases for the hydrolysis of the methyl **23** and ethyl **24** esters, the process was repeated for the ethyl thio ester **25** (**Scheme 18**) in order to identify a suitable biocatalyst for the kinetic resolution.



Scheme 18: Lipase catalysed enantioselective hydrolysis of the racemic ester **25** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **25b**.

Table 12 highlights the results obtained for the initial screening experiments for a range of lipases used in the enzyme catalysed enantioselective hydrolysis of the DL-α-chloro ethyl thioester **25** to the enantiomeric D-α-chloro acid **22a** and it is clear that, of the three racemic ester substrates studied so far, the ethyl thio ester **25** can be selectively hydrolysed by a large number of the lipases evaluated.

Clearly, lipase Amano D could be discounted immediately, as although the conversion of ester to acid is less than 50% after 24 hr (an indication that the enzyme could be enantioselective), it is clear from the corresponding ee (-5.3%) that the enzyme has preference towards either enantiomer of the ester substrate and is in fact almost totally unselective. Likewise, lipase Amano PS was unsuitable for the enantioselective hydrolysis as both the ee_D and ester conversion is low after 24 hr. In the case of lipases AE07 and AE011, the conversion of ester to acid is not significantly higher than 50% after 24 hr (62 and 52% for lipases AE07 and AE011 respectively) and the ee_D value is acceptable (21 and 34%) and was potentially only compromised as a result of the reaction not being stopped at the required point. However, it is lipase AE08, which at this stage would appear to be the most enantioselective lipase with respect to the selective hydrolysis of the racemic α-chloro ethyl thio ester, yielding the highest ee_D value of 72% with a conversion of ester to acid of 26%, after 24 hr. Although the rate of reaction seems low, a slow rate is often the compromise for a product with high enantiomeric excess.

Lipase	Conversion to Acid / %	Acid / % ee_D
AE04	36	51
AE07	62	21
AE08	26	72
AE011	52	34
Amano PS	57	12
Amano AS	36	40
Amano D	28	-5

Table 12: Lipase catalysed enantioselective hydrolysis of the racemic ester **25** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro thioester **25b**. Reaction conditions; 40 ^{o}C, *ca.* 100mg lipase, 1mL substrate (0.02 M in t-BuOH/water (90: 10)) for 24 hr in batch.

From these observations, lipases AE04, AE07, AE08, AE011 and Amano AS were utilised in further kinetic resolution experiments of the thioester **25** and underwent analysis by chiral HPLC at 2, 4 and 24 hr intervals.

TOA was added to the hydrolysis reactions involving the racemic substrates **23** and **24**, in an attempt to increase the conversion of ester to enantiomeric acid, by maintaining a pH as close to 7 as possible. Whilst it is believed that TOA is not a strong enough base to racemise enantiomeric esters, such as the ones reported in **Sections 3.4.2.1** and **3.4.2.2**, as they are less acidic than thioesters, it is well known in the literature that TOA is able to racemise enantiomeric thioesters (Um & Drueckhammer, 1998), which would have an overall effect on the hydrolysis reaction results. For this reason, prior to adding TOA to the second stage screening studies for this racemic thioester substrate, as was carried out previously, an additional kinetic resolution experiment, with and without the addition of TOA (1 eq.), was carried out using the DL-α-chloro ethyl thioester substrate and one of the lipases under investigation (lipase AE011). The composition of the L-ester peak was used as measure of how the reaction was proceeding (bearing in mind that it is the D-ester which is hydrolysed with the lipase), the results of which can be seen in **Table 13**.

Time / hr	L-Ester / %	
	With TOA	Without TOA
1	52	58
2	31	43
3	24	38
4	18	34

Table 13: Lipase catalysed enantioselective hydrolysis of the racemic ester **25** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro thioester **25b**. Reaction conditions; 40 ^{o}C, *ca.* 100mg lipase, 1mL substrate (0.02 M in t-BuOH/water (90: 10)), with and without the addition of TOA (1eq) for 24 hr in batch.

It can be seen from **Table 13** that with the addition of TOA to the reaction, the proportion of L-ester in the reaction mix decreases more rapidly *cf.* the analogous reaction conducted in the absence of the base, suggesting that TOA has the ability to racemise the L-ester as the hydrolysis reaction proceeds. It would seem that as the D-ester is consumed in the hydrolysis reaction, the TOA, in an attempt to re-establish an equimolar mixture of the ester substrate, is slowly racemising the less reactive L-ester and consequently, the composition of L-ester in the reaction mixture decreases as the racemate forms. Alternatively, the TOA may be acting as a base catalyst to the hydrolysis reaction and the L-ester composition is decreasing as it is converted to the corresponding acid. Additional investigations would be required in order to identify whether the L-ester was being converted to the acid or racemised. However, with respect to the investigation herein, the fact that the addition of TOA was having an effect on the results, TOA will not be added to the reaction mixture but the base will be considered in the future when investigating racemising agents in order to establish a DKR set up. The results obtained for the detailed screening of lipases for the kinetic resolution of racemic thioester **25** are presented overleaf in **Table 14**.

Lipase	Conversion to Acid / %			Acid / % ee_D		
	2	**4**	**24**	**2**	**4**	**24**
AE04	14	18	36	79	76	51
AE07	47	48	62	39	33	21
AE08	1	1	26	-	-	72
AE011	42	45	52	73	57	34
Amano AS	1	4	36	-	-	40

Table 14: Lipase catalysed kinetic resolution screening reactions of the DL-α-chloro ethyl thioester racemate **25**, with the most 'promising' lipase enzymes (AE04, AE07, AE08, AE011 and Amano AS), monitored at 2, 4 and 24 hr intervals. Reaction conditions; 40 ^oC, *ca.* 100mg lipase, 1mL substrate (0.02M in t-BuOH/water (90: 10)) for 24 hr in batch.

When the results from the initial screening experiment (**Table 12**), using the racemic α-chloro ethyl thioester as a substrate are compared to those presented in **Table 14**, where more frequent analysis was conducted, it is clear that carrying out the additional screening, with extra analysis is of high importance. From the initial screening experiments (**Table 12**), lipase AE08 appeared to be the most suitable lipase of those investigated, where even though the rate of hydrolysis was slow the excellent selectivity of the lipase compensated for this. However, upon completion of the second screening experiment, it is clear form the results displayed in **Table 14**, that lipase AE011 is by far the most suited biocatalyst for the enantioselective hydrolysis of this substrate, yielding a high conversion of ester to acid (42%), with a good enantiomeric excess (73%), after only 2 hr.

The importance of frequent reaction monitoring is evident here as the $\%ee_D$ of the acid significantly decreases over time when the L-ester becomes involved in the hydrolysis process. It is this that lead to the misleading results for this enzyme (52% conversion, 34% ee_D acid) initially observed after 24 hr. Lipase AE08, although yielding an impressive ee_D value after 24 hr, has a reaction rate that is far too slow, especially in comparison to most of the other lipases studied. In fact, lipases AE04 and AE011 resulted in similar $\%ee_D$ values (79 and 73% respectively) in a much shorter time frame. Although lipase AE04 resulted in an ee_D value comparable to lipase AE011 after only 2 hr, the rate of reaction was much slower, with only 14% conversion of ester to acid after 2 hr, 18% after 4 hr and still only 36% conversion after the full 24 hr evaluation period.

Of the other two lipases investigated, although lipase AE07 hydrolysed the α-chloro ethyl thioester at an acceptable rate, with 47% after 2, the corresponding ee_D was poor (39%). Lipase Amano AS on the other hand had an extremely slow rate of reaction (only 4% conversion of ester to acid after 4 hr) with regards to the hydrolysis of the ester in conjunction with an overall poor ee_D value (40% after 24 hr).

Following the screening experiments, lipase AE011 was employed in the batch scale hydrolysis of substrate **25** in order to synthesise a sufficient quantity of the enantioenriched L-ester for subsequent racemisation studies (**Table 15**).

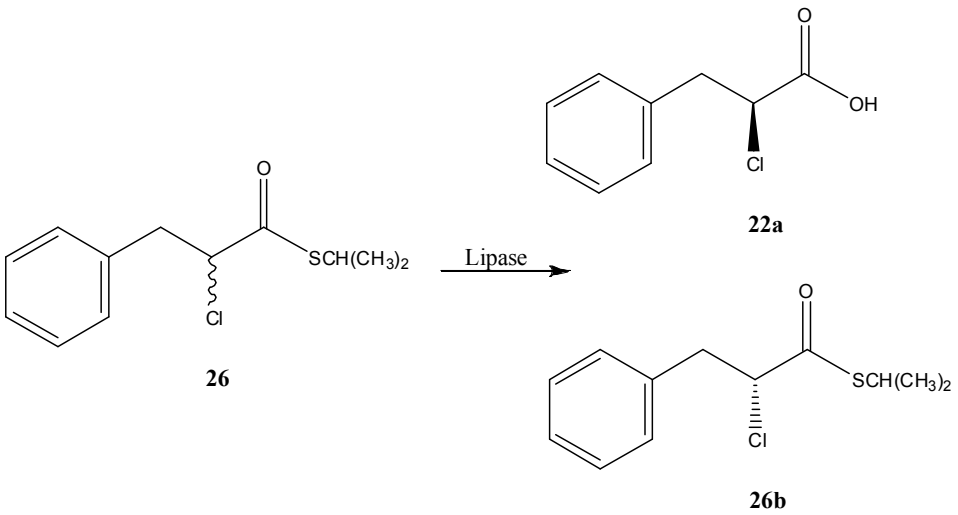
Time / hr	Ester / %	Acid / %
1	64	36
1.5	64	36
3	64	36
5	64	36
8	64	36

Table 15: Large scale, lipase catalysed, enantioselective hydrolysis of racemic α-chloro ethyl ester **25** to yield the D-α-chloro acid **22a**. Reaction conditions; 40 ^{o}C, 1g lipase AE011 g ester^{-1}, 0.02M ester in t-BuOH/water (90: 10) for 24 hr in batch, with a constant pH of 7.

The reaction was stopped after 8 hr when it appeared that no further reaction was occurring and the reaction worked up using the standard procedure detailed in **Section 3.5**, to afford the D-acid with an excellent 94%ee_D and enantioenriched α-chloro ethyl thioester with an equally impressive 90%ee_L.

3.4.2.4 RACEMIC DL-α-CHLORO i-PROPYL THIOESTER

The identification of a suitable biocatalyst for the enantioselective resolution of a sterically hindered ester, DL-α-chloro i-propyl ester **26**, to yield the desired enantiomeric acid **22a** and residual L-enantiomer, **26b** (**Scheme 19**) was subsequently investigated and the results are summarised in **Table 16**.



Scheme 19: Lipase catalysed enantioselective hydrolysis of the racemic ester **26** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **26b**.

Enzyme	Conversion to Acid / %	Acid / %ee_D
AE04	0	-
AE07	58	24
AE08	0	-
AE011	21	29
Amano PS	61	21
Amano AS	4	-
Amano D	0	-

Table 16: Lipase catalysed enantioselective hydrolysis of the racemic ester **26** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro thioester **26b**. Reaction conditions; 40 ^{o}C, *ca.* 100mg lipase, 1mL substrate (0.02 M in t-BuOH/water (90: 10)) for 24 hr in batch.

It can be seen from the preliminary screening results detailed in **Table 16** that out of the nine lipases investigated, four of them (lipases AE04, AE08, Amano AS and Amano D) were unable to catalyse the resolution illustrated in **Scheme 19**. An observation that is attributed to the steric bulk of the i-propyl ester ($CH(CH_3)_2$), making it more difficult for this substrate to be incorporated into the enzymes active site, resulting in little or no conversion of ester to acid. Likewise, lipase AE011 resulted in a low conversion of the racemic substrate to the acid product after 24 hr (21%) with a poor enantiomeric excess for the D-acid (29%).

In comparison, lipases AE07 and Amano PS gave the highest conversions after the 24 hr reaction period, under the conditions studied (58 and 61% respectively) but again, the $\%ee_D$ values were somewhat disappointing (24 and 21%). Despite this, lipases AE011, AE07 and Amano PS were investigated further in additional screening experiments, in an attempt to identify the most suitable biocatalyst for the reaction under investigation. Prior to performing the screening, the effect of TOA (1 eq.) was again investigated and found to result in the rapid racemisation of the L-ester and therefore was not employed in further reactions of thio esters.

Lipase	**Conversion to Acid / %**			**Acid / $\%ee_D$**		
	2	**4**	**24**	**2**	**4**	**24**
AE07	43	49	58	59	39	24
AE011	0	0	21	-	-	29
Amano PS	10	32	61	57	38	21

Table 17: Lipase catalysed kinetic resolution screening reactions of the DL-α-chloro i-propyl thioester racemate **26**, with the most 'promising' lipase enzymes (AE07, AE011 and Amano PS), monitored at 2, 4 and 24 hr intervals. Reaction conditions; 40 ^oC, *ca.* 100mg lipase, 1mL substrate (0.02 M in t-BuOH/water (90: 10)) for 24 hr in batch.

It can be seen from the results detailed in **Table 17** that lipase AE07 was the most suitable enzyme for the hydrolysis of the racemic α-chloro i-propyl thioester **26**, where after 2 hr, an excellent conversion of ester to acid of 43% was observed with a respectable corresponding ee_D value for the acid (59%). Despite lipase AE011 being ideal for the enantioselective hydrolysis of the racemic α-chloro ethyl thioester **25** the rate of hydrolysis for this substrate, under analogous conditions, was extremely slow with no conversion of ester to acid seen after 4 hr; further illustrating the need for a rapid screening tool.

Lipase Amano PS resulted in similar $\%ee_D$ values for the acid (57% after 2 hr) to lipase AE07 (59%) but the rate of hydrolysis under the conditions studied was somewhat slower with only 10% conversion observed after 2 hr *cf.* 43% using lipase AE07. The screening summarized in **Table 17** therefore identified lipase AE07 as the most suitable enzyme for the enantioselective hydrolysis of the α-chloro propyl thioester **26** and used for the synthesis of enantioenriched L-ester for racemisation studies (**Table 18**).

Time / hr	L-Ester / %	Acid/ %
1	49	8
2	51	12
3	49	16
4	51	14
5	50	21
6	52	30
7	52	31
8	50	31
9	47	37

Table 18: Large scale, lipase catalysed, enantioselective hydrolysis of racemic α-chloro i-propyl thioester **26** to yield the D-α-chloro acid **22a**. Reaction conditions; 40 ^{o}C, 1g lipase AE07 g ester^{-1}, 0.02M ester in t-BuOH/water (90: 10) for 24 hr in batch, with a constant pH of 7.

From the results shown in **Table 18**, it would seem that *cf.* the results obtained in the second screening experiment (**Table 17**) the rate at which lipase AE07 catalysed the ester hydrolysis is somewhat slower. The selectivity however is greater, with no unwanted enantiomer (L-ester) being consumed until after 8 hr. This trend is consistent with that seen for the batch enantioselective hydrolysis of the racemic α-chloro methyl ester **23**, discussed earlier and are again attributed to the increased reaction control through the use of an auto titrator to maintain pH.

After 9 hr the results would suggest that the L-ester enantiomer is being consumed in the hydrolysis reaction (a decrease from 50 to 47% of the reaction mixture), despite there being some D-ester remaining (16%). Therefore, as this was an attempt to yield some enantioenriched L-ester and D-acid, with high enantiomeric excess, the reaction was stopped after 9 hr and worked up accordingly to afford the D-acid with an excellent 91%ee_D and enantioenriched α-chloro i-propyl thioester with a good 60 %ee_L.

The results of these four kinetic resolution reactions afforded the desired hydrolysis product **22a** in excellent enantiomeric excess (> 90%ee_D in all cases) and workable to excellent ee_L figures (between 30 and 90%) for the recovered L-ester. Therefore, an

investigation into the racemisation of the unwanted, enantiomeric esters to enable the establishment of DKR reactions was conducted.

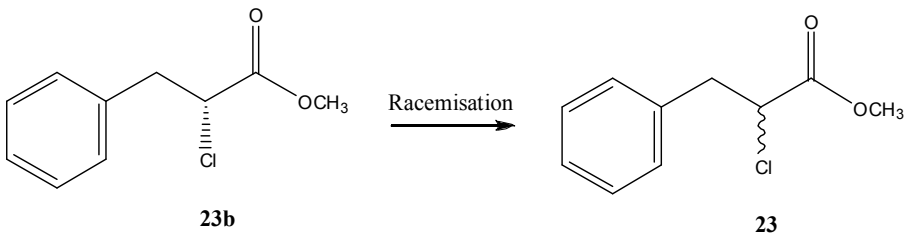
3.4.3 RACEMISATIONS

As discussed previously, DKR is governed by the generation of a continuous equilibration (racemisation) of both enantiomers. Therefore, the racemisation step is an important parameter that needed to be investigated, prior to attempting a DKR reaction. With good procedures in hand and enantioselective lipases identified for the kinetic resolutions of the four racemic esters attention was turned to the selective racemisation of the enantioenriched esters.

As base catalysed racemisation is well known and probably the most frequently used method for the racemisation of optically pure organic substrates, a range of bases of varying strength were investigated, including; pyridine (PY), dicyclohexylamine (DCHA), *tert*-butylamine (TEBA), TOA, tri-butylamine (TRBA), *N,N*-diisopropylethylamine (DIPEA), 1,4-diazabicyclo[2.2.2]octane (DABCO), proton sponge (PS) and 1,8-diazabicycloundec-7-ene (DBU). The results are again presented separately for each ester, starting with the enantioenriched α-chloro methyl ester **23b**.

3.4.3.1 ENANTIOENRICHED α-CHLORO METHYL ESTER

The racemisation of L-α-chloro methyl ester **23b** to yield the desired DL-racemate **23** (**Scheme 20**) was investigated using a range of racemising bases and the results are presented in **Table 19**.



23b

% ee_L = 60

23

% ee_L = 0

Scheme 20: Racemisation of enantioenriched α-chloro methyl ester **23b** to yield the racemic ester **23**.

Unfortunately, within experimental error, none of the organic bases evaluated were found to cause racemisation of L-α-chloro methyl ester **23b**. This observation suggested that the free bases were not of sufficient strength to catalyse the racemisation process of the enantioenriched α-chloro methyl ester **23b**. For DBU, it appears to be the case that whilst this particularly strong organic base is not capable of racemising the enantioenriched α-chloro methyl ester, it has caused some breakdown of the ester substrate. Almost immediately, a decrease in the ester peak was seen on the reverse phase HPLC trace with an increase in size of an additional peak appearing on the reverse phase HPLC trace, at 4.2 min, over time. It is thought that this could be a possible elimination reaction leading to the formation of an alkene, where deprotonation does occur, followed by very rapid elimination of the chloride, in conjunction with acid catalysed hydrolysis.

Racemising Base	**Ester / %ee$_L$ over time**				
	1hr	**2hr**	**4hr**	**6hr**	**24hr**
PY	59	61	60	60	59
DCHA	60	60	61	59	60
TEBA	59	61	60	59	59
TOA	60	61	61	61	59
TRBA	59	60	60	59	60
DIPEA	59	59	59	61	60
DABCO	61	59	61	59	60
PS	Base peak co-eluted with ester peaks				
DBU	Breakdown of ester				

Table 19: Base catalysed racemisation reactions of the enantioenriched α-chloro methyl ester enantiomer **23b**, with a range of free bases. Reaction conditions; 2 equivalents of base, 1mL substrate (0.02M in t-BuOH/water (90: 10) at 40 ^{o}C in batch.

Based on literature precedent, a range of chlorides were employed as racemising agents, proposing racemisation *via* the S_N2 reaction mechanism route (halide by halide displacement). With examples of this type involving chloride, bromide and iodide been reported (Jones & Williams, 1998; Carrea & Riva, 2000; Huerta et al., 2001; Caddick & Jenkins, 1996). Work also carried out by Badjic *et al*. (Badjic *et*

al., 2001) using a Wittig reagent (triphenylphosphonium chloride immobilised to Merrifield resin) demonstrated successful racemisation of chloro propionate and Haugton & Williams (2001), used a series of ammonium chloride salts in water at 40 ^{o}C for 16 hr to racemise bromo substituted esters

The chlorides studied therefore were, Aliquat 336, tetrabutyl ammonium chloride, tetraphenyl phosphonium chloride, tributylmethyl phosphonium chloride (polymer bound, 1.4 mmol Cl g^{-1} resin), chloride (polymer bound, 4 mmol Cl g^{-1} resin), Merrifield polymer (4.3 mmol Cl g^{-1} resin), and triphenylphosphonium chloride (polymer bound, 4 mmol Cl g^{-1} resin). The results obtained for the chlorides are presented in **Table 20**. Again, within experimental error, no racemisation was observed.

Racemising Chloride	**Ester / %ee$_L$**
Aliquat 336	67
Tetrabutyl ammonium chloride	67
Tetraphenyl phosphonium chloride	59
Tributylmethyl phosphonium chloride	65
Polymer bound chloride	59
Merrifield polymer	57
Triphenylphosphonium chloride	55

Table 20: Chloride catalysed racemisation reactions of the enantioenriched α-chloro methyl ester enantiomer **23b**, with a range of free and immobilised chlorides. Reaction conditions; 2 equivalents of chloride, 1mL substrate (0.02M in t-BuOH/water (90: 10)) at 40 ^{o}C in batch, for 24 hr.

As none of the racemisation experiments using the enantioenriched α-chloro methyl ester **23b** had been successful using organic bases or chlorides, the experiments were repeated employing a range of organic solvents of varying polarities. This would enable us to evaluate if the reaction solvent was retarding racemisation. Using the same free bases (**Table 19**) and chlorides (**Table 20**), the racemisation experiments were repeated, in five additional solvents; hexane, toluene, DCM, ACN and DMSO and the results summarised in **Table 21** and **22**.

Racemising Base	Ester / %ee$_L$				
	Hexane	**Toluene**	**DCM**	**ACN**	**DMSO**
PY	59	61	61	60	59
DCHA	61	60	60	60	59
TEBA	62	60	59	59	59
TOA	61	62	59	59	61
TRBA	60	59	59	59	60
DIPEA	59	61	60	60	60
DABCO	59	61	61	58	59
PS	Base peak co-eluted with ester peaks				
DBU	Breakdown of ester				

Table 21: Base catalysed racemisation reactions of the enantioenriched α-chloro methyl ester enantiomer **23b** with a range of free bases. Reaction conditions; 2 equivalents of base, 1mL substrate (0.02M in a range of solvents at 40 ^oC in batch.

It can be seen from **Table 21** that racemisation of ester **23b** *via* enolate formation is not possible using any of the organic bases and solvent combinations studied. It would seem that the α-hydrogen atom is not sufficiently acidic enough for the reaction to take place. In comparison however it can be seen that altering the solvent system, resulted in successful racemisation with a range of chlorides (highlighted in the table in bold italic).

Racemising Chloride	Ester / %ee$_L$				
	Hexane	**Toluene**	**DCM**	**ACN**	**DMSO**
Aliquat 336	***1***	***14***	***36***	***4***	***9***
Tetrabutyl ammonium chloride	62	59	59	60	59
Tetraphenyl phosphonium chloride	64	60	60	59	60
Tributylmethyl phosphonium chloride	***1***	***43***	58	***27***	***25***
Polymer bound ammonium chloride	***15***	54	55	56	***22***
Merrifield polymer	60	59	59	62	60
Triphenylphosphonium chloride	63	***47***	***47***	***39***	***16***

Table 22: Chloride catalysed racemisation reactions of the enantioenriched α-chloro methyl ester enantiomer **23b** with a range of free and immobilised chlorides. Reaction conditions; 2 equivalents of chloride, 1mL substrate (0.02M in a range of solvents) at 40 ^oC in batch, for 24 hr.

Chlorides Aliquat 336, Tributylmethyl phosphonium chloride, polymer bound ammonium chloride and polymer bound triphenylphosphonium chloride would appear to be the most effective racemising chlorides investigated and capable of initiating, to different extents, S_N2 displacement and hence racemisation when utilised in an appropriate solvent.

As **Section 3.4.2.1** detailed, the kinetic resolution of racemic DL-α-chloro methyl ester **23** using lipase AE04 was achieved in 1 hr; consequently, for DKR to be successful, the racemisation step should ideally be much faster than the kinetic resolution ($k_r >> k_D >> k_L$). Although it is not yet known whether the extent or speed of racemisation is sufficient for a DKR reaction, further investigations using these materials were conducted at intervals of 4 and 24 hr (**Table 23**).

Racemising Chloride	**Ester / %ee$_L$**									
	4 hr					**24 hr**				
	Hexane	**Toluene**	**DCM**	**ACN**	**DMSO**	**Hexane**	**Toluene**	**DCM**	**ACN**	**DMSO**
Aliquat 336	***23***	58	61	56	***21***	***1***	14	36	4	***9***
Tributylmethyl phosphonium chloride	45	58	-	53	55	1	43	-	27	25
Polymer bound ammonium chloride	58	-	-	-	60	15	-	-	-	22
Triphenylphosphonium chloride	-	58	58	56	***31***	-	47	47	39	***16***

Table 23: Chloride catalysed racemisation reactions of the enantioenriched α-chloro methyl ester enantiomer **23b** with a range of free and immobilised chlorides. Reaction conditions; 2 equivalents of chloride, 1mL substrate (0.02M in a range of solvents) at 40 ^oC in batch.

From this investigation, it was clear that Aliquat 336 in hexane or DMSO and polymer bound triphenylphosphonium chloride also in DMSO resulted in perhaps sufficiently rapid racemisation of the enantioenriched α-chloro methyl ester substrate **23b** to the corresponding DL-racemate **23** (highlighted in **Table 23** in bold italics). Aliquat 336 showed the most promising results of the two chlorides with a decrease in %ee$_L$ of the enantioenriched ester from 60% to 23% and 1% after 4 and 24 hr respectively in hexane and similarly from 60% to 21% and 9% after 4 and 24 hr respectively in the polar solvent DMSO, demonstrating no link between solvent polarity and the rate of racemisation.

At this stage it is important to point out that even if the racemisation step proceeds at a fast enough rate in a certain solvent, if the kinetic resolution cannot also be achieved in the same solvent, then the DKR will not be possible. Although theoretically it would be possible to conduct the kinetic resolution step in one solvent, remove this solvent and then carry out the racemisation process in the second required solvent and cycle these steps the sufficient number of times, this would be extremely laborious and very difficult to conduct on both a batch and miniaturised scale.

Therefore, as ultimately this racemisation process would be used in a DKR set-up in an attempt to achieve the D-acid in 100% yield with as close to 100% ee_D as possible, the kinetic resolution reactions of racemic DL-α-chloro methyl ester **23**, using the most selective lipase enzyme, identified from **Section 3.4.2.1** (AE04) were repeated in hexane and DMSO, which had proved successful for the racemisation step; the results are summarised in **Table 24**.

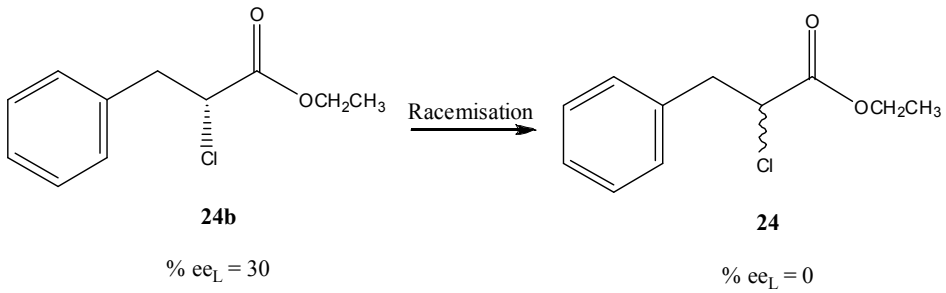
Solvent: Water (90: 10)	Conversion to Acid after 24 hr / %
Hexane	0
DMSO	23
t-BuOH	53

Table 24: Lipase catalysed enantioselective hydrolysis of the racemic ester **23** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro methyl ester **23b**. Reaction conditions; 40 ^{o}C, *ca.* 100mg lipase, 1mL substrate (0.02 M in hexane, DMSO or t-BuOH/ water (90: 10)) for 24 hr in batch.

It can be seen that the kinetic resolution of racemic α-chloro methyl ester **23** to yield the acid product, does not proceed to any extent in the non polar solvent hexane and although successful in DMSO the reaction is slow *cf.* t-BuOH / water. These results indicate that the kinetic resolution reaction does proceed, even if somewhat slowly, in the DMSO and that Aliquat 336 and polymer bound triphenylphosphonium chloride are capable of racemising the L-ester enantiomer in the same solvent. Therefore, DKR reactions using these two chlorides in DMSO were conducted using lipase AE04 and the results are detailed in **Section 3.4.4**.

3.4.3.2 ENANTIOENRICHED α-CHLORO ETHYL ESTER

Having identified two suitable racemising agents for the methyl ester, the racemisation of L-α-chloro ethyl ester **24b** to yield the desired DL-racemate **24** (**Scheme 21**) was investigated using the same racemising agents.



24b

% ee_L = 30

24

% ee_L = 0

Scheme 21: Racemisation of enantioenriched α-chloro ethyl ester **24b** to yield the racemic ester **24**.

It can be seen from **Table 25**, that excluding experimental error, no significant racemisation was observed for the nine organic bases studied. Again this suggests that the bases were not of sufficient strength to catalyse the racemisation process, with DBU seen to promote decomposition of the ester.

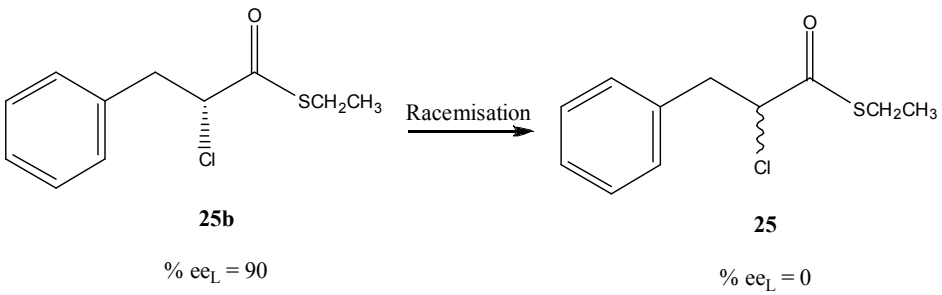
Racemising Base	**Ester / %ee_L over time**				
	1hr	**2hr**	**4hr**	**6hr**	**24hr**
PY	32	30	35	30	33
DCHA	30	28	30	32	30
TEBA	29	29	32	33	34
TOA	31	30	29	29	32
TRBA	30	31	32	30	31
DIPEA	29	29	34	30	30
DABCO	30	31	31	30	31
PS	Base peak co-eluted with ester peaks				
DBU	Breakdown of ester				

Table 25: Base catalysed racemisation reactions of the enantioenriched α-chloro methyl ester enantiomer **24b** with a range of free bases. Reaction conditions; 2 equivalents of base, 1mL substrate (0.02M in t-BuOH/water (90: 10)) at 40 ^oC in batch.

As these results show a great deal of similarity to those obtained for the base catalysed racemisation of the enantioenriched α-chloro methyl ester **23b** reported earlier (**Section 3.4.3.1**). It was decided that the slight difference between the two esters (only one extra CH_2 group in the alkyl chain in this example) is not significant enough to justify repeating the volume of work conducted with regards to the α-chloro methyl ester **23b** (investigating a range of chlorides and solvents). This was because it is more than likely that the results obtained would be similar, if not identical. The similarity between these two compounds is supported by the fact that the most suitable enzyme for the lipase catalysed kinetic resolution of these two racemic esters (**23** and **24**) was identified to be AE04. Therefore, only α-chloro methyl ester **23** will be used in the remainder of the investigation.

3.4.3.3 ENANTIOENRICHED α-CHLORO ETHYL THIOESTER

To identify those reaction conditions suitable for the racemisation of L-α-chloro ethyl thioester **25b** to the desired DL-racemate **25** (**Scheme 22**), nine organic bases were investigated in t-BuOH / water as illustrated in **Table 26**.



25b

% ee_L = 90

25

% ee_L = 0

Scheme 22: Racemisation of enantioenriched α-chloro ethyl ester **25b** to yield the racemic ester **25**.

Of the bases investigated, the enantioenriched α-chloro ethyl thioester **25b** was found to be inert to pyridine (PY); however six of the organic bases (DCHA, TEBA, TOA, TRBA, DIPEA and DABCO) were found to racemise α-chloro ethyl thioester at varying rates. TOA resulted in the slowest racemisation, with 27% of the L-ester ee_L remaining after 24 hr. Likewise, TRBA appeared to have a particularly slow racemisation rate, but did manage to achieve a racemic mixture (2%ee_L) of the α-chloro ethyl thioester after the full 24 hr at 40^oC. It is believed however, that this rate would be too slow for a dynamic kinetic resolution set up to be achieved, where

as discussed previously (**Section 3.3.2.3**), the rate of racemisation needs to be rapid with respect to the rate of hydrolysis ($k_{rac}>>k_D$). DCHA, TEBA, DIPEA and DABCO all had fast rates of racemisation of the enantioenriched L-ester. However, despite DABCO appearing to be a suitable racemising base with respect to the enantioenriched α-chloro ethyl thioester, it was noticed upon analysis that a solid precipitate was formed as the reaction was proceeding. Bearing in mind that ultimately this work will be transferred to a miniaturised flow reactor system, where precipitates can cause blockages in the reactor and hence be problematic, DABCO was eliminated from further investigations.

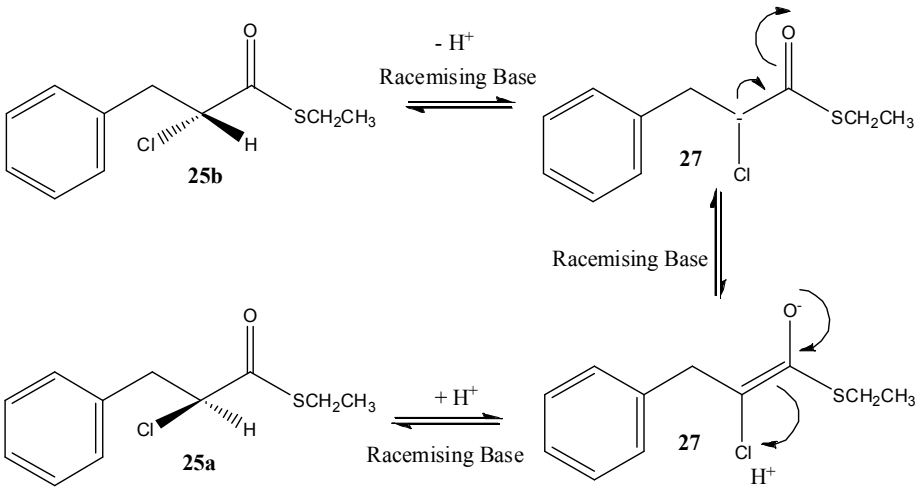
Racemising Base	**Ester / %ee$_L$ over time**				
	1hr	**2hr**	**4hr**	**6hr**	**24hr**
PY	90	92	92	91	92
DCHA	7	0	4	2	-4
TEBA	2	3	4	-6	-18
TOA	90	83	47	40	27
TRBA	61	44	19	7	2
DIPEA	2	2	3	-4	-13
DABCO	1	2	2	-3	-8
PS	Base peak co-eluted with ester peaks				
DBU	Breakdown of ester				

Table 26: Base catalysed racemisation reactions of the enantioenriched α-chloro ethyl ester enantiomer **25b** with a range of free bases. Reaction conditions; 2 equivalents of base, 1mL substrate (0.02M in t-BuOH/water (90: 10) at 40 ^oC in batch.

As was observed when investigating the racemisation of the enantioenriched α-chloro methyl and ethyl esters (**23b** and **24b**), the HPLC peak generated for the proton sponge (PS) co-eluted with the ester peaks generated on the chiral HPLC trace and hence the extent and rate of racemisation could not be determined. The racemisation reactions with bases DCHA, TEBA, TOA, TRBA, DIPEA and DABCO showed some success and the lack of any extra peaks on the reverse phase HPLC traces, even after 24 hr, suggested no break down of the ester or formation of any other compounds unlike the use of DBU. Using the organic bases identified (DCHA,

TEBA, DIPEA) DKR reactions were conducted, the results of which can be found in **Section 3.4.4**.

From the results shown in **Sections 3.4.3.1** and **3.4.3.2** it was clear that, under the reaction conditions studied, the two enantioenriched α-chloro esters **23b** and **24b** were inert to racemisation using any of the organic bases investigated. Thioesters however, such as the one presented here (enantioenriched α-chloro ethyl thioester **25b**), are much more acidic than esters incorporating oxygen atoms. This is because the p-orbitals of the sulfur atom do not overlap with the p-orbitals of the neighbouring carbon atom, resulting in the carbonyl group of the thioester being more polar. Consequently, thioester reactivity towards nucleophiles (in this case the organic bases) is enhanced and they are easier to enolise than *O*-esters Base catalysed racemisation relies on a stereogenic centre in an α-position to the carbonyl group, bearing an enolisable proton. Whereby, deprotonation takes place at the α-carbon, followed by reprotonation (**Scheme 23**). In order for the racemisation of an enantiomer to take place using an organic base, a certain degree of strength of the base is required for the enolate intermediate **27** to be formed.



Scheme 23: Proposed mechanism for base catalysed racemisation of enantioenriched α-chloro ethyl thioester **25b** *via* an enolate formation.

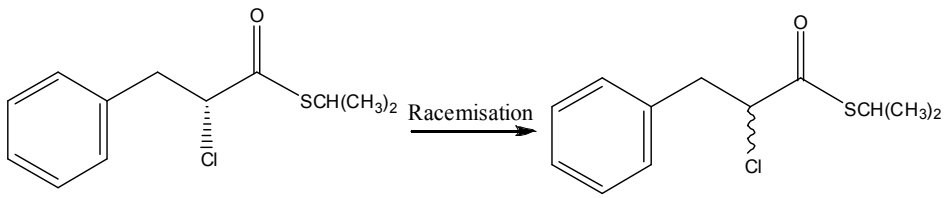
With this in mind, the free bases DCHA and TEBA, which are stronger than PY but weaker than TOA and TRBA would not be expected to racemise the enantiomeric ester as efficiently. In fact however, it is clear from the results obtained (**Table 26**)

that these two free bases (DCHA and TEBA) are excellent racemising agents with respect to this enantiomer. One possible explanation for this is that the weaker organic bases (DCHA and TEBA) are forming a dimer in the reaction mixture, which in turn is acting as a stronger free base and hence catalysing the racemisation step quickly and effectively.

Three (DCHA, TEBA and DIPEA) of the nine organic bases investigated were identified as being possible racemising agents for the enantioenriched α-chloro ethyl thioester **25b**, with varying rates and degrees of racemisation. Therefore, no subsequent racemisation investigations were conducted with regard to this substrate.

3.4.3.4 ENANTIOENRICHED α-CHLORO i-PROPYL THIOESTER

Based on previous results, for the enantioenriched esters **23b**, **24b** and **25b**, organic bases PY, PS and DBU can be eliminated from the investigation, focusing on the remaining six organic bases for the racemisation of L-α-chloro i-propyl thioester **26b** (**Scheme 24**).



26b

% ee_L = 80

26

% ee_L = 0

Scheme 24: Racemisation of enantioenriched α-chloro ethyl ester **26b** to yield the racemic ester **26**.

Racemising Base	Ester / %ee$_L$ over time				
	1hr	2hr	4hr	6hr	24hr
DCHA	20	14	7	6	4
TEBA	2	3	3	3	2
TOA	80	78	71	63	33
TRBA	60	50	28	18	4
DIPEA	3	3	4	4	4
DABCO	2	1	1	-6	-13

Table 27: Base catalysed racemisation reactions of the enantioenriched α-chloro i-propyl thioester enantiomer **26b** with a range of free bases. Reaction conditions; 2 equivalents of base, 1mL substrate (0.02M in t-BuOH/water (90: 10)) at 40 ^oC in batch.

As was seen for the previous enantioenriched thioester **25b** (**Section 3.4.3.3**), all six of the remaining free bases; DCHA, TEBA, TOA, TRBA, DIPEA and DABCO demonstrated the ability to racemise the L-ester enantiomer at different reaction rates (**Table 26**). Observations made using organic bases TEBA, DIPEA and DABCO were consistent with those seen in the experiment using the enantioenriched α-chloro ethyl thioester **25b** where the racemisation rate was rapid with a racemic mixture being achieved after as soon as 1 hr. Again however, the use of organic base DABCO as the racemising agent resulted in a solid precipitate being formed, eliminating its use in future experiments. The main difference which is apparent when comparing the two sets of data for the thioester substrates (**Tables 26** and **27**) is that for the racemisation of the enantioenriched α-chloro ethyl thioester **25b** using bases DCHA, TEBA, DIPEA and DABCO, suspected inversion takes place. This is where the predominant enantiomer of the mixture changes resulting in a negative %ee$_L$ value. However, in the case of the α-chloro propyl thioester **26b** this phenomenon only appears to be apparent when utilising one of the stronger bases (DABCO) as the racemising agent. The reasons for this are not known and further investigations to identify if inversion is taking place are required. However, as it is the racemisation step which is required for a DKR set-up, which has been achieved, further studies investigating the suspected inversion, are not of importance for this work.

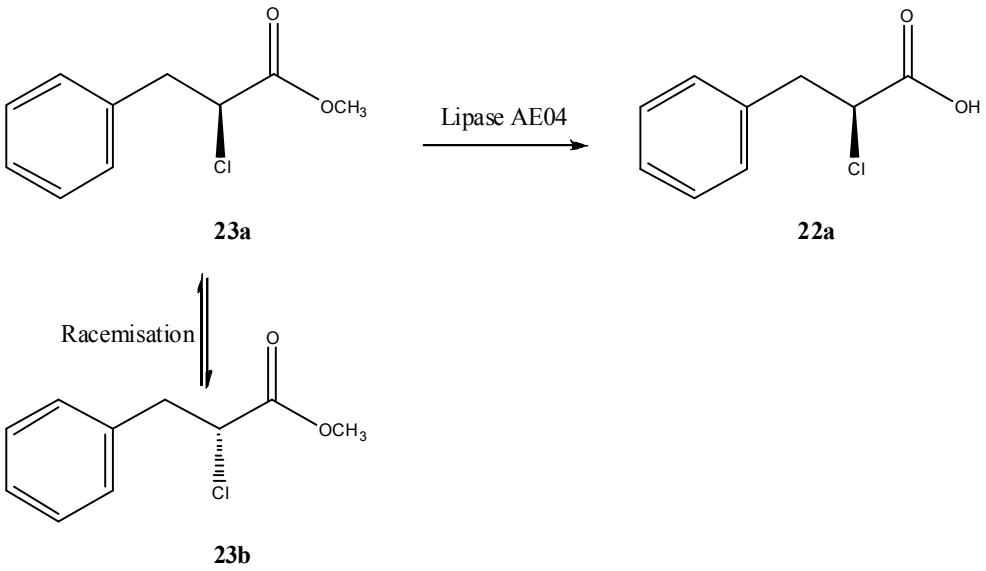
Three (DCHA, TEBA and DIPEA) of the nine free bases investigated were identified as being possible racemising agents for the enantioenriched α-chloro ethyl thioester **26b**. Therefore, no subsequent racemisation investigations were conducted with regard to this substrate. DKR reactions incorporating these three organic bases (DCHA, TEBA, TRBA and DIPEA) and the appropriate enantioselective lipase (AE07) were carried out. Details of which can be found in the dynamic kinetic resolution section of the report (**Section 3.4.4**).

3.4.4 DYNAMIC KINETIC RESOLUTION REACTIONS

Lipase catalysed kinetic resolution reactions in t-BuOH/water (90: 10) have been successfully completed for all four racemic DL-α-chloro ester substrates **23**, **24**, **25** and **26** to yield the corresponding D-acids (**Section 3.4.2**). Likewise, successful racemisation protocols appear to be in place for the two enantioenriched thioesters **25b** and **26b** in the same solvent system (**Sections 3.4.3.3 and 3.4.3.4**). Possible racemisation methods have also been highlighted for the enantioenriched α-chloro methyl ester **23b** in DMSO (**Section 3.4.3.1**). Consequently, the two steps were combined in a single pot to evaluate if the lipases and organic bases or chloride were compatible, therefore allowing successful DKR reactions to be established for the three racemic substrates; DL-α-chloro methyl ester **23**, DL-α-chloro ethyl thioester **25** and DL-α-chloro i-propyl thioester **26**.

3.4.4.1 RACEMIC α-CHLORO METHYL ESTER

The chemo-enzymatic dynamic kinetic resolution of DL-α-chloro methyl ester **23** to yield the desired, corresponding D-acid **22a** (**Scheme 25**) was investigated initially using lipase AE04 and two racemising chlorides.



Scheme 25: DKR of racemic α-chloro methyl ester **23** to yield enantiomeric α-chloro acid **22a**.

Although the kinetic resolution reaction of DL-α-chloro methyl ester **23** to yield the corresponding D-acid (**Section 3.4.2.1**) had proved successful, with a conversion of 52% (24 hr) when using t-BuOH/water (90: 10), racemisation studies had failed in this solvent. However, as **Table 28** illustrates the racemisation reactions were successful in DMSO.

Racemising Chloride	**Ester / %ee$_L$ after 24 hr**	
	Hexane	**DMSO**
Aliquat 336	1	9
Triphenylphosphonium chloride	-	16

Table 28: Chloride catalysed racemisation reactions of the enantioenriched α-chloro methyl ester enantiomer **23b**, with a free and immobilised chloride. Reaction conditions; 2 eq. of chloride, 1mL substrate (0.02M in a range of solvents) at 40 ^{o}C in batch.

Consequently, investigations were carried out to determine if the lipase catalysed kinetic resolution of DL-α-chloro methyl ester could be successfully conducted in either of these solvents, with **Table 24** showing only moderate reaction in DMSO/water (90: 10). The lack of enzyme activity for lipase AE04 in hexane, eliminated this solvent from further studies; therefore DKR reactions utilising either Aliquat 336 or polymer bound triphenylphosphonium chloride in DMSO/water (90: 10) were the only remaining combination at this stage (**Table 29**).

Racemising Chloride	Conversion to Acid / %	
	2 hr	24 hr
Aliquat 336	0	0
polymer bound triphenylphosphonium chloride	0	0

Table 29: Chemo-catalytic DKR reactions of the racemic α-chloro methyl ester **23** using lipase AE04 as the enantioselective biocatalyst and Aliquat 336 or triphenylphosphonium chloride as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, 2 equivalents of chloride, 1mL substrate (0.02M in DMSO/water (90: 10)) at 40 ^{o}C in batch.

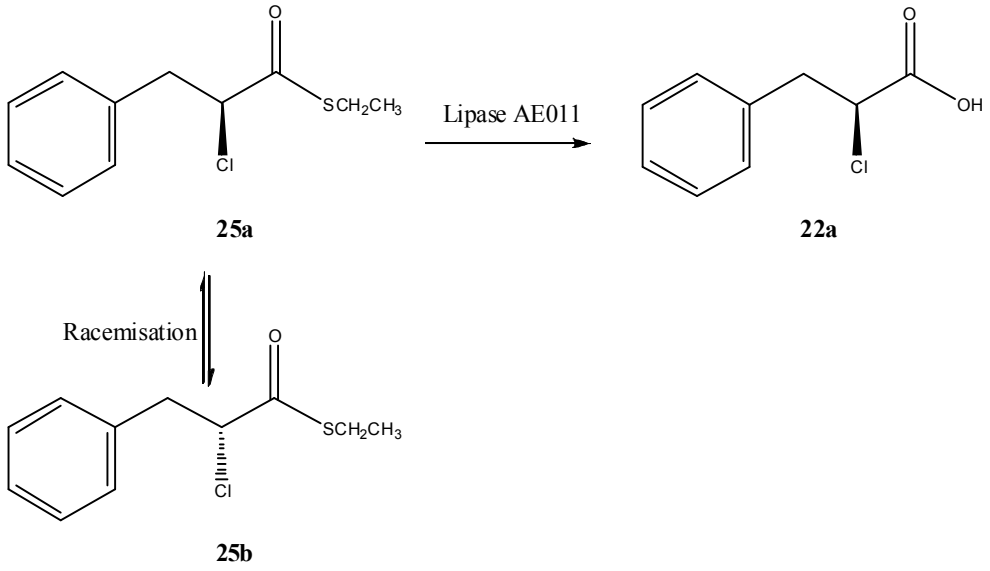
When the enzymatic kinetic resolution experiment of racemic α-chloro methyl ester **23** was conducted (**Section 3.4.3.1**), a conversion of D-ester to the corresponding acid of 23% was observed after 24 hr, in the solvent DMSO using lipase AE04. However, it can be seen from **Table 29** that when the enzyme is combined with either one of the possible racemising chlorides, resolution of the ester to the corresponding acid is not achievable to any extent. These results would suggest that the presence of the chlorides have rendered the enzyme inactive. As a result, no further investigations into the DKR of DL-α-chloro methyl ester **23** (or similarly DL-α-chloro ethyl ester **24**) were conducted.

Despite the lack of success with regards to the DKR of the DL-α-chloro methyl ester substrate, this scenario does provide a good example of where biocatalyst and racemising agent are incompatible and therefore the need for a separated two step process in some cases (**Chapter 4**).

The remaining focus of this Chapter therefore, is with regards to the two thioester substrates; DL-α-chloro ethyl thioester **25** and DL-α-chloro i-propyl thioester **26** which were successfully racemised using organic bases.

3.4.4.2 RACEMIC α-CHLORO ETHYL THIOESTER

The chemo-enzymatic dynamic kinetic resolution of DL-α-chloro ethyl thioester **25** to yield the desired, corresponding D-acid **22a**, (**Scheme 26**) was investigated using lipase AE011 and a range of organic bases, in t-BuOH/ water (90: 10).



Scheme 26: DKR of racemic α-chloro ethyl thioester **25** to yield enantiomeric α-chloro acid **22a**.

From the lipase catalysed kinetic resolution studies of the racemic α-chloro ethyl thioester substrate **25**, lipase AE011 was identified as the most suitable biocatalyst. A 42% conversion of the DL-ester to the corresponding D-acid product was observed with a high ee_D of 73%, after only 2 hr (**Section 3.4.2.3**). With equal success, three possible organic bases (DCHA, TEBA and DIPEA) were identified from the racemisation studies for the efficient racemisation of the L-α-chloro ethyl thioester **25b** back to the corresponding racemate **25** (**Section 3.4.3.3**). An attempt was therefore made to drive the reaction to 100% conversion of DL-ester to D-acid, whilst maintaining the high $\%ee_D$ value of the acid product **22a**. Lipase AE011 and the racemising base under investigation (DCHA, TEBA or DIPEA) were combined in one pot, batch, chemo-enzymatic DKR reactions. The results of which are shown in **Table 30**.

Racemising Base	Conversion to Acid / %	Acid / $\%ee_D$
DCHA	> 99	48
TEBA	> 99	91
DIPEA	> 99	81

Table 30: Chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **25**, using lipase AE011 as the enantioselective biocatalyst and DCHA, TEBA or DIPEA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, 2 eq. of racemising base, 1mL substrate (0.02M in t-BuOH/ water (90: 10)) at 40 ^oC in batch.

When lipase catalysed kinetic resolution of the DL-ester is coupled with base catalysed racemisation of the slow reacting L-enantiomer of the ester substrate, the enantioselective hydrolysis of DL-α-chloro ethyl thioester **25** to the corresponding D-α-chloro acid **22a** is increased from approximately 50% to >99% after 24 hr, under the reaction conditions studied.

Whilst these conversion results suggest success in so far as the chemo-catalytic DKR reaction is concerned, with no obvious compatibility issues between racemising agent and biocatalyst, the degree of this success is dependant upon the resulting $\%ee_D$ of the chiral acidic product. When DCHA was employed as the racemising base, although the conversion of ester to acid increased to >99%, the $\%ee_D$, and hence the chiral purity of the acid, was relatively low (48%). Despite this however, this enantiomeric excess value is higher and therefore an improvement on that which was seen after 24 hr in the corresponding kinetic resolution experiment (34%, **Section 3.4.2.3**, **Table 12**). The figure would suggest that whilst DCHA does have a role as a racemising agent, the rate at which this base does so is too slow to ensure a constant supply of D-α-chloro ethyl thioester and in fact DCHA may also be acting as a base catalyst for the hydrolysis reaction of ester to acid. This is feasible as out of the three possible racemising bases, DCHA illustrated the slowest rate of conversion of enantiomeric α-chloro ethyl thioester **25b** back to the corresponding racemate **25** (90% to $7\%ee_L$ ester in 1 hr) as shown in **Section 3.4.3.3**. When TEBA and DIPEA were used as the racemising agents however, excellent $\%ee_D$ values (91 and 81% respectively) were observed for the chiral D-acid product in conjunction with the high conversions of ester to acid. These values, in particularly that seen for organic base TEBA would suggest that the rate of racemisation (k_r) is significantly higher than the rate at which lipase AE011 hydrolyses the D-enantiomer of DL-α-chloro ethyl thioester, thereby allowing the reaction to proceed to >99% conversion with excellent selectivity. Based upon the previous racemisation studies, where these two organic bases racemised the enantiomeric ester back to the racemate at a rapid rate (90% to $1\%ee_L$ in 1 hr) these DKR results are what would be predicted. Thus, a successful chemo-enzymatic DKR set up has been achieved for the enantioselective hydrolysis of DL-α-chloro ethyl thioester **25** when lipase AE011 is employed as the biocatalyst with *in situ* racemisation of the ester using TEBA (or DIPEA), under the reaction conditions studied.

With this in mind, our attentions were turned to the optimisation of this DKR process for this ester (α-chloro ethyl thioester **25**). Initially (**Section 3.4.2.3**) a range of eight lipases were screened for the lipase catalysed enantioselective hydrolysis of the DL-α-chloro ethyl thioester to the corresponding D-acid. Lipase AE011 resulted in an impressive conversion (42%) of racemic ester to enantioenriched acid with a high $\%ee_D$ value (73%) after only two hour (**Table 14**). Consequently, this biocatalyst was successfully incorporated into a chemo-enzymatic DKR reaction with *in situ* racemisation of the ester. With this DKR procedure in hand, an additional range of lipase enzymes from various sources were investigated in further DKR experiments, in an attempt to perhaps improve the $\%ee_D$ values of the acid product already obtained (**Table 30**). Lipases studied in addition to those already considered were; lipase AE05, lipase from wheat germ, lipase from porcine pancreas, Amano AK, Amano M, Amano G and Julich RN. Racemising base TEBA, resulted in the most impressive DKR reaction when lipase AE011 was used as the biocatalyst, with both high conversion (>99%) and enantiomeric excess values ($91\%ee_D$). Therefore subsequent chemo-catalytic DKR reactions utilised TEBA as the *in situ* racemising agent. The results obtained for these additional DKR reactions are shown in **Table 31**.

Lipase	Conversion to Acid / %	Acid / % ee_D
AE04	66	34
AE07	> 99	28
AE08	40	9
AE011	***> 99***	***89***
Amano PS	39	3
Amano AS	66	1
Amano D	46	< 1
AE05	40	13
Wheat Germ	39	8
Porcine Pancreatic	81	12
Amano AK	35	44
Amano M	42	4
Amano G	40	3
Julich RN	43	1

Table 31: Chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **25** using a range of lipases as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, 2 equivalents of racemising base, 1mL substrate (0.02M in t-BuOH/ water (90: 10)) at 40 ^oC in batch.

Of the fourteen lipases investigated for the chemo-enzymatic DKR of DL-α-chloro ethyl thioester **25**, lipase AE011 (**Table 31** in bold italic), when coupled with *in situ* racemisation using organic base TEBA, results in the best outcome with a >99% conversion of DL-ester to enantioenriched acid product in 89%ee_D. There was a distinct lack of selectivity exhibited by the other lipases, despite some good conversions. Consequently, for all subsequent DKR reactions involving DL-α-chloro ethyl thioester **25**, lipase AE011 remains the biocatalyst of choice, coupled with TEBA as the racemising base.

The impact of temperature on the reaction was studied at room temperature, 40 ^oC, and 75 ^oC. Room temperature as this is ideal from an environmental, energy, cost and ease of reactor set up perspective, 40 ^oC because this is the believed optimum operating temperature of lipase AE011 and 75 ^oC to represent a higher temperature where deactivation could occur. Chemo-catalytic DKR reactions using lipase AE011

as the enantioselective biocatalyst and organic base TEBA as the racemising agent were conducted at a range of temperatures. The results are detailed in **Table 32**.

Time / hr	Conversion to Acid / %			Acid / %ee_D		
	RT	40 ^oC	75 ^oC	RT	40 ^oC	75 ^oC
1	54	79	20	92	95	>99
2	68	86	25	89	89	90
3	78	97	22	94	87	90
4	83	95	18	91	86	91
24	> 99	>99	23	85	83	90

Table 32: Chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **25** using lipase AE011 as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, 2 equivalents of racemising base, 1mL substrate (0.02M in t-BuOH/ water (90: 10)) at a range of reaction temperatures, in batch.

It would seem that in batch mode, chemo-enzymatic DKR of DL-α-chloro ethyl thioester **25** to yield the corresponding chiral acid product is most successful when carried out at the recommended operating temperature of 40 ^oC, when utilising lipase AE011 as the enantioselective biocatalyst and TEBA as the racemising agent. Where, under such conditions 97% conversion of racemic ester to chiral acid is achieved after only 3 hr, with the acid in excellent %ee_D (87%). These results, obtained at 40 ^oC confirm the success already seen for this reaction (Tables **30** and **31**) where after 24 hr >99% conversion of ester to acid with the acid product in 89%ee_D was achieved. At ambient temperature, a high conversion of DL-ester to enantioenriched acid (>99%) was observed after 24 hr, under the reaction conditions studied. However, it is clear from the results shown in **Table 32** that the rate of the reaction is somewhat slower than when the lipase catalysed hydrolysis was carried out at 40 ^oC. This is likely to be due to the higher activity of the enzyme at 40 ^oC. Clearly, when the chemo-enzymatic DKR of DL-α-chloro ethyl thioester **25** is carried out at the elevated temperature of 75 ^oC there is a significant decrease in the achievable conversion of ester to acid. At 75 ^oC it would seem, from the results shown in **Table 32**, that the optimum conversion of DL-α-chloro ester (*ca.* 20%) had been achieved after 1 hr. It is believed that denaturation of the lipase, caused by the high temperature resulted in this result. Whereby, the active site of the enzyme has been altered and the D-ester substrate can no longer be incorporated into the active site and

subjected to hydrolysis. The conversion of ester to acid did not improve, even after 24 hr. This would suggest that initially the high temperature had only slightly changed the structure of the enzyme, still allowing the reaction to proceed to some extent. However, a temperature of 75 ^{o}C would seem to very quickly cause the structure of the enzyme to be altered such that no further reaction takes place.

From **Table 32** it can be seen that the batch, chemo-enzymatic DKR reaction of the DL-α-ethyl ester **25** utilising lipase AE011 as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent is better carried out at 40 ^{o}C. However, the results obtained at ambient temperature are by no means disappointing. In fact, after 4 hr, the percentage conversion of ester to acid was only 12% less at room temperature than it was at the optimal temperature of 40 ^{o}C whilst the %ee$_D$ of the chiral product after the same time was similar (91% compared to 86% at 40^{o}C).

Having evaluated the effect of temperature, the effect of racemising agent was subsequently investigated, employing a range of equivalents (0.5-10eq.) *cf.* previous experiments where 2 eq. were employed. Lipase AE011 was again the enantioselective biocatalyst with TEBA (in varying amounts) as the *in situ* racemising base for the reaction, which was conducted at the optimum reaction temperature of 40 ^{o}C. The results for the percentage conversion of ester to acid over time can be seen in **Figure 16** with corresponding trends for the %ee$_D$ values shown in **Figure 17**.

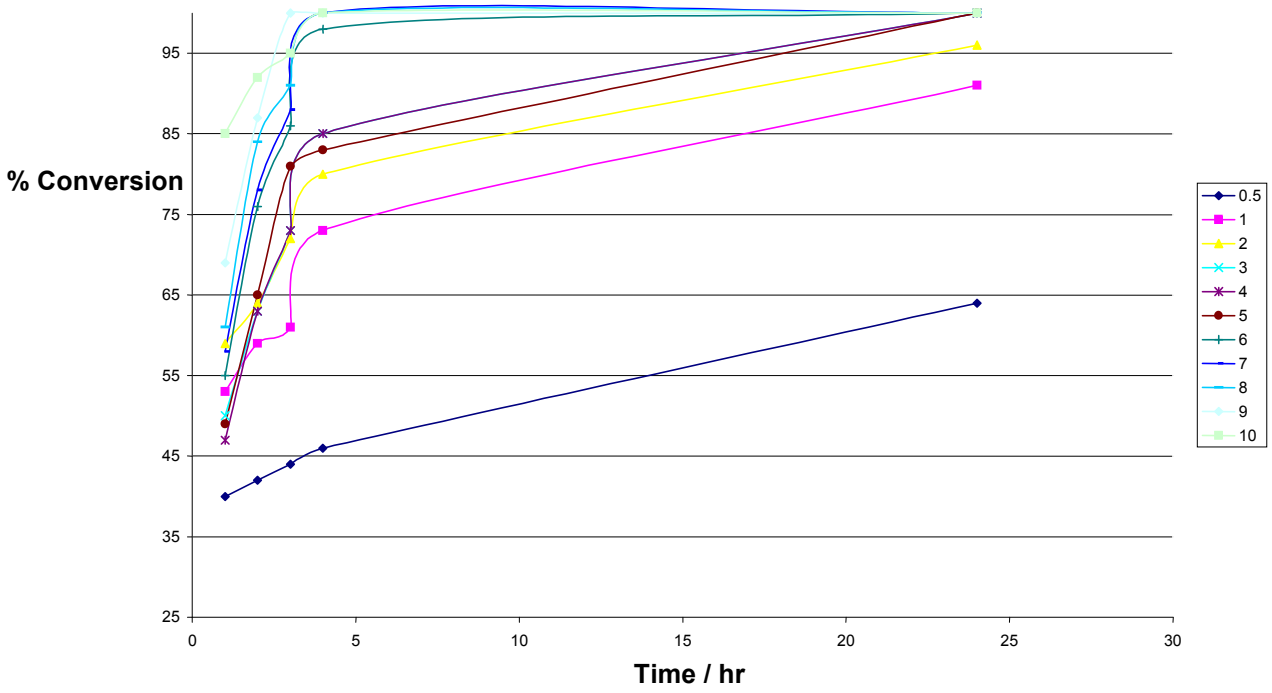


Figure 16: Chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **25** using lipase AE011 as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, varying amounts of racemising base, 1mL substrate (0.02M in t-BuOH/ water (90: 10)) at 40°C, in batch.

It is obvious from the results shown in **Figures 16** and **17** that 0.5eq. of racemising base is insufficient to achieve racemisation. Even after the full 24 hr reaction time, only 64% conversion has been achieved with a $\%ee_D$ of approximately 30% for the acid product. These results are not dissimilar to those seen when the kinetic resolution experiment was conducted without the addition of an *in situ* racemising base (**Section 3.4.2.3**). This would suggest that whilst the lipase catalysed kinetic resolution reaction is proceeding, the rate of racemisation of the L-α-chloro ethyl thioester is negligible. In fact, the slight increase seen in conversion of ester to acid, compared to the kinetic resolution reaction would suggest that the TEBA in 0.5eq. is acting as an additional catalyst for the hydrolysis of the DL-α-chloro thioester to the corresponding acid. Between 1 and 10 eq. of base, the changes in conversion and $\%ee_D$ of the product are not as obvious as when 0.5 eq. of base were used but the differences are still significant.

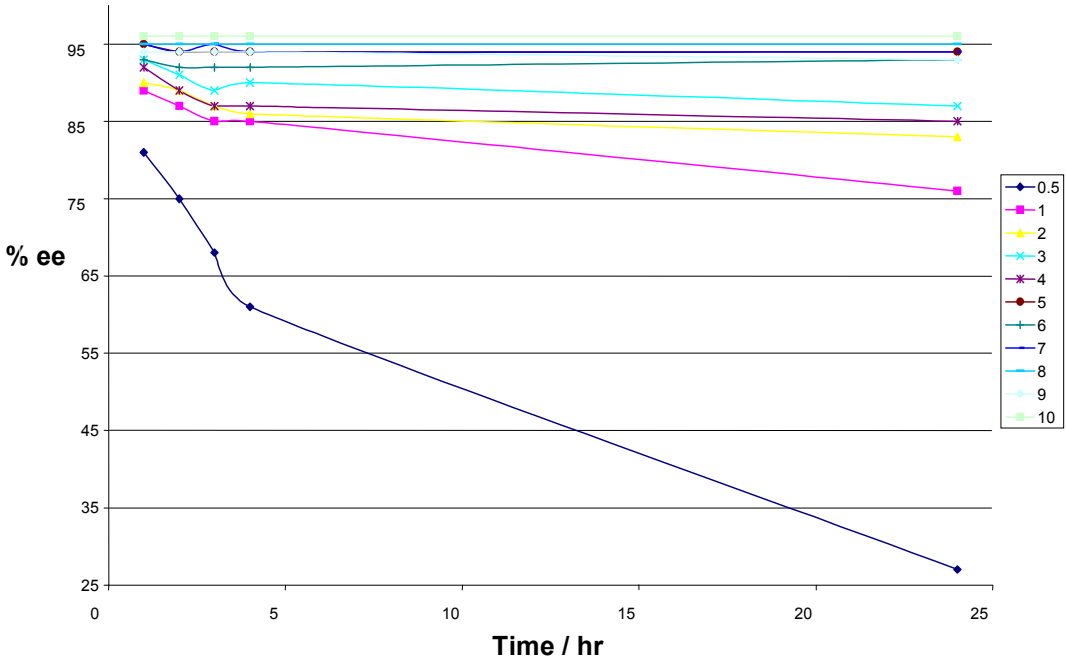


Figure 17: Chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **25** using lipase AE011 as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, varying amounts of racemising base, 1mL substrate (0.02M in t-BuOH/ water (90: 10)) at 40°C, in batch.

It can be seen from **Figure 17** that the amount of base has a significant effect on enantioselectivity, with 1 eq. of TEBA resulting in a significant decrease in $\%ee_D$ of the product from 90% to 76%. Whilst this decrease is not so marked for 2, 3 and 4 eq. of base, the drop in $\%ee_D$ over time (8, 6 and 7% respectively) still affects the purity of the chiral product. This would imply that whilst the organic base (TEBA) is catalysing the racemisation of the slow reacting enantiomer (L-α-chloro ethyl thioester) to some extent, it is not at a rate sufficient enough to maintain a constant supply of the preferred enantiomer of the ester substrate (D-α-chloro ethyl thioester). Hence, the $\%ee_D$ decreases with time, despite an increase in conversion of ester to acid (**Figure 16**).

The ideal concentration of racemising base would result in a DKR set up that resulted in a steady increase in conversion of ester to acid at a reasonable rate with no significant decrease in $\%ee_D$ of the product over time. This would mean that the rate of racemisation was faster than the rate of hydrolysis of the ester whilst ensuring the racemising base is not also acting as a catalyst for the hydrolysis reaction. When 5 eq. of base was utilised in the chemo-enzymatic DKR reaction of DL-α-chloro ethyl

thioester, a steady increase in percentage conversion of ester to acid was observed over the 24 hr with no decrease in $\%ee_D$ of the chiral product, as shown in **Table 33**.

Time / hr	Conversion to Acid / %	Acid / $\%ee_D$
1	49	95
2	65	94
3	81	94
4	83	94
24	>99	94

Table 33: Chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **25** using lipase AE011 as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, 5eq. of racemising base, 1mL substrate (0.02M in t-BuOH/ water (90: 10)) at 40°C, in batch.

The results obtained when employing 5 eq. of base are an improvement upon the chemo-enzymatic DKR reaction results achieved when 2 eq. of base were utilised in a reaction previously carried out and monitored in the same way (**Table 32**), where a $\%ee_D$ of 83% was obtained after 24 hr, compared to 94% seen here. Above 5 eq. (6-10eq) the $\%ee_D$ values for the acid product remained constant (within 2% for each experiment) and high (>90%) over time, in all cases (**Figure 17**). However, the rate of hydrolysis (**Figure 16**) was much faster than when less than 6 eq. of base were used in the chemo-enzymatic DKR reaction. This increase rate of reaction seen for concentrations of organic base (TEBA) above 5 eq. would again suggest that the base is acting as an additional catalyst in the hydrolysis of the ester. This phenomenon does not appear to be having an effect upon the $\%ee_D$ of the chiral acid, probably because the rate of racemisation is quicker than the rate of either the base catalysed or lipase catalysed hydrolysis. To ensure that it is definitely the lipase which is catalysing the hydrolysis reaction and that the base is only catalysing racemisation of the slow reacting enantiomer, 5 eq. of TEBA was determined to be the optimum concentration of organic base. This set up afforded a steady conversion of racemic ester to chiral acid for the chemo-catalytic DKR reaction of DL-α-chloro ethyl thioester **25** over time, with consistently high $\%ee_D$ values (**Table 33**).

Finally, a range of solvent/water (90: 10) compositions were investigated as the reaction medium for the chemo-enzymatic DKR of DL-α-chloro ethyl thioester **25**.

Solvents representing a range of polarities (hexane, toluene, DCM, ACN, DMSO) were investigated (and a repeat of t-BuOH for completeness). Lipase AE011 was used as the enantioselective enzyme and TEBA (optimised 5eq.) was the racemising base for the batch, chemo-enzymatic DKR reaction of DL-α-chloro ethyl thioester **25** at 40°C. The results of which are shown in **Table 34**.

Solvent / Water (90: 10)	Conversion to Acid / %	Acid / % ee_D
Hexane	18	64
Toluene	12	63
DCM	9.7	61
ACN	62	80
DMSO	52	15
t-BuOH	***> 99***	***96***

Table 34: Chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **25**, using lipase AE011 as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, 5eq. of racemising base, 1mL substrate (0.02M in a range of solvents (90: 10)) at 40°C, in batch.

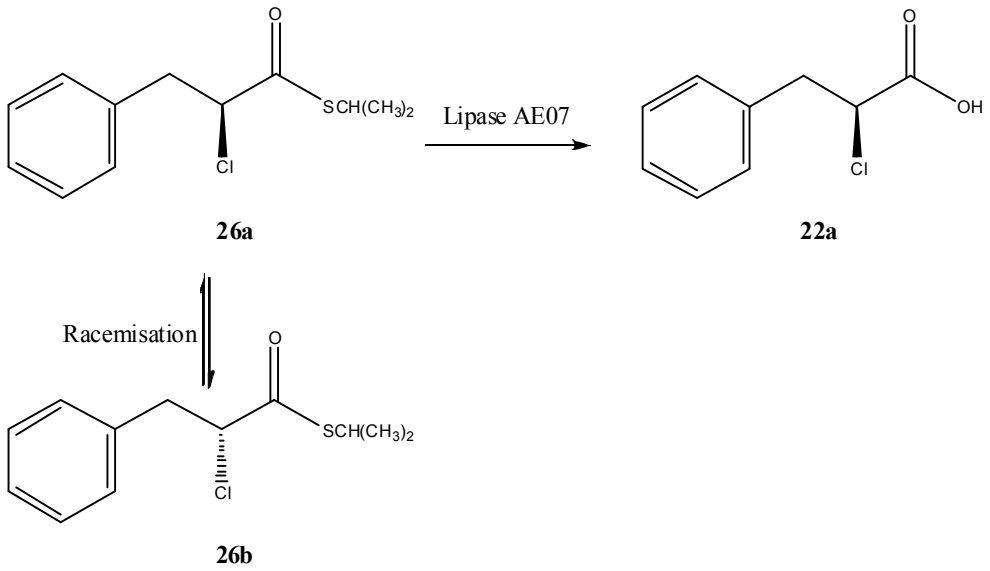
For the most successful batch, chemo-enzymatic DKR of DL-α-chloro ethyl thioester **25** to be established, from the parameters investigated, it would seem that the reaction should be conducted at 40°C, with lipase AE011 and 5 eq. of TEBA in a reaction solvent of t-BuOH/ water (90: 10). The reaction was repeated under these conditions, the results of which can be seen in **Table 35**.

Time / hr	Conversion to Acid / %	Acid / % ee_D
2	62	95
4	82	95
24	> 99	93

Table 35: Optimised chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **25** using lipase AE011 as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, 5eq. of racemising base, 1mL substrate (0.02M in t-BuOH/ water (90: 10)) at 40°C, in batch.

3.4.4.3 RACEMIC α-CHLORO i-PROPYL THIO ESTER

Following the successful chemo-enzymatic dynamic kinetic resolution of DL-α-chloro ethyl thioester **25** to yield the desired, corresponding D-acid **22a** (**Section 3.4.4.3**) in high optical purity (93%ee_D) and with excellent conversion of ester to acid (>99%), the process was transferred to the DKR of DL-α-chloro i-propyl thioester **26** (**Scheme 27**).



Scheme 27: DKR of racemic α-chloro propyl thioester **26** to yield enantiomeric α-chloro acid **22a**.

Originally (**Section 3.4.2.4**), lipase AE07 was identified as the most enantioselective lipase with respect to the hydrolysis of the DL-α-chloro i-propyl ester to D-acid (43% conversion with the acid product in 59%ee_D after 2 hr, **Table 17**). This %ee_D of the acid is low and therefore the chiral acid is impure. Therefore, additional screening experiments incorporating a wider range of lipases than were originally studied (**Section 3.4.2.4**) were conducted, under DKR conditions. All other parameters (40 ^oC, 5 eq. of TEBA base and a solvent system of t-BuOH/ water (90: 10)) were consistent with those identified to be the optimum in **Section 3.4.2.4**. The results of the DKR screening experiments, with a wide range of lipase enzymes are shown in **Table 36**.

Lipase	Conversion after 24 hr / %	Acid / % ee_D
AE04	37	20
AE07	***78***	***79***
AE08	54	13
AE011	55	22
Amano PS	44	18
Amano AS	19	-1
Amano D	34	17
AE05	36	6
Wheat Germ	22	18
Porcine Pancreatic	68	34
Amano AK	36	6
Amano M	26	4
Amano G	28	5
Julich RN	20	8

Table 36: Chemo-catalytic DKR reactions of the racemic α-chloro ethyl thioester **26** using a range of lipases as the enantioselective biocatalyst and TEBA as the *in situ* racemising agent. Reaction conditions; *ca*. 100mg lipase, 2 eq. of racemising base, 1mL substrate (0.02M in t-BuOH/ water (90: 10)) at 40 ^{o}C in batch.

Of the fourteen lipases investigated for the chemo-enzymatic DKR of DL-α-chloro i-propyl thioester **26**, lipase AE07 (**Table 36** in bold italic), when coupled with *in situ* racemisation using organic base TEBA, results in the highest conversion of ester to acid. Although, the results are not as impressive as those obtained for the DL-α-chloro ethyl thioester substrate **25** (**Section 3.4.4.2**), a 78% conversion of DL-α-chloro i-propyl ester **26** to enantioenriched acid product **22a** in 79%ee_D was achieved under the same optimised reaction conditions.

3.5 EXPERIMENTAL

3.5.1 MATERIALS

All of the solvents and reagents used were either HPLC or standard laboratory reagent grade, purchased from Fisher Scientific UK Limited, Loughborough, Leicestershire, UK, or Sigma Aldrich, Steinheim, Germany. The water used throughout was deionised.

The series of Alphamerix enzymes used; AE04 *Achromobacter spp*, AE07 *Ps. stutzeri*, AE08 *Rhizopus spp*. and AE011 *Alcaligines spp*, were obtained from Alphamerix Limited, Cambridge, UK. Amano Pharmaceuticals Limited, Nagoya, Japan, supplied Amano AS *A. niger* and Amano D *R. oryzae*, whilst Europe Bioproducts Limited, Cambridge, UK, provided Amano PS *Ps. cepacia*. Sigma Aldrich, Steinheim, Germany was the source of *C. rugosa* lipase, Amano lipase AK (*Ps. fluorescens*), Amano lipase G (*P. camembertic*), Amano lipase M (*M. javanicus*), lipase from porcine pancreas (type II) and lipase from wheat germ. Novozyme 435 (*C. antarctica* B immobilised to macro porous resin), Lipozyme RM (*Rh. miehei*) and Lipozyme TM (*Th. langinisa*) were supplied by Novo, Nordisk, Denmark and Julich Fine Chemicals supplied lipase RN (*Rh. niveus*).

Pyridine (>99 %), poly(allylamine) 20 wt. % solution in water, dicyclohexylamine (99 %), *tert*-butylamine (98 %), trioctylamine (98 %), *N,N* diisopropylethylenediamine (DIPEA) (99 %), 1,4 diazabicyclo[2.2.2.]octane (DABCO) (98 %), Proton Sponge (99 %), 1,8 bis (dimethylamino) naphthalene (99 %), *N,N,N,N* tetramethylguanidine (99 %) were from Sigma Aldrich, Steinheim, Germany. Arcos Organics, New Jersey, USA / Geel, Belgium were the source of tributylamine (99 %), and Lancaster, East Gate, White Lund, Morecambe, UK, supplied the 1,8 diazabicyclo [5.4.0] undec-7-ene (DBU) (97 %).

Sigma Aldrich, Steinheim, Germany supplied the triphenylphosphonium chloride on polymer (0.7-1.3 mmol / g loading), tetraphenylphosphonium chloride (98 %), Aliquat 336 and tetrabutylammonium chloride hydrate (98 %).

Tributylmethylphosphonium chloride polymer bound (1.4 mmol cl / g resin), chloride on polymer support (4 mmol chloride / g resin) from ordered from Fluka, Steinheim, Germany.

3.5.2 INSTRUMENTATION

3.5.2.1 HPLC INSTRUMENTATION

The development of accurate methods for the determination of enantiomeric purity, which began in the late 1960's, has been crucial for the assessment of enantioselective synthesis. Thus, a prerequisite in the enzyme catalysed kinetic resolution of racemates is a precise and reliable assessment of the degree of enantioselectivity, enantiomeric excess (ee) and conversion. Among these methods are polarimetric methods, gas chromatographic methods, liquid chromatographic (LC) methods and nuclear magnetic resonance (NMR) spectroscopy. The most convenient and accurate method used is chiral HPLC. HPLC methods are sensitive, quick and simple to carry out. The analysis is usually unaffected by the presence of any soluble impurities in the sample, therefore isolation and purification of the analysed sample is not required. Also, a small sample volume is required for analysis, meaning reactions can be done on a very small scale, which is very important when the work is transferred to a miniaturised reactor set up. This method is based on the fact that molecular association may lead to an efficient chiral recognition, leading to enantiomeric separation when a chiral stationary phase is used in the LC. The liquid mobile phase is carrying the chiral analyte through the stationary phase. The enantiomers to be analysed undergo rapid and reversible diastereomeric interactions with the chiral stationary phase and hence may be eluted at different times. Polar and non volatile compounds can be analysed. The measurement of ee using LC is linked with a high degree of precision so that reliable data may be obtained.

Reverse phase HPLC analysis was conducted using a HPLC system equipped with an ACE-115-1503 3-phenyl column (D_f 3μm, length 150mm, ID 3mm) supplied by HiChrom. Analyte detection was carried out using a UV/ Vis detector at a wavelength, λ of 220nm. All analysis was carried out at 40 ^{o}C in gradient mode using a solvent system of A (10% acetonitrile in water with 0.1% TFA v/v) and B

(10% water in ACN with 0.1% TFA v/v) as the mobile phase, at a flow rate of 1.0mL min^{-1}. The method followed was such that at T=0, the mobile phase was 5% B (95% A), increased to 60% B (40% A), over a ten min period, reaching 80% B (20% A) at 15 min and then decreasing back to 5% B (95% A) at the end time of 17 min.

Chiral HPLC analysis was determined on a Gilson HPLC system equipped with a Gilson pump coupled to either a ChiralCel® OJ (D_F 10µm, length 250mm and ID 4.6mm) or a ChiralPak® ADH (D_F 5µm, length 250mm and ID 4.6 mm) column (**Figure 18**), manufactured by Chiral Technologies, Europe. Analyte detection was carried out at a wavelength, λ of 220nm using a Gilson UV/Vis detector. All analysis was carried out at 40 ^oC using an external column heater, supplied by Jones Chromatography, in isocratic mode using a solvent system of iso-hexane, ethanol and trifluoroacetic acid (TFA), either in the ratio of 85: 15: 0.1 or 95:5: 0.1 for the OJ and ADH columns respectively, as the mobile phase, at a flow rate of 1.0Ml min^{-1}, with a run time of 15 min.

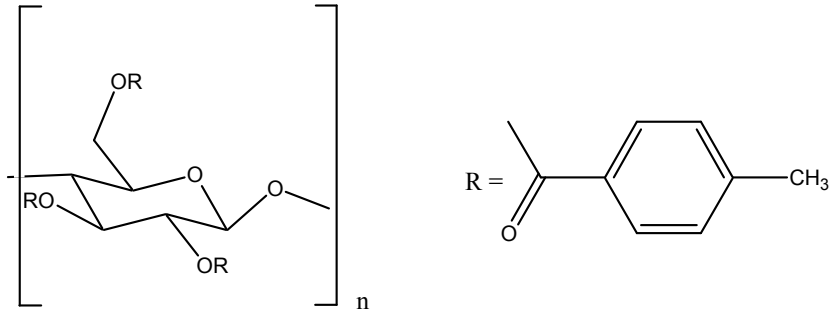
The ee of the ester substrates or acid product was determined from areas of the two enantiomer peaks shown on the OJ or ADH column respectively, calculated using the equation shown in **Equation 1**.

$$\text{Enantiomeric Excess (\% ee)} = \frac{\text{(Peak Area A – Peak Area B)}}{\text{(Peak Area A + B)}} \times 100$$

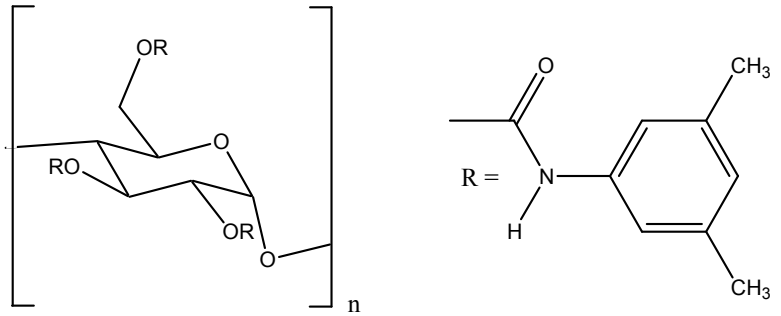
Where A = Major enantiomer

B = Minor enantiomer

Equation 2: Calculation of enantiomeric excess (% ee).



OJ column packing composition:
Cellulose Tris (4-methylbenzoate) coated on silica gel



ADH column packing composition:
Amylose tris (3,5-dimethylphenylcarbamate) coated on silica gel

Figure 18: Packing compositions of the ChiralCel® OJ and ChiralPak® ADH columns on silica gel.

3.5.2.2 PROTON NUCLEAR MAGNETIC RESONANCE

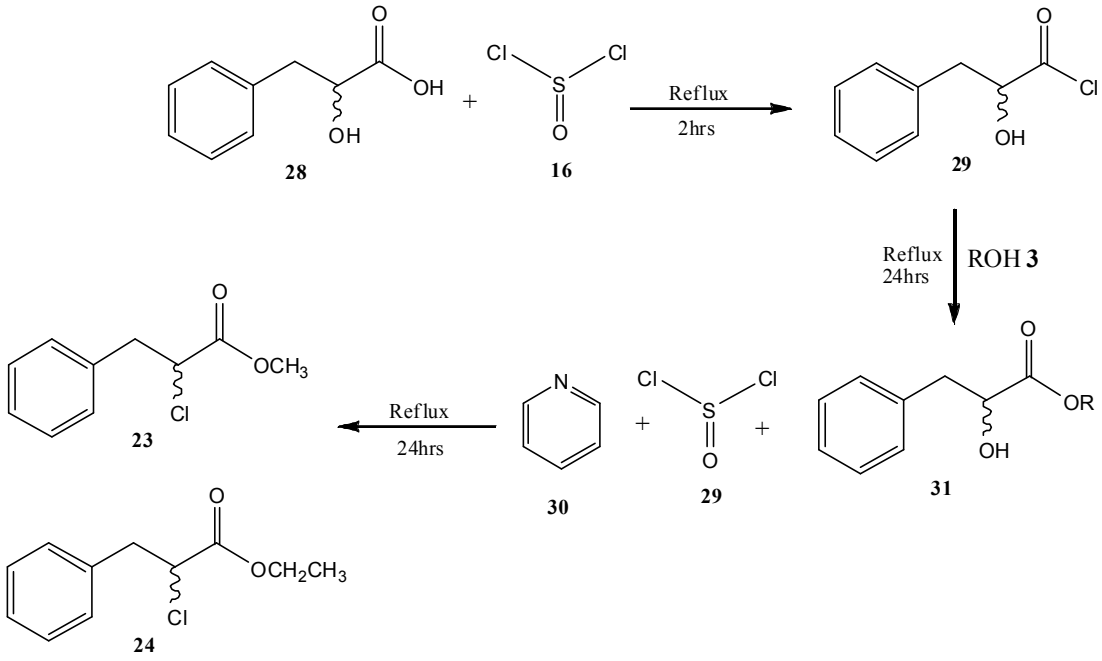
^{1}H NMR spectra were recorded on Varian Unity lNOVA-300 Fourier transform spectrometers at 300MHz using $CDCl_3$ or DMSO as a solvent. Chemical shifts (δ) are quoted in ppm using tetramethylsilane (TMS) as internal reference (δ = 0.00ppm), and coupling constants (J) are quoted in Hz.

3.5.3 METHODS

3.5.3.1 PREPARATION OF MATERIALS

Preparation of DL-α-chloro esters **23** and **24** (**Scheme 28**)

A solution of commercially available DL-phenyllactic acid **28** (20mmol, 3.3g) and thionyl chloride **16** (3 eq., 4.4mL) in dichloromethane (200mL) was heated to reflux at 75 ^{o}C for 2 hr, to yield the acyl chloride intermediate **29**. Without isolation of the acyl chloride intermediate, an excess of alcohol **3** (methanol or ethanol) (30mL) was added to the reaction and the mixture was left stirring at 70 ^{o}C under reflux for 24 hr. The reaction mixture was concentrated *in vacuo* and the mixture was analysed by reverse phase HPLC to confirm that the esterification step had been successful. Conversion of the α-hydroxy group to α-chloro was achieved by adding thionyl chloride (4 eq.) and pyridine **30** (4 eq.) to a solution of the hydroxy intermediate **31** (~0.1 M) in DCM (200 mL) and allowing the mixture to reflux at 70 ^{o}C with stirring for another 24 hr. After cooling, the mixture was quenched by the addition of saturated ammonium chloride (10 g) in water (200mL) and the resulting racemic DL-ester substrates (**23** and **24**), were extracted into DCM (3 x 200mL). The combined DCM layers were washed with water (3 x 50mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield the products as yellow, sweet smelling oils. Product confirmation was carried out by NMR and reverse phase and chiral HPLC.



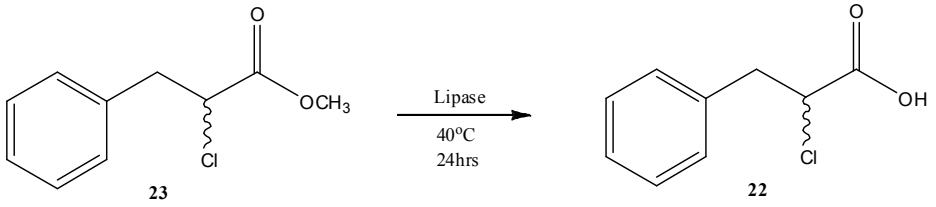
Scheme 28: Preparation of racemic DL-α-chloro ester substrates **23** and **24**.

DL-α-chloro methyl ester racemate **23**, 198.65 g mol^{-1}, R_T; reverse phase HPLC, 7.2 min, OJ column chiral HPLC, 7.2 and 8.9 min (L- and D- enantiomers respectively), ADH column chiral HPLC, 4.7 min. ^{1}H NMR in $CDCl_3$ solution: 7.29 (5H, m, **Ar**); 4.45 (1H, m, C**H**Cl); 3.64 (3H, s O**CH_3**); 3.38 (1H, dd, J 7.0 and 14.0, C**H**H); 3.16 (1H, dd, J 7.0 and 14.0, CH**H**).

DL-α-chloro ethyl ester racemate **24**, 212.65 g mol^{-1}, R_T; reverse phase HPLC, 7.9 min, OJ column chiral HPLC, 5.7 and 6.3 min (L- and D- enantiomers respectively), ADH column chiral HPLC, 4.3 min. ^{1}H NMR in $CDCl_3$ solution: 7.26 (5H, m, **Ar**); 4.40 (1H, t, C**H**Cl); 4.15 (2H, q O**CH_2**); 3.37 (1H, dd, J 7.4 and 13.9, C**H**H); 3.14 (1H, dd, J 7.4 and 13.9, CH**H**); 1.26 (3H, t, CH_2C**H_3**).

Preparation of DL-α-chloro acid, **27** (**Scheme 29**)

Four lipases; Novozyme 435, Lipozyme RM (*Rh. miehei*), Lipozyme TL (*Th. langinisa*), and *C. rugosa*, were screened in an attempt to hydrolyse the DL-α-chloro ester **23** to the DL-α-chloro acid racemate **22**. Lipase (100 mg) was added to a solution of DL-α-chloro methyl ester **23** (1 mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate was analysed by reverse phase HPLC.



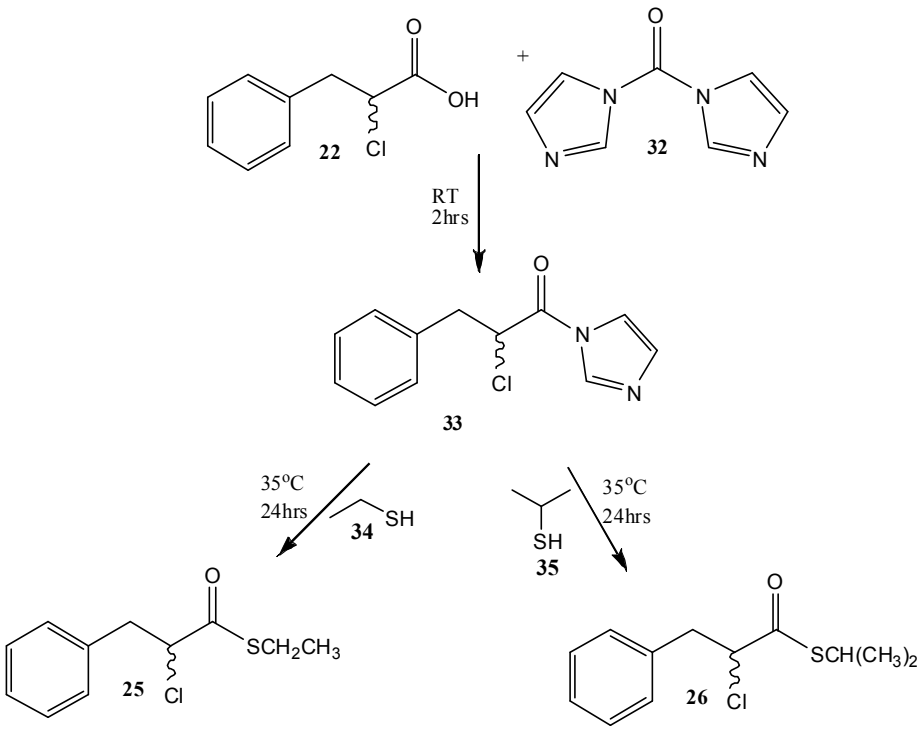
Scheme 29: Enzymatic hydrolysis of DL-α-chloro methyl ester **23** to yield DL-α-chloro acid **22**.

In order to generate a useable quantity of the racemic DL-acid **22**, Novozyme 435 (1g) was added to a stirred solution of **23** (70mL, 0.15M) in *t*ButOH / water (90: 10) and the mixture was allowed to stir at 40 ^{o}C for 24 hr. The pH of the reaction mixture was monitored continuously and sufficient quantities of base (10% ammonia in t-BuOH / water (90: 10)) were added automatically when required, to ensure the pH remained at 7. After 24 hr, when optimum conversion of **23** to **22** had been achieved, the Novozyme 435 was filtered from the reaction mixture and washed with acetonitrile (2 x 30mL). The filtrate was concentrated *in vacuo* to yield a yellow oil (residual ester **23**) mixed with the white solid **27**. Ethyl acetate (2x50mL) was added to extract the remaining ester, followed by Buchner filtration which yielded clean DL-acid **22a**, as a white crystalline solid. The acid was washed with ethyl acetate (50mL) and dried under suction prior to analysis by reverse phase and chiral HPLC and NMR.

DL-α-chloro acid racemate **22**, 184.65 g mol^{-1}, R_T; reverse phase HPLC, 4.9 min, OJ column chiral HPLC, 7.3, ADH column chiral HPLC, 7.9 and 7.2 min (L- and D-enantiomers respectively). ^{1}H NMR in DMSO solution: ^{1}H NMR in $CDCl_3$ solution: 7.38 (5H, m, **Ar**); 4.81 (1H, s, O**H**); 4.53 (1H, t, C**H**Cl); 3.38 (1H, m, C**H**H); 3.21 (1H, m, CH**H**).

Preparation of DL-α-chloro thioesters **25** and **26** (**Scheme 30**).

A solution of **22** (0.55g, 3mmol, 0.2M) and carbonyldiimidazole **32** (1.2equivalents, 0.58g) in DMF (15mL) was allowed to sir continuously at room temperature for 2 hr, to generate the unstable acyl imidazole **33**. Without isolation of the imidazole intermediate, ethane thiol **34** or 2-propane thiol **35** (1.2 eq.) was added to the reaction mixture, which was allowed to stir continuously at 35 ^oC for 24 hr. Removal of the DMF was achieved by quenching the reaction with copious amounts of water and extracting the crude thioester **25** or **26**, into DCM (3 x 200mL). The combined DCM layers were washed with water (3 x 50mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to yield the crude products as yellow oils. Purification of the products was done by flash chromatography using an eluent of 98: 2 hexane/ ethyl acetate. The appropriate fractions, analysed by thin layer chromatography (TLC) were combined and concentrated *in vacuo* to yield colourless oils of the thioesters. Analysis of the product was carried out by reverse phase HPLC and NMR.



Scheme 30: Preparation of racemic DL-α-chloro thioester substrates **25** and **26**.

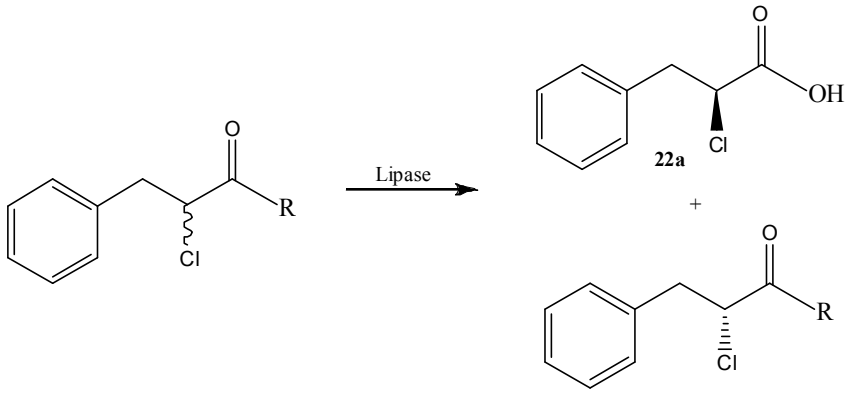
DL-α-chloro ethyl thioester racemate **25**, 228.74 g mol^{-1}, R_T; reverse phase HPLC, 9.5 min, OJ column chiral HPLC, 5.9 and 6.6 min (L- and D- enantiomers respectively, ADH column chiral HPLC, 4.6 min. ^{1}H NMR in $CDCl_3$ solution: 7.29 (5H, m, **Ar**); 4.50 (1H, m, C**H**Cl); 3.41 (1H, m, C**H**H); 3.11 (1H, m, CH**H**); 2.90 (2H, q, OC$\mathbf{H_2}$); 1.25 (3H, t, $CH_2C\mathbf{H_3}$).

DL-α-chloro i-propyl thioester racemate **26**, 241.77 g mol^{-1}, R_T; reverse phase HPLC, 10.3 min, OJ column chiral HPLC, 4.8 and 5.5 min (L- and D- enantiomers respectively, ADH column chiral HPLC, 4.3 min. ^{1}H NMR in $CDCl_3$ solution: 7.28 (5H, m, **Ar**); 4.48 (1H, m, C**H**Cl); 3.61 (1H, m, C**H**$(CH_3)_2$); 3.40 (1H, dd, J 8.4 and 14.1, C**H**H); 3.17 (1H, dd, J 8.4 and 14.1, CH**H**); 1.30 (6H, m, $(C\mathbf{H_3})_2$).

3.5.3.2 KINETIC RESOLUTIONS

Primary screening kinetic resolutions

Lipase (*ca.* 100mg) from a range of sources (AE04, AE07, AE08, AE011, Amano PS, Amano AS and Amano D) was added to a solution of racemic ester **23**, **24**, **25** or **26** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr (**Scheme 31**). After which, the enzyme was filtered off through cotton wool and celite and the filtrate (a mixture of acid product **22a** and ester) was analysed by reverse phase and chiral HPLC.



R = OCH_3 (**23**),OCH_2CH_3 (**24**), SCH_2CH_3 (**25**) or $SCH(CH_3)_2$ (**26**)

Scheme 31: Lipase catalysed hydrolysis of DL-α-chloro ester substrates to yield D-α-chloro acid **22a**.

Secondary screening kinetic resolutions

DL-α-chloro methyl ester **23**.

Either lipase AE04 or AE08 (*ca.* 100mg) was added to a solution of racemic ester **23** (1mL, 0.02M) in t-BuOH / water (90: 10) with TOA (1eq.) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate (a mixture of acid product **22a** and enantioenriched ester) was analysed by reverse phase and chiral HPLC.

DL-α-chloro ethyl ester **24**.

Lipase AE04, AE07, AE08 or Amano AS (*ca.* 100mg) was added to a solution of racemic ester **24** (1mL, 0.02M) in t-BuOH / water (90: 10) with TOA (1eq.) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate (a mixture of acid product **22a** and enantioenriched ester) was analysed by reverse phase and chiral HPLC.

DL-α-chloro ethyl thioester **25**.

Lipase AE07, AE011 or Amano PS (*ca.* 100mg) was added to a solution of racemic ester **25** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate (a mixture of acid product **22a** and enantioenriched ester) was analysed by reverse phase and chiral HPLC.

DL-α-chloro i-propyl thioester **26**.

Lipase AE04, AE07, AE08 or Amano AS (*ca.* 100mg) was added to a solution of racemic ester **26** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate (a mixture of acid product **22a** and enantioenriched ester) was analysed by reverse phase and chiral HPLC.

Large scale kinetic resolutions

Lipase AE04, AE07 or AE011 (1g per 1g ester) was added to a solution of racemic ester **23**, **24**, **25** or **26** (0.1M) in t-BuOH/ water (90: 10) and the reaction mixture was stirred at 40 ^{o}C until approximately 50% of the ester substrate had converted to the enantiomeric α-chloro acid **22a** or the less reactive enantiomer of the ester (L) started to be consumed. The reaction was monitored by chiral HPLC for up to 24 hr. The pH of the reaction mixture was monitored continuously and sufficient quantities of base (10% ammonia in t-BuOH/ water (90: 10)) were added automatically when

required, to ensure the pH remained at 7. After which, the enzyme was filtered off, washed with ACN and the filtrate was concentrated *in vacuo*. Ethyl acetate (100mL) was then added to the concentrated filtrate (containing some white solid (acid) and an orange oil (ester)) before the mixture was subjected to evaporation again *in vacuo*. As the acid product was not fully soluble in ethyl acetate, yet the ester substrate was, in order to remove the majority of the acid from the mixture, the ester was extracted into additional ethyl acetate (3 x 50mL) whilst the enantioenriched acid was filtered off and dried under suction. The organic portion was then concentrated *in vacuo* to yield the enantioenriched ester as oil. Upon analysis it was noticed that the acid product was partially soluble in the organic solvent and that some residual acid remained in the ester portion. Therefore, a further work up of the enantioenriched ester was required in order to remove this acid and so an acid base extraction was carried out. Aqueous sodium hydroxide (5M, 20mL) was added to a solution of the contaminated enantioenriched ester in DCM (20mL). The solution was transferred to a separating funnel, shaken vigorously and the layers allowed to separate. After which, the organic layer was further washed with additional aqueous sodium hydroxide (5M, 2 x 20mL) before being dried over magnesium sulphate and concentrated *in vacuo* to yield the pure, acid free, enantioenriched ester. The enantiomeric excess of the unreacted ester enantiomer of the starting material (L) was then determined using chiral HPLC.

3.5.3.3 RACEMISATIONS

Racemisations with organic bases

A range of organic bases, in varying strength, were investigated, as outlined in **Table 37**.

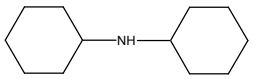
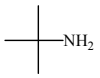
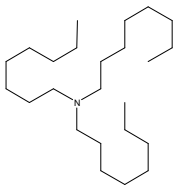
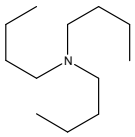
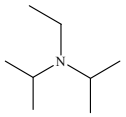
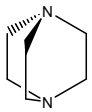
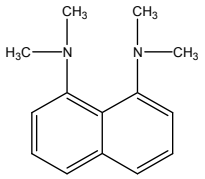
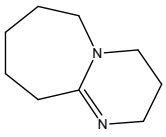
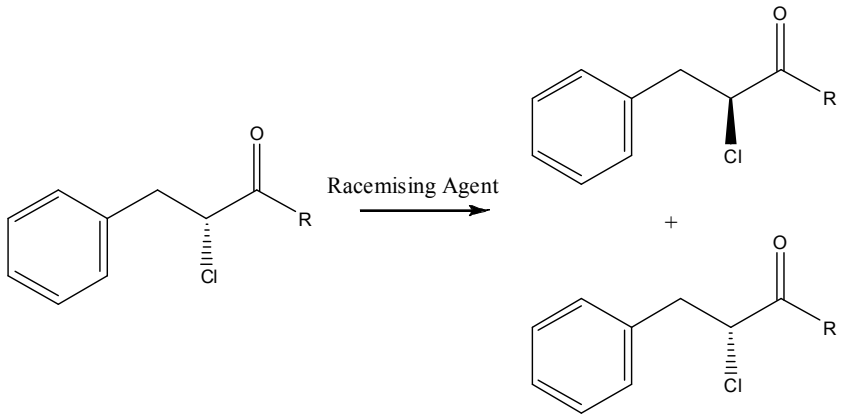
Base	Acronym	Structure
Pyridine	PY	
Dicyclohexylamine	DCHA	
Tert-Butylamine	TEBA	
Tri-Octylamine	TOA	
Tri-Butylamine	TRBA	
N,N-Diisopropylethylamine	DIPEA	
1,4-Diazabicyclo[2.2.2]octane	DABCO	
Proton Sponge	PS	
1,8-Diazabicycloundec-7-ene	DBU	

Table 37: Range of organic bases studied in the racemisation experiments of the enantioenriched esters.

Organic base PY, DCHA, TEBA, TOA, TRBA, DIPEA, DABCO, PS, or DBU (2eq.) was added to a solution of the enantioenriched ester **23**, **24**, **25** or **26** (1mL, 0.02M) in t-BuOH/water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr (**Scheme 32**). After which, the mixture was analysed by reverse phase and chiral HPLC.



R = OCH_3 (**23**), OCH_2CH_3 (**24**), SCH_2CH_3 (**25**) or $SCH(CH_3)_2$ (**26**)

Scheme 32: Racemisation of enantioenriched α-chloro esters to yield the corresponding racemic esters.

Racemisation with chlorides in t-BuOH / water

Aliquat 336, tetrabutylammonium chloride, tetraphenylphosphonium chloride, tributylmethylphosphonium chloride (polymer bound), ammonium chloride (polymer bound), Merrifield polymer, or triphenylphosphonium chloride on polymer (2eq.) was added to a solution of the enantioenriched ester **23** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the mixture was analysed by reverse phase and chiral HPLC.

Racemisation with chlorides in a range of solvents

Aliquat 336, tetrabutylammonium chloride, tetraphenylphosphonium chloride, tributylmethylphosphonium chloride (polymer bound), ammonium chloride (polymer bound), Merrifield polymer, or triphenylphosphonium chloride on polymer (2eq.) was added to a solution of the enantioenriched ester **23** (1mL, 0.02M) in t-BuOH / water (90: 10), hexane, toluene, DCM, ACN or DMSO and the mixture was allowed

to stir continuously at 40 ^{o}C for 24 hr. After which, the mixture was analysed by reverse phase and chiral HPLC.

3.5.3.4 DYNAMIC KINETIC RESOLUTION REACTIONS

DL-α-chloro methyl ester **23**

Lipase AE04 (*ca.* 100mg) and Aliquat 336 or polymer bound triphenylphosphonium chloride (2eq.) was added to a solution of racemic ester **23** (1mL, 0.02M) in DMSO and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate was analysed by reverse phase and chiral HPLC.

DL-α-chloro ethyl thioester **25**

Lipase AE011 (*ca.* 100mg) DCHA, TEBA, or DIPEA (2eq) was added to a solution of racemic thioester, **25** (1mL, 0.02 M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate was analysed by reverse phase and chiral HPLC.

DL-α-chloro i-propyl thioester **26**

Lipase AE07 (*ca.* 100 mg) and DCHA, TEBA or DIPEA (2eq.) was added to a solution of racemic thioester **26** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate was analysed by reverse phase and chiral HPLC.

Optimisation of DKR reaction with respect to the lipase source

Lipase (*ca.* 100mg) from a range of sources (AE04, AE07, AE08, AE011, Amano PS, Amano AS, Amano D, AE05, wheat germ, porcine pancreas, Amano AK, Amano M, Amano G or Julich RN) and TEBA (2eq.) was added to a solution of racemic thioester, **25** or **26** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate was analysed by reverse phase and chiral HPLC.

Optimisation of DKR reaction with respect to temperature

Lipase AE011 (*ca.* 100mg) and TEBA (2eq.) was added to a solution of racemic thioester **25** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at RT, 40^{o}C or 75 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate was analysed by reverse phase and chiral HPLC.

Optimisation of DKR reaction with respect to amount of organic base

Lipase AE011 (*ca.* 100mg) and TEBA (0.5-10eq.) was added to a solution of racemic thioester **25** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate was analysed by reverse phase and chiral HPLC.

Optimisation of DKR reaction with respect to amount of solvent/ water mixture

Lipase AE011 (*ca.* 100mg) and TEBA (0.5-10eq) was added to a solution of racemic thioester **25** (1mL, 0.02M) in a range of solvent (hexane, toluene, DCM, ACN)/ water compositions (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr. After which, the enzyme was filtered off through cotton wool and celite and the filtrate was analysed by reverse phase and chiral HPLC.

3.6 CONCLUSIONS

Enantiomers of racemic compounds can often exhibit diverse pharmacological and therapeutic properties. In fact, different drugs usually have their biological activity based mainly on one enantiomer. Consequently, research into the preparation of enantiomerically pure chiral compounds is increasing in interest. Dynamic kinetic resolution is emerging as a potentially efficient process, whereby, standard enzymatic kinetic resolution is coupled with *in situ* racemisation of the slow reacting starting substrate, using a suitable racemising catalyst. A lipase catalysed, enantioselective hydrolysis process with continuous *in situ* racemisation of substrate, using TEBA as an organic base, was developed for the production of D-α-chloro acid from racemic α-chloro thioesters, in t-BuOH/ water (90: 10).

The D-forms of racemic ester and thioester substrates were found to be substrates for the lipase catalysed hydrolysis to the corresponding chiral acids, affording excellent optical purity (>99%ee_D). Racemisation studies, with a range of organic bases and racemising chlorides, suggested that the L-enantiomers of the esters (L-α-chloro methyl **23** and L-α-chloro ethyl ester **24**) were inert to racemisation back to the corresponding DL-racemates, under the reaction conditions studied. However, three organic bases (DCHA, TEBA and DIPEA) were identified as suitable racemising catalysts for the L-enantiomers of the thioester substrates. Combination of the KR and racemisation reactions for the two thioesters, allowed for successful DKR processes to be established. Investigations on the parameters indicated that 5 eq. of *tert*-butylamine as the *in situ* racemising agent and an operating temperature of 40^oC gave optimum conversions and optical purity in the dynamic kinetic resolution reactions. Excellent to good conversions (>99% and 78% for the ethyl and propyl thioesters respectively) of racemic ester to L-acid were achieved, with the resulting chiral acid in high to moderate optical purity (93%ee_D and 79%ee_D for the ethyl and propyl thioesters respectively) after 24 hr in batch.

4 IMPROVING KINETIC RESOLUTION REACTIONS

4.1 AIMS

Efficient methods for the enantioselective hydrolysis of a series of esters, using a range of lipases, have been developed (**Chapter 3**, **Section 3.4.2**). The D-forms of the racemic ester and thioester substrates were found to undergo lipase catalysed enantioselective hydrolysis to the corresponding chiral acids, in excellent optical purity (>99%ee_D). Base catalysed racemisation studies showed that the ester substrates could not be racemised to any extent, under the reaction conditions studied. However, three organic bases were identified as suitable racemising catalysts for the L-enantiomers of the two thioester substrates under investigation. The combined reactions allowed for a DKR process to be established for the biocatalytic production of enantioenriched D-α-chloro acid from two racemic α-chloro thioester substrates (**Chapter 3**, **Section 3.4.4**). Whereby, the lipase catalysed enantioselective hydrolysis of the D-ester, coupled with TEBA catalysed *in situ* racemisation of the slow reacting enantiomer afforded excellent to good conversions and enantioselectivies.

Miniaturising chemical processes results in a number of advantages (discussed in **Chapter 1**, **Section 1.3**) and with this in mind, successful DKR processes are to be investigated with the aid of miniaturised reactor technology, as a means of improving the technique further. It would therefore be possible to determine if those advantages associated with the miniaturisation of biocatalytic synthetic reactions, observed in **Chapter 2** can be harnessed for the enantioselective chemistry reported in **Chapter 3** also.

Additionally, as discussed in **Chapter 3** (**Section 3.3.2.3**) there are often compatibility issues surrounding one-pot, chemo-enzymatic dynamic kinetic resolution reactions (Martin-Matute & Backvall, 2007). That is, the presence of the racemising agent often affects the activity of the enantioselective enzyme. In fact, the unsuccessful DKR reaction of the racemic α-chloro methyl ester substrate, observed in **Chapter 3** (**Section 3.4.4.1**) would appear to be an example of this, where the presence of the *in situ* racemising chloride in the reaction pot, rendered the

biocatalyst inactive. Similarly, Roengpithya *et al*. (2006) experienced compatibility issues with regards to the racemising agent and biocatalyst in a DKR reaction, where the presence of racemising bases in the one pot reaction resulted in a less enantiospecific KR process. It is proposed that if the kinetic resolution and racemisation steps can be successfully achieved separately in the packed bed, miniaturised, continuous flow reactor (described in **Chapter 2**), then the reduction in enzyme activity or enantiospecificity, in the presence of a racemising agent, would be overcome. It is therefore believed that by combining an immobilised biocatalyst and an immobilised base, operating in separate reaction capillaries, this approach would overcome compatibility issues whilst maintaining the inherent advantages of miniaturisation. Using this approach, the ester under investigation would pass through the immobilised enzyme reactor where enantioselective hydrolysis would occur and then through the immobilised base where racemisation of the residual ester would occur. The reaction products could then be re-circulated through the enzyme to attain the desired acid in excellent yield and enantiopurity. **Chapter 4** therefore is a combination of the miniaturisation work investigated in **Chapter 2** and the lipase catalysed enantioselective resolution experiments discussed in **Chapter 3**.

4.2 RESULTS & DISCUSSION

4.2.1 ENZYME IMMOBILISATIONS

It is important when utilising a packed bed, miniaturised continuous flow reactor, that the catalysts of interest are utilised in their immobilised form (discussed in **Chapter 1**). Therefore, prior to conducting the lipase catalysed enantioselective hydrolysis reactions in the packed bed, miniaturised set up, immobilised forms of the lipase enzymes and racemising agents of interest needed to be obtained.

As is detailed in **Chapter 3** (**Section 3.4.2**), free lipases AE04, AE011 and AE07 were identified as enantioselective enzymes for the biocatalytic hydrolysis of racemic α-chloro methyl **23** (AE04), α-chloro ethyl **24** (AE04), α-chloro ethyl thio **25** (AE011) and α-chloro propyl thio **26** (AE07) esters.

Prior to investigating techniques for the immobilisation of these three lipases (AE04, AE011 and AE07), it is important to consider the fact that lipases exhibit a very complex catalytic mechanism that implies important conformational changes in the zones near to the active site. Lipases can exist in two different conformations; a closed inactive conformation, where the active site is secluded by an oligopeptide chain and an open conformation, where this so called 'lid' is displaced. It is for this reason that the use of different immobilisation techniques involving different areas of the enzyme, generating a certain microenvironment can result in different functional properties of the biocatalyst, including activity, stability and enantioselectivity. As is discussed in **Chapter 1**, enzymes can be immobilised using different supports and protocols to obtain insoluble preparations. It is important that if the free enzyme is successful as an enantioselective catalyst then the catalytic properties of the immobilised biocatalyst are not so different that this feature is lost or, in the worse case scenario, complete inactivation of the enzyme results. Additionally, as the enzymes in question are to be utilised in a packed bed, miniaturised continuous flow reactor, a support that would result in minimum swelling and therefore avoid problems with the generation of back pressure within the reactor capillary, must be chosen.

As is discussed in great detail in **Chapter 1** (**Section 1.3.3**), many chemists have studied several different approaches for the immobilisation of an enzyme within the microreactor. The approaches discussed include immobilisation of an enzyme onto silica or polymer microparticles (which are then packed into the reactor channel of the miniaturised reactor), immobilisation of a biocatalyst directly onto the inner walls of the reactor, utilising membranes or incorporating a monolith into miniaturised reactor channels. Each method hosts a range of advantages and disadvantages, discussed in **Chapter 1** (**Section 1.3.3**). However, the size of the reactor capillary used for the work presented herein is not significantly small (30 x 1.65mm). It was decided therefore, that if particles of the support matrix of choice were small enough to easily pack into these dimensions, pre-immobilisation of the biocatalyst to a support, which would not swell under the reaction conditions, would be the most feasible option for immobilisation of the biocatalyst. Likewise, many commercially available lipases could be also used in the miniaturised flow reactor in this way. In so far as the support matrix of choice is concerned, controlled porous glass (CPG) provides an attractive solution due to its resistance towards solvents at a high pH, durability and zero swelling characteristics in flow systems.

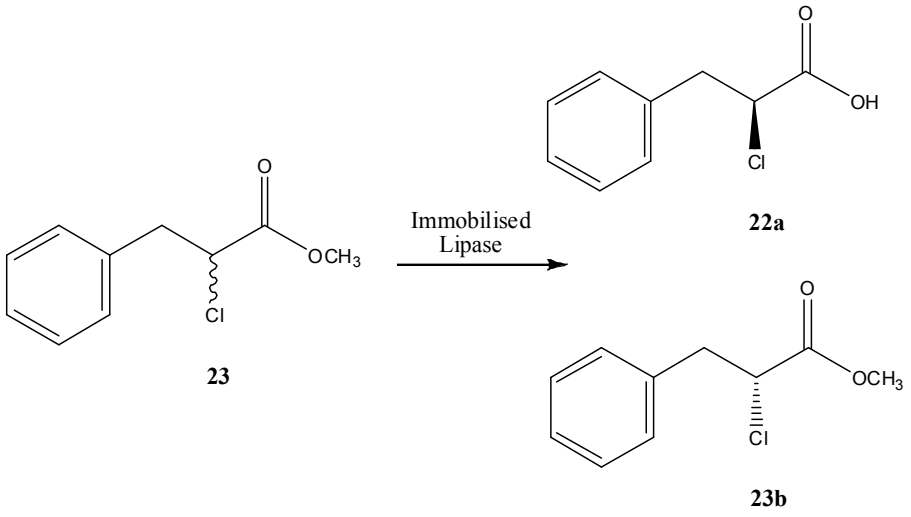
Due to time limitations, enzyme immobilisation *via* adsorption to the insoluble support matrix (controlled pore glass) was chosen. Although yields of enzyme bound per unit of adsorbent tend to be typically low, and the biocatalyst may be partially or totally inactivated, enzyme immobilisation *via* adsorption is exceedingly easy to perform, where typically, the adsorbent and enzyme are simply stirred together for a period of time. It is important to mention that without time constraints, alternative immobilisation techniques would have been investigated, such as immobilisation by covalent binding, entrapment or using perhaps cross-linked enzyme crystals (discussed in **Chapter 1**, **Section 1.2.1**).

4.2.2 BATCH KINETIC RESOLUTIONS

An attempt was made to immobilise the soluble forms of the three lipases (AE04, AE07 and AE011) *via* adsorption to controlled pore glass. After which, batch, kinetic resolution experiments for the four ester substrates **23**, **24**, **25** and **26**, to yield the chiral acid **22a**, were conducted utilising the immobilised enzymes (**Section 4.2.2**). These additional batch reactions, which were identical to those conducted using the free forms of lipase, would determine if the biocatalysts had been successfully immobilised onto the CPG support and remained active.

4.2.2.1 RACEMIC DL-α-CHLORO METHYL ESTER

The immobilised lipase catalysed enantioselective resolution of DL-α-chloro ethyl thioester **23** to yield the desired enatiomeric acid product **22a** and residual L-enantiomer, **23b** (**Scheme 33**) was investigated using the in-house prepared immobilised form of lipase AE04, to determine if immobilisation of the biocatalyst had been successful.



Scheme 33: Immobilised lipase catalysed enantioselective hydrolysis of the racemic ester **23** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **23b**.

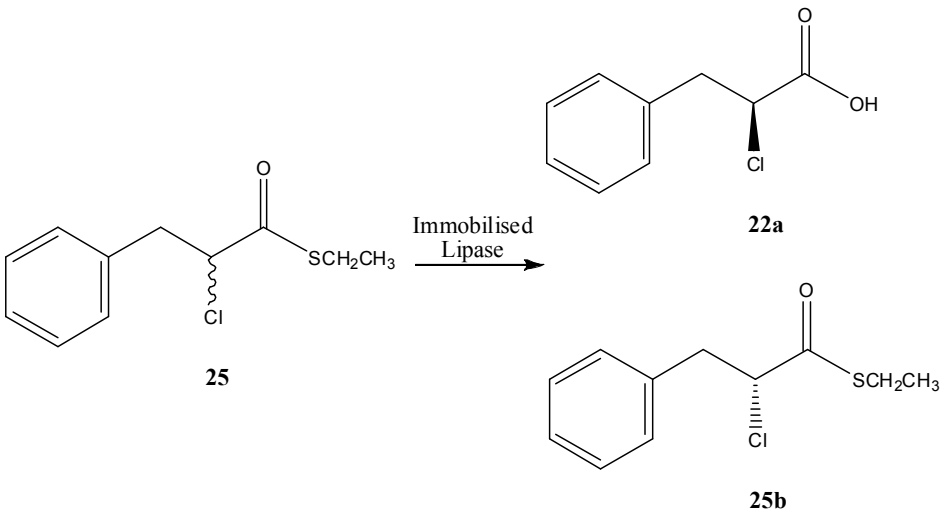
Under the reaction conditions studied, there was no conversion of racemic α-chloro methyl ester **23** to acid **22a**, even after 48 hr at 40 ^{o}C when the immobilised form of lipase AE04 was utilised. A successful KR reaction using the free form of AE04, conducted at the same time and under the exact same conditions, confirmed that the

lack of conversion of ester to acid seen when utilising the in-house immobilised lipase was due to issues with the immobilisation of lipase AE04 onto the CPG support. This would suggest that either immobilisation of the lipase had been unsuccessful *via* the adsorption protocol followed or that the activity of the biocatalyst had reduced significantly as a result of immobilisation.

The enantioselective hydrolysis of racemic α-chloro ethyl ester was also most successful with lipase AE04 (**Chapter 3**, **Section 3.4.2.1**). However, as a successful KR process utilising immobilised AE04 had not been achieved, due to suspected inactivation of the enzyme as a result of immobilisation, no kinetic resolution experiment with the immobilised lipase was conducted for this additional substrate (α-chloro ethyl ester **24**). In fact, until a suitable immobilised biocatalyst can be identified for the KR of these two esters (α-chloro methyl **23** and α-chloro ethyl **24**), requiring a significant amount of extra time, no further studies involving either of these two ester substrates were conducted with regards to the packed bed, miniaturised flow reactor. Our attentions were then turned to the two thioester substrates of interest (α-chloro ethyl **25** and α-chloro i-propyl **26**).

4.2.2.2 RACEMIC DL-α-CHLORO ETHYL THIOESTER

The immobilised lipase catalysed enantioselective resolution of DL-α-chloro ethyl thioester **25** to yield the desired enatiomeric acid product **22a** and residual L-enantiomer, **25b** (**Scheme 34**) was investigated using the in-house prepared immobilised form of lipase AE011, to determine if immobilisation of the biocatalyst had been successful. In addition to the in-house prepared immobilised biocatalysts, two commercially immobilised forms of lipase AE011 were obtained and investigated. Lipase AE175 and lipase AE176 were commercial forms of lipase AE011 adsorbed on to granulated and fined silica respectively. The suitability of these lipases was assessed, whilst providing a benchmark for the efficiency of the immobilised lipases in the packed bed, miniaturised flow reactor.



Scheme 34: Immobilised lipase catalysed enantioselective hydrolysis of the racemic ester **25** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **25b**.

It can be seen from the results shown in **Table 38** that, as one would expect, the kinetic resolution experiment utilising free lipase AE011 yielded similar results to those obtained previously (**Chapter 3**, **Section 3.4.2.3**), where the conversion of ester to acid was 52% with a 34%ee_D of the chiral product.

It is widely reported that immobilisation of an enzyme very often results in a decrease in enzyme activity and the reaction detailed here would appear to support this fact. When used in the free form, lipase AE011 resulted in 54% conversion after 24 hr, under the reaction conditions studied. However, when used in the immobilised state, irrespective of the support, the same enzyme afforded considerably lower conversions of acid to ester, under the same reaction conditions. In fact, in the case of the in-house immobilised lipase, the conversion of ester to acid after 24 hr was some 16% less. The larger surface area of the fined silica support and hence increased enzyme/substrate contact in the reaction pot, is believed to be the reason for an improved percentage conversion (45%) of racemic ester **25** to acid product after 24 hr, compared with the conversions reached when the support was CPG or granulated silica (38 or 40% respectively).

What is most obvious from the results shown in **Table 38** however, is the massive improvement in enantiomeric excess of the acid product when utilising an immobilised form of the resolution biocatalyst, in particular, when the support in

question is controlled pore glass. It is documented in the literature that the enantioselectivity of an enzyme can increase significantly as a result of immobilisation (Cao, 2005) and it would appear from the results obtained herein that the choice of support plays an important role also. The commercially obtained immobilised AE011 enzymes (AE175 and AE176) provided increased conversions of racemic ester **25** to acid **22** (40 and 45% respectively) in comparison to the conversion achieved using the in-house immobilised lipase (38%). However, it would appear from the $\%ee_D$ values shown that when lipase AE011 is immobilised to CPG as opposed to granulated or fined silica, the selectivity of the enzyme is dramatically increased. An improvement from an average of $48\%ee_D$ acid with the silica supported enzymes to an impressive $90\%ee_D$ acid when CPG was employed is observed. It is likely that the method by which the enzyme was immobilised to the support is the reason for this. Whereby, adsorption of the lipase to different supports incorporates alternative areas of the enzyme and results in a net change in selectivity.

Lipase	Conversion to Acid / %	Acid / % ee_D
Immobilised in-house	38	90
AE175	40	49
AE176	45	47
AE011	54	29

Table 38: Lipase catalysed enantioselective hydrolysis of the racemic ester **25** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **25b**. Reaction conditions; 40 ^oC, *ca.* 100mg lipase, 1mL substrate (0.02M in t-BuOH/water (90: 10)) for 24 hr in batch.

Ultimately, the enzymes are to be used in a packed bed, miniaturised continuous flow reactor which would ideally operate at room temperature. Therefore, it was decided to repeat the immobilised lipase catalysed KR experiments at room temperature as opposed to 40 ^oC. This additional investigation resulted in some interesting data, detailed in **Table 39**.

Lipase	Conversion to Acid / %	Acid / % ee_D
Immobilised in-house	25	> 95
AE175	35	> 95
AE176	35	> 95
AE011	38	81

Table 39: Lipase catalysed enantioselective hydrolysis of the racemic ester **25** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **25b**. Reaction conditions; RT, *ca.* 100mg lipase, 1mL substrate (0.02M in t-BuOH/water (90: 10)) for 24 hr in batch.

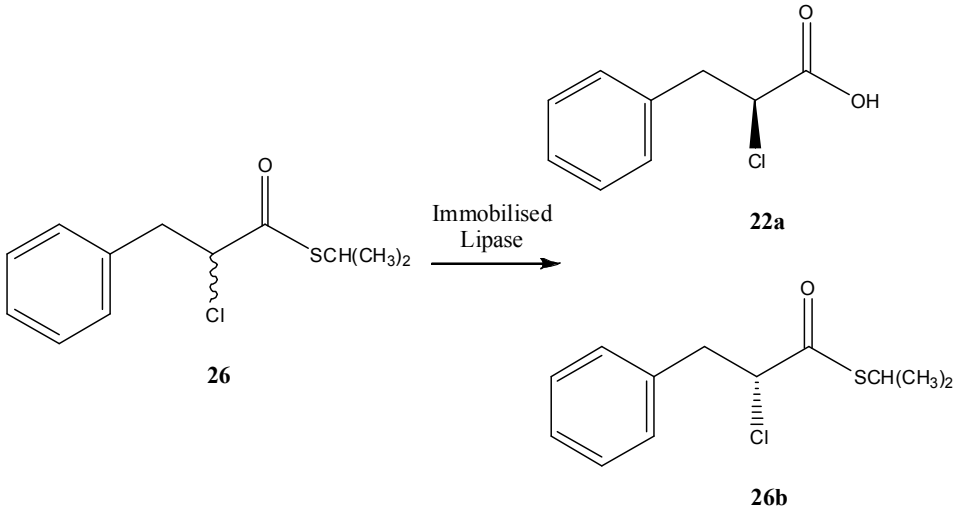
It can be seen from the results shown in **Table 39** that, as one would expect, the trend in terms of conversion of racemic ester **25** to chiral acid **22a**, for the different forms of AE011 is consistent with that observed when the reactions were conducted at 40 ^oC. The immobilised lipase prepared in-house showed the lowest activity and hence gave the lowest conversion of ester to acid (25%). Improved conversions of ester to acid were observed when the commercially available lipase enzymes immobilised to granulated (35%) or fined silica (35%) were utilised. The KR reaction catalysed using the free form of lipase AE011 yielded the highest conversion of acid to ester (38%), confirming that the greatest level of enzyme activity is achieved when the biocatalyst has not been immobilised. Unlike the results obtained for the comparable reaction conducted at 40 ^oC, here there is no apparent difference between the use of granulated and fined silica as a support for lipase AE011 in so far as percentage conversion of ester to acid is concerned.

What is more interesting however, are the values obtained for $\%ee_D$ of the chiral acid product following the KR reactions. The results would suggest that the overall selectivity of the lipase enzyme in the kinetic resolution reaction is improved when the reaction is carried out at room temperature. At 40 ^oC, the optimum $\%ee_D$ of the product after the KR reaction was 90% when the in-house prepared immobilised lipase was used. A much poorer average %eeD of 48% was observed for the acid product when lipases AE175 and AE176 were used in the KR reactions at 40 ^oC, after 24 hr. The acid was only obtained in a purity of 29%ee_D after 24 hr of the KR reaction when lipase AE011 in the free form was utilised at 40 ^oC. When the same series of KR reactions were conducted at room temperature however, the purity of the chiral acid product was excellent in all cases. In fact, the poorest result was still

high at 81%ee_D when free lipase AE011 was used and when the biocatalyst was employed in the immobilised forms, the chiral purity of the product was >95% in all cases, following the enantioselective hydrolysis of α-chloro ethyl thioester **25** in batch, at room temperature, after 24 hr. This would suggest that the slower rate of hydrolysis seen at ambient temperature allows for improved selectivity towards the hydrolysis of the D-α-chloro ethyl thioester. In addition to this, where at 40 ^oC, the CPG supported lipase showed the most impressive %ee_D acid values, those obtained using the silica supported lipase are equally as good here. Again, an improvement in reaction control and hence enzyme selectivity is thought to be the reasoning for this, where if the reaction is proceeding slower, the enzyme has more chance to catalyse the preferred reaction with respect to the racemic ester substrate. These results provide scope for additional work investigating the reactions and enzyme kinetics in more detail. However, it is sufficient for the purpose of the work described here, to conclude that immobilisation of lipase AE011 *via* adsorption to CPG has been a success and results in a biocatalyst with an activity not dissimilar to that seen for commercially available forms of the immobilised enzyme or even, the enzyme in its free form. Transfer of the kinetic resolution reaction of racemic α-chloro ethyl thioester **25**, utilising these immobilised lipases, to the packed bed, miniaturised continuous flow reactor could now take place. This work, detailed in **Section 4.2.3**, allowed us to determine if the inherent advantages associated with miniaturisation, discussed in **Chapter 1** could improve the KR technique developed in **Chapter 3**.

4.2.2.3 RACEMIC DL-α-CHLORO i-PROPYL THIOESTER

The immobilised lipase catalysed enantioselective resolution of DL-α-chloro i-propyl thioester **26** to yield the desired enantiomeric acid product **22a** and residual L-enantiomer, **26b** (**Scheme 35**) was investigated using the in-house prepared immobilised form of lipase AE07, to determine if immobilisation of the biocatalyst had been successful.



Scheme 35: Immobilised lipase catalysed enantioselective hydrolysis of the racemic ester **26** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **26b**.

Under the reaction conditions studied, there was no conversion of racemic α-chloro propyl thioester to acid, even after 48 hr at 40 ^{o}C when the immobilised form of lipase AE07 was utilised. A successful KR reaction using the free form of AE07, conducted at the same time and under the exact same conditions, confirms that the lack of conversion of ester to acid seen when utilising the in-house immobilised lipase was due to issues with the immobilisation of lipase AE04 onto the CPG support. This would suggest that either immobilisation of the lipase had been unsuccessful *via* the adsorption protocol followed or that the activity of the biocatalyst has reduced significantly as a result of immobilisation.

Lipase AE011 has been successfully immobilised onto CPG *via* adsorption. This immobilised biocatalyst has been utilised, with success, in a batch, kinetic resolution reaction of racemic α-chloro ethyl thio ester. The results of which were comparable to, and in the case of selectivity of the enzyme towards the L-ester an improvement upon, the reactions conducted using either commercially available forms of the immobilised lipase or the free equivalent. Lipases AE04 and AE07 did not result in the same success and it would seem from the results that immobilisation of these two enzymes renders the biocatalyst inactive or there is no immobilisation of the enzyme. Collaboration with the supplier of the immobilised forms of lipase AE011 (AE175 and AE176) confirmed that the immobilisation of lipases AE04 and AE07 is problematic and hence commercial equivalents are not currently available either.

Factors such as pore size, available surface area, internal structure and particle size of the glass are possible reasons for unsuccessful immobilisation with lipases AE04 and AE07 but not AE011. Whilst the immobilisation of these two enzymes would form the basis for some additional work, it was not important to focus on this for the work presented herein. This is because AE011 has been successfully immobilised whilst retaining a good amount of activity and a high amount of selectivity. Therefore, the remainder of the work discussed in **Chapter 4** focuses on enantioselective hydrolysis reactions of DL-α-chloro ethyl thioester that utilises lipase AE011.

4.2.3 MINIATURISED KINETIC RESOLUTIONS

Successful batch KR reactions of α-chloro ethyl thioester **25** utilising immobilised forms of lipase AE011 (on CPG, fined or granulated silica) at both 40 ^{o}C and ambient temperature have been carried out (**Section 4.2.2.2**). Consequently, the process was transferred to the packed bed, miniaturised reactor to determine if the inherent advantages of miniaturisation such as faster reactions due to an increase in the surface to volume area ratio can be realised for the KR reaction presented here. The conditions used were based on the batch kinetic resolution experiments, where all experiments were carried out using lipase AE011 immobilised to CPG as the biocatalyst (packed into the reactor capillary), t-BuOH/water (90: 10) as the solvent and at ambient temperature.

The flow rate of the racemic ester through the miniaturised reactor was investigated using the enantioselective hydrolysis of DL-α-chloro ethyl thioester **25** to yield the chiral acid **22a** (**Scheme 2**). It can be seen from the results shown in **Table 40** that as the flow rate through the miniaturised, continuous flow reactor is decreased an increase in percentage conversion of racemic ester to acid is observed. At 25 and 1μLmin^{-1}, conversions of 4 and 32% were observed respectively, after collection of the product stream for 10 min.

Flow Rate / μLmin-1	Conversion to Acid after 10 min / %	Acid / % ee_D
25.0	4	>99
10.0	7	>99
4.0	13	>99
2.0	25	>99
1.0	32	>99

Table 40: Immobilised lipase catalysed enantioselective hydrolysis of the racemic ester **25** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **25b**. Reaction conditions; RT, *ca.* 10mg lipase, 1mL substrate (0.02M in *t*-BuOH/water (90: 10)) in the packed bed, miniaturised continuous flow reactor.

It would seem that at $1\mu Lmin^{-1}$ the optimum percentage conversion has been reached and further decreases in the flow rate do not appear to increase the conversion any further (at a flow rate of $0.5\mu Lmin^{-1}$ a conversion of 32% was also attained). The decrease in percentage ester conversion observed at the higher flow rates is due to a reduced residence time of the reactants in the capillary and hence lower conversion of starting materials to product. Whereas upon decreasing the flow rate, the residence time of the reactants over the catalyst bed increases and provides more opportunity for reaction, resulting in higher conversions of racemic ester to acid. Therefore, flow rates of $1\mu Lmin^{-1}$ were maintained for the remainder of the work.

One of the disadvantages associated with the use of packed bed, continuous flow reactors is that access to the reactor is limited. As a result, this type of reactor is difficult to recharge with the enzyme making it not ideally suited for use with enzymes with a short operational half life. Although immobilisation through adsorption is one of the most widely used methods of enzyme immobilisation, adsorbed enzymes are not very useful for employment in high water activity systems because of the possibility of desorption. As there is a significant degree of polarity in the reaction mixture presented here (t-BuOH/ water (90: 10)) and desorption is possible, a further study, investigating the activity of immobilised AE011 onto CPG was conducted over a period of 8 hr.

It was found that the conversion of racemic ester **25** to chiral acid **22a**, within the limits of experimental error, did not vary from 30% over a period of 8 hr for the enantioselective hydrolysis of DL-α-chloro ethyl thioester in the miniaturised,

continuous flow reactor with lipase AE011 immobilised onto CPG. This would suggest that the actual efficiency of the enzyme does not appear to diminish for up to 8 hr at least, in the reaction conditions studied and thus enzyme desorption is not a problem in this particular system.

Following optimisation of the miniaturised, continuous flow reactor experiment, with respect to flow rate, and the successful preparation of chiral acid **22a** in a reasonable conversion (32%) and with excellent optical purity (>99%ee_D), using lipase AE011 immobilised to CPG, a comparison of a standard bench top, one pot, batch reaction (**Section 4.2.2.2**) and the miniaturised reaction was carried out. This would determine if the inherent advantages of miniaturised reaction systems such as faster reactions due to increased surface to volume area ratio can be realised. The reaction conditions were identical in batch (0.02M reaction mixture in t-BuOH/ water (90: 10), at room temperature using lipase AE011 immobilised to CPG) and samples were taken every 10 min to mimic the miniaturised reaction and analysed using the same analytical methodology.

It is evident from the results shown in **Figure 19** that miniaturisation of the enantioselective hydrolysis reaction of DL-α-chloro ethyl thioester **25** has several advantages with respect to the rate of the reaction. After 10 min in the batch reaction only 14% conversion, of the maximum theoretical 50% for KR reactions, was achieved compared to 32% in the miniaturised reaction set up. Although the percentage conversion of ester to acid in the batch reaction increased with time, it was not until 60 min that a result comparable to the miniaturised set up (32%) was reached. This improved efficiency in the miniaturised reactor set up is attributed to the smaller reaction environment in the miniaturised capillary. This means that, compared with batch reactions, the effective concentration of enzyme in the miniaturised flow reactor is higher due to a high surface to volume ratio, resulting in a greater percentage conversion of ester to acid, in a shorter amount of time. In all cases, the optical purity of the chiral acid was excellent (>99%ee_D).

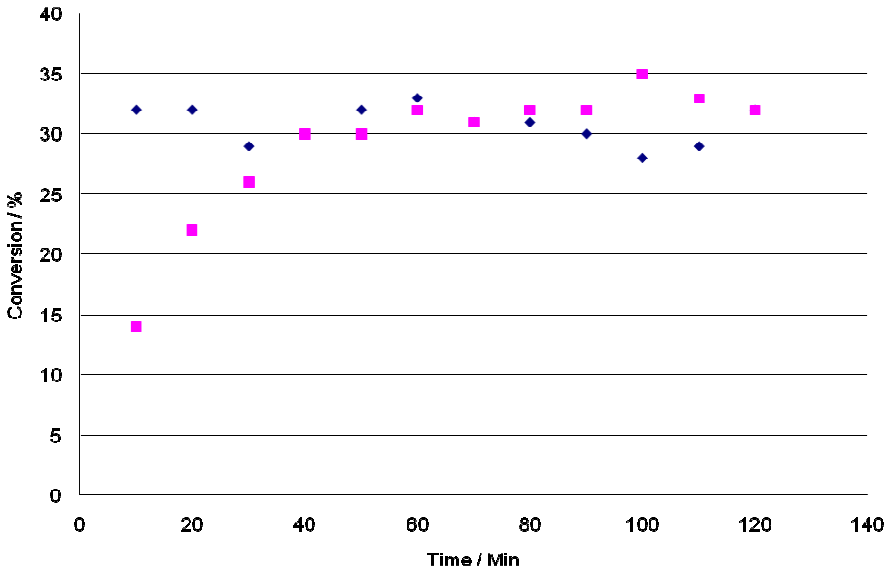


Figure 19: Immobilised lipase catalysed enantioselective hydrolysis of the racemic ester **25** to yield the enantiomeric D-α-chloro acid **22a** and residual enantioenriched L-α-chloro ester **25b**. Reaction conditions; RT, lipase AE011 immobilised to CPG, 1mL substrate (0.02M in t-BuOH/water (90: 10)) in a miniaturised flow reactor (♦) at 1μLmin-1 and in batch (■).

4.2.4 MINIATURISED RACEMISATIONS

Unfortunately, immobilisation of lipase AE04 onto a solid support for incorporation into the packed bed, miniaturised flow reactor was unsuccessful. Therefore, an immobilised lipase for the enantioselective hydrolysis of the two DL-α-chloro ester substrates has not been identified at this stage. Consequently, within the time constraints of the work presented, at least, a two stage DKR process is not going to be established for the DL-α-chloro ester substrates. Whilst Aliquat 336 and polymer bound triphenylphosphonium chloride were found to be potential racemising agents for these two substrates (**Chapter 3**, **Section 3.4.3**), further investigations to identify an immobilised chloride capable of racemising the slow reacting L-α-chloro ester were omitted from the work presented. However, this area could be the focus of additional work in the future.

As is described in **Chapter 3** (**Section 3.4.3**), DCHA, TEBA and DIPEA would appear to be capable of racemising the L-α-chloro ethyl **25** and L-α-chloro i-propyl **26** thioesters, to different degrees and at varying rates, in t-BuOH/ water (90: 10).

TEBA was identified as being the most suitable *in situ* racemising base for the DKR reactions of the DL-α-chloro thioesters (**Chapter 3.4.4**). As a successful KR protocol is in place for the DL-α-chloro ethyl thioester utilising lipase AE011 immobilised onto CPG, techniques into the immobilisation of TEBA (and other possible racemising bases) were investigated, in collaboration with Charlotte Wiles. If successful, this would allow the two steps of the DKR process to be conducted separately using two isolated reactor capillaries in the packed bed, miniaturised continuous flow reactor. In addition to the investigation to immobilise the TEBA racemising base, a more extensive range of commercially available immobilised racemising agents were also studied.

It can be seen from the results detailed in **Chapter 3** (**Section 3.4.3.3**) that TEBA is capable of racemising L-α-chloro ethyl thioester **25b** at a suitably rapid rate. Where, after 2 hr in a batch reaction, at 40 ^{o}C in t-BuOH/water (90: 10), the %ee$_L$ of the enantioenriched ester had decreased from 90% in the chiral mixture to 2% when the corresponding racemate had been achieved. As is reported in **Chapter 3** (**Section 3.4.4.2**) this success allowed TEBA to be utilised, in free form, as an *in situ* racemising catalyst for the DKR of DL-α-chloro ethyl thioester **25** to yield the corresponding acid in high conversion (>99%) and with high optical purity (91%ee$_D$). However, following in-house immobilisation of *tert*-butylamine (**Section 4.3.3.4**), it was found that, when utilised in the packed bed, miniaturised, continuous flow reactor set up described, the immobilised form of this otherwise suitable racemising agent, does not catalyse racemisation of L-α-chloro ethyl thioester **25b** to any extent. Additional batch reactions, in conjunction with elemental analysis, confirmed that there is indeed some level of activity for this immobilised base. However, it would seem that when employed under the reaction conditions studied, in the packed bed, miniaturised flow reactor, immobilised *tert*-butylamine is not a suitable racemising agent. It is believed that the reduction in operating temperature from 40 ^{o}C in batch to ambient in the miniaturised set up and/or a low activity of the base, as a result of immobilisation, are the causes for this lack of racemisation, at any rate fast enough to aid DKR.

In a further attempt to identify a suitable agent for the racemisation of L-α-chloro ethyl thioester **25b** to the corresponding DL-α-chloro ethyl thioester **25b** racemate,

eight commercially available immobilised bases were also investigated in the packed bed, miniaturised continuous flow reactor. 3-Dimethylamino-propylfunctionalised gel appeared to be capable of some level of racemisation of the chiral L-α-chloro thioester substrate (90 to 74%ee_L) after 10 min in the packed bed, miniaturised continuous flow reactor in t-BuOH/ water (90:10) at room temperature. However, this rate is too slow to enable a DKR process to be established. Unfortunately, none of the other immobilised bases studied for the racemisation of the L-α-chloro ester in the packed bed, miniaturised continuous flow reactor; polymer bound diisopropylamine, polystyrene-*co*-divinyl benzene amine, polymer bound *N*-benzylamine, polystyrene bound phosphazene, piperidinomethyl polystyrene resin and TBD methyl polystyrene, showed any signs of successfully racemising the optically pure L-ester to the corresponding DL-racemate. Consequently, combination of an immobilised biocatalyst and an immobilised base, operating in separate reaction capillaries, to overcome compatibility issues associated with standard DKR reactions, whilst maintaining the inherent advantages of miniaturisation has not been possible.

4.3 EXPERIMENTAL

4.3.1 MATERIALS

All of the solvents and reagents used were either HPLC or standard laboratory reagent grade, purchased from Fisher Scientific UK Limited, Loughborough, Leicestershire, UK or Sigma Aldrich, Steinheim, Germany. The water used throughout was deionised.

Mann Associates, Cambridge, UK supplied 2g quantities of the Alphamerix enzyme AE011 (*Alcaligenese spp*.) on both granulated and fined silica (AE0175 and AE0176 respectively). Controlled pore glass (500-200 mesh) was from Sigma Aldrich, Steinheim, Germany.

Immobilised bases; 3-dimethylamino propyl functionalised silica gel (200-400 mesh), polymer bound diisopropylamine (50-90 mesh, 1% cross-linked), polystyrene-*co*-divinyl benzene amine functionalised (70-90 mesh, macroporous, 1.5-3.0 mmol g^{-1}) and polymer bound *N*-benzylamine (100-200 mesh, 1% cross-linked) were purchased from Sigma Aldrich, Steinheim, Germany. Fluka, Steinheim, Germany was the source of the polystyrene bound phosphazene (2.2 mmol g^{-1}). Calbiochem-Novabiochem, Laufelfingen, Switzerland supplied both the piperidinomethyl polystyrene HL resin (200-400 mesh, 2% DVB, 4 mmol g^{-1}) and TBD Methyl polystyrene (2% DVB).

4.3.2 INSTRUMENTATION

Reverse phase HPLC analysis, chiral HPLC analysis and calculations of enantiomeric excess (%ee) was conducted according to the instrumentation experimental details detailed in **Chapter 3**, **Section 3.5.2**.

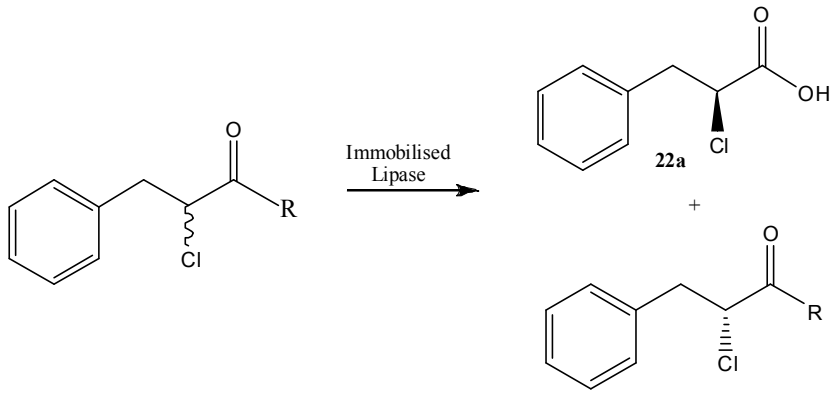
4.3.3 METHODS

4.3.3.1 ENZYME IMMOBILISATION

CPG (1g) was added to a solution (20mL) of AE011 (20mg mL^{-1}) in Tris HCl buffer (0.1M, pH 7) and the mixture was allowed to stir continuously, at room temperature for 24 hr. After which, the CPG was filtered off, washed with buffer (3 x 20mL) followed by deionised water (3 x 20mL) and dried under vacuum for 24 hr. The resulting, now beige powder, was then stored in an air tight container until ready for use.

4.3.3.2 BATCH KINETIC RESOLUTIONS

Lipase (AE04, AE07 or AE011) (*ca.* 100mg) immobilised to a range of supports (CPG, AE175 or AE176) or the free form of the lipase was added to a solution of racemic ester **23**, **25** or **26** (1mL, 0.02M) in t-BuOH / water (90: 10) and the mixture was allowed to stir continuously at 40 ^{o}C for 24 hr (**Scheme 36**). After which, the enzyme was filtered off through cotton wool and celite and the filtrate (a mixture of acid product **22a** and ester) was analysed by reverse phase and chiral HPLC.



R = OCH_3 (**23**),OCH_2CH_3 (**24**), SCH_2CH_3 (**25**) or $SCH(CH_3)_2$ (**26**)

Scheme 36: Lipase catalysed hydrolysis of DL-α-chloro ester substrates to yield D-α-chloro acid **22a**.

4.3.3.3 MINIATURISED KINETIC RESOLUTIONS

A miniaturised continuous flow reactor set up (**Figure 20**) was used where the immobilised enzyme (*ca.* 10mg) was incorporated into a small glass capillary (1.65mm diameter x 30mm length) resistant to the solvents and reagents required. In a similar way to work carried out by Peterson (2005), the immobilised enzyme was trapped within the channel to ensure they were not flushed out with operation, using a micro porous silica frit fabricated (Christensen *et al*., 1998) at the end of the capillary. This ensured that the immobilised enzyme was held in place whilst the reaction solution was pumped over the biocatalyst. A pressure driven, hydrodynamic syringe pump set up which could operate at flow rate ranges between 0.5 and 100µLmin-1 was used. The glass capillary reactor portion of the set up was connected to a glass luer lock syringe using combined standard HPLC finger tight fittings (1/16″) with PEEK tubing (360µm outer and 150µm internal diameters) from Upchurch Scientific and female-female luers interfacing the tubing and syringes, supplied by Supelco.

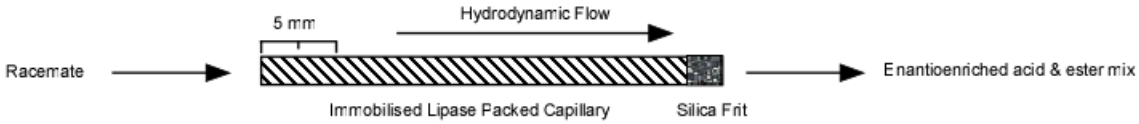


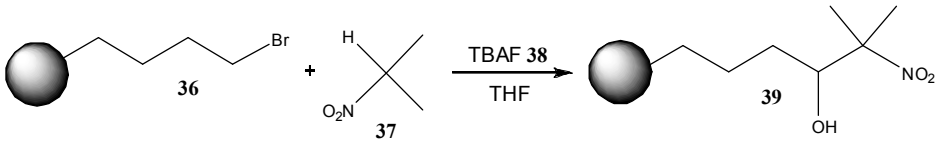
Figure 20: Set up of a miniaturised, packed bed, continuous flow reactor.

A solution of DL-α-chloro ethyl thioester in t-BuOH/ water (90: 10) (500µL, 0.02M) was passed over (25-1µLmin^{-1}) the packed bed, continuous flow reactor containing lipase AE011 immobilised on to CPG (*ca.* 10mg) to yield the chiral acid product. Following the reaction, the product mixture, collected in a small glass vial outlet, was removed every 10 min over a period of 2-8 hr and analysed by chiral HPLC.

4.3.3.4 BASE IMMOBILISATION

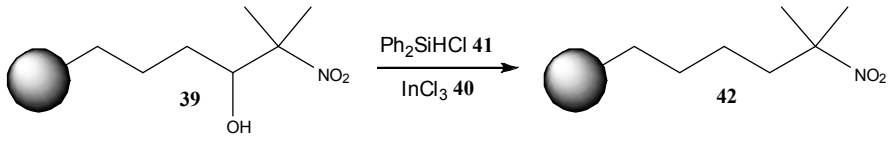
This work was carried out in conjunction with Charlotte Wiles and details the protocol followed in order to prepare an immobilised form of TEBA.

To a suspension of bromopropyl functionalised polystyrene **36** (1.50g, 3.20mmol Br g^{-1}) in nitropropane **37** (5mL), under N_2, was added tetrabutylammonium fluoride (TBAF) **38** (24.0mL, 1.0M in THF, 24.0mmol) and the resulting orange reaction mixture stirred at room temperature (**Scheme 37**). After 48 hr, the reaction mixture was filtered under suction and the resin was with DI water (50mL), acetone (50mL) and diethyl ether (50mL) prior to drying in an oven at 60 °C, to afford the desired beige, free flowing solid **39** (1.61g): elemental analysis found 2.73 % N, which equates to a loading of 1.95mmol N g^{-1}.



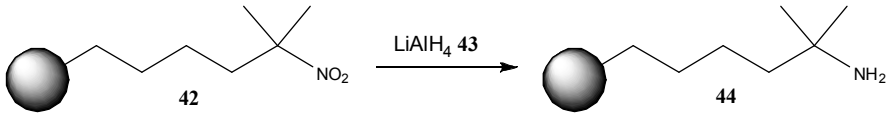
Scheme 37: Nitro aldol (Henry) reaction (Klein and Schkeryantz, 2005).

The polymer-supported Henry adduct **39** (0.50g, 0.98mmol) was dispersed in a solution of indium (III) chloride **40** (0.014g, 0.06 mol) and chlorodiphenylsilane **41** (0.251mL, 1.96mmol) in DCM (20 mL) and heated to reflux for 4 hr (**Scheme 38**). Upon cooling the reaction mixture was filtered under suction and the resin washed with DI water (50mL) and acetone (50mL) prior to drying in an oven at 60 °C, to afford a free flowing solid **42** (0.48g).



Scheme 38: Reduction of the hydroxyl functionality (Klein and Schkeryantz, 2005).

In the final step (**Scheme 39**), reduction of the nitro moiety was achieved by treating the polymer-supported nitro derivative **42** (0.21g, 0.41mmol) in diethyl ether (5 mL) with lithium aluminium hydride **43** (2.00mL, 1.0M solution in diethyl ether, 2.00mmol), the reaction mixture was subsequently stirred for 5 hr at 0 °C. Prior to filtration, excess $LiAlH_4$ **43** was carefully quenched with aq. HCl (10mL, 10%v/v) and the resulting solution neutralised with aq. $NaHCO_3$ (sat). The reaction mixture was then filtered under suction and washed with aq. HCl (100 ml, 10 % v/v) to remove any aluminium residues, followed by aq. $NaHCO_3$ (sat) (1000 ml) to afford the free amine. The resin was further washed with DI water (100mL) and acetone (50 mL), prior to oven drying at 60 °C to afford the desired polymer-supported amine **44** as a pale yellow, free flowing solid (0.17g). IR analysis confirmed quantitative reduction of the nitro group to afford the desired *tert*-amine functionality.



Scheme 39: Reduction step

In order to determine the loading of the polymer-supported *tert*-butylamine **44**, a portion of the material was derivatised using 4-dimethylaminobenzaldehyde, followed by elemental analysis.

Derivatisation of the polymer-supported *tert*-butylamine

Having quantified the proportion of immobilized nitrogen on the polymer, the material was subsequently derivatised in order to quantify the proportion of free amine groups on the material. The polymer-supported *tert*-butylamine **44** (0.050 g) was added to a stirred solution of 4-dimethylaminobenzaldehyde (0.075 g, 0.50 mmol) in MeCN (5 ml) and stirred for 24 hr prior to filtration. Upon washing with MeCN (50 ml), a free flowing orange powder was obtained: elemental analysis found 2.73 % N (pre-derivatisation) and 5.24 % post-derivatisation = 1.79 mmol *tert*-butylamine g^{-1}.

A second immobilisation strategy was investigated using Merrifield peptide resin:

To a suspension of Merrifield peptide resin (1.00g, 2.30mmol Cl g^{-1}) and lithium carbonate (0.85g, 11.50mmol) in THF (150mL) was added *tert*-butylamine (0.48mL, 4.60mmol) in THF (10mL) and the resulting reaction mixture heated to reflux for 24 hr. Upon cooling the resin was filtered under suction and washed with DI water (50mL), DCM (50mL) and acetone (50 mL) prior to drying in an oven (60 °C) to afford a pale yellow, free flowing powder **45** (**Figure 21**) (1.11 g): elemental analysis found 2.34 % N = 1.67 mmol N g^{-1}.

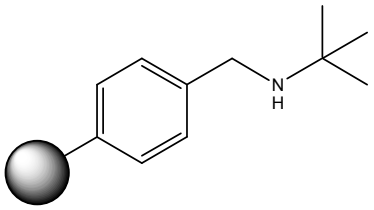
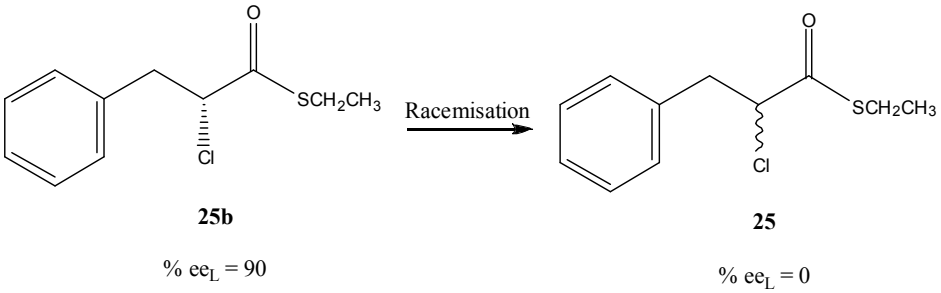


Figure 21: *tert*-Butylamine immobilised to Merrifeid peptide resin **45**.

4.3.3.5 MINIATURISED RACEMISATIONS

A range of immobilised bases, in varying strength, were investigated, including those prepared in-house (**Section 4.3.3.4**).

Immobilised base (*ca.* 10mg) was placed in the reactor capillary of the packed bed, miniaturised continuous flow reactor. A solution of L-α-chloro ethyl thioester (90%eeL) in t-BuOH/ water (90: 10) (500µL, 0.02M) was passed over (1µLmin^{-1}) the packed bed, continuous flow reactor containing immobilised base. Following the reaction (**Scheme 40**), the product mixture, collected in a small glass vial outlet, was removed every 10 min over a period of 2 hr and analysed by chiral HPLC.



25b

% ee_L = 90

25

% ee_L = 0

Scheme 40: Racemisation of enantioenriched α-chloro ethyl ester **25b** to yield the racemic ester **25** utilising a range of immobilised bases.

4.4 CONCLUSIONS

Chapter 4 reports the investigation of improving previously developed, successful DKR processes, with the aid of miniaturised reactor chemistry. The work described details a combination of miniaturised reactor technology and biocatalytic resolution reactions. Additionally, in an attempt to overcome compatibility issues, with regards to the enzyme and racemising agent in the DKR process, the work presented discusses the development of a separated DKR reaction.

It is important, when utilising a packed bed, miniaturised continuous flow reactor, that the catalysts of interest are utilised in their immobilised form, to provide a cost effective mode of operation. The successful immobilisation of lipase AE011 *via* adsorption to controlled pore glass has been carried out. In so far as conversion of ester to acid is concerned, the efficiency of this immobilised lipase was found to be comparable to commercially available forms of the immobilised biocatalyst. However, it would seem from the results obtained that in fact, the in-house prepared immobilised enzyme shows a greater degree of enantioselectivity to the lipase catalysed hydrolysis of the racemic α-chloro ethyl thioester substrate. This successful immobilisation of the required biocatalyst allowed the KR process to be transferred to the packed bed, miniaturised continuous flow reactor. **Chapter 4** therefore, successfully demonstrated that miniaturisation can be advantageous over bench top, one pot batch reactions for enantioselective biocatalytic reactions. Advantages include; an increased throughput, where high percentage ester conversions are obtained more rapidly than in a batch reaction, due to a high surface to volume ratio in the capillary; lower associated costs, as less reagents are required; increased environmental control, reducing the chance of the enzyme catalysing the reverse reaction; and a more benign process as smaller volumes of reagents are required.

Combination of an immobilised biocatalyst and an immobilised base, operating in separate reaction capillaries, to overcome compatibility issues associated with standard DKR reactions, whilst maintaining the inherent advantages of miniaturisation would be an ideally operating set up for a DKR reaction. Using this

approach, the ester under investigation would pass through the immobilised enzyme reactor, where enantioselective hydrolysis would occur, and then through the immobilised base where racemisation of the residual ester would occur. The reaction products could then be re-circulated through the enzyme to attain the desired acid in excellent yield and enantiopurity. However, issues surrounding the activity of the immobilised bases employed as racemising catalysts did not allow for the DKR reaction to be achieved separately.

5 CONCLUSIONS

Conventional chemical processes often require a series of complex synthesis steps that utilise a great deal of energy and raw materials. There is a huge commitment by the chemical industry to search for "green", environmentally more friendly processes. Biocatalysis, using enzymes to catalyse a chemical reaction, is emerging as an economical and ecological production route for a wide range of fine chemicals, pharmaceuticals and food ingredients. In addition to biocatalysis, miniaturised chemistry has also become of interest and significance to the chemical industry over the last decade. Flow reactors allow for the synthesis of small volumes of compounds quickly and in high yield, with fast optimisation of conditions. Additionally, when used with immobilised biocatalysts, these miniaturised devices can enable high throughput screening of a wide range of enzymes and their substrates. Despite this however, the development of applications in the field of miniaturised enzymatic processes are still in the initial stages.

Chapter 2 explored the feasibility of combining biocatalysis within continuous flow reactors, using the enzymatic synthesis of methyl, ethyl and butyl esters in a packed bed, continuous flow reactor. Initially, benchmarking of the reaction conditions in batch was carried out. Parameters including temperature, enzyme concentration, reaction media and enzyme concentration were investigated. Under the optimised conditions, it took up to 24 hours to achieve reasonable percentage conversion of methanol and octanoic acid to methyl octanoate in the batch reaction. The process of enzymatic esterification, utilising a cheap, commercial, readily available enzyme (Novozyme 435), was then transferred to a packed bed, continuous flow reactor. The successful synthesis of methyl laurate in the miniaturised reactor system allowed the study to be extended to the synthesis of nine esters. It would seem however, that the extent of the reaction using this lipase, under these conditions, is affected by the chain lengths of the acid and alcohol. Biocatalyst specificity problems with respect to the substrates, enzyme inhibition by the larger product molecules, or adsorption of some of the products onto the enzyme support matrix, resulting in lower percentage ester conversions, are possible reasons for the differences observed between conversions of the esters. Despite this, the conversion of acid and alcohol to ester, at ten minute intervals, and even after two hours, was in all cases greater than 65%. In fact, in the case of butyl hexanoate, excellent (>90%), consistent percentage ester

conversions were achieved. The work detailed in **Chapter 2** successfully demonstrated that miniaturisation can be advantageous over batch reactions for biocatalytic synthesis. Advantages include; an increased throughput, where high percentage ester conversions are obtained more rapidly than in a batch reaction, due to a high surface to volume ratio in the capillary; increased environmental control, so there is less chance of the enzyme catalysing the reverse reaction; and a more benign process as smaller volumes of reagents are required for optimisation. Additionally, the work described in **Chapter 2** demonstrated the ability to screen the enzyme for substrate specificity. Consequently, this concept could be applied to other more complicated processes that involve enantioselective reactions or utilise expensive enzymes, where miniaturisation is of high importance.

As discussed in **Chapter 3**, in chemistry, most molecules exist as racemic mixtures. Enantiomers of racemic compounds can often exhibit diverse pharmacological and therapeutic properties. In fact, different drugs usually have their biological activity based mainly on one enantiomer. Consequently, research into the preparation of enantiomerically pure chiral compounds is increasing in interest. Dynamic kinetic resolution is emerging as a potentially efficient process, whereby standard enzymatic kinetic resolution is coupled with *in situ* racemisation of the slow reacting starting substrate, using a suitable racemising catalyst. A lipase catalysed enantioselective hydrolysis process under continuous *in situ* racemisation of substrate, by using *tert*-butylamine as an organic base, was developed for the production of optically active D-α-chloro acid from racemic α-chloro thioesters, in t-BuOH/ water (90: 10). The D-forms of racemic ester and thioester substrates were found to be substrates for the lipase catalysed hydrolysis to the corresponding chiral acids, affording excellent optical purity (>99%ee_D). Racemisation studies, with a range of organic bases and nucleophilic chloride salts, suggested that the L-enantiomers of the esters were inert to racemisation back to the corresponding DL-racemates, under the reaction conditions studied. However, three organic bases (DCHA, TEBA and DIPEA) were identified as suitable racemising catalysts for the L-enantiomers of the thioester substrates. Combination of the kinetic resolution and racemisation reactions the two thioesters, allowed for successful dynamic kinetic resolution processes to be established. Investigations on the parameters indicated that 5eq. of *tert*-butylamine as the racemising agent and an operating temperature of 40^oC gave optimum

conversions and optical purity in dynamic kinetic resolution. Excellent to good conversions (>99% and 78% for the ethyl and i-propyl thioesters respectively) of racemic ester to L-acid were achieved, with the resulting chiral acid in high to medium optical purity (93%ee_D and 79%ee_D for the ethyl and i-propyl thioesters respectively) after 24 hours in the batch reaction.

The successful development of dynamic kinetic resolution processes for two thioester substrates allowed for an investigation into improving the technique with the aid of continuous flow chemistry. **Chapter 4** therefore, detailed a combination of the miniaturised reactor technology described in **Chapter 2** and the biocatalytic resolution reactions presented in **Chapter 3**. The purpose here was to determine if the inherent advantages associated with miniaturisation, discussed in **Chapter 2**, could be observed for enzymatic resolution set ups. Additionally, in an attempt to overcome compatibility issues, with regards to the enzyme and racemising agent in the DKR process, as discussed in **Chapter 3**, **Chapter 4** discusses the development of a separated dynamic kinetic resolution reaction. It is believed that by combining an immobilised biocatalyst and an immobilised base, operating in separate reaction capillaries, this approach would overcome compatibility issues whilst maintaining the inherent advantages of flow reactor technology.

There is, without doubt, an obvious advantage to miniaturising a biocatalytic reaction. When the lipase catalysed esterification of butyl hexanoate was conducted in the batch reactor (**Chapter 3**), only a 3% ester conversion was seen after 10 min. However, when the same reaction was conducted in the miniaturised reactor set up, a massively improved conversion of 93% was observed, due to the high surface to volume ratio in the reactor capillary. Similarly, when the enantioselective hydrolysis of DL-α-chloro ethyl thioester was investigated in the same set up, a conversion of ester to acid of 32% was seen after 10 min compared to only a 14% conversion after the same time in the packed bed reactor.

Kinetic resolution of a range of α-chloro esters was successfully achieved in the miniaturised reactor, utilising an immobilised lipase. Unfortunately however, issues surrounding the activity of the immobilised bases, employed as racemising agents, did not allow for the full dynamic kinetic resolution reaction to be achieved

separately. Additional racemising agents in the immobilised form would need to be further investigated for completion.

The aim of the work presented in this thesis was to develop and improve biocatalytic reactions. The use of miniaturised reactor chemistry to improve enzymatic esterification reactions has been demonstrated with impressive results. The conversion of acid to ester increases significantly in the miniaturised set up compared to the same reaction after the same amount of time in the batch reactor. Additionally, issues regarding water production that are associated with batch enzymatic esterification appear to be overcome as the small concentrations of water generated are being continuously removed from the reaction stream. KR reactions for the enantioselective hydrolysis of a range of α-chloro esters of interest to the pharmaceutical industry have been developed. Similarly, in the case of two thioester substrate, this resolution has been improved with further development of a DKR process for the same enantioselective hydrolysis reaction. Improvement of the KR process of one of the thioester substrates has been achieved with the aid of flow reactor technology, yet no successful improvement to the DKR process, with the aid of miniaturisation and separation of the two steps, has been established.

Following the development of biocatalytic reactions in batch, the packed bed, miniaturised flow set up described herein has demonstrated the preparation of racemic and chiral products *via* biocatalytic methodologies, with obvious advantages over larger scale reactions. Consequently, this technique could now be applied to a range of reactions which utilise expensive enzymes and/or when only a small amount of high value material is required. Similarly, the set up could be applied in a screening manner, where high throughput and low volumes of material required to identify suitable biocatalysts for a reaction make it preferential over traditional batch biocatalytic methodologies.

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APPENDIX

PUBLICATIONS

Woodcock, L.L., Wiles, C., Greenway, G.M., Watts, P., Wells, A. and Eyley, S. 'Enzymatic synthesis of a series of alkyl esters using Novozyme 435 in a packed bed, miniaturised, flow reactor.' *Biocatalysis and Biotransformation*, **26**:**6**, 466-472 (2008).

CONFERENCE PROCEEDINGS

Annual Conference
ProBio Faraday Partnership
Scarman House, University of Warwick
8-9th November, 2004

Biochemical Society Focused Meeting
Biocatalysis: Enzymes, Mechanisms and Bioprocesses
Manchester Conference Centre
Manchester, UK
21-22nd November, 2005

Royal Society of Chemistry (RSC)
Analytical Research Forum
University of Plymouth, UK
18-20th July, 2005

24th SCI Process Development Symposium
Churchill College, Cambridge UK
6-8th December 2006

International Conference
Biocatalysis 2007 "Structure, Functions & Applications"
Moscow, St Petersburg, Russia17-22nd
June, 2007