THE USE OF ENZYMES IN ORGANIC SOLVENTS IN POLYTRANSESTERIFICATION REACTIONS

ADRIAN JAMES WIGGETT

DOCTOR OF PHILOSOPHY

ASTON UNIVERSITY

September 1998

ASTON UNIVERSITY

THE USE OF ENZYMES IN ORGANIC SOLVENTS IN POLYTRANSESTERIFICATION REACTIONS

Submitted for the degree
of Doctor of Philosophy

Adrian James Wiggett
September 1998

SUMMARY

The aim of this research project was to identify the factors affecting the porcine pancreatic lipase (PPL)-catalysed polytransesterification of a diester and a diol in organic solvents. It was hoped that by modifying reaction conditions a commercially acceptable polymer molecular weight (Mn) of 20,000 daltons might be attained.

Exploratory investigations were carried out using 1,4-butanediol/bis(2,2,2-trichloroethyl) adipate and glutarate systems in diethyl ether, with and without molecular sieves. It was found that molecular sieves promoted the reaction by reducing hydrolysis of the ester end-groups, resulting in polymer molecular weights between 1.2 and 2.2 times greater than those obtainable without molecular sieves.

Investigations were then concentrated on the PPL-catalysed polytransesterification of 1,4-butanediol with divinyl adipate. The particular advantage of this system is that the reaction is irreversible.

The effects of varying substrate concentration, mass of drying agent, reaction solvent, reaction temperature, mass of enzyme and also enzyme immobilisation on the 1,4-butanediol/divinyl adipate system were investigated.

The highest molecular weight polymer obtained for the PPL-catalysed polytransesterification of 1,4-butanediol with divinyl adipate in diethyl ether was Mn ~8,000. In higher boiling ether solvents molecular weights as high as Mn ~9,200 were obtained for this system at elevated temperatures. It was found that the major factor limiting polymerisation was the low solubility of the polymer in the solvent which resulted in precipitation of the polymer onto the surface of the enzyme.

Keywords porcine pancreatic lipase, enzyme, polytransesterification, organic solvent, vinyl ester.

2

DEDICATION

To my family

ACKNOWLEDGEMENTS

CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

LIST OF ABBREVIATIONS

ASP	-	Aspartic acid
CCL	-	*Candida cylindracea* lipase
CRL	-	*Candida rugosa* lipase
δ	-	Chemical shift, ppm
DCC	-	1,3-Dicyclohexylcarbodiimide
DHU	-	1,3-Dicyclohexylurea
DMAP	-	4-Dimethylaminopyridine
DMI	-	Dimethyl isosorbide
GC	-	Gas chromatography
GPC	-	Gel permeation chromatography
HIS	-	Histidine
HPLC	-	High performance liquid chromatography
NMR	-	Nuclear magnetic resonance
PPL	-	Porcine pancreatic lipase
SER	-	Serine
THF	-	Tetrahydrofuran
TLC	-	Thin layer chromatography
WMS	-	With molecular sieves
WOMS	-	Without molecular sieves

CHAPTER 1
INTRODUCTION

1.0 <u>INTRODUCTION</u>

1.1 <u>Enzymes</u>

Enzymes are naturally occurring, very efficient biological catalysts, which **usually operate in aqueous media.**[1,2] Their 3D-structure and the presence of an 'active site' allows them to catalyse a wide range of reactions with high stereo- and regiospecificity.[3,4] Enzymes are biodegradable and most operate at quite low temperatures, between 20-50°C, which makes them attractive as mild environmentally benign catalysts.

The 'active site' is held in its conformation by various intramolecular forces.[1] The primary, secondary and tertiary structure varies from enzyme to enzyme which enables them to catalyse very specific reactions between specific substrates. The interaction between the enzyme and the substrate has often been likened to the fit of a key into a lock. However, a more realistic view[5] is that of the 'Induced-Fit Model' which assumes that during the formation of the enzyme-substrate complex, the approach of a substrate causes the enzyme to change its conformation so as to wrap around the substrate as illustrated below :-
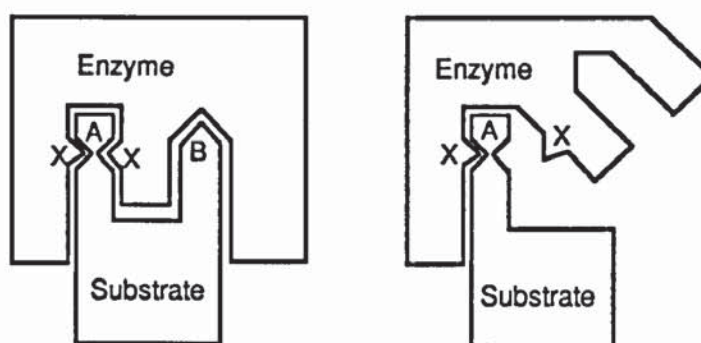
Scheme 1. Schematic representation of the 'Induced-Fit' mechanism. Redrawn from reference 5.

In **Scheme 1**, 'A' represents the reactive group of the substrate and 'X' represents the complementary reactive group(s) of the enzyme - the 'chemical

operator'. Part 'B' of the substrate forces the enzyme to adapt an active conformation (the 'induced-fit'). This in turn positions both 'X' active groups of the enzyme into the correct orientation to effect catalysis. If part 'B' is not present then no conformational change takes place and thus the chemical operators stay in their inactive state.

More than 2,000 different enzymes are known and there is a large body of published work relating to their structure and activity. Enzymes are categorised into six main classes based on which particular type of reaction they catalyse.[1] The classes are Lyases, Ligases, Isomerases, Transferases, Oxidoreductases and **Hydrolases** (the class which has been used in the work reported in later chapters).

1) Oxidoreductases   -   catalyse oxidation reduction reactions.

2) Transferases   -   transfer of a group.
     e.g. acyl, methyl, phosphoryl.

3) Hydrolases   -   catalyse the hydrolysis/formation of esters, amides, nitriles, lactones, lactams, epoxides.
     e.g. esterases, glucosidases, peptidases, proteases, phospholipases, and lipases.

4) Lyases   -   addition-elimination reaction of small molecules to and from C=C, C=N, C=O bonds.

5) Isomerases   -   catalyse structural or geometric changes within a molecule.
     e.g. racemases.

6) Ligases   -   formation-cleavage of C-N, C-O, C-S, C-C bonds with accompanying hydrolysis of a pyrophosphate bond in ATP or a similar triphosphate.

Since there are so many different enzymes within each of the classes they are each given a four part Enzyme Commission (E.C.) when they are registered in accordance with the recommendations of the 'Nomenclature Committee of

IUB'. The code identifies the class to which the enzyme belongs and the nature of the reaction it catalyses in aqueous media.

The E.C. code is explained as follows;

E.C. 3.1.1.3   The first of the four numbers represents the class of the enzyme (in this example it is the Hydrolase class); the second number indicates the substrate type or type of molecule transferred; the third number represents the nature of the co-substrate; the fourth number is the individual enzyme number.

Enzymes are specific and highly versatile catalysts and are used in a wide variety of aqueous based industries including washing powders, baking, cheese manufacture, wine making, brewing and distillation, pharmaceuticals, leather tanning, paper manufacture, adhesives, sewage disposal, animal feeds, diagnostic test strips, clinical and environmental analysis and are extensively used to make highly complex pharmaceuticals.[6] **Hydrolase** enzymes have been widely used because of the diverse range of reactions they catalyse.

Hydrolases are enzymes which break down long chain molecules into smaller units by hydrolysis. Unlike the other five classes of enzyme, hydrolases are self sufficient and do not require substances called 'co-factors' which promote catalytic activity in the enzyme. There are many types of hydrolase enzyme, for example proteases break down long chain proteins into peptide fragments, whilst **lipases** break down fats and oils (triglycerides) *in vivo* and *in vitro* to di-glycerides, mono-glycerides and fatty acids, in aqueous media.[2]

$$
\begin{array}{c}
RCO_2CH_2 \\
RCO_2CH \\
RCO_2CH_2
\end{array}
\quad + \; 2H_2O \;
\xrightarrow[\text{aqueous media}]{\text{(enzyme)}}
\quad
\begin{array}{c}
CH_2OH \\
HCO_2CR \\
CH_2OH
\end{array}
\quad + \; 2RCO_2H
$$
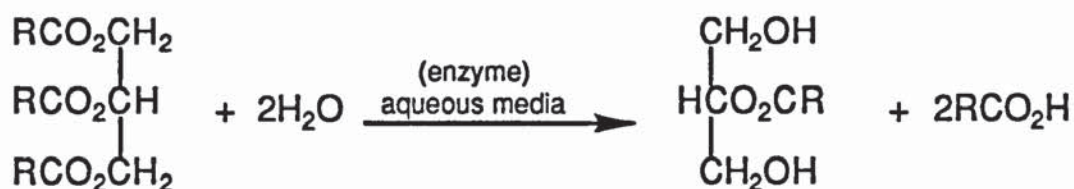
Scheme 2. Hydrolysis of triglycerides to monoglycerides and fatty acids using lipase enzyme in aqueous media.

1.2 Lipases

Lipases differ from each other in their source, substrate specificity, catalytic activity and cost. The cheapest and most widely available lipases are *Candida rugosa* lipase (CRL) (formerly known as *Candida cylindracea* lipase (CCL)) and porcine pancreatic lipase (PPL).

Porcine pancreatic lipase operates in **aqueous media** at the oil-water interface,[7,8] and hydrolyses triglycerides to non-branched monoglycerides and fatty acids containing between 14-22 even numbers of carbon atoms.[7-9]

Crude PPL is a low activity enzyme (110-220 units/mg protein, using olive oil substrate) preferentially hydrolysing esters of primary alcohols.[8] Crude PPL accepts a broad spectrum of substrates enabling it to hydrolyse many different types of aromatic and alkyl esters.[8,10]

Temperature and pH are the factors which have a marked effect on enzyme activity.[9] For PPL,[9] optimum enzyme activity is achieved in a temperature range between 45-55°C and in a pH range of 7.0 to 8.5, see **Figure 1.** Even though the enzyme exhibits optimum performance under these conditions, use of the enzyme at the higher end of this temperature range results in a shortened life span of the enzyme activity,[9] and hence for quantitative reactions and constant performance, reactions should be carried out at or below the lower end of the range.

pH Activity curve   **Temperature Activity curve**
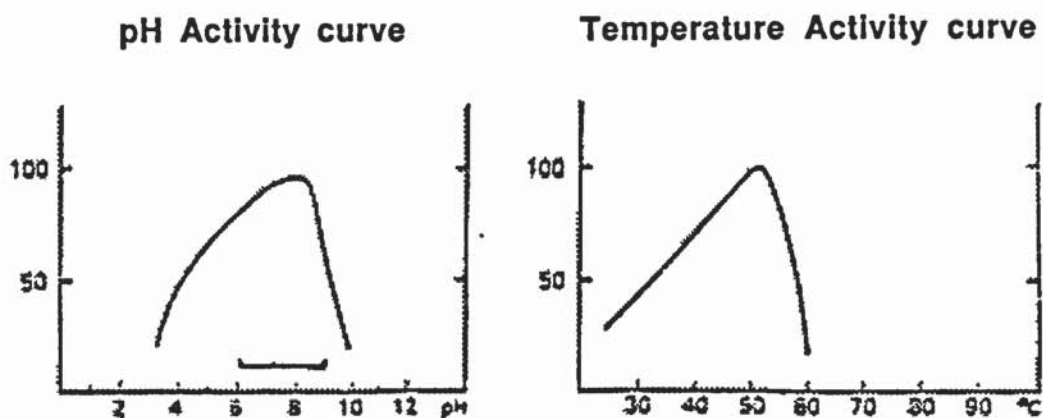
Figure 1. Graphs of pH activity and temperature activity for porcine pancreatic lipase.

Porcine pancreatic lipase like other lipases does not show normal Michaelis-Menten kinetics in aqueous solutions, displaying little activity and hence low reaction rate (*v*) with soluble substrates. However, a sharp increase in activity is observed when the substrate concentration is increased beyond its critical micelle concentration.[11-14] This increase in lipase activity at the lipid-water interface led to the suggestion that soluble lipases might undergo conformational changes at the oil-water interface, involving the opening of a short helical lid covering the active site, prior to substrate binding. [15,16]

The inexpensive and commercially available porcine pancreatic lipase is a very crude extract from the pig pancreas and may contain some, if not all, the enzymes shown in the table below. [17]

Table 1. Typical enzyme composition for crude PPL.

Enzyme	E.C Code	Uses	Enzyme Molecular weight, M_r
α-Amylase	3.2.1.1	Conversion of starch to maltose	50,000
Carboxy peptidase	3.4.17.2	Hydrolysis of hippuryl-L-arginine	34,400
Cholesterol Esterase	3.1.1.13	Hydrolysis of cholesterol esters	440,000
Elastase	3.4.21.11	Hydrolysis of peptides	25,000
PPL	3.1.1.3	Hydrolysis of oleic acid esters	50,000
Phospholipase A	3.1.1.4	Hydrolysis of Phospholipids at the 2-acyl linkage	14,000
Chymotrypsin	3.4.21.4	Cleavage of proteins to peptides	23,000
Trypsin	3.4.21.4	Hydrolysis of rapeseed proteins	23,800

Enzyme molecular weights (M_r) are normally quite large since they are very complex three-dimensional molecular structures consisting of different combinations of the 20 common amino acid residues and peptide bonds, which ultimately give rise to different structures and active site structures. As can be seen from the above table, crude porcine pancreatic lipase contains not only lipases but also proteases such as chymotrypsin and trypsin.

1.2.1 Structure and Mode of Action

Surprisingly, even though lipases and proteases are different groups of enzymes, they operate in much the same fashion because they have very similar 'active sites'.

27

Illustrated below is a computer generated 3D-structure of porcine pancreatic lipase showing two 'active site' triads.[18]
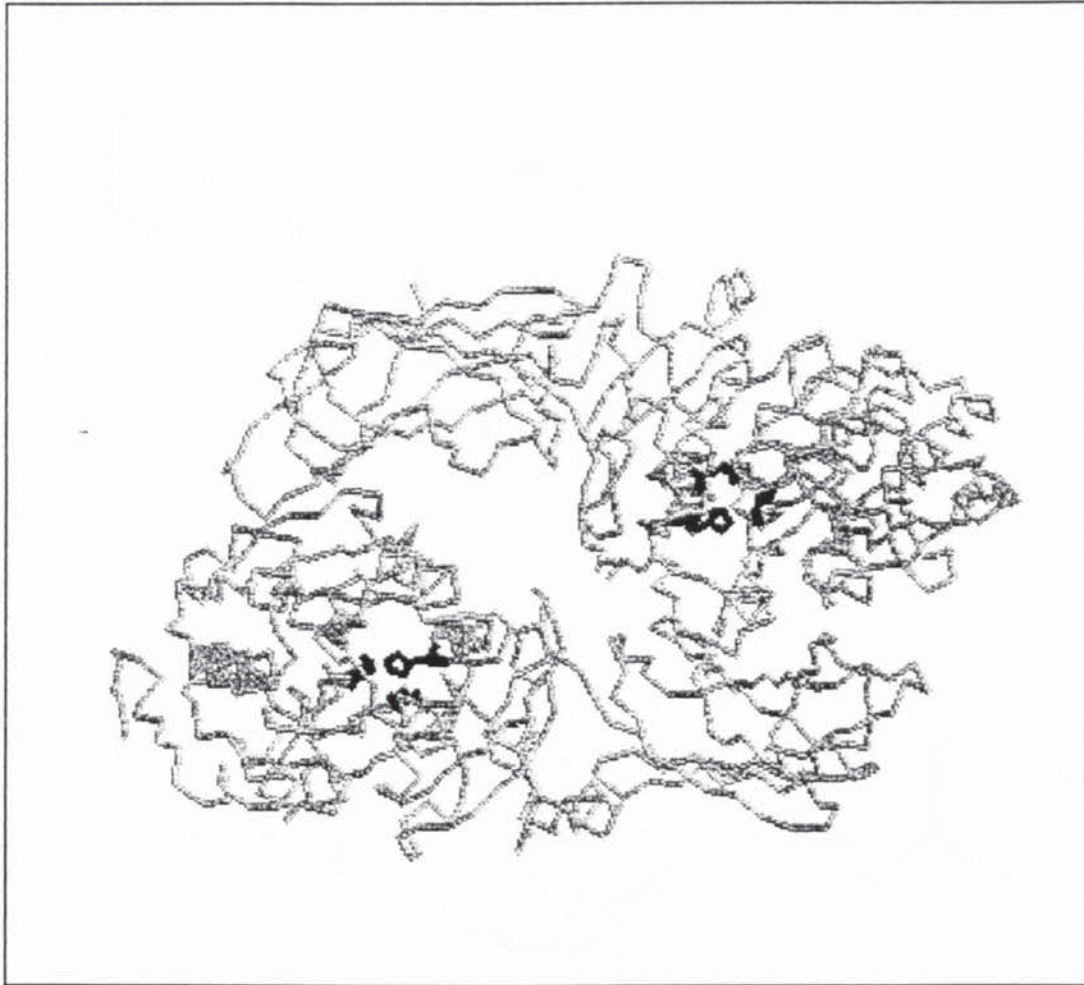
Figure 2. Computer generated 3D-structure of porcine pancreatic lipase (PPL) with 'active site' triads (Ser 152), (His 263), (Asp 176) highlighted in black.

The PPL active site[19-21] consists of three amino acids - serine, histidine and aspartic acid. The hydroxy group of the serine (Ser 152) is the oxygen nucleophile which attacks the carbonyl of the ester being cleaved. Histidine (His 263), through its imidazole ring, functions as a base to enhance the nucleophilicity of the serine hydroxyl group. Aspartic acid (Asp 176), the third amino acid, is not directly responsible for ester hydrolysis at the active site. However, X-ray and NMR data show that the carboxylate of aspartic acid is hydrogen bonded to the histidine enhancing the basicity of the imidazole ring and thereby increasing the activity of the enzyme.[22]

A mechanism for the catalytic hydrolysis of esters by PPL is outlined below in **Scheme 3.**

Step 1

Enzyme acylation

(ester substrate)

Where R^1 and R^2 = alkyl, ary

Step 2

Regenerated enzyme
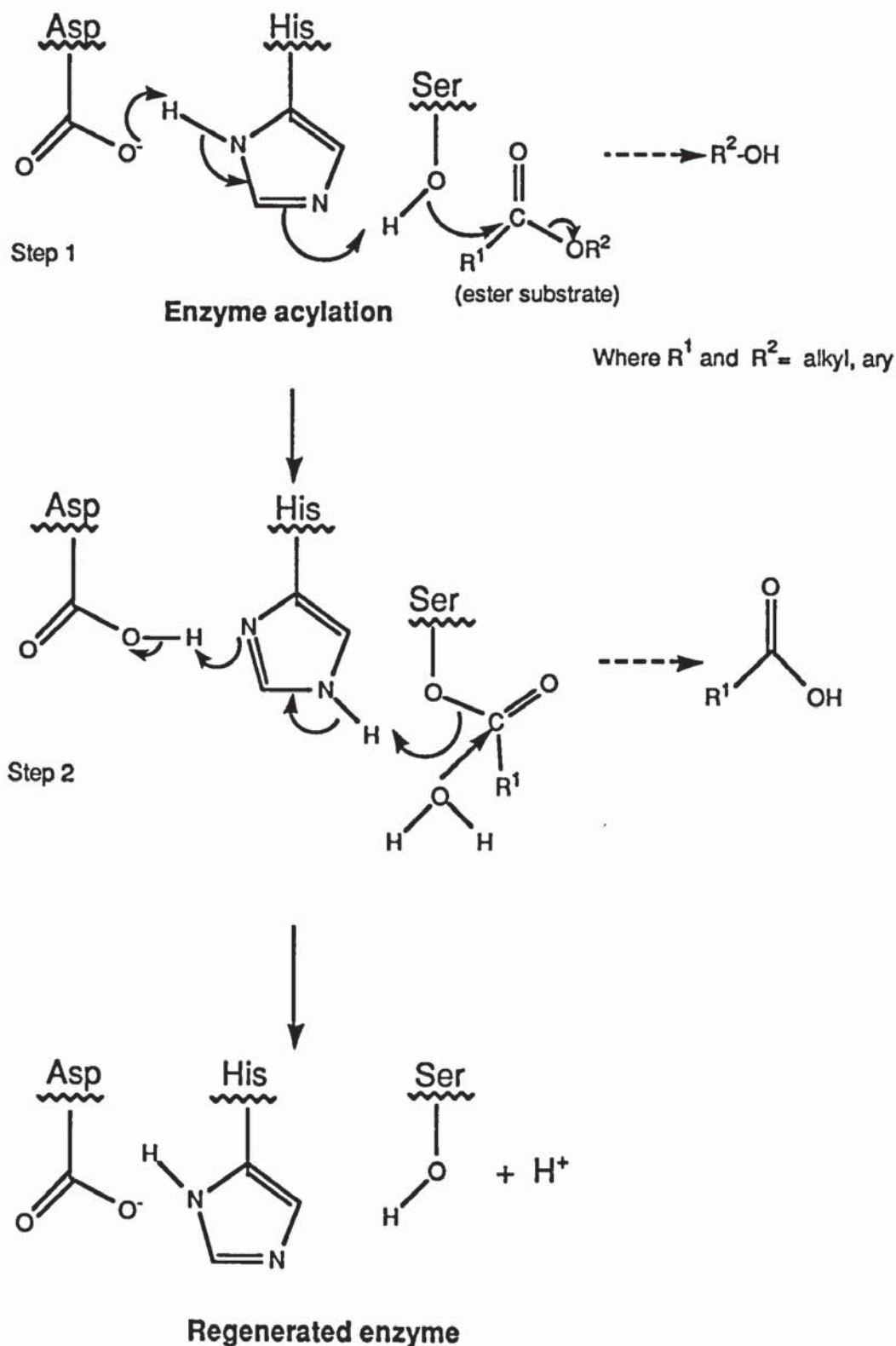
Scheme 3. The mechanism for the catalytic hydrolysis of esters by PPL. Redrawn from reference 5.

1.3 Enzymes In Aqueous Media

For many years enzymes[23] were employed by chemists in aqueous based organic molecular reactions both for the detection[24] and modification[24] of simple and complex molecules.

Examples of early practical applications of enzymes in aqueous systems have been reported by Mann and Saunders.[24] Some of these applications are outlined below;

Diastase was used to catalyse the hydrolysis of starch to a mixture of maltose and dextrin,

$$(C_6H_{10}O_5)_n \quad + \quad H_2O \quad \rightarrow \quad C_{12}H_{22}O_{11} \quad + \quad (C_6H_{10}O_5)_{n-2}$$

whilst *invertase* catalysed the hydrolysis of sucrose to glucose and fructose.

$$C_{12}H_{22}O_{11} \quad + \quad H_2O \quad \rightarrow \quad C_6H_{12}O_6 \quad + \quad C_6H_{12}O_6$$

Mann and Saunders highlighted the versatility of enzymes and the ease with which enzymes could be purified.[24] They identified *urease* as an important enzyme with the chief sources being (a) the jack bean (b) the soy (or soya) bean. Crystalline urease was obtained by stirring jack bean meal with 30% aqueous acetone. The slurry was filtered and the filtrate was then allowed to stand at 0°C for several hours. The urease which crystallised out was separated by centrifuging, and then recrystallised. This enzyme is of great value in the medical field for detecting and estimating urea in samples. The action of urease on urea is specific, the reaction catalysed being;

$$(NH_2)_2CO \quad + \quad 2H_2O \quad \rightarrow \quad (NH_4)_2CO_3$$

The ammonium carbonate formed causes the pH of the test solution (preset to pH 7) to rise to over 8; this change is noted by the use of phenol red indicator.

Mann and Saunders also described the early practical use of *emulsin* and its ease of extraction from ground almonds with water.[24] *Emulsin* naturally hydrolyses β-methylglucosides to glucose and methanol. This is a reversible

reaction and they suggested that dried *emulsin* could be used to synthesise β-methylglucoside from glucose in an excess of anhydrous methanol.

$$C_6H_{11}O_5 - OCH_3 \quad + \quad H_2O \quad \underset{\xrightarrow{\hspace{1cm}}}{\overset{Emulsin}{\rightleftharpoons}} \quad C_6H_{12}O_6 \quad + \quad CH_3OH$$

β-methylglucoside   glucose
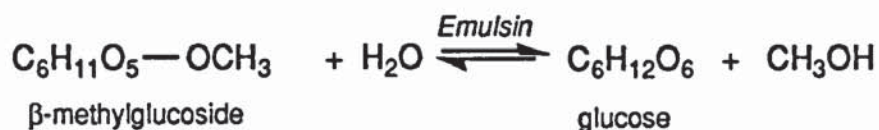
The hydrolysis of triglycerides to produce fatty acids is an important industrial process, which traditionally uses high temperatures (250°C) and pressures (45 bar). More recently, the use of immobilised lipases for the enzymatic hydrolysis of these triglycerides has been investigated[25] as a more economical alternative to the present process. The enzyme-catalysed reaction can be performed at much lower temperatures (30-50°C), at atmospheric pressure and the immobilised enzymes can be used repeatedly.

Among these lipase based reactions, PPL has been employed over the last decade to carry out selective hydrolysis of esters in complex molecules for the preparation of pharmaceuticals and other useful precursor compounds.[26] (See **Scheme 4**).
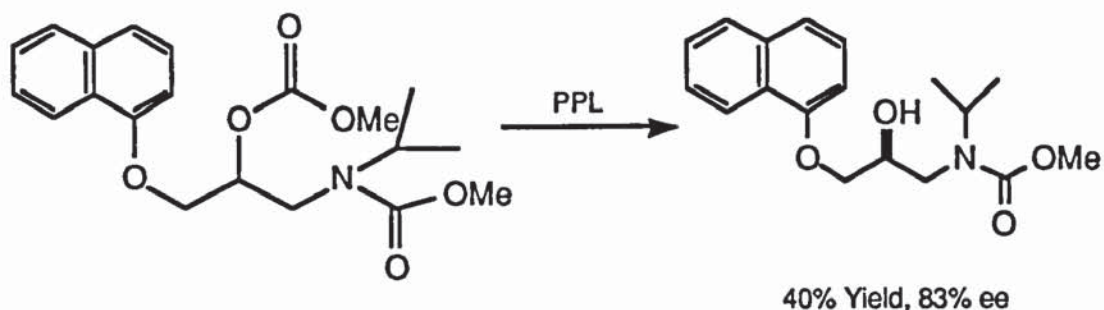
Scheme 4. Stereoselective hydrolysis of an ester function in a complex molecule catalysed by PPL.

Organic solvents are necessary for many organic reactions; however, the main limitation in the early application of enzymes, such as *emulsin* and PPL, to reactions in organic solvents, was that it was assumed that enzymes required predominantly aqueous media. A number of approaches were used to try to overcome this problem.

1.4 Enzymes In Organic Media

The early use of organic solvents in enzyme-catalysed transformations was in the form of biphasic systems. The systems were composed of an aqueous and an organic phase, which were immiscible. The enzyme dissolved in the aqueous phase where it catalysed the reaction between water soluble substrates to produce organic products, which preferentially dissolved in the organic phase.[27] This allowed for easier organic product recovery. Many organic biotransformations were carried out using such systems [28,29] including transesterification of esters and alcohols. Some are still used today, for example[6] in the synthesis of water insoluble peptides from soluble substrates. The biphasic system was found to be limiting since water sensitive reactions were impossible in such media; hence research was centred on the potential use of enzymes in monophasic organic solvents.

Until about 15 years ago, it was assumed that enzymes could only function in an aqueous environment. However, pioneering work by Klibanov *et al.* [23,29-32] demonstrated that **lipases** and other enzymes such as chymotrypsin could operate in nearly anhydrous monophasic organic solvents without the need for stabilisation. It was found that for the enzyme to function it required only a thin hydration layer which was tightly bound to the enzyme and acted as a buffer between the enzyme and the bulk reaction medium. The enzyme molecule was not affected beyond this layer and for that reason enzymes could function in organic media. This unexpected discovery stimulated much research with potential applications in both laboratory and industrial synthetic processes.[31] Water sensitive reactions which were deemed to be impossible in biphasic systems were now possible, opening up a new range of enzyme reactions.[32]

There are numerous advantages[33-46] to using enzymes in anhydrous organic solvents;

- the enzyme is insoluble which enables easy recovery of the catalyst.

- the enzyme is still "locked" in its proper orientation for catalytic activity due to the presence of the tightly bound monolayer of water.

- enzyme denaturing by microbial contamination and proteases is eliminated.

- substrates are more soluble in organic solvents than in water.

- easier product recovery from low boiling solvents.

- enzyme processes in organic solvents can be coupled directly with traditional chemical synthesis.

- the natural hydrolytic mode of action of the lipase is **reversed** and so esterification becomes the major reaction (shown in **Scheme 5**).

Scheme 5. The reversibility of lipase-catalysed reactions.

The **reversed operation is highly advantageous** since reactions which are impossible in water such as esterification and polytransesterification can be carried out successfully. At present, it is not clear what actually happens when an enzyme is placed in an organic solvent but there are some suggestions that the enzyme unfolds and exposes the hydrophobic interior and 'active site', promoting reaction.[23]

The application of enzymes in organic solvents offers a potential route to biodegradable/biocompatible materials with specific functionality and/or stereochemistry, characteristics which are much sought after in the synthesis of new materials and are often difficult to achieve using traditional chemical methods. The enzyme catalyst can also be fully recovered and therefore avoids contamination of the product. For this reason,[47] there is a growing interest in the biocatalytic synthesis of materials; for example Unichema use lipases to manufacture esters of C2-C8 alcohols such as isopropyl myristate, isopropyl palmitate and 2-ethylhexyl palmitate on a scale of several thousand tonnes per annum. These materials have applications in **biomedical and cosmetic products**. It is in these areas that one sees a great potential for growth since the use of traditional chemical catalysts may result in contamination of the product by the catalyst and produce allergic reactions.

Enzymes in organic solvents are now routinely used in the laboratory synthesis of esters.[48] For industrial processes there are obvious economic advantages in the use of organic solvents, since they require mild conditions and unlike water they can be removed easily and cheaply. The compatibility of enzymatic catalysis in organic solvents with existing chemical processes is a further advantage,[49] particularly if enzyme-catalysed regio- and stereo-selective synthesis could be coupled directly with chemical synthesis. To date, particular attention has been paid to lipases, because they have a large range of substrate specificities and can selectively react with esters of primary or secondary alcohols, but as with all processes there are certain criteria which lead to successful reaction systems.

1.5 Factors Affecting Enzymes In Organic Media

Enzyme catalysis in organic solvents is one of the most important synthetic techniques developed over the last decade. There is usually insufficient understanding to be able to predict the ideal conditions for any particular reaction. Finding the right conditions for a particular reaction requires experience, knowledge and a great deal of experimental work. The factors which govern the enzyme reactions in aqueous media such as reaction pH, temperature, inhibition and enzyme source are also important in organic

media.[50] A significant factor in anhydrous organic solvent based reactions is the water content of the enzyme.[51,52]

1.5.1 Water Content

The productivity of lipases in organic solvents is dependent upon the water content associated with the total system.[37] Too much water in the system will lead to hydrolysis of the products and too little may lead to reduced enzyme activity and poor reaction. No-one has yet come to any conclusions as to the optimum amount of water required, but there are numerous suggestions. [37,53-55] Zaks and Klibanov[56] carried out an extensive investigation and reported that crude PPL could operate successfully in organic solvents with a water content between 3.6% and 0.3 %, but the rate of reaction under these drier conditions was very low indeed. In many cases, the optimum water content required for reaction in an organic solvent has to be determined by experimentation.

Enzymes can be dried to a low water content (~ 0.3%) by freeze-drying. Freeze-drying is a non-destructive technique adopted by bio-related industries to remove water absorbed by the enzyme (which usually facilitates protein denaturisation under ambient temperatures), so that the life span of biological materials and enzymes during storage is prolonged. It is an ideal method of drying an enzyme prior to its use as a catalyst in an anhydrous organic solvent.

1.5.2 Drying Agents

In many cases, drying agents can be added to condensation reactions to remove ancillary water to provide near anhydrous reaction conditions.[42,57-61] Roberts *et al.* [58] used molecular sieves to absorb water generated in a lipase-catalysed esterification reaction which was carried out in two vessels interconnected by a porous sintered disc. The substrates and enzyme were placed in one of the chambers whilst molecular sieve was placed in the other. The enzyme was stirred and polymeric products were obtained from the reaction.

1.5.3 Reaction pH

As highlighted earlier with PPL (Figure 1), an enzyme in aqueous media exhibits optimum activity only at a particular pH. The pH controls the conformation and the ionisation state of catalytically active groups. These in turn control the activity and also the specificity of the enzyme.

The enzyme structure adopted at optimum activity is maintained by lyophilising (freeze-drying) the enzyme from a buffered aqueous solution. The enzyme can then be placed in anhydrous organic media where it still maintains its conformation and hence the high catalytic specificity and activity. [32]

1.5.4 Choice of Organic Solvent

The choice of organic solvent for a particular biocatalytic system has been shown to be important. Klibanov and other workers have reported that enantioselectivity varied with a change in organic solvent.[45,62-66] Laane et al. [50] have reported that it is possible to categorise organic solvents for biocatalysis into three groups; poor, good, and excellent in terms of their log P (the partition coefficient between octanol and water). It was suggested that it is possible to use this data to predict whether a biocatalytic reaction will work well in the chosen solvent. Log P values calculated from the hydrophobic fragmental constants according to Harnisch[67] were listed for some solvents such as acetonitrile (-0.33), tetrahydrofuran (0.49), pyridine (0.71), diethyl ether (0.85), diisopropyl ether (1.9), chloroform (2.0), toluene (2.5), hexane (3.5) and heptane (4.0). The expected biocatalytic activity within the log P ranges are shown in **Table 2** below.

Table 2. Expected biocatalytic activity of enzymes in various organic solvents with particular log P values.

Biocatalytic Activity	log P	Solubility in water (20°C) wt. %
Poor	≤ 2	>0.4
Good	$2 < \log P < 4$	0.04 - 0.4
Excellent	≥ 4	<0.04

General biocatalysis in organic solvents was low in polar solvents having a log $P \leq 2$, moderate in solvents having a log P between 2 and 4, and high in apolar solvents having a log $P \geq 4$. However, it was found that the only exception to the log P rule was PPL since it appeared to be active in all solvents chosen.[50]

36

1.5.5 Temperature Stability

In aqueous media, enzyme activity is dependent on temperature and they normally operate (as shown earlier in **Figure 1**) between 20 and 50°C. Above this range, the enzyme 'denatures' and becomes irreversibly inactive. Below this temperature range the enzyme activity is not destroyed but is seriously reduced. Enzymes can be moderately protected from the rigours of increasing temperature and chemical inactivation in aqueous media by immobilisation.[68,69]

Enzymes in an organic system can operate within a broader temperature range than in water without immobilisation. Zaks and Klibanov[56] reported that PPL could survive temperatures of 100°C or more in an organic solvent, and the activity of PPL was found to be five times greater at 100°C than at 20°C. This was only possible after the water content of the PPL had been reduced to a free water content of approximately 0.48% by freeze-drying. PPL with this reduced water content and operating at high temperature had only a limited lifetime and the broad substrate specificity was reduced.

1.5.6 Reaction Inhibition

'Inhibition' of an enzyme occurs when a substrate or a product remains bound within the enzyme active site, preventing further reaction and reducing the activity of the enzyme.[1] This is commonplace in aqueous reactions and is also expected to occur in organic solvent systems. The degree of inhibition may be reduced by careful consideration of the reaction conditions and choice of reaction solvent.

1.5.7 Choice of Enzyme

As with aqueous systems, the source of an enzyme governs its activity and selectivity in organic solvent based reactions. In our investigations[70-72] we found that transesterification reactions proceeded better with crude PPL whilst CRL was best for esterification. A similar observation has been made previously.[73]

1.5.8 Pressure

Work by Garcia and workers[74] showed that pressure had a negative effect on the ester yield in reactions catalysed by an immobilised lipase from *Mucor miehei*. When the reactor pressure was increased from 60

to 710mmHg the ester yield decreased. The effect of pressure has also been studied with other enzyme systems.[75] Chaudhary *et al.* used pressure to control the molecular weight of the polymer produced during the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) adipate by 1,4-butanediol in supercritical fluids such as carbon dioxide and fluoroform. Varying the pressure of the system altered the solubility of the polymer in the supercritical fluid causing a polymer of a particular molecular weight to precipitate. These polymer fractions were found to have lower polydispersity than those produced using a conventional enzyme-based system in organic solvent at atmospheric pressure.[75]

1.5.9 Enzyme Immobilisation

Enzymes are usually immobilised on supports to make them more robust and prevent deactivation by organic solvents and substrates. Immobilisation[68,69] can be achieved by either physical or chemical methods, and each has its advantages. Enzyme immobilisation can be carried out by chemical attachment, simple adsorption or entrapment.[28] The nature of the support and the method of immobilisation influences the activity of the enzyme.[76-78] Chemical immobilisation is preferred in aqueous media, but for use in organic media adsorption is sufficient as the enzyme is insoluble in the organic solvent. Chemical immobilisation makes covalent bonds between the support and the enzyme, but it can also cause deactivation of the enzyme in some cases. [51,79]

Mustranta *et al.* [80] found that using hexane in place of aqueous buffer for the immobilisation of lipases on support material, actually enhanced the activity of high activity lipases, such as *Candida rugosa* lipase and also improved their temperature stability. They also found that an anionic exchange resin was a superior support material to other commercially available supports, such as Celite.

1.6 Esterification Reactions

Lipase-catalysed esterifications are fairly facile reactions and can usually be forced to completion through careful choice of the alcohol. In many instances,

the alcohol is used as the solvent for the reaction in order to shift the equilibrium in favour of ester synthesis.[81,82]

$$RC(O)OH + R'OH \; \overset{\substack{\text{(enzyme)} \\ \text{organic solvent}}}{\underset{\text{aqueous media}}{\rightleftharpoons}} \; RC(O)OR' \; + \; H_2O$$
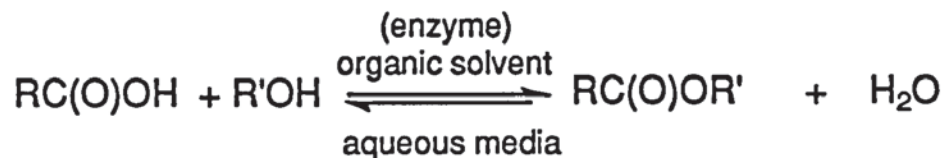
Scheme 6. The reversibility of the enzyme-catalysed esterification of an acid by an alcohol in an organic solvent.

This reaction has the disadvantage that water is produced which promotes the reverse reaction and hence addition of a water removing agent is required to force the reaction to completion. Transesterification differs from esterification in that an alcohol is produced in the reaction rather than water.

1.6.1 Transesterification Reactions

Lipase-catalysed transesterifications[29,81,83] have been employed by the flavours and essences industry for the synthesis of products such as ethyl isovalerate, heptyl oleate, geranyl acetate and citronellyl acetate.[84]

$$RC(O)OR' + R''OH \; \overset{\substack{\text{(enzyme)} \\ \text{organic solvent}}}{\underset{}{\rightleftharpoons}} \; RC(O)OR'' \; + \; R'OH$$
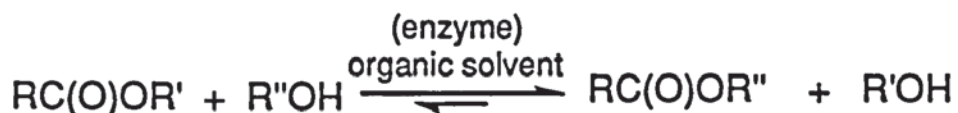
Scheme 7. The enzyme-catalysed transesterification of an ester by an alcohol in an organic solvent.

Esterification and transesterifications are generally much slower than hydrolysis, and hence addition of a drying agent is usually required to remove ancillary water, and retard the back reaction.[41]

Another way to partially overcome the reversibility in lipase-catalysed transesterification reactions is to use activated esters as acylating reagents to facilitate the forward reaction and to make it kinetically irreversible. Several types of activated esters such as 2,2,2-trichloroethyl or 2,2,2-trifluoroethyl esters,[81,85] cyanomethyl esters,[86]

enol esters,[42,87-90] thioesters,[91] oxime esters,[92,93] and vinyl carbonates[94] have been used in enzyme-catalysed transesterification reactions. These reactions are driven to completion because the leaving group released from the ester is a poor nucleophile. Their rates of catalysis are about one to two orders of magnitude faster than those of non-activated methyl or ethyl esters.[29]

In transesterification reactions, 2,2,2-trichloroethyl esters produce weakly nucleophilic alcohols which slows down the reverse reaction. Even though the 2,2,2-trichloroethyl esters are less expensive than trifluoroethyl esters, the weakly nucleophilic 2,2,2-trichloroethanol by-product is more difficult to remove (bp 151°C cf. 77-80°C).[84] 2-Chloroethanol is easier to remove (bp 130°C), but the degree of activation is relatively low.[84] Some examples of the use of 2,2,2-trichloroethyl esters in enzyme-catalysed transesterification are shown below.[95,96]

(+) >98% ee, 20% yield

95% ee, 90% yield
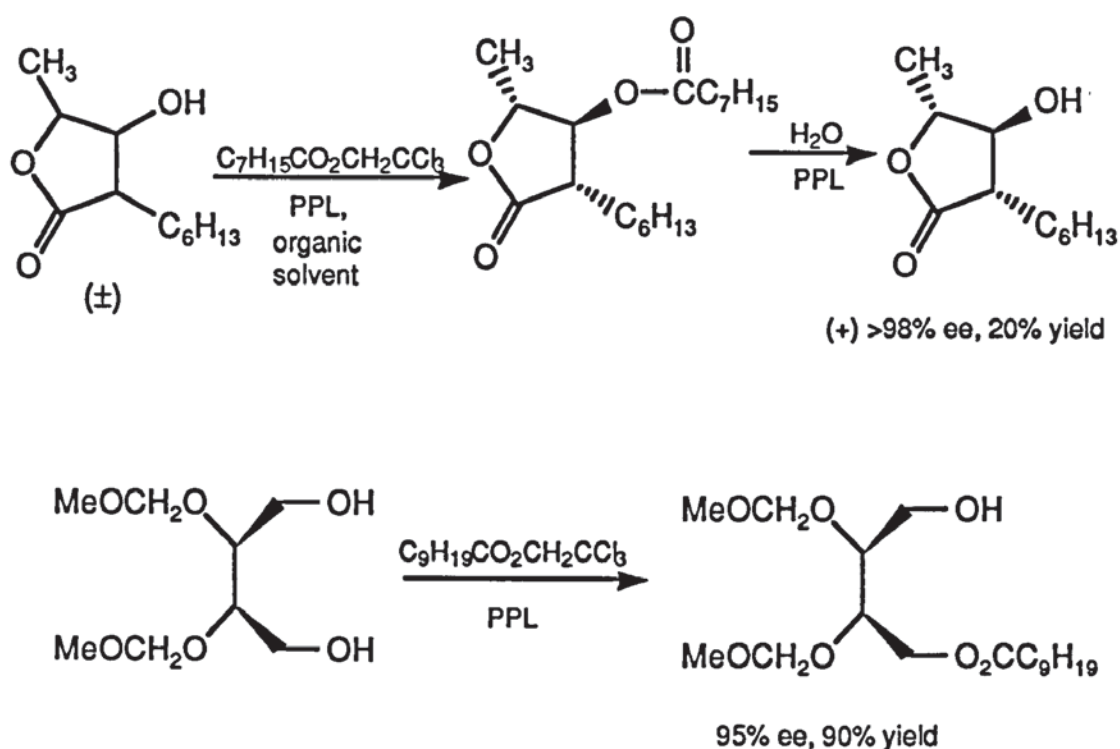
Scheme 8. Transesterification reactions of 2,2,2-trichloroethyl alkanedioate esters catalysed by PPL in organic solvent.

40

1.6.2 Irreversible Transesterification using Vinyl Esters

Irreversible transesterification[57] can be achieved by using enol esters which liberate unstable enols. These enols tautomerise readily to aldehydes or ketones, making the reaction completely irreversible.

The acetaldehyde liberated from reactions with enol esters may inactivate the enzyme due to the formation of a Schiff's base with the enzyme's lysine residues. This problem may be avoided by immobilisation of the enzyme on a support.[97]

The use of vinyl esters was pioneered by Wong and co-workers.[84,88,89] Examples of the work carried out this area are shown below in **Schemes 9 and 10;**

$$ROH \; + \; \underset{R'\;\;\;O\;\;\;R''}{\text{(enol ester)}} \; \xrightarrow[\text{organic solvent}]{\text{lipase}} \; R'CO_2R \; + \; \underset{H_3C\;\;\;R''}{\text{(carbonyl)}}$$

R'	R"	
CH$_3$	CH$_3$	isopropenyl acetate
CH$_3$(CH$_2$)$_3$	CH$_3$	isopropenyl valerate
CH$_3$	H	vinyl acetate
CH$_3$CH$_2$	H	vinyl propionate
CH$_3$(CH$_2$)$_3$	H	vinyl valerate
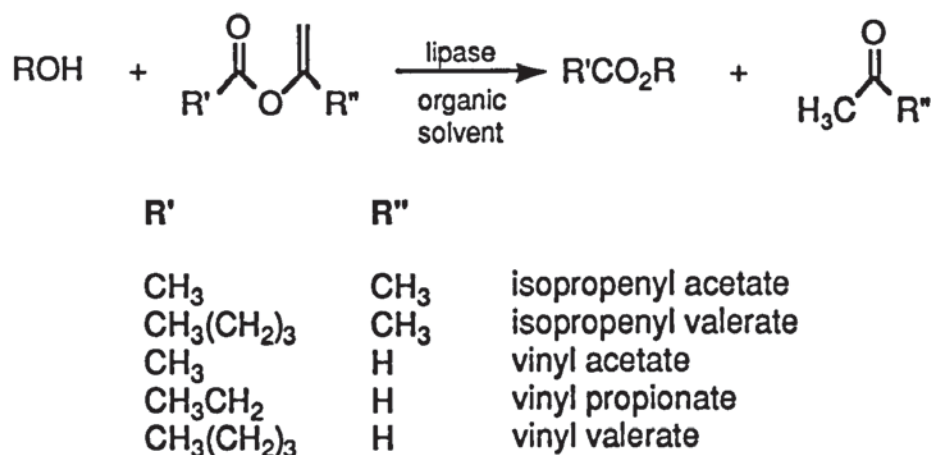
Scheme 9. Use of vinyl esters in transesterification reactions.

Vinyl acetate can be readily utilised as the acylating agent in lipase-catalysed transesterification reactions to resolve racemic alcohols. An example is illustrated below.[98]

R= -(CH$_2$)$_6$CO$_2$Me
    -(CH$_2$)$_2$-CH=CH(CH$_2$)$_2$CO$_2$Me
    -(CH$_2$)C≡C(CH$_2$)$_2$CO$_2$Me
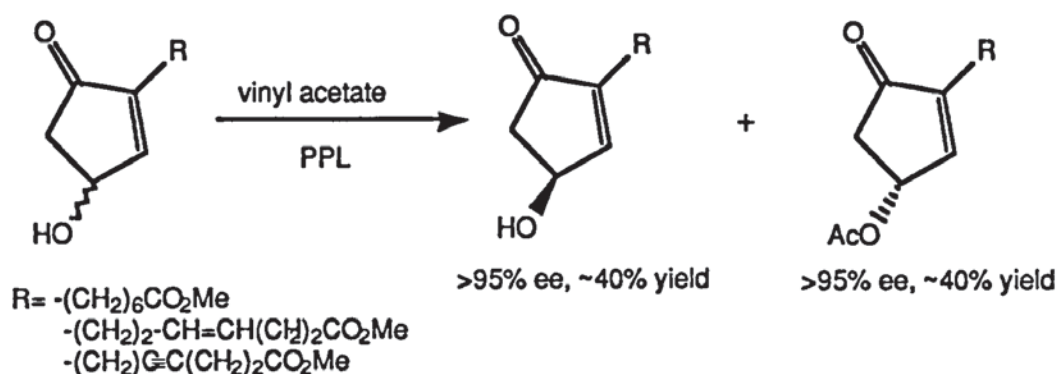
Scheme 10. Lipase-catalysed acylation of a racemic alcohol by vinyl acetate.

Enzyme-catalysed transesterification has also been employed in the area of polyester formation over the last 15 years as highlighted in the following section. Such mild bioprocesses offer both advantages and disadvantages compared to existing traditional industrial processes.

1.7 Industrial Synthesis of Polyesters and Polyamides

Traditional chemical-catalysed industrial syntheses of polyesters and polyamides require high temperatures and pressures. Polyesters[99] such as poly(ethylene terephthalate) are usually prepared by melt polycondensation of dicarboxylic acids or diesters with diols, under high vacuum and at elevated temperatures above the melting points of the resulting polyesters. The reversibility of these esterification reactions do not favour the formation of high molecular weight polymers unless eliminated by-products such as water or alcohol are removed from the reaction.

Activated diesters[99] which produce weak nucleophiles can be employed to promote the polyesterification. For example, bulk polycondensation of diphenyl esters is carried out at temperatures above 200°C, but even under these severe conditions, high molecular weight polymers (Mw ~100,000) are difficult to obtain and the polymer products have relatively high polydispersities.

Nylon-6 is a polyamide which is prepared commercially by the high temperature ring opening polymerisation of ε-caprolactam. Water is used as an initiator and small amounts of acetic acid are added to control chain length.

42
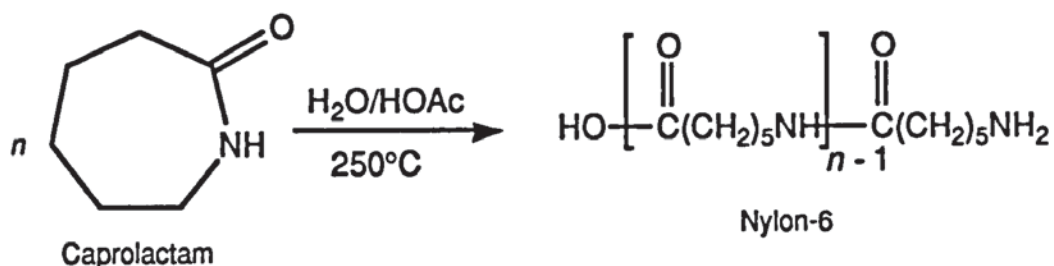
Scheme 11. Industrial preparation of Nylon-6.

Another polyamide, Nylon-66 is prepared by reaction of equimolar quantities of hexamethylenediamine and adipic acid in an aqueous methanol medium. A solution of the "nylon salt" precipitates on mixing of the monomers and is then heated under pressure to about 270°C to produce the polymer.
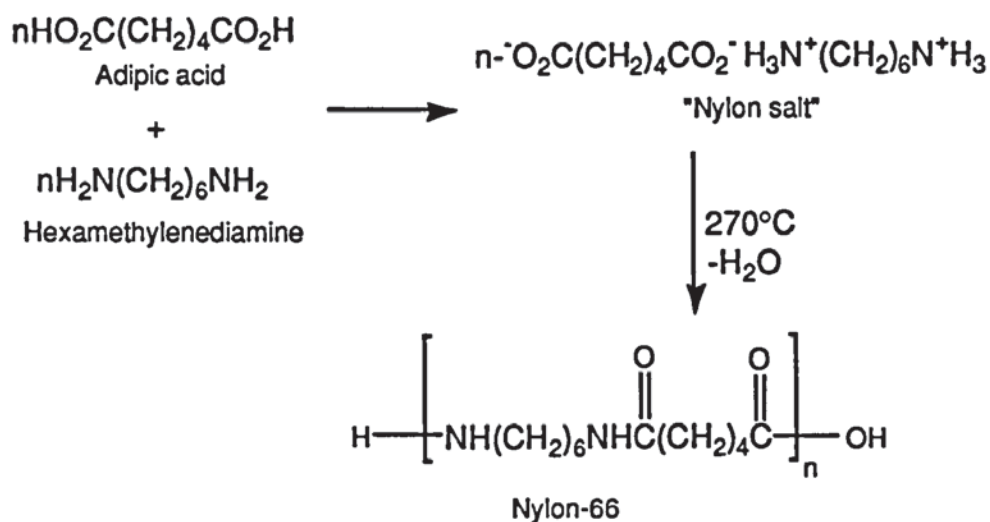
Scheme 12. Industrial preparation of Nylon-66.

Polyesters obtained from high temperature polymerisations of activated esters and diols typically have molecular weights (Mw) in the range of 10,000 to 20,000 daltons, and usually have polydispersities between 1.8-2.0. Compared with other commercial polymers e.g. acrylates, these polymers are of fairly low molecular weight. This is because the chain growth required to produce high molecular mass material becomes increasingly difficult as the reaction proceeds. There are a number of factors which limit molecular weight :-

i)   A precise stoichiometric ratio of starting materials is required.

ii) As the reaction proceeds, the concentration of reactive functional groups decreases.

iii) The increase in viscosity as the reaction proceeds reduces the diffusion rate.

Values for molecular weights of polymer in 'ideal situations' can be calculated by the following equations.[100] However, the ideal situation is difficult to achieve due to the factors described above.

1.7.1 Average Molar Mass

A prediction for ideal values for number average (Mn) and weight average (Mw) molar masses can be calculated from the following equations[100] (where M_0 is taken as the molar mass of the repeat unit, and p is taken as fractional reactant conversion). Thus;

$$Mn = N_x \, \Sigma(M_0 N_x)/N = M_0/(1-p),$$

$$\text{and} \quad Mw = M_0(1+p) \, / \, (1-p).$$

By calculation, the expected ideal polydispersity index (Mw/Mn) of the polymer when $p = 1$ (100% conversion) equates to;

$$(Mw/Mn) = \left\{ M_0(1+p)/(1-p) \right\} \left\{ (1-p)/M_0 \right\} = 2$$

In practice $p=1$ is rarely achieved, giving rise to a range of polydispersities for a given system.

The effects of the degree of polymerisation and starting material purity on the polymer molecular weight attainable is best illustrated by the Carothers equation.

1.7.2 Carothers Equation

W. H. Carothers (the pioneer of step growth polymerisations) proposed a number of simple equations to illustrate the problems associated with polymer reactions.

44

The first of these relates fractional conversion, '*p*', to the degree of polymerisation, 'x_n' for linear polycondensations.

If $2N_0$ is the original number of molecules present in an AA-BB monomer system and 'N' is the number of all molecules remaining after time '*t*' then the total number of functional groups of either A or B which have reacted is (N_0-N). At time '*t*' the fractional reactant conversion, '*p*' is given by

$$p = (N_0 - N)/N_0 \quad \text{or} \quad N = N_0(1-p);$$

substituting for $x_n = N_0/N$ in the above gives

$$x_n = 1/(1-p)$$

The degree of polymerisation 'x_n' varies significantly depending upon the percentage conversion of reactants. For example, in a reaction where *p* =0.95 (95% conversion), the degree of polymerisation is 20, compared to a reaction which has reached 99% conversion, *p*=0.99, 'x_n' increases to 100.

(When using the Carothers equation for an A-A, B-B system, half the average molar mass of the [A-AB-B] repeat unit is used to calculate the degree of polymerisation.)

An imbalance in the reaction stoichiometry has a profound effect on the degree of polymerisation, x_n which is expressed as;

$$x_n = (1+r)/(1+r-2rp)$$

(where 'r' is the ratio of the number of molecules of the substrates (N_{AA}/N_{BB}))

For a stoichiometric reaction where 'r'=1 and '*p*' approximates to 0.999 (99.9% conversion), x_n =1000. However, in a non-stoichiometric reaction between, for example, N molecules of A-A and 1.05N molecules of B-B, the value of 'r' (1/1.05) approximates to **0.952** with '*p*' still approximating to 99.9% conversion. The degree of polymerisation, x_n therefore equates to,

$$x_n = (1+0.952)/(1+0.952-2 \times 0.999 \times 0.952) \approx 39$$

The purity of the starting materials is also crucial and if one of them contains only 95 per cent of the di-functional material, then immediately, r=0.95 and so the maximum attainable value of x_n will be 40.

In practice, *p*=1 is rarely achieved and is difficult to obtain.

All the above factors are applicable and have to be considered in the case of the enzyme-catalysed polytransesterification reactions described below.

1.8 Enzyme-Catalysed Synthesis of Polyesters In Organic Solvents

Synthetic routes to polyesters are of considerable commercial importance. Polyesters have a wide variety of applications from clothing to fibres to plastic film.[20] There is an increasing need to impart selectivity in synthesis. This is particularly important in the synthesis of pharmaceuticals and biomaterials where regioselective introduction of functionality and stereoselectivity is required. The application[101-104] of enzymes in organic solvents to polyester synthesis offers a potential route to biodegradable/biocompatible materials with specific functionality and/or stereochemistry, characteristics which are often difficult to achieve using traditional chemical methods.

Enzymatic synthesis has an advantage over chemical catalysis, as the insoluble enzyme catalyst can be removed easily from the reaction. Products from chemical-catalysed polymerisations often contain some catalyst which may have undesirable effects on the products in down-stream processes.[105]

For example, in industrial polyurethane manufacture, organometallic catalysts are employed.

$$nHO(CH_2)_4OH \quad + \quad n\ O=C=N-(CH_2)_6-N=C=O$$

Tertiary amine or organotin
or organozinc catalyst

$$H\text{-}\!\!\sim\!\!\left[O(CH_2)_4-O-\overset{\displaystyle O}{\overset{\|}{C}}-\underset{\underset{H}{|}}{N}-(CH_2)_6-\underset{\underset{H}{|}}{N}-\overset{\displaystyle O}{\overset{\|}{C}}\right]_n\!\!\sim\!\!N=C=O$$
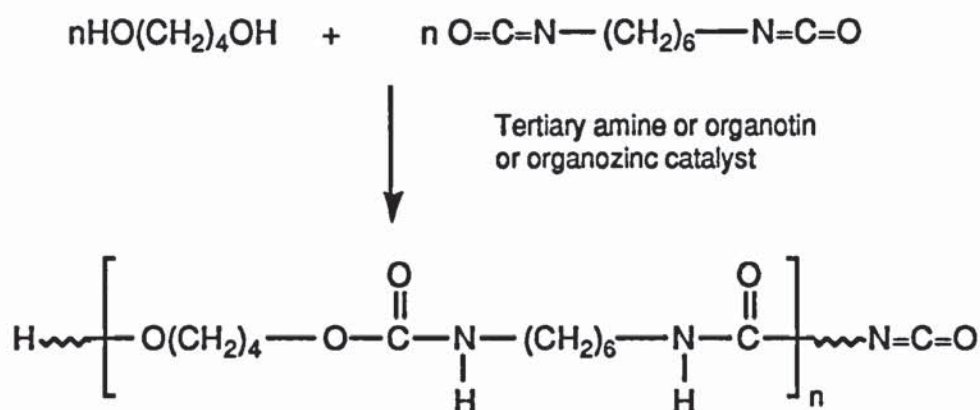
Scheme 13. Synthesis of a polyurethane (Perlon U).

Removal of all the organometallic catalyst (mainly organotin and organozinc compounds) is very important because the presence of any catalyst residue catalyses the hydrolytic degradation of the formed polyurethane.[105]

The use of enzymes in organic solvents in polyurethane manufacture has potential commercial importance, since the linear hydroxy-terminated low molecular weight aliphatic oligoesters can be used as intermediates for polyurethane synthesis.

So far, a number of groups have attempted to use enzymes as polymerisation catalysts for the synthesis of polyesters in organic solvents, with varying degrees of success.[58,59,61,101,102,106-110]

Roberts et al.[58] studied the lipase-catalysed polyesterification of adipic acid with 1,4-butanediol in organic solvents. Lipozyme IM-20 (a lipase from *Mucor miehei* immobilised on a macroporous anion exchange resin) was found to be a more robust and superior catalyst to PPL in diisopropyl ether at ambient temperature because the PPL was completely deactivated after 21h due to mechanical damage. Lipozyme IM-20 produced oligomeric residues with Mw 769 and Mn 527. Roberts et al. subsequently found that higher molecular weights (Mw 4,600 and Mn 4,200) could be obtained by employing a novel two-chambered vessel system, which allowed for the agitation of added molecular sieves in one chamber without the risk of abrasive damage to the enzyme in the other chamber.
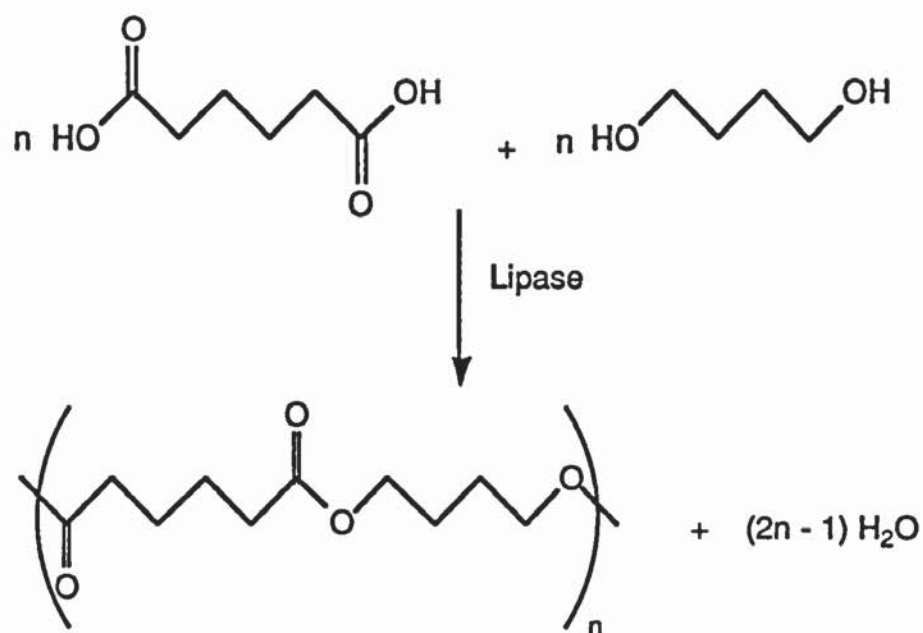
Scheme 14. Polycondensation of adipic acid and 1,4-butanediol by PPL in diisopropyl ether.

O'Hagan *et al.* [59] produced a polymer with an Mn of 9,300 and Mw of 12,000 by incubating 10-hydroxydecanoic acid in hexane at 55°C with *Candida cylindracea* lipase (now known as *Candida rugosa* lipase) in the presence of 3Å molecular sieves (as illustrated below in **Scheme 15**). The polymer molecular weight was higher than that obtained by Kobayashi,[107] but similar to that achieved by Knani.[106] O'Hagan was also able to prepare a polymer with an Mw of 35,000 using 11-hydroxyundecanoic acid, again with the aid of molecular sieves.[59-61]
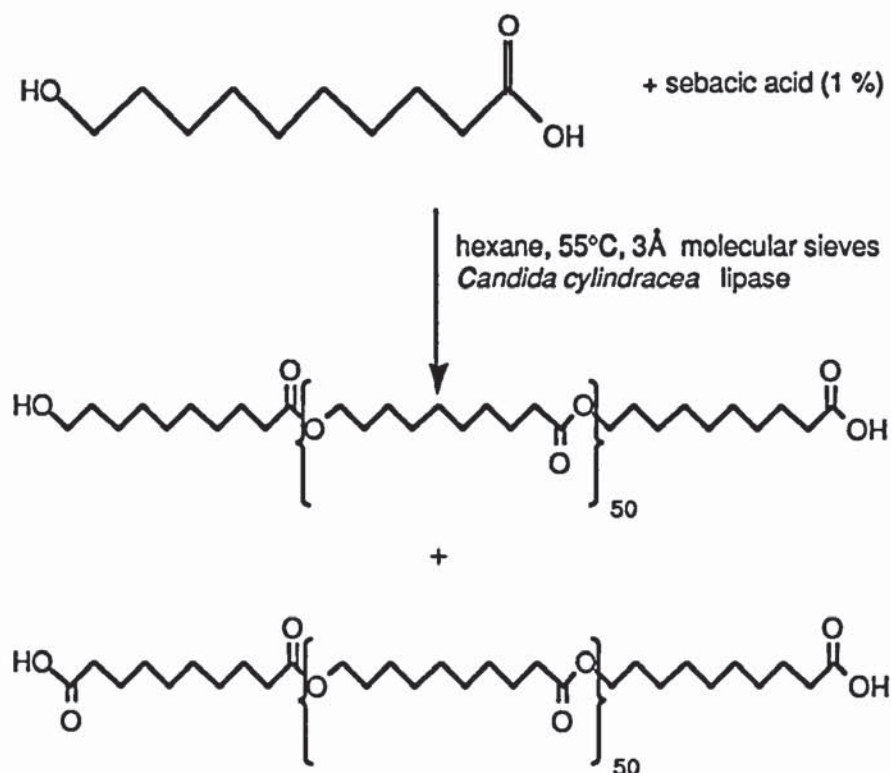
Scheme 15. Polymerisation of 10-hydroxydecanoic acid with *Candida cylindracea* lipase. Redrawn from reference 59.

There are many reports in the literature describing lipase-catalysed polytransesterification in organic solvents, some of which are outlined in the following examples. [23,102,111-116]

A characteristic feature of these reactions is that they produce relatively low molecular weight products. This is because the enzyme must continually generate an acyl-enzyme intermediate at the active site by reaction with the terminal ester moiety of the growing polymer. The efficiency of this process drops rapidly with increasing molecular weight due to the inherent diffusional limitations. [117]

Workers have investigated the use of activated esters such as 2,2,2-trichloroethyl, [101,102] 2,2,2-trifluoroethyl [109,114,118,119] and vinyl [109] as monomers using various enzymes.

Wallace and Morrow [101] prepared relatively high molecular weight polymers by the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) alkanedioates and diols in organic solvents. The polymer products were

found to give Mn values of 1,300-8,200 daltons by end group NMR analysis. Mw values of 2,800-14,900 daltons were obtained by GPC.

Roberts[58] noted that whilst the use of bis(2,2,2-trichloroethyl) alkanedioates gave excellent results, it could not easily be applied in an industrial context, since the starting materials are expensive, and are often not commercially available. In addition, the halogeno alcohols formed as by-products are toxic and present disposal problems.

An alternative approach to [A-B] polymers which has been investigated is the ring opening polymerisation of lactones.[107,120]

Kobayashi et al. [107] studied the synthesis of polyesters by the lipase-catalysed ring opening of ε-caprolactone. Three lipases, *Pseudomonas fluorescens* lipase, *Candida cylindracea* lipase(CCL) and porcine pancreatic lipase (PPL) were employed. The reaction involved stirring the lactone with each lipase at 60°C for 10 days. *Pseudomonas fluorescens* lipase was the most effective catalyst producing a polymer with an Mn of 7,000 and polydispersity 2.2, whilst PPL gave a polymer with an Mn of 2,500 and polydispersity 1.9.

Rather higher molecular weights were achieved in the PPL-catalysed polycondensation of linear ω-hydroxyesters and in the ring opening polymerisation of ε-caprolactone by Knani et al. [106] who obtained film forming polyesters with a degree of polymerisation (DP)>100 and Mw values up to 12,000 from the PPL-catalysed condensation of methyl 6-hydroxyhexanoate in hexane at 69°C.

It is known that small ring lactones e.g. ε-caprolactone which possess strained rings can be polymerised readily. In contrast, large ring lactones in which the ring is unstrained polymerise very slowly if at all.[121] However, one of the surprising features of enzyme-catalysed ring opening polymerisation of lactones is that large unstrained lactones react more rapidly than smaller ring lactones such as ε-caprolactone. It has been suggested[122,123] that the large rings bind more efficiently to the active site than do the smaller rings. Ring opening polymerisation has the advantage that molecular weight can be controlled by the quantity of initiator and that no alcohol by-products are produced. Indeed, after work on this project had finished, papers[124,125] appeared which reported the highly successful lipase-catalysed ring-opening polymerisation of ω-pentadecalactone and trimethylene carbonate to give

polymers with Mns of 62,000 and 24,400 respectively. This result shows that high molecular weight polymers can be synthesised using enzyme catalysis.

One of the most important features of enzyme-catalysed reactions is their stereo- and regioselectivity. This has been used to good effect in the synthesis of several polyester systems.

Wallace and Morrow used 2,2,2-trichloroethyl esters in the stereoselective synthesis of polyesters.[102] Using this approach, they prepared an optically active polyester from 1,4-butanediol and racemic epoxy-diester, as illustrated in **Scheme 16** below.

$$Cl_3CCH_2O_2CH_2CH\text{----}CHCH_2CO_2CH_2CCl_3$$

$$+ \quad HO(CH_2)_4OH$$

$$\xrightarrow[\text{Et}_2\text{O}]{\text{PPL}} \quad H\left[O_2CCH_2\text{-----}CH_2CO_2(CH_2)_4\right]_n OH$$
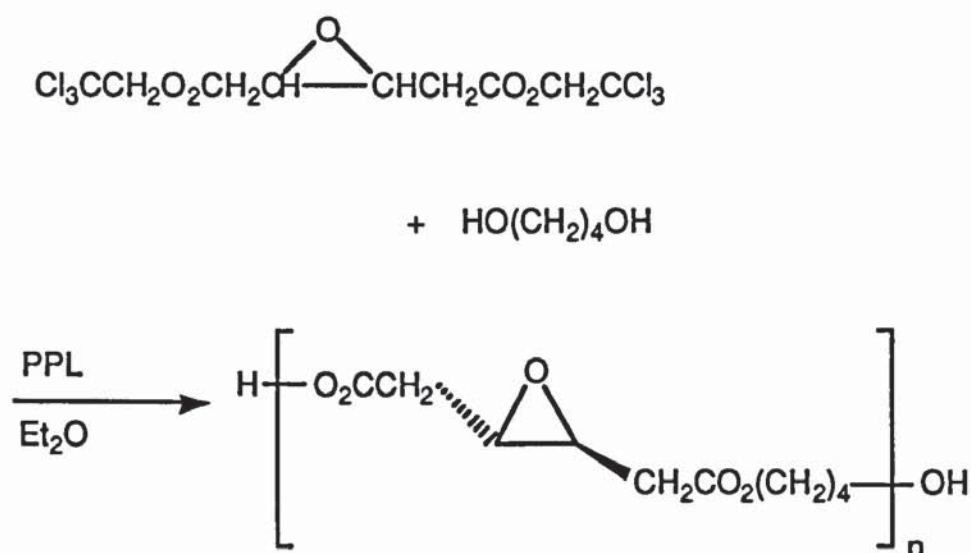
Scheme 16. Polymerisation of a racemic diester monomer with 1,4-butanediol.

Dordick *et al.* have synthesised biodegradable sucrose-adipate copolymers by the protease-catalysed condensation of carbohydrate (the diol monomer) with bis(2,2,2-trifluoroethyl) and vinyl adipate esters in anhydrous pyridine.[108,109,118,126] Remarkably, this process was carried out selectively in one step without the need for protecting groups. The poly(sucrose-adipate) has potential applications as a water adsorbent and is completely biodegradable.
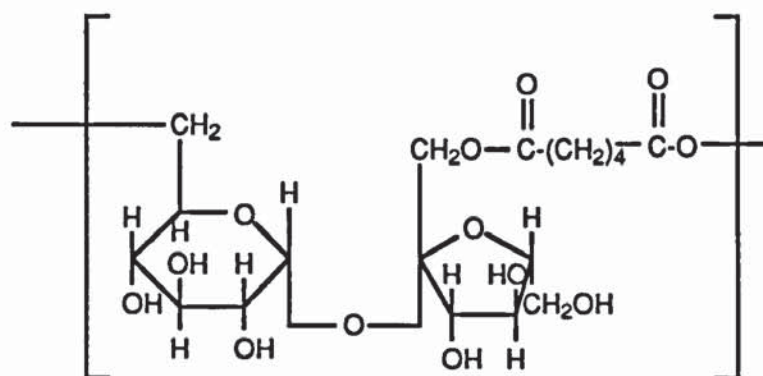
Scheme 17. Molecular structure of poly(sucrose adipate).

A common feature of enzyme-catalysed polytransesterification reactions is that they are relatively slow often requiring reaction times of many hours or even several days to achieve the highest molecular weights. [29,40,42,75 101,102,106,114,118]

Even though there are now reports that high molecular weights can be achieved by the ring opening polymerisation of lactones,[127] at the time this work was inaugurated, the highest molecular weights had been achieved with [AA-BB]$_x$ systems. It appeared logical to investigate these systems in order to develop efficient enzymatic routes to these materials.

1.9 PPL-Catalysed Polytransesterification In Organic Solvents - Project Aim

PPL was of special interest to this project since it has a broad substrate specificity and was specific for primary hydroxy groups only. PPL catalyses the polytransesterification between primary diols and diesters to form linear polyesters as shown in **Scheme 18.**

52

$$(CH_2)_x \underset{CO_2R}{\overset{CO_2R}{<}} \quad + \quad (CH_2)_4 \underset{OH}{\overset{OH}{<}} \quad \xrightarrow{\text{Enzyme}} \quad RO\text{-}[\text{-}\overset{O}{\overset{\|}{C}}(CH_2)_x\overset{O}{\overset{\|}{C}}\text{-}O(CH_2)_4O\text{-}]_n\text{-}H$$
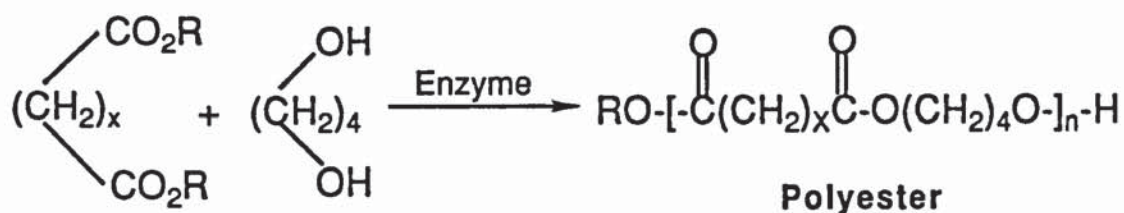
Polyester

Scheme 18. Lipase-catalysed polytransesterification of a diester with 1,4-butanediol in an organic solvent to form a linear aliphatic polyester.

A number of workers have reported the enzyme-catalysed synthesis of polyesters which have high Mw values (between 769 and 35,000 daltons) but low Mn values (between 527 and 9,300 daltons). [58,59,61,75,78,84,99,101-103,107-109,118,119,126]

The aim of this research project was;

I)   to identify the factors controlling the PPL-catalysed polytransesterification of activated diesters of primary alcohols by a primary diol in organic solvents.

II)   to prepare a commercially acceptable polymer with an Mn of 20,000 daltons using the enzyme system shown in **Scheme 19** below:-

$$R-O\overset{O}{\overset{\|}{C}}-(CH_2)_x-\overset{O}{\overset{\|}{C}}O-R \quad + \quad HO-(CH_2)_4-OH$$

PPL
organic
solvent

| R= Cl₃CCH₂- |
| or |
| R= CH₂=CH- |

$$R-O-[-\overset{O}{\overset{\|}{C}}-(CH_2)_x-\overset{O}{\overset{\|}{C}}-O-(CH_2)_4-O-]_n-H \quad + \quad ROH$$
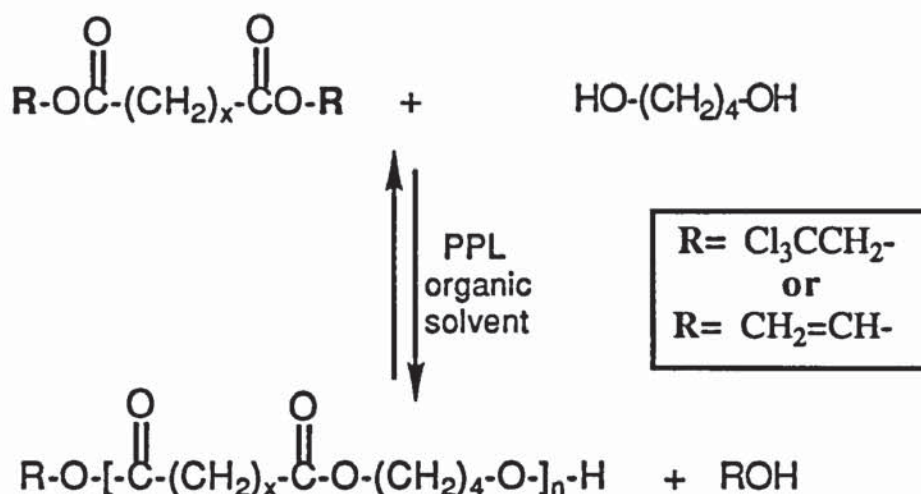
Where x = 3 or 4

Scheme 19. The PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) and divinyl alkanedioates in organic solvent.

The effects of varying reaction temperature, type and concentration of substrate, enzyme mass, drying agent, reaction solvent and modification of the enzyme on the reaction above (**Scheme 19**) were investigated. The results of these investigations are detailed in the following four chapters.

The first of these chapters, (chapter 2) details the effects of reaction time and molecular sieve on the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) alkanedioate esters with 1,4-butanediol in anhydrous diethyl ether. The effects of employing substrates with different chain length on reaction rate is also considered. The influence of the reversibility of the reaction, the mass of enzyme, the presence of molecular sieve and the nature of the substrates are discussed in chapter 3. Chapter 4 details the effects of varying the reaction solvent and reaction temperature on both PPL and immobilised catalyst systems, whilst chapter 5 deals with the modification of the enzyme catalyst. The main conclusions drawn from this work are discussed in chapter 6. For cross-referencing purposes, the numbers in bold in brackets shown throughout chapters 2-6 relate directly to the relevant experiments which are detailed in chapter 7.

CHAPTER 2

ENZYME-CATALYSED POLYTRANSESTERIFICATION OF BIS(2,2,2-TRICHLOROETHYL) ALKANEDIOATES IN ORGANIC SOLVENT

2.0 ENZYME-CATALYSED POLYTRANSESTERIFICATION OF BIS(2,2,2-TRICHLOROETHYL) ALKANEDIOATES IN ORGANIC SOLVENT

2.1 Background

Exploratory studies of the enzyme-catalysed polytransesterification of bis(2,2,2-trichloroethyl) alkanedioates (adipate and glutarate) in organic solvents have been carried out previously in these laboratories by another worker.[70] These studies were initiated to gain experience of using enzymes in organic solvents for the production of polyesters. It was anticipated that a thorough understanding of these reactions would allow this procedure to be applied to systems containing more than one type of hydroxyl group. One of the great advantages of using enzymes is their specificity, and it was thought that it might be possible to react primary hydroxyl groups but leave the secondary hydroxyl groups intact. A number of problems were encountered in these early studies and it took some time and modification of the system to achieve molecular weights comparable to those quoted by Wallace and Morrow, who calculated polymer molecular weights by NMR end-group analysis.[101,102] The original lack of experimental success in our laboratory was probably due mainly to two factors. Firstly, the reactions were carried out for 10 days, and secondly the system was not kept sufficiently dry. This was problematic because the presence of moisture promoted hydrolysis instead of synthesis of the forming polymer. It emerged from these observations that the PPL-catalysed polytransesterification in organic solvents was rather more rapid than had previously been thought. Both these factors gave products with relatively low molecular weights due to hydrolysis. In the case of the glutarate system the highest molecular weights were obtained between 24-36 hours and for the slower adipate reaction between 72-100 hours. It was found that the systems had to be kept very dry and that the highest molecular weights were obtained with added molecular sieve. It was also observed that Mn values calculated using the NMR end group analysis technique were often very different from the values obtained by GPC analysis. Analysis of the polymer products from long reaction times showed that the Mn values determined by NMR were generally much higher than those obtained by GPC.[70]

The further work on the 2,2,2-trichloroethyl systems reported here was carried out to consolidate the previous work and to establish the reproducibility of the PPL- catalysed polytransesterification in organic solvents. Further NMR analysis of the products were also carried out. It was hoped to obtain from these studies some information about the structure of the polymer and discover why the molecular weights reached a maximum and then declined. It was anticipated that with such information it might be possible to modify the reaction in order to produce higher molecular weight products.

2.2 PPL-Catalysed Polytransesterification of Bis(2,2,2-trichloroethyl) Adipate and Glutarate with 1,4-Butanediol in Anhydrous Diethyl Ether

The PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) glutarate and adipate with 1,4-butanediol in diethyl ether was studied over various time periods in our laboratory to determine the relative effects of monomer chain length on the polymer molecular weight. The effect of added molecular sieve was also studied. It has been reported both by Roberts *et al.* and O'Hagan *et al.* that molecular sieve promotes the polyesterification process.[58-61]

$$Cl_3CCH_2-O-\overset{O}{\overset{\|}{C}}-(CH_2)_x-\overset{O}{\overset{\|}{C}}-O-CH_2CCl_3 \quad + \quad HO-(CH_2)_4-OH$$

PPL
organic
solvent

(where x = 3 or 4)

$$Cl_3CCH_2-O-[-\overset{O}{\overset{\|}{C}}-(CH_2)_x-\overset{O}{\overset{\|}{C}}-O-(CH_2)_4-O-]_n-H \quad + \quad Cl_3CCH_2-OH$$
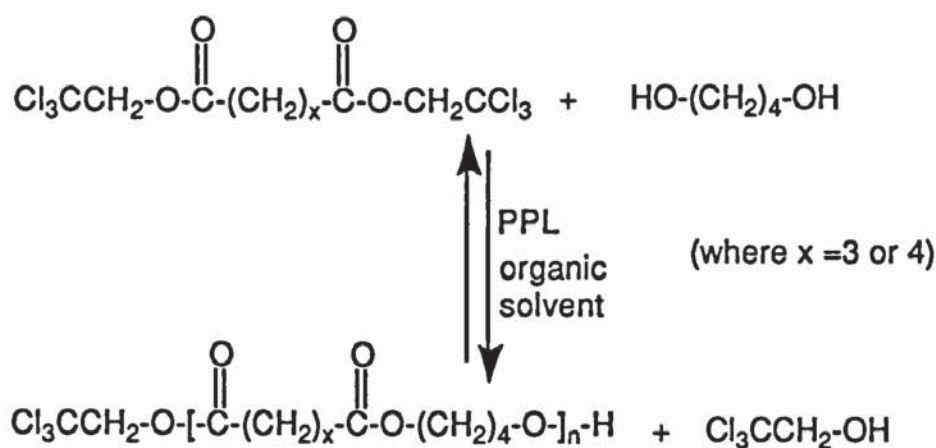
Scheme 20. The porcine pancreatic lipase (PPL)-catalysed polytransesterification of bis(2,2,2-trichloroethyl) alkanedioates (glutarate and adipate) with 1,4-butanediol in an organic solvent.

The products obtained after various time intervals from the PPL-catalysed reaction of 1,4-butanediol with bis(2,2,2-trichloroethyl) adipate and glutarate

with and without 4Å molecular sieve in diethyl ether were analysed by GPC and ^{1}H NMR. The results of these analyses are presented in **Tables 3** and **4**.

Note: The data presented in **Tables 3** and **4** are obtained from polymers produced in individual reactions and are not sequential samples from a single, timed reaction. The long term reproducibility of GPC analysis was quoted by RAPRA Technology Ltd., Shrewsbury as being "poor". To this end, a reference polymer ("Diolpate" a poly(1,4-butylene adipate)) was run along with the samples under exactly the same conditions to detect long term variation in the GPCs.

Table 3. Progress of the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) adipate with 1,4-butanediol in anhydrous diethyl ether with and without molecular sieves.

Reaction no.	Molecular Sieve	Time (h)	Mn (GPC)	Mn (NMR)	Mw (GPC)	Mw/Mn
1	absent	8	1,000	1,600	2,900	2.9
2	"	24	2,100	1,700	3,000	1.4
3	"	72	3,600	3,400	5,000	1.4
4	"	120	4,500	4,700	6,100	1.4
5	"	168	3,600	4,900	5,600	1.6
6	"	240	2,100	4,100	6,100	2.9
7	present	8	1,800	2,700	6,600	3.7
8	"	24	4,500	4,200	6,500	1.4
9	"	72	6,500	4,600	9,400	1.4
10	"	120	5,600	5,300	8,100	1.4
11	"	240	2,200	4,300	8,900	4.0

The above results for Mn (GPC) with and without molecular sieves can be represented graphically as shown below:-
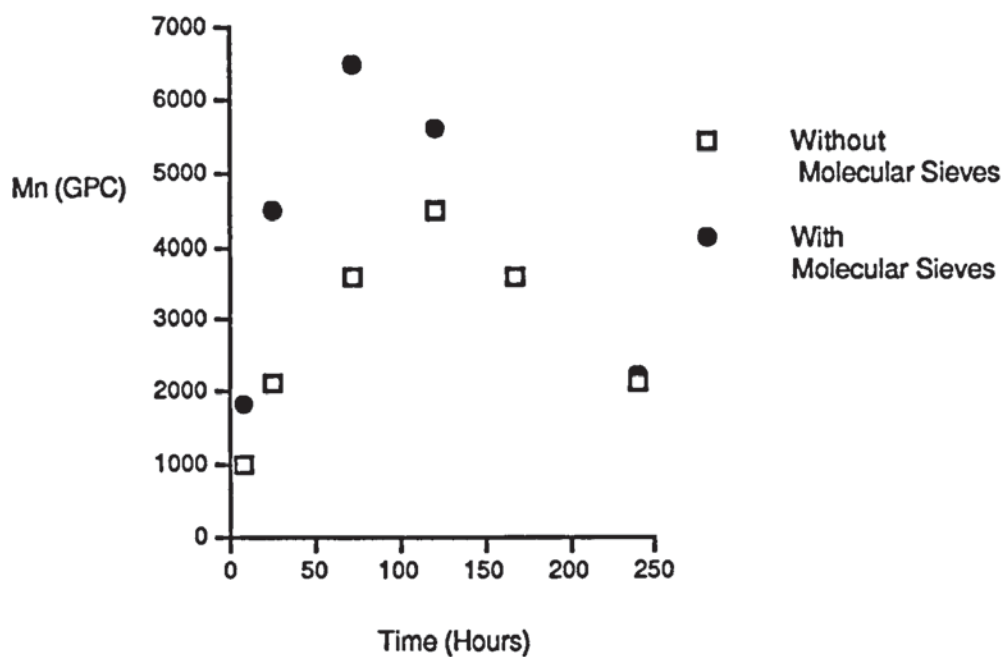
Figure 3. The variation of Mn (GPC) with time in the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) adipate with 1,4-butanediol in anhydrous diethyl ether with and without molecular sieves.

Table 4. Progress of the PPL-catalysed polytransesterification of bis (2,2,2-trichloroethyl) glutarate with 1,4-butanediol in anhydrous diethyl ether with and without molecular sieves.

Reaction no.	Molecular Sieve	Time (h)	Mn (GPC)	Mn (NMR)	Mw (GPC)	Mw/Mn
12	absent	8	3,200	2,200	5,500	1.7
13	"	24	4,100	2,900	6,100	1.4
14	"	72	3,500	3,500	6,100	1.7
15	"	240	1,600	1,800	5,300	3.3
16	present	8	4,000	3,000	8,000	2.0
17	"	24	5,700	5,100	9,500	1.4
18	"	48	5,000	4,600	9,400	1.9
19	"	72	4,600	5,200	8,000	1.7
20	"	240	2,000	2,700	7,300	3.6

The above results for Mn (GPC) with and without molecular sieves can be represented graphically as shown below:-
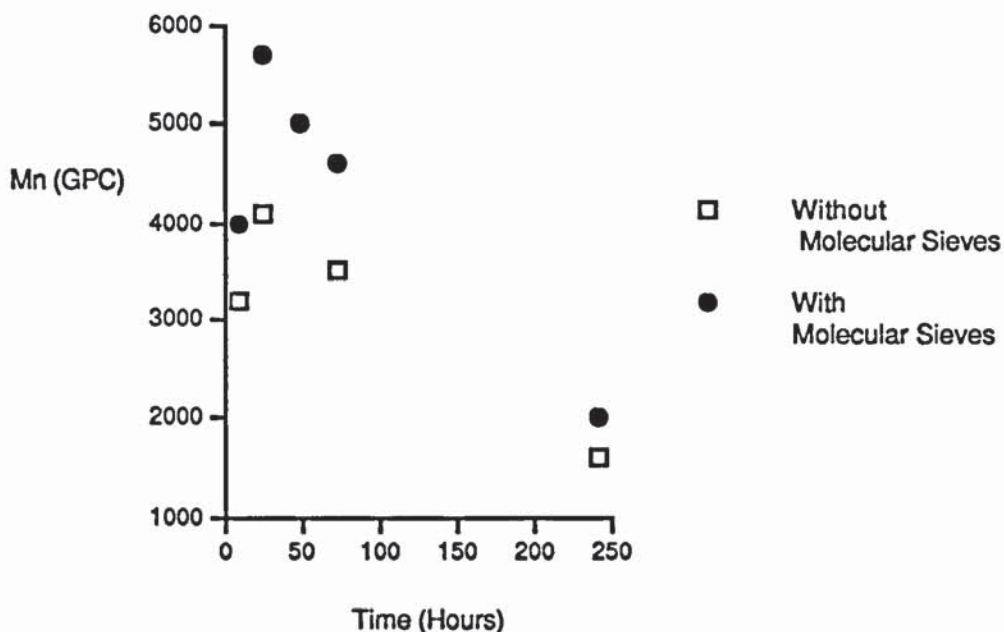
Figure 4. The variation of Mn (GPC) with time in the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) glutarate with 1,4-butanediol in anhydrous diethyl ether with and without molecular sieves.

It can be seen from **Tables** (3 and 4) and **Figures** (3 and 4) above that there is a peak maximum molecular weight for both systems. This may be due to hydrolysis competing with the polytransesterification, thereby limiting the molecular weight of the polymer. Consequently, reactions were carried out in the presence of molecular sieve together with a shorter reaction time to minimise the degradation of the polymer. The polydispersity values for the polymers vary quite considerably, and tend to be higher over the early and latter stages of each of the systems. This is possibly because initiation produces a large number of polymer chains of different lengths.

Recovery of the polymer involved rotary evaporation of the dichloromethane from the filtrate followed by precipitation of the polymer from the remaining oil by addition of diethyl ether. On addition of the diethyl ether there appeared to

61

be two different products. First, a free floating white precipitate formed. After standing, the residual oil eventually solidifed.

It was found that for the bis(2,2,2-trichloroethyl) adipate polyesters the polymer molecular weight of the solidified oil was twice that of the free floating precipitate. For the bis(2,2,2-trichloroethyl) glutarate polymer however, the molecular weights were almost exactly the same for both solids.

2.3 The Effect of the Addition of Molecular Sieves

It was observed by O'Hagan,[59] and Roberts and co-workers[58] that molecular sieve promoted the enzyme catalysed polyesterification reactions between acids and alcohols. This is scarcely surprising since water produced in these reactions would promote the hydrolysis of any oligomeric or polymeric species formed.

Morrow *et al.*[114,115] reported that the alcohol liberated in polytransesterification promoted release of the enzyme-bound water which hydrolyses the activated ester end groups. So although the polytransesterification reaction itself does not produce water it was thought that addition of molecular sieve might promote reaction by removal of this ancillary water.

It is apparent from **Table 3** that addition of molecular sieve alleviates the effect of the water and promotes the polymerisation reaction. In the case of the adipate, the Mn values after 8h (Mn 1,800), 24h (Mn 4,500) and 72h (Mn 6,500) reactions with molecular sieve (wms) were about twice as high as for the same reactions (8h (Mn 1,000), 24h (Mn 2,100) and 72h (3,600)) without molecular sieve (woms)). The differences for the other reaction times with and without molecular sieve were less significant. For instance, the 120h reaction (Mn 5,600 wms cf. Mn 4,500 woms) whilst for the 240h reaction (Mn 2,200 wms cf. Mn 2,100 woms).

The addition of molecular sieve had a smaller influence on the Mn values for the glutarate than for the comparable adipate reactions. For the more reactive glutarates the Mn values for the 8 hour (Mn 4,000 cf. Mn 3,200) and 24 hour (Mn 5,700 cf. Mn 4,100) reaction were about 30% higher when molecular

sieve was present whereas for the 8 hour (Mn 1,800 cf. Mn 1,000) and 24 hour (Mn 4,500 cf. Mn 2,100) reactions of the adipate, the addition of molecular sieve approximately doubled the Mn of the polymer. The influence of the molecular sieve on the glutarate reaction may be smaller because the relatively faster glutarate-diol condensation reaction may be able to compete more effectively with the accompanying hydrolysis process than the slower adipate reaction.

It is likely that the main action of the molecular sieve is to prevent hydrolysis of the active 2,2,2-trichloroethyl end groups and there is ^{1}H NMR evidence to support this proposition (see **Table 5**). It was observed that for reactions carried out in the absence of molecular sieve the products were mainly hydroxy-terminated; the -CH_2OH (δ 3.7) : -CH_2CCl_3 (δ 4.8) ratio increasing with time suggesting the possibility that the ester groups might be being consumed in a side reaction. In contrast, the products from the reactions carried out with molecular sieve were predominantly 2,2,2-trichloroethyl terminated. Here again it was apparent from the -CH_2OH:-CH_2CCl_3 ratios that the relative proportion of ester-terminated polymers decreased with time so even with molecular sieve present hydrolysis may still be occurring.

As with the adipate reaction, the glutarate products formed in the absence of molecular sieve were mainly hydroxy-terminated, and the products from the reactions carried out with molecular sieve were predominantly ester-terminated as shown in **Table 5**. The proportions of ester and hydroxy-terminated groups with and without molecular sieve respectively was relatively greater for the glutarate than the adipate system.

Table 5. The relative proportions of hydroxy- and ester-terminated functions in the products formed from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) adipate and glutarate with 1,4-butanediol with (wms) and without molecular sieves (woms) in anhydrous diethyl ether.

	Glutarate hydroxyl:ester		Adipate hydroxyl:ester	
Time(h)	wms	woms	wms	woms
8	1:3	2:1	1:11	4:1
24	1:18	7:1	1:10	7:1
72	1:10	20:1	1:6	9:1
120	-	-	2:1	11:1
240	3:1	-	4:1	-

A possible explanation for the observed high proportions of (2,2,2-trichloroethyl) end group functions with molecular sieves present is that the molecular sieve dries the system sufficiently, reducing hydrolysis of both the bis(2,2,2-trichloroethyl) ester monomer and the (2,2,2-trichloroethyl) ester-terminated oligomers. This maintains the active end groups for subsequent reaction and hence propagation continues to produce relatively high polymer molecular weights.

In systems without molecular sieves there is a greater risk of both the bis(2,2,2-trichloroethyl) ester monomer and (2,2,2-trichloroethyl) ester end-capped oligomers being hydrolysed. Hence there will be an increase in the hydroxyl end group concentration. This leads to an earlier termination of the propagation of the polymer and hence lower polymer molecular weights, as we have observed.

^{1}H NMR studies by Wallace and Morrow also showed an excess of hydroxy terminated chains. They concluded that this was due to the oligomers reacting more rapidly with 1,4-butanediol than did the diester monomer.[102] From our observations the addition of molecular sieves reduced the occurrence of hydrolysis and suggested that the presence of an excess of hydroxyl terminated chains was due to ester hydrolysis.

Athawale and Gaonkar[128] studied the lipase-catalysed polytransesterification of bis(2,3-butanedione monoxime) alkanedioate with a range of primary diols.

The diol disappeared within 4-5 hours but about 20-25% of the diester remained. The complete disappearance of the diester occurred after 20-25 hours. This indicated that the oxime acetate groups of the dimers and oligomers were transesterified faster than those of the starting material.

2.4 The Effect of Varying Alkanedioate-Glutarate vs. Adipate

As was observed previously in our laboratory,[70,71] the PPL-catalysed condensation of the adipate with the 1,4-butanediol (Table 3) was significantly slower than the comparable glutarate reaction. After only 8 hours the reaction without molecular sieve produced an oligomer with Mn = 1,000 and Mw = 2,900, and the 120 hour reaction gave the highest observed molecular weights without molecular sieve Mn = 4,500 and Mw = 6,100.

The PPL-catalysed condensation of the glutarate and 1,4-butanediol (Table 4) was significantly faster than the adipate reaction (Table 3). The maximum Mn values of the polyesters isolated from the glutarate-diol system occurred after 24 hours with (Mn 5,700) and without molecular sieves (Mn 4,100). These are comparable to those observed for the adipate system after 72 hours with (Mn 6,500) and without molecular sieve (Mn 3,600).

2.5 Difference Between NMR and GPC Results

The Mn values determined by NMR analysis in some cases were close to those obtained by GPC. Proton NMR analysis allowed us to calculate the number average molecular weight by comparing the ratios of the end groups to the butyl groups within the polymer chains.

The molecular weights of the polymers were determined by GPC (Mn and Mw) and ^{1}H NMR (Mn). The number average molar mass Mn is defined by

$$M_n = \frac{\sum N_i M_i}{\sum N_i}$$

and the weight average molar mass Mw is defined by

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i}$$

where Ni is the number of molecules of species i of molar mass Mi

It is apparent from **Tables 3** and **4** that there are significant differences in the Mn values of the diol-ester polymers determined by GPC and NMR. In some cases the NMR values are greater than the GPC and vice versa. Comparison of Mn values obtained by the two methods shows no consistent pattern. It has been suggested that hydrolysis of the terminal trichloroethyl groups[109,110] might be one factor responsible for the differences in the molecular weights obtained by NMR end group analysis and by GPC; however, agreement between the two methods is no better for those reactions carried out in the presence of molecular sieve where hydrolysis of the trichloroethyl end groups occurs to a much lesser extent.

2.6 Comparison of Conventional and Viscosity GPC Results

All the polymer test samples were analysed by conventional GPC and are represented in this report as 'polystyrene equivalent molecular weights'. However, the use of a viscosity detector allows an accurate determination of the molecular weight. For completeness, some of the samples were analysed by GPC/viscosity and are compared with standard GPC results as shown below:-

66

Table 6. Polymer molecular weights as determined by conventional and viscosity GPC analysis.

Polymer	GPC Viscosity			GPC Conventional		
	Mn	Mw	Mw/Mn	Mn	Mw	Mw/Mn
Diolpate	4,800	8,800	1.8	4,500	8,000	1.8
4	3,400	4,600	1.4	4,500	6,100	1.4
9	4,700	6,100	1.3	6,500	9,400	1.4
13	2,500	15,600	6.1	4,100	6,100	1.4
17	4,400	7,200	1.7	5,700	9,500	1.4

The results indicate that the 'true' (GPC/Viscosity determined) molecular masses of the samples are generally somewhat lower than those expressed as polystyrene equivalent molecular weights but both GPC/viscosity and conventional GPC show the similar differences between the samples. Therefore the results suggest that it is possible to adequately assess the differences between the samples using conventional results.

It was thought that the inconsistency of the GPC results could be due to enzyme becoming entrapped in the polymer. This was investigated by taking polymer from a polytransesterification reaction and testing it in a protein assay to determine if any enzyme was present. The polymer was found to contain no protein and therefore no enzyme.

2.7 Conclusions

Our observations indicate that the PPL-catalysed condensations of 1,4-butanediol with alkanedioates are not particularly slow as would appear from previous observations.[101,102] Under our conditions, maximum M_n values were reached at around 24 and 72 hours for the glutarate and adipate respectively. One factor limiting the molecular weight could be the poor solubility of the forming polymer in the reaction solvent, which causes the polytransesterification reaction to terminate prematurely. A reason for this may be that the polymer forming on the enzyme surface eventually encapsulates the enzyme preventing further penetration of the monomer to the enzyme.

It is apparent from these results and previous studies[70,71] that the moisture level is a very important factor in controlling reactivity and molecular weights. It was found that addition of molecular sieve to the alkanedioate reaction system promoted the condensation and the M_n values of the polymers produced with added molecular sieve are from 1.2-2.2 times greater than those obtained from the comparable molecular sieve free reactions. Presumably removal of moisture retards the competing hydrolysis and subsequent removal of the active ester functions. (Table 5) It would also appear from the variations in the $-CH_2OH:-CH_2CCl_3$ ratios that the influence of the molecular sieve decreases with time.

Even though enzyme reactions are fairly rapid, most of the early work on enzyme-catalysed reactions gave relatively low molecular weight products. There are several possible explanations to account for the relatively low molecular weights obtained. One, that polyester synthesis is relatively slow compared to the rate of hydrolysis. Earlier observations[102,114] suggested that another factor limiting molecular weight of the polymers was the reversibility of the reaction. It seemed possible that employing an irreversible reaction system such as vinyl esters, in conjunction with molecular sieves, may lead to higher molecular weight polymers by a slightly more rapid process.

68

CHAPTER 3

USE OF VINYL ESTERS IN ENZYME-CATALYSED POLYTRANSESTERIFICATION IN ORGANIC SOLVENTS

3.0 USE OF VINYL ESTERS IN ENZYME-CATALYSED POLYTRANSESTERIFICATION IN ORGANIC SOLVENTS

3.1 Background

The use of enol esters and other activated diesters in enzyme catalysed transesterification reactions in organic solvents has been extensively investigated especially by Wong and co-workers.[42,84,87-90] The enol esters have two advantages over other esters. Firstly, they react one or two orders of magnitude faster than non-activated methyl or ethyl esters. Secondly, enol esters are among a number of systems which have been used to completely eliminate reversibility in the transesterification reaction. The enol liberated in the reaction rapidly tautomerises to the highly volatile acetaldehyde which evaporates from the system as illustrated below (**Scheme 21**) ;

$$RC(O)OCH=CH_2 + R'OH \xrightarrow[\text{Organic solvent}]{\text{Enzyme}} RC(O)OR' + CH_2=CHOH$$

$$CH_2=CHOH \downarrow$$

$$CH_3CHO$$
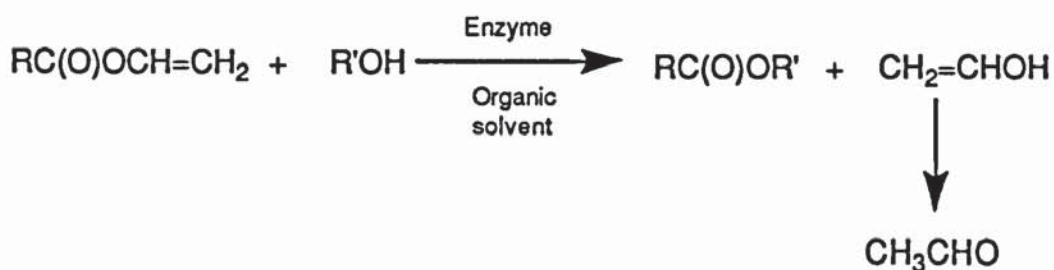
Scheme 21. The enzyme-catalysed transesterification of a vinyl ester with an alcohol in an organic solvent.

Chiral resolutions can be achieved by enzyme-catalysed transesterification. In the example below, (shown in **Scheme 22**) one enantiomer can be prepared from racemic 2-O-benzylglycerol through lipase-catalysed transesterification. The use of an enol ester as the transesterification reagent makes the process irreversible.[88]

70
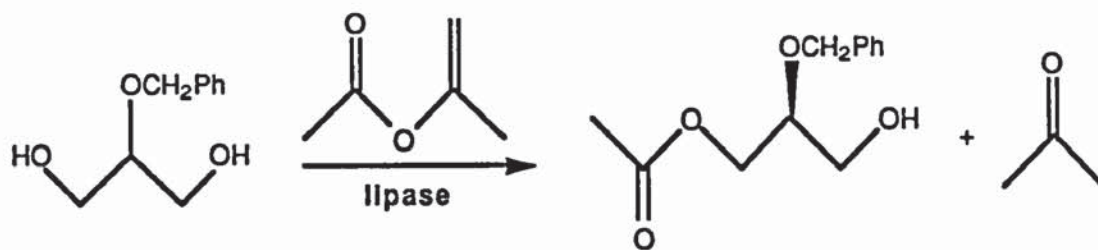
Scheme 22. Transesterification using an enol ester to obtain one enantiomer.

It was decided that we would investigate alternative diester systems since neither we nor other workers[101,129] had been able to achieve Mn values >8,000 daltons using the bis(2,2,2-trichloroethyl) systems for polytransesterification. The bis(2,2,2-trichloroethyl) esters had been chosen originally because the 2,2,2-trichloroethyl group was a good leaving group and the 2,2,2-trichloroethanol formed was weakly nucleophilic; thus, the synthetic reaction would be promoted and the reverse reaction would be retarded. It was thought that achievement of an equilibrium could be a major factor limiting polymerisation, since even with the poorly nucleophilic trihaloethanols,[101,113,114] the reverse reaction might compete with the forward polymerisation process. Evidence to support this observation has been obtained by Morrow *et al.*[114] who found that much higher molecular weights could be achieved by removing the 2,2,2-trifluoroethanol from the system by periodically placing the reaction mixture under reduced pressure. They were able to prepare polymers with Mw values between 20,000-40,000 daltons using this rather inconvenient procedure. These workers concluded that the release of the alcohol had a doubly adverse affect on the polymerisation process; it not only promoted the reverse reaction but also accelerated the release of the enzyme bound water which then hydrolysed the activated ester end groups. Wang *et al.* [73] used a similar approach in the *Mucor miehei* lipase-catalysed polytransesterification of bis(2,2,2-trifluoroethyl) dodecanedioate with 1,4-butanediol in diphenyl ether as the solvent. The polymers obtained had Mw values of up to 34,750 which they achieved under reduced pressure. Although high molecular weight materials were obtained in both of these studies, the solvents and the techniques employed were far from ideal.

It appeared from these previous observations that the major disadvantages of enzymatic polyester synthesis were the relatively slow rates of reaction and

71

the low average molecular weights that could be achieved, except under highly specialised conditions.

It was thought that reversibility might be a limiting factor in the enzymatic polytransesterification process and for that reason it was decided to investigate the use of dienol esters. A known disadvantage of these systems is that the acetaldehyde liberated in the transesterification process could inactivate the enzyme due to the formation of a Schiff's base with the enzyme's lysine residues. It was thought that this problem could be avoided by immobilisation of the enzyme.

In 1994, Uyama and Kobayashi[130] reported the synthesis of polyesters by the lipase-catalysed polytransesterification of divinyl esters with diols in diisopropyl ether at 45°C. They used *Pseudomonas fluorescens* lipase successfully, but failed to achieve products using PPL. In our laboratory we had already found that PPL could produce relatively high molecular weight products (Mn ~6,500) with the (2,2,2-trichloroethyl) system at ambient temperature, and so we decided to investigate the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol and study the effects of a wide range of factors on the polymerisation process, as detailed in the following sections.

3.2 A Study of the PPL-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol in Anhydrous Diethyl Ether

The effect of changing the following variables on this reaction was studied;

  i) Time
  ii) Molecular sieve
  iii) Temperature
  iv) Enzyme mass
  v) Enzyme substrate
  vi) Substrate concentration
  vii) Agitation rate

Control reactions run in the absence of PPL enzyme gave no products.

3.3 The Effects of Varying Reaction Time

Note: The results presented in **Table 7** below are obtained from individual reactions and are not sequential samples from a single timed reaction.

Table 7. Progress of the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether with and without molecular sieves.

Reaction no.	Molecular Sieve	Time (h)	Mn (GPC)	Mn (NMR)	Mw (GPC)	Mw/Mn
21	absent	4	900	1,000	1,500	1.7
22	"	8	2,000	3,100	2,900	1.5
23	"	24	3,900	9,000	5,100	1.3
24	"	48	4,400	5,600	5,500	1.3
25	"	72	4,800	5,600	7,200	1.5
26	"	240	3,100	6,400	5,200	1.7
27	present	8	2,400	2,800	4,300	1.8
28	"	24	4,300	7,800	5,500	1.3
29	"	48	4,800	9,500	8,200	1.7
30	"	72	8,000	11,000	13,000	1.6
31	"	240	5,700	13,000	9,300	1.6

The above results for Mn (GPC) with and without molecular sieves can be represented graphically as shown below:-

73
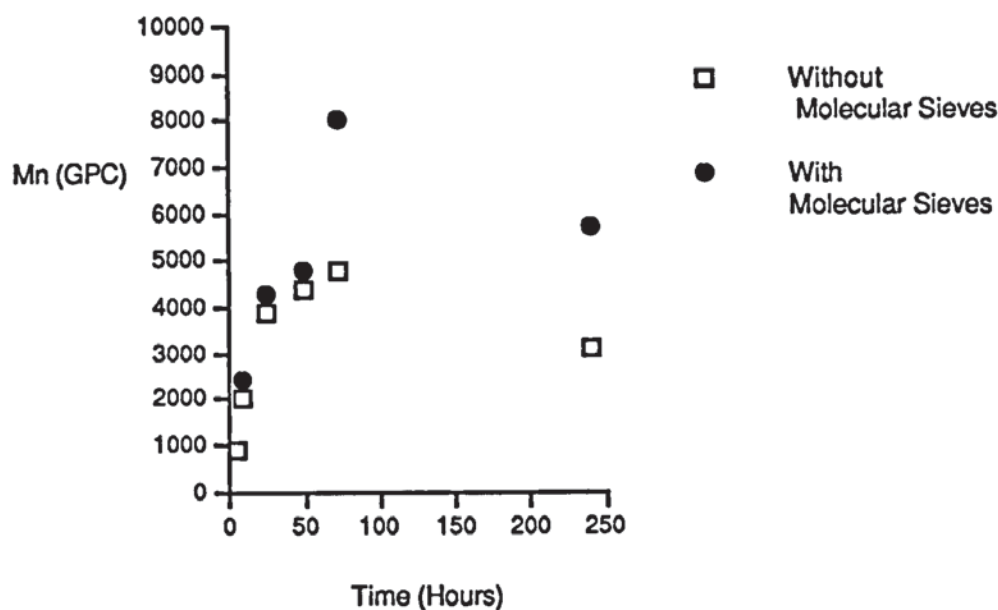
Figure 5. The variation of Mn (GPC) with time in the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether with and without molecular sieves.

3.4 The Effect of Limiting Reaction Reversibility

In contrast to the report by Kobayashi[130] which stated that PPL was not a suitable catalyst for the polymerisation of divinyl adipate and 1,4-butanediol, we obtained polymeric materials under a wide variety of conditions. Comparing the data in **Table 3 (Section 2.2)** and **Table 7 (Section 3.3)** it can be seen that the effect of changing from the 2,2,2-trichloroethyl ester to the vinyl ester was to produce a polymer with a higher maximum molecular weight (Mn 8,000) after 72 hours (with molecular sieves (wms)) compared with the reaction of bis(2,2,2-trichloroethyl) adipate which gave Mn 6,500 after 72 hours (wms). The results obtained indicate that polytransesterification with the vinyl ester was slightly faster than for the bis(2,2,2-trichloroethyl) reaction.

However, it was realised that use of the enzyme in the vinyl system would cause the enzyme to lose activity due to the liberated

acetaldehyde forming Schiff's bases with the lysine groups of the enzyme. Indeed the formation of a yellow substance (Schiff's base) was observed emanating from the enzyme after a very short period of contact with the substrates. It was thought that this could be a factor in limiting molecular weight. In order to eliminate this problem, immobilisation of the enzyme was considered, as detailed later in **Sections 5.0 to 5.7.**

It is apparent from **Table 7** of **Section 3.3,** that there are significant differences in the Mn values of the polymers determined by GPC and by NMR. Mn values determined by NMR are generally greater than those determined by GPC. This would suggest that ester end groups are being removed by some competing process. Since reversibility was eliminated, hydrolysis by water would appear to be the most likely competing reaction, but further work was needed to elucidate this problem.

These results appeared promising and it was thought that further modification of the system might give higher molecular weight products.

3.5 The Effects of Added Molecular Sieve

Since the addition of molecular sieve had a significant effect on the reactions of both bis(2,2,2-trichloroethyl) and divinyl esters it was thought that modification of the sieves might also have a profound effect on the polytransesterification process. The effect of variations in the ratio of the mass of the sieves to enzyme mass (0:2.5, 3:2.5, 6:2.5, 9:2.5), their pore size (3Å and 4Å) and physical state (macerated and pelleted) were investigated and the results are discussed below;

3.5.1 Presence versus Absence of Molecular Sieves
From **Table 7** of **Section 3.3,** it can be seen that polymer from reactions with and without molecular sieves attained maximum molecular weight after about 3 days. With molecular sieve present the condensation reaction was promoted, a higher maximum molecular weight was achieved and the polymer product was whiter than that obtained in the reaction without molecular sieve. This latter observation

was probably due to the acetaldehyde being absorbed by the molecular sieves. Thus Schiff's base formation was prevented. It was also noticed that added molecular sieves partially coagulated the insoluble 1,4-butanediol. Shaking the flask dispersed the pellets. No polymer was obtained from the control reactions, (with and without molecular sieves) where the enzyme was absent.

Table 8. The relative proportions of hydroxyl- and ester-terminated functions in the products formed from the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol with (wms) and without molecular sieve (woms) in anhydrous diethyl ether.

	Hydroxyl:Ester	
Time(h)	wms	woms
8	-	18:1
24	3:2	3:1
48	4:1	16:1

It was observed previously for the (2,2,2-trichloroethyl) system, that the reaction in diethyl ether in the absence of molecular sieves produced a polymer with a substantial majority of hydroxyl end groups. The vinyl system showed a similar effect in that with molecular sieve present, the hydroxyl:ester ratio was reduced, relative to the molecular sieve free system. However, even with added molecular sieve the hydroxyl:ester ratio was significantly higher than was observed for the trichloroethyl system which would suggest that the vinyl ester groups are more rapidly hydrolysed than the 2,2,2-trichloroethyl esters. Therefore, the presence of molecular sieves does not seem to have such a profound effect on the vinyl reaction as it did on the 2,2,2-trichloroethyl system.

Chaudhary et al. [131] also studied the enzyme-catalysed polymerisation of divinyl adipate. They stated that the rapid disappearance of vinyl groups from the monomer in the system was due to hydrolysis by the water intimately bound to the enzyme. It was stated that the degree of hydrolysis is ultimately dependent upon the dryness of the enzyme. MacDonald et al. [132] also identified hydrolysis of the monomers and the presence of water in the enzyme system as a limiting factor in the

76

attainment of high polymer molecular weights in the ε-caprolactone ring-opening polymerisations. It seems that since this is the case then successful attainment of high polymer molecular weights is dependent upon vigorous drying of the apparatus, reagents and the enzyme.

From our observations the addition of molecular sieve reduces the occurrence of the hydrolysis reaction.

3.5.2 The Reproducibility of PPL-Catalysed Reactions with Molecular Sieve

It was found that duplicate reactions carried out simultaneously were in good agreement for the divinyl adipate system, but generally, the divinyl adipate reactions with molecular sieves proved to be fairly inconsistent over the time-scale of this project. This contrasted with the 2,2,2-trichloroethyl system where reproducible results were obtained even with different operators and different batches of enzyme.

PPL has a storage life of one year, according to the suppliers, if it is refrigerated and desiccated. Each fresh batch of PPL was opened, desiccated and refrigerated until required. The enzyme was then vacuum desiccated daily for at least 3 weeks prior to use. The performance of a sample of the fresh batch of enzyme was compared to the previous batch of enzyme in separate polytransesterification reactions in diethyl ether. The results of these reactions were then analysed to check that the polymer molecular weights obtained were consistent. For each of the individual reactions, the date of the batch from which the enzyme came was recorded. This has enabled us to characterise the performance of the enzyme, both seasonally and by batch.

77

Table 9. The polymer molecular weights from different batches of PPL enzyme in the polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether with molecular sieves at ambient temperature.

Reaction No.	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn	PPL Batch Dated	Date of reaction at ambient temp.
30*	72	13,000	8,000	1.6	21/6/95	14/8/95
61	72	5,900	4,100	1.4	29/11/95	16/1/96
52	72	3,300	2,400	1.4	20/2/96	20/2/96
48	72	6,400	4,500	1.4	14/6/96	28/6/96
47	72	6,200	4,700	1.3	20/2/96	28/6/96
33	72	5,400	4,200	1.3	1/10/96	11/2/97
62	72	4,000	3,000	1.3	1/10/96	7/4/97
32	72	4,200	3,300	1.3	19/3/97	11/4/97
95	72	3,200	2,500	1.3	1/10/96	11/4/97

*This reaction suffered loss of approximately 75% reaction solvent through evaporation.

Results in **Table 9** show some seasonal variation in the molecular weights obtained because the laboratory was not air conditioned and so the slightly hotter months gave rise to slightly higher molecular weight polymer. However, this variable was investigated and discussed in a later **Section 3.6**. When reactions were carried out in diethyl ether at ambient temperature, 27°C and at reflux, surprisingly it was found that there was little impact made by temperature on the overall molecular weights.

3.5.3 Comparison of Conventional and Viscosity GPC Results
As with the 2,2,2-trichloroethyl system in **Chapter 2**, viscosity GPC was also carried out on polymers formed in the vinyl adipate system.

Table 10. Polymer molecular weights as determined by conventional and viscosity GPC analysis.

Polymer	GPC Viscosity			GPC Conventional		
	Mn	Mw	Mw/Mn	Mn	Mw	Mw/Mn
"Diolpate" reference	4,800	8,800	1.8	4,500	8,000	1.8
67	7,400	15,400	2.1	10,400	24,000	2.3
104	7,300	12,900	1.8	10,400	19,600	1.9

The results indicate that the 'true' (GPC/Viscosity determined) molecular masses of the samples are somewhat lower than those calculated as polystyrene equivalent molecular weights, but both GPC/viscosity and conventional GPC show similar differences between the samples. Therefore the results suggest that it is possible to adequately assess the differences between the samples using conventional results.

3.5.4 The Effect of Varying the Mass of Pelleted Molecular Sieve

The mass of the molecular sieve used initially in the divinyl adipate reactions was chosen arbitrarily at 3g. Other workers[59-61,74,82,103] had used varying amounts of both finely macerated molecular and pelleted sieves in their reactions with reported success. It was decided to examine the effect of the mass of sieve on the divinyl adipate reaction. The results of these studies are shown in **Table 11.**

Table 11. The effects of varying the mass of pelleted molecular sieve on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether.

Reaction No.	Mass of Pelleted Molecular Sieves (g)	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn
34	9	72	3,300	2,700	1.2
35	6	72	5,300	4,100	1.3
36	3	72	4,300	3,400	1.2
37	0.5	72	4,000	3,200	1.3
38	9	96	5,300	3,200	1.6
39	6	96	3,500	2,800	1.3
40	3	96	3,800	3,000	1.3
41	1	96	3,600	2,900	1.2

The reactions carried out for the shorter period of 72 hours (reactions **34-37**) gave slightly better polymer molecular weights than the reactions carried out for 96 hours (reactions **38-41**). It would seem that the effectiveness of the molecular sieve as a drying agent is reduced after 72 hours.

The results indicate that there was a very slight increase in polymer molecular weight obtained by employing 6g of molecular sieve in the 72 hour reaction compared with using 3g of molecular sieve. However, use of 9g of molecular sieve in the 72 hour reaction resulted in a decrease in the polymer molecular weight obtained. This might be due to more of the 1,4-butanediol being adsorbed onto the larger surface area of the molecular sieves, thus reducing the concentration and availability of the diol in the vicinity of the enzyme. On the otherhand, the results indicate that employing a reduced mass of 0.5g of molecular sieves in the 72 hour reaction gave slightly poorer polymer molecular weight to that obtained using 3g of molecular sieves. It seems that the smaller mass is less effective than 3g of molecular sieve in preventing hydrolysis.

3.5.5 The Effect of Intimate Mixing between PPL and Molecular Sieve

In order to test whether the enzyme was being damaged by the molecular sieve, a reaction was carried out in a two chambered vessel as used by Roberts *et al.* [58] The two chambers were connected by an integral sintered disc which allowed for the flow of solvent but prevented mixing of the molecular sieves and the enzyme (42). Comparing this reaction with a single vessel reaction (35) showed that intimate mixing gave a higher molecular weight product in better yield. After 72 hours the results showed an expected trend of higher molecular weight with increasing quantities of sieve upto 6g. This can be accounted for by the reduction in hydrolysis by the larger quantities of sieve. The lower molecular weight for the 9g reaction could be due to enzyme damage as observed previously by Roberts. However, after 96 hours, the reactions gave products with very similar molecular weights. Consequently no overall conclusion is possible.

Table 12. The effect of intimate mixing between PPL and molecular sieves on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether.

Reaction No.	Mass of Molecular Sieves (g)	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn	Reaction Type
35	6	72	5,300	4,100	1.3	Single Flask
42	6	72	4,900	3,500	1.4	Dual Chamber

3.5.6 The Effect of Varying the Molecular Sieve Pore Size

The effect of changing the pore size of the molecular sieves from 3Å (43) to 4Å was studied (29). Similar results were obtained for both sieve systems.

Table 13. The effects of varying the molecular sieve pore size on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether.

Reaction No.	Mass of Molecular Sieve (g)	Molecular Sieve Porosity	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn
2 9	3	4Å	48	8,200	4,800	1.7
4 3	3	3Å	48	8,200	4,600	1.8

3.5.7 The Effect of Changing the Physical Form of Molecular Sieve

It was assumed that not only the mass of molecular sieves, but also the physical form (especially the surface area) might influence the polymerisation reactions. To test this hypothesis macerated and pelleted sieves were compared.

The effects of using equal masses of the macerated (44) and pelleted sieve (36), were compared. A 72 hour reaction with 3g of macerated molecular sieve produced no solid product. This may be due to the fact that the 1,4-butanediol was more readily adsorbed by the macerated sieve, agglomerated and effectively removed it from reaction. It was thought that the reaction time might be a factor and a shorter reaction time of 17 hours was used. However, this reaction likewise produced no polymer. It was then decided to use a smaller amount of macerated sieve, (1g (44)). The reaction produced a low yield of polymer with a lower molecular weight than the standard reaction using 3g of pelleted molecular sieve.

82

Table 14. A comparison of the effect of macerated molecular sieve and pelleted molecular sieve on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether.

Reaction No.	Mass of Molecular Sieve (g)	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn	Form of Molecular Sieves
36	3	72	4,300	3,400	1.3	Pellet
44	1	72	3,300	2,700	1.2	Macerated

3.5.8 The Effect of Varying the Type of Drying Agent

It was thought that the presence of an alternative drying agent might enhance the reaction. Reactions were carried out with sodium chloride, sodium metabisulphite and also magnesium sulphate in the presence of molecular sieves.

Table 15. The effect of employing different drying agents on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether.

Reaction No.	Mass and Type of Drying Agent	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn
45	10g NaCl	72	2,900	2,300	1.3
46	6.2g of Molecular Sieves and 10.8g of MgSO$_4$	72	7,600	5,400	1.4

Neither the sodium chloride or the addition of magnesium sulphate to the molecular sieve improved the molecular weight of the product. It was hoped that the metabisulphite might facilitate the reaction by trapping the acetaldehyde. However, no product at all was obtained from this reaction.

83

It appeared from these observations that changing the mass, pore size and physical state of the molecular sieve gave lower molecular weight polymers than the original reaction conditions (Mn of 8,000). Since the high molecular weight had been achieved during the heat wave of August 1995 and the molecular sieve modified reactions had been carried out in mid-winter it was thought that the change in temperature might be responsible for the decline in molecular weight.

This prompted us to look at the effect of temperature on the reaction.

3.6 The Effect of Varying Reaction Temperature

Since all previous reactions were carried out at ambient temperature it was necessary to determine the effects of a slight increase in temperature on the reaction to mimic seasonal temperature variation.

Table 16. The effects of varying reaction temperature on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether.

Reaction No.	Mass of Molecular Sieves (g)	Time (h)	Temperature	Mw (GPC)	Mn (GPC)	Mw/Mn
29	3	48	Ambient	8,200	4,800	1.7
30*	3	72	Ambient	13,000	8,000	1.6
47	3	72	Ambient	6,200	4,700	1.3
48	3	72	Ambient	6,400	4,500	1.4
49	3	72	27°C	6,800	4,500	1.5
50	3	48	Reflux	5,400	3,100	1.7

* This reaction suffered loss of approximately 75% reaction solvent through evaporation.

A reaction (49) was carried out at 27°C, and the result shows little difference compared to those at ambient temperature (48), (47) which were carried out concurrently.

84

An experiment carried out at reflux (50) for 48 hours gave lower molecular weight product than the analogous reactions carried out at ambient temperature (29). It was observed with the reflux reaction that the polymer caused agglomeration of the PPL. This may have terminated the reaction. A similar reaction was stopped after 8 hours but this gave no product.

3.7 The Effects of Using Denatured PPL

In order to ascertain whether the PPL-catalysed polytransesterification reaction was a result of "active site enzyme catalysis" or external surface interactions, an amount of denatured PPL (denatured by heating in an oven at 65°C for 14 days, followed by 14 days storage in a desiccator) was used in place of active PPL. The reaction using denatured PPL yielded no product, suggesting that there was no surface catalysis nor was there any other catalytically active species remaining in the crude PPL. This observation was contrary to Kobayashi et al.[133] who stated that there was polymer formed in a polytransesterification reaction using PPL which had been denatured in water at 100°C for several hours. There was insufficient time to repeat this or make further tests involving blocking inhibitors to confirm the experiments.

3.8 The Effect of Increasing the Substrate Concentration

A series of reactions were carried out with different substrate concentrations relative to PPL.

Table 17. The effects of varying substrate concentration on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether with molecular sieve.

Reaction No.	Divinyl Adipate (mol.dm^{-3})	1,4-Butanediol (mol.dm^{-3})	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn
51	0.4	0.4	72	5,300	4,200	1.2
52	0.2	0.2	72	3,300	2,400	1.4
53	1.0	1.0	72	No polymer recovered by usual		
54	0.4	0.4	144	method.		
55	0.4*	0.4*	72 + 72 woms	3,700	2,900	1.3
56	0.4*	0.4*	72 + 72	3,600	3,000	1.2

* The initial concentration of reactants was 0.2 mol.dm^{-3} which was then increased to 0.4 mol.dm^{-3} after 72 hours.

In reaction (51) where the concentration of reactants was initially double that in reaction (52), a good yield of polymer was produced after a period of 72 hours, with a higher than average molecular weight than that obtained from reaction (52).

It can be seen that a five-fold (53) increase in the initial substrate concentration actually inhibited the reaction and failed to produce polymer.

However, when reactions were carried out with the addition of double the concentration of reactants in two batches, (the second batch being added after an initial reaction period of 3 days) (55) and (56), the polymer which was produced after the 6 days had a molecular weight which was similar to that obtained in a 3 day reaction (52). This was possibly due to the fact that the polymer formed from the first batch of reactants was insoluble in the diethyl ether, causing the reaction to

terminate. The second batch of reactants would then be polymerised in the same way as the first.

3.8.1 PPL-Catalysed Polymer Modification In Diethyl Ether

It was thought that adding initial substrates to the terminal groups in pre-formed polymer might produce higher molecular weight products.

NMR analysis of the polymer products from the polytransesterification reactions indicated that in the presence of molecular sieve the reaction gave predominantly ester-terminated polymer, whereas in the absence of molecular sieve hydroxy-terminated polymer was produced. It was thought that the polymer molecular weight could be increased by reacting these polymer products with more reactants.

Polymer which was ester-terminated (57) was reacted with 1,4-butanediol (58). After reaction, the polymer was recovered and was found to have a higher molecular weight than the original ester-terminated polymer.

Similarly, polymer which was hydroxy-terminated (59) was reacted with divinyl adipate (60). The polymer recovered from this reaction had a similar molecular weight to the original hydroxy-terminated polymer.

Table 18. The effects of extending hydroxy- or ester- terminated polymer with the conjugate esterification reagent on the polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether.

Reaction No.	Divinyl Adipate (mmol)	1,4-Butanediol (mmol)	Mw (GPC)	Mn (GPC)	Mw/Mn	Original terminal end group
57	10	10	11,500	5,100	2.2	Ester
58	0	10	12,800	8,900	1.4	-
59	10	10	6,600	4,200	1.6	Hydroxyl
60	10	0	7,000	4,300	1.6	-

These results suggest that it may be possible to increase the molecular weight of some types of polymer by this addition process.

3.9 The Effects of Varying Degree of Agitation

It was observed that in some reactions using magnetic stirrers that the PPL enzyme agglomerated. To determine whether the agglomeration had an adverse effect, a reaction was carried out using a mechanical stirrer.

Table 19. The effect of the degree of stirring on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether with 3g of molecular sieve at ambient temperature.

Reaction No.	Time (h)	Mass of PPL (g)	Mw (GPC)	Mn (GPC)	Mw/Mn	Stirrer Type
61	72	2.5	5,900	4,100	1.4	Magnetic
62	72	2.5	4,000	3,200	1.3	Mechanical

The more efficient mechanical stirring gave a lower molecular weight product which could be accounted for by damage to the enzyme.

3.10 Conclusions

One important conclusion that can be drawn from our observations of the vinyl system in diethyl ether, is that the reproducibility of this system was subject to a wider variation in terms of molecular weight, compared with the 2,2,2-trichloroethyl system, where the reactions were more reproducible over a wide range of times, and from operator to operator.

Even though the vinyl system in diethyl ether suffered from lack of reproducibility it still on occasion produced higher molecular weight polymer (Mn ~8,000 and Mw ~13,000) than that obtained using the 2,2,2-trichloroethyl system. Interestingly, as with the 2,2,2-trichloroethyl system, the polymers were also insoluble in this solvent.

Solvent dryness was again an important factor. Much improved molecular weights were observed when molecular sieves were added to the reactions, but even so, the polymers were predominantly hydroxy-terminated, indicating that hydrolysis was still occurring.

On the basis of our observations it would appear that the two main factors still limiting the molecular weight are;

a) the competing hydrolysis due to release of water intimately associated with the enzyme and

b) the insolubility of the polymer in the ether solvent, since once the polymer is precipitated out of solution, the condensation reaction is terminated.

It would appear that a change in reaction solvent to one which would dissolve the polymer as it forms on the enzyme, might lead to an improved molecular weight. Equally, use of a higher boiling ether solvent at an increased reaction temperature might increase the polymer solubility, leading to polymers of higher molecular weight.

CHAPTER 4

THE EFFECT OF CHANGING THE ORGANIC SOLVENT ON THE LIPASE-CATALYSED POLYTRANSESTERIFICATION OF DIVINYL ADIPATE WITH 1,4-BUTANEDIOL

4.0 THE EFFECT OF CHANGING THE ORGANIC SOLVENT ON THE PPL-CATALYSED POLYTRANSESTERIFICATION OF DIVINYL ADIPATE WITH 1,4-BUTANEDIOL

4.1 Background

All the reactions so far had been carried out in diethyl ether. It was decided to investigate the use of other solvents especially higher boiling ethers since it was thought that a higher temperature might improve the solubility of the polymer.

4.2 The Effect of Using Alternative Ether Solvents

A number of alternative ether solvents were investigated including diisopropyl ether, *tert*-butyl methyl ether, ethylene glycol diethyl ether and 1-*tert*-butoxy-2-methoxyethane. Each solvent was tested with standard substrates at ambient temperature for 3-4 days initially to check for enzyme compatibility.

The results of these reactions for diisopropyl ether (63) and *tert*-butyl methyl ether (64, 65) are shown in **Table 20**.

Ethylene glycol diethyl ether and 1-*tert*-butoxy-2-methoxyethane failed to produce solid at ambient temperature and were therefore not investigated further.

Table 20. The effect of various ether solvents on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol at ambient temperature.

Reaction No.	Ether Solvent	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn
63	diisopropyl ether	72	2,800	2,400	1.2
64	*tert*-butyl methyl ether	72	4,300	3,000	1.4
65	*tert*-butyl methyl ether	96	3,400	2,500	1.3

The molecular weight of the products obtained from either of these solvents was no better than that from reactions employing diethyl ether.

4.3 The Effects of Using High Boiling Ethers at an Elevated Temperature

It was observed that with diethyl ether as a solvent the polymer precipitated. It was thought that this might cause a termination of the condensation reaction. Since it was likely that the solubility of the polymer would increase with an increase in reaction temperature, it seemed possible that increasing the temperature might give higher molecular weight products. Some attempts had been made to carry out reactions with diethyl ether at higher temperature (27°C and reflux). They had not given improved products. However, with diisopropyl ether (bp 68-69°C) and *tert*-butyl methyl ether (bp 55-56°C) a wider range of temperatures was possible.

4.3.1 Diisopropyl Ether (bp 68-69°C)

Kobayashi had reported that the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol at 45°C in diisopropyl ether gave no product.[130] We observed that a diisopropyl ether/PPL system with added molecular sieve gave polymeric products after 72 hours at 43°C (see **Table 21**).

Table 21. The effect of changing the reaction temperature on the 72 hour PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diisopropyl ether with molecular sieves.

Reaction No.	Reaction Temp. (°C)	Polymer Yield (g)	Mw (GPC)	Mn (GPC)	Mw/Mn
63	Ambient	0.16	2,800	2,400	1.2
66	43	0.16	10,000	6,400	1.6

It was expected that an increase in the reaction temperature might produce higher molecular weight products. However, the molecular weights were comparable to the higher range obtained for the diethyl ether system. No polymeric products were obtained from a control reaction at this elevated temperature, in the absence of enzyme.

4.3.2 *tert*-Butyl Methyl Ether (bp 55-56°C)

Table 22. The effect of changing the reaction temperature on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous *tert* -butyl methyl ether with molecular sieves.

Reaction No.	Reaction Temp. °C	Time (h)	Mw (GPC)	Mn (GPC)	Mw/Mn	Comment
64	Ambient	72	4,300	3,000	1.4	-
65	Ambient	96	3,400	2,500	1.3	-
67	43	72	23,000	8,600	2.8	Argon purge
68	43	72	8,500	3,700	2.3	No purge

One of the reactions (67) using an argon purge at 43°C produced polymer with a higher molecular weight than that which had been obtained in diisopropyl ether. The probable reason for this increase in molecular weight is a result of acetaldehyde being efficiently removed from the system.

93

4.3.3 The Effect of Varying Substrate Concentration on the PPL-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol in Anhydrous *tert*-Butyl Methyl Ether at 43°C

Table 23. The effect of changing the molarity of the substrates on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous *tert*-butyl methyl ether at 43°C.

Reaction No.	Time (h)	Mass of PPL (g)	Mw (GPC)	Mn (GPC)	Mw/Mn	Substrate Conc. (mol.dm^{-3})
69	24	2.5	13,000	7,300	1.8	0.2
70	24	2.5	44,000	9,200	4.7	0.1
71	72	2.5	22,500	5,900	3.8	0.06

A decrease in concentration led to the isolation of the highest molecular weight products so far observed, although this was accompanied by an increase in polydispersity.

4.4 The Effect of Using Hydrocarbon Solvents

It has been reported that high molecular weight cyclic products can be obtained using hexane as a reaction solvent for the lipase-catalysed esterification of hydroxy acids.[59,61,106] As a result some reactions were carried out in hydrocarbon solvents (n-hexane and n-heptane) but these resulted in agglomeration of the enzyme and gave quite low yields of low molecular weight products. A similar agglomeration of enzyme in hexane has been observed.[106,134] These solvents were not investigated further.

4.5 The Effect of Using Polymer Solubilising Solvents

4.5.1 THF

THF seemed to be the ideal solvent for the system since it dissolved the polymer and the 1,4-butanediol and had also been used successfully in this solvent by other workers.[42]

Table 24. The PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in HPLC grade THF with and without molecular sieves.

Reaction no.	Molecular Sieve	Time (h)	Mn (GPC)	Mn (NMR)	Mw (GPC)	Mw/Mn
72	absent	2	1,800	2,500	3,500	1.9
73	"	4	2,000	3,100	2,500	1.5
74	"	24	2,100	2,700	2,500	1.2
75	"	72	2,500	4,200	3,000	1.2
76	"	320	2,100	3,700	2,700	1.3
77	present	4	2,000	2,400	3,400	1.7
78	"	24	2,600	5,000	3,500	1.4
79	"	48	2,500	3,100	3,000	1.2
80	"	320	2,800	9,500	3,500	1.3

The above results for Mn (GPC) with and without molecular sieves can be represented graphically as shown below:-
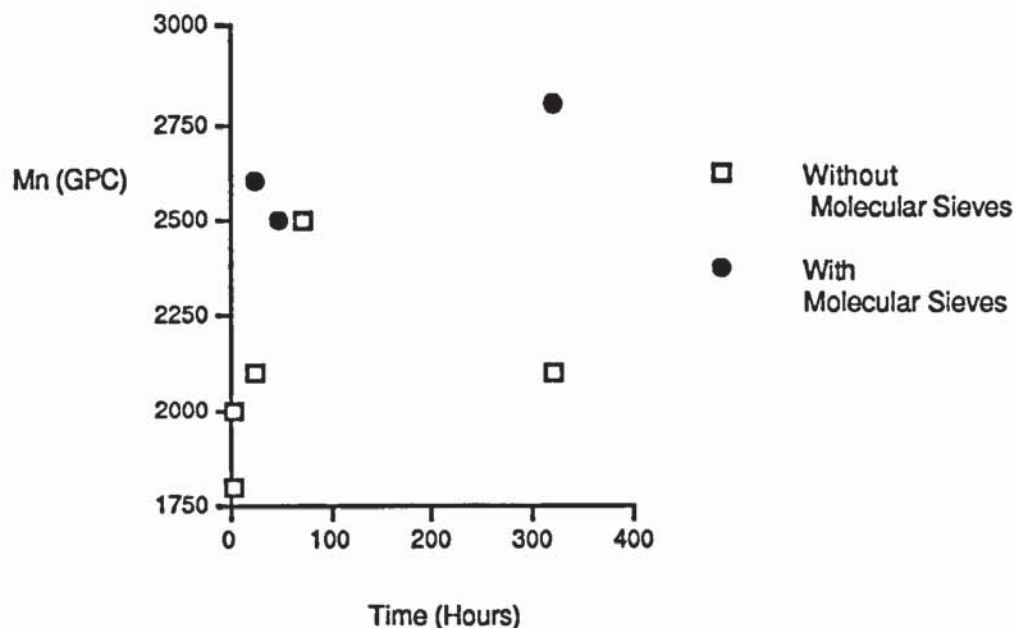
Figure 6. The variation of Mn (GPC) with time in the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in HPLC Grade THF with and without molecular sieves

The initial rate of polymer formation was rapid in THF, but the Mn value remained constant over extended periods. However, this maximum molecular weight was lower than that obtained using anhydrous diethyl ether and further study of this solvent showed that there was no increase in the Mn beyond 3,000 even after extended reaction periods with molecular sieve. Qualitative lipase activity tests of the enzyme after reaction in THF surprisingly showed that the enyzme still maintained 40% of the initial enzyme activity even after 317 hours of reaction time. (see **Table 25**)

Table 25. PPL activity after its use as catalyst in polytransesterification reactions.

Enzyme Sample (15mg/ml)	0.05M NaOH Titres (cm^3) (Bromo Thymol Blue Indicator)	Lipase Activity (units/mg protein)
THF/vinyl system (Reaction time : 317 hours)	0.5	80
Crude PPL	1.3	200
Water control	-	-

As was observed when diethyl ether was used as a solvent, the hydroxyl:ester ratio was lower for reactions with molecular sieve than for those without molecular sieve (see **Table 26**).

The initial reaction rate in THF was far higher than that of the other solvents studied. This was probably due to the increased solubility of 1,4-butanediol. The decrease in enzyme activity was probably caused by the hydrophilic THF removing the water intimately associated with the enzyme.

Table 26. The relative proportions of hydroxyl- and ester-terminated functions in the products formed from the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol with (wms) and without molecular sieve (woms) in THF.

	THF	
	Hydroxyl:Ester	
Time(h)	wms	woms
4	2:1	-
24	1:1	12:1
72	1:1	17:1
320	1:1	no ester

4.5.2 Dimethyl Isosorbide (DMI)

DMI is a high boiling ether which readily dissolved the polymer and the diol. DMI is an inert, completely water miscible and a consumer safe solvent used extensively to prepare commercial pharmaceuticals and body creams.

The DMI was used in two reactions. One with untreated solvent and the other after it had been dried over $MgSO_4$. It 'wetted' the enzyme well just like THF but produced poor products which were inherently difficult to recover due to the solvent's high boiling point.

4.5.3 Dichloromethane

Reactions in dichloromethane were unsuccessful.

4.5.4 THF and Diethyl Ether Mixtures

Finally consideration was given to mixtures of diethyl ether and THF in varying proportions. Linko et al. [134] tried to maintain polymer solubility using a mixed solvent system. They studied the polytransesterification of bis(2-chloroethyl) succinate with 1,4-butanediol in a mixed organic solvent system with 70% diisopropylether and 30% chloroform. The polymer had a maximum Mw 1,500 with a degree of polymerisation of 8 units obtained after 72 hours.

Table 27. The effect of employing THF/diethyl ether mixtures on the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol at ambient temperature with molecular sieves.

Reaction No.	Time (h)	Mass of molecular sieves(g)	THF:diethyl ether	Mw (GPC)	Mn (GPC)	Mw/Mn
8 1	72	3	50:50	3,200	2,600	1.2
8 2	72	3	10:90	4,800	3,600	1.3

Mixed diethyl ether/THF solvents gave products with Mn comparable to those in THF.

4.5.5 Acetonitrile

Acetonitrile was chosen as a potential solvent towards the end of this project. It gave polymer with a molecular weight similar to the diethyl ether system (Mn 2,600 and Mw 3,600) (83). However, there was insufficient time left to investigate this solvent further.

4.6 Conclusions

It was found that the PPL-catalysed polytransesterification of 1,4-butanediol with vinyl adipate in diisopropyl ether gave polymers with Mn values in the range of 2,400 to 6,400 daltons. This was contrary to the observations of Kobayashi et al. [130] who reported that PPL was not active in this system.

It was apparent from these solvent studies that a relatively successful solvent was one in which the 1,4-butanediol was insoluble. It is known that in aqueous media PPL operates only at an oil/water interface, but whether the reaction was interfacial in organic solvents was unknown. However, we found that solvents such as THF, DMI, and diglyme which dissolved both the polymer and the 1,4-butanediol did not give product that could be isolated.

Work to improve the polymer molecular weight achieved in diethyl ether was centred around increasing the reaction temperature of the enol system by replacing the reaction solvent with higher boiling point ethers. It was found that use of higher boiling point ether solvents such as *tert*-butyl methyl ether at an elevated reaction temperature resulted in a slight increase in molecular weight but also a large increase in polydispersity from 1.2 to 4.4. We were unable to produce polymers with Mn values greater than the maximum already achieved using diethyl ether at ambient temperature; however, *tert*-butyl methyl ether gave consistently high molecular weight material. This peak in attainable molecular weight might be attributed to the insolubility of the polymer in these ether solvents, but even when a mixed, polymer soluble/polymer insoluble ether solvent system such as THF and diethyl ether was employed, no improvement in Mn was observed.

CHAPTER 5
ENZYME MODIFICATION

5.0 ENZYME MODIFICATION

5.1 Background

Enzymes in their native crude form are susceptible to denaturisation due to external factors such as temperature, pH, aldehydes and some organic solvents. It has been shown that modification of the enzyme in a variety of ways aids performance in reactions in both aqueous and organic solvents.[49,69,77,79,80]

In the case of enol esters, enzyme immobilisation increases enzyme activity and prevents deactivation by agents such as acetaldehyde.[97] One type of immobilised enzyme preparation is that of Lipozyme IM (from Novo Nordisk) which supports a lipase from a *Mucor miehei* strain on the surface of a macroporous anion exchange resin of a phenolic type. Lipozyme IM is strongly bonded to the carrier by adsorption. In commercial reactions it is used for esterifications and interesterifications involving the 1 and 3 ester bonds in triglycerides. Lipozyme IM can be used in inert organic solvents such as petroleum-ether and n-hexane.[135]

5.2 The Effect of Enzyme Immobilisation on the Lipozyme IM-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol In Anhydrous Diethyl Ether

Lipozyme IM was assessed for its effects on polymer molecular weight at ambient temperature and at elevated temperatures. It was used as supplied and was desiccated at all times and used in the same way as PPL. Since the preparation was more active than PPL, an arbitrary smaller mass was used in the reactions (0.5g cf. 2.5g PPL).

Reactions were carried out for 4, 24, 48, 96 and 120 hours and the results are shown in **Table 28**.

Table 28. Progress of the Lipozyme IM-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether at ambient temperature without molecular sieves.

Reaction No.	Time (h)	Mass of Lipozyme (g)	Mw (GPC)	Mn (GPC)	Mw/Mn
84	4	0.5	3,900	1,500	2.6
85	24	0.5	4,400	2,200	2.0
86	48	0.5	3,100	1,800	1.7
87	96	0.5	3,400	1,900	1.8
88	120	0.5	4,600	2,500	1.8

During the reactions, white polymer could be seen forming in suspension, becoming more granular with time.

As can be seen from the data presented above using this immobilised system, the Mn of the polymer was lower than that obtained with PPL.

5.2.1 The Effect of Increasing Substrate Concentration

Table 29. The effect of substrate concentration on the Lipozyme IM-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether at ambient temperature without molecular sieves.

Reaction No.	Substrate Conc. (mol.dm^{-3})	Time (h)	Mass of Lipozyme (g)	Mw (GPC)	Mn (GPC)	Mw/Mn
87	0.2	96	0.5	3,400	1,900	1.8
88	0.2	120	0.5	4,600	2,500	1.8
89	0.4	72	0.5	5,400	3,700	1.5

It seems that doubling the concentration of the substrates increased the polymer molecular weight (Mn). However, it did not exceed that already obtained using the PPL system. This suggests that it is the precipitation of the polymer in diethyl ether which is limiting the molecular weight. Hence further consideration was given to solvents which dissolved the

polymer as it formed. THF was then investigated as an alternative solvent since it had afforded some polymer with PPL.

5.3 The Effect of Using Polymer Solubilising Solvent

A reaction using Lipozyme IM was carried out for 6 hours in THF (which dissolved both the polymer and the 1,4-butanediol). Surprisingly no product was obtained from this reaction. This was probably due the hydrophilic THF removing the water intimately associated with the enzyme resulting in conformational changes. Additionally, the acetaldehyde released by the polytransesterification reaction would react with the lysine groups of the enzyme. Both of these factors would cause the enzyme to lose catalytic activity.

In order to reduce the effects of the THF a small amount of the THF was mixed with diethyl ether in the ratio of (10:90 (THF:diethyl ether)) which was subsequently employed as the reaction solvent. The polymer produced after a period of 6 hours (90) was of a poorer molecular weight (Mn 1,300 and Mw 3,900) than the polymer obtained from the polymerisation reaction using PPL as the enzyme catalyst (Mn 3,600) in the same solvent mix (10:90 (THF:diethyl ether)).

In view of the fact that the reaction may need an insoluble substrate such as the diol, hydrocarbon solvents were investigated.

5.4 The Effect of Using Hydrocarbon Solvents

It had been previously observed by O'Hagan[59,61] and co-workers that use of hexane as a solvent for enzyme-catalysed polymerisation was very successful in producing polymers with high Mn values. As a result, hexane was investigated along with toluene.

With both toluene and hexane the Lipozyme particles adhered together and gave low molecular weight polymers from toluene (Mn 2,200 and Mw 2,600) and hexane (Mn 1,900 and Mw 2,600). These results were poorer than had been obtained with diethyl ether.

Since high boiling ethers had resulted in some success with the PPL system their effects were also considered for the Lipozyme system.

5.5 The Effect of Employing High Boiling Ethers

Ethylene glycol diethyl ether (bp 121°C) (1,2-diethoxyethane) (dried over $MgSO_4$)) was used in place of diethyl ether at ambient temperature and diisopropyl ether (distilled over Na wire) was used in place of diethyl ether at an elevated reaction temperature of 43°C.

Table 30. The effect of changing the reaction solvent and reaction temperature on the Lipozyme IM-catalysed polytransesterification of divinyl adipate with 1,4-butanediol.

Reaction No.	Time (h)	Reaction Temp.	Solvent	Mass of Lipozyme (g)	Mw (GPC)	Mn (GPC)	Mw/Mn
88	120	Ambient	Diethyl ether	0.5	4,600	2,500	1.8
91	120	Ambient	Ethylene glycol diethyl ether	0.5	7,000	4,300	1.6
92	48	43°C	Diisopropyl ether	0.5	4,000	2,800	1.4

Ethylene glycol diethyl ether at ambient temperature (91) gave the highest molecular weight product, but the yield was relatively poor (22%) compared with diethyl ether (82%).

Diglyme (an ether solvent which dissolved the polymer) was also tested as a reaction solvent, but it failed to produce product.

5.6 The Effect of Immobilising PPL on Silica on the Lipase-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol

PPL was immobilised on to the silica gel using a simple slurry technique in which the minimum amount of phosphate buffer (pH 7.2) was added to a dry mix of crude PPL (2.5g) and silica gel (12.5g), followed by drying on a watch glass at ambient temperature in a vacuum desiccator. This technique had been successfully employed in another system, in our laboratory.[72]

Use of this dried preparation (1.5g≅0.25g of PPL) in a polytransesterification reaction yielded no product. When silica gel was simply added to a standard polytransesterification reaction,(93) the reaction produced polymer with a molecular weight (Mn 3,700 and Mw 4,700) similar to that achieved previously for the PPL-catalysed reaction in diethyl ether.

5.7 The Effect of Water Content on the PPL-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol

Considering both the substantial effect of molecular sieves on the reaction and the volume of literature[23,32,33,35-37,39-41,43,49-52,55,56,59-61,63,68,76,84,99,130] devoted to the effect of water content on enzyme catalysed reactions in organic solvents, it was apparent that water content might be a major factor in the transesterification process.

In order to determine the minute quantity of water present in this system, each reaction component was analysed by Karl Fischer Titration 'for liquids'. The enzyme was analysed by an indirect method because it was a solid. The method involved absorbing water from the enzyme with anhydrous ethanol (dried over 3Å molecular sieves).

Table 31. Substrate water content determined by Karl Fischer Titration.

Sample	Water Content
Ethanol	0.60 %w/w
1,4-Butanediol	0.28 %w/w
Diethyl ether (Sodium wire distilled)	106.4 ppm
Divinyl Adipate	342.4 ppm
Vacuum Desiccated Enzyme (PPL)	0.07 %w/w

The results above indicated that, in the absence of molecular sieve, a standard anhydrous reaction involving diethyl ether, vacuum desiccated PPL enzyme, 1,4-butanediol and divinyl adipate, had a total water content of 0.02%w/w.

It was thought that the presence of this small amount of water could be causing the polymer molecular weight to plateau due to hydrolysis of the polymer. Our investigations initially involved vacuum lyophilised PPL (as supplied) and freeze-dried PPL (freeze-dried slurry of vacuum desiccated PPL in buffer). Both enzymes were tested for lipase activity to detect if there was any detrimental effects from freeze-drying. The activities were found to be almost identical (see **Table 32**).

Table 32. Enzyme activities of PPL before and after freeze-drying.

Reaction No.	Enzyme Drying Method	Average 0.05M NaOH Titre (cm^3) (BromoThymol Blue Indicator)	Lipase Activity (units/mg protein)
94	Freeze Dried PPL + buffer	2.4	380
95	Vacuum Desiccated PPL	2.3	370

Each enzyme batch was then employed in a polytransesterification reaction. On this occasion 1,3-propanediol rather than 1,4-butanediol was used. The polymer products obtained were compared (see **Table 33**).

Table 33. A comparison of the polymer molecular weights attainable under different conditions of enzyme dryness.

Reaction No.	Mass of 1,3-propanediol (g)	Mass of PPL (g)	Mw (GPC)	Mn (GPC)	Mw/Mn	Enzyme Drying Method
94	0.76	3.67	5,200	3,500	1.5	Freeze Dried
95	0.76	2.5	4,200	3,300	1.3	Vacuum Desiccated

The results indicated that there was very little difference between the two levels of dryness and showed that there was little advantage in using the drier freeze-dried PPL.

It was thought that exposure of the enzyme to the laboratory atmosphere for longer than usual during the assembly of the apparatus and the charging with reactants might be the factor responsible for the lack of consistency in the results from different experiments. In order to gain a feel for the amount of moisture absorbed a test was carried out

which involved desiccating a sample of PPL under vacuum over phosphorous pentoxide, weighing it and then exposing it to the laboratory atmosphere for a period of 3 hours. The enzyme sample was then re-weighed and the gain in weight calculated as being 0.8%w/w. Compared to the moisture content of the desiccated enzyme this figure was quite large and it appeared possible that this absorption of additional moisture by the enzyme might be responsible for some hydrolysis of the polymer.

5.8 PPL-Catalysed Polymer Degradation in Aqueous Media

Poly(1,4-butylene adipate) is well known for its biodegradability in an aqueous environment.[118,136] If a polymer can be formed using PPL then it should be possible for it to be degraded by PPL in an aqueous phase. A test was carried out to determine the extent of hydrolysis. Some pre-formed polymer from experiment (69) was placed in a sealed container along with PPL in aqueous buffer at ambient temperature for a period of 2 months. At the end of this time the polymer (96) was filtered from the enzyme and washed with water. The GPC of the polymer was then compared against a re-run GPC of the original polymer.

Table 34. PPL-catalysed polymer degradation in aqueous media.

Reaction No.	Mass and Polymer Source	Mw (GPC)	Mn (GPC)	Mw/Mn
69	-	12,500	6,500	1.9
96	0.2g polymer from 69	12,200	5,300	2.2

The treated polymer (96) was found to have a molecular weight lower than the original and a higher polydispersity, which was interesting and indicated that some degradation had occurred. In hind-sight it was thought that the polymer needed to be dissolved in a solvent for

complete degradation since the rate of hydrolysis in the aqueous media was slower than expected.

5.9 Enzyme Purification

The crude PPL preparation contains other enzymes, any of which could be catalysing the reaction. Purification of the crude preparation would perhaps lead to a more active preparation and the identity of the active species.

In the early stages part of the work was carried out in collaboration with a co-worker[137] who suggested that PPL might be separated into water soluble and insoluble fractions.

5.9.1 Separation of PPL into Soluble and Insoluble Fractions

When the crude PPL preparation was separated into water soluble and insoluble fractions it was found that about 10% of the total mass was insoluble material. The presence of lipase activity in the soluble fraction was confirmed by a lipase activity test (see **Table 35**).

Table 35. Lipase activity assay results for crude PPL, freeze-dried soluble and insoluble fractions.

Enzyme Sample (15mg/ml)	0.05M NaOH Titres (cm^3) (BromoThymol Blue Indicator)	Specific Lipase Activity (units/mg protein)
Crude Lipase	1.4	220
Soluble fraction	1.0	160
Insoluble fraction	0.55	90
Water control	-	-

A protease activity test was carried out which confirmed the presence of proteases in both fractions (see **Table 36**).

110

Table 36. Protease activity assay results for crude PPL, freeze-dried soluble and insoluble fractions.

Sample	Protease activity (arb units litre^{-1} hour^{-1})
Crude PPL	4,100
Insoluble Fraction	10,100
Soluble Fraction	5,200

It was found that there was twice the amount of protease activity in the insoluble fraction compared to the soluble fraction. This was surprising since it was thought that all the enzymes present would be washed into the soluble fraction.

Discussions with a cell biologist[138] revealed that proteases permanently adhere to insoluble cell material which acts as a support and increases catalytic activity. Also, according to our source[138] proteases can also be excreted into the supernatant liquid (soluble fraction). This observation presented us with a problem in that we would find it difficult to separate the proteases and lipases. Also, proteases operate within three different pH ranges making it difficult to artificially inhibit them.

It was also suggested that the lipases might adhere onto the inside of the glass reaction flask. This was tested by taking a flask used in a PPL catalysed polytransesterification reaction and rinsing it out with distilled water. A lipase activity test was then carried out on the rinsed flask and we found no lipase activity.

5.9.2 Soluble and Insoluble Fractions in Organic Polytransesterification Reactions.

Comparative organic polytransesterification reactions on the freeze-dried insoluble and soluble fractions showed some rather interesting results.

Table 37. The polymer molecular weights of the products from the polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether using the freeze-dried insoluble fraction of crude PPL.

Reaction No.	Time (h)	Mass of freeze dried insoluble fraction (g)	Mw (GPC)	Mn (GPC)	Mw/Mn
9 7	48	0.25	4,600	3,500	1.3
9 8	48	0.25	3,300	2,200	1.5

Use of the soluble fraction gave no polymer whilst surprisingly the insoluble fraction gave solid polymer with a molecular weight comparable to the product obtained from a reaction using 2.5g of crude PPL. This would indicate that it was the insoluble fraction in the PPL which was catalysing the reaction and not the total crude PPL preparation. This would reduce the amount of enzyme preparation required in a reaction by 90%.

Since the soluble fraction gave no product it was thought that it must require a surface on which to adsorb to become catalytically active. Addition of oven dried silica to the reaction had no effect. Work on the soluble material was not pursued any further and attention was given to the insoluble material.

The make-up of the insoluble material was unknown and so pure samples of protease enzymes which were known to be present in the crude PPL were used in polytransesterification reactions in an attempt to help elucidate the active species.

5.9.3 The Effect of Using Proteolytic Enzymes

Protease enzymes such as trypsin and pancreatin were tested in place of crude PPL in standard reactions with molecular sieve. Both enzymes were dried and stored in exactly the same way as PPL.

Addition of trypsin to the usual divinyl system in anhydrous diethyl ether resulted in no polymer product. However, it was later thought that a solvent which dissolved the diol and diester should have been used

since, unlike PPL, trypsin operates with soluble phases. A number of reactions were carried out with trypsin, varying the mass between 2.5g and 0.25g, but all yielded no product. Even addition of dried silica failed to enhance the reaction.

Pancreatin gave no polymer product in the usual divinyl system in anhydrous diethyl ether.

There was insufficient time and money to fully elucidate the active species present in crude PPL using this test technique since a large number of different enzymes would have had to be used.

It was observed by another worker[139] in our laboratory that PPL contained a high percentage of sucrose. Investigations were carried out to determine whether sucrose played any supporting role in the reactions.

5.9.4 Determination of Sucrose in Crude PPL

A test sample of 0.05g of the soluble enzyme fraction in 10ml of water (0.5%w/w in solution) was analysed against a 1%w/w standard sucrose solution by HPLC.

Table 38. HPLC data obtained for test and standard solution sucrose samples.

Sample	Retention time (minutes)	Peak Area Units
1%w/w Sucrose standard	11.17	14397367
0.5%w/w Test Sample	11.17	4527820

By calculation, the test sample peak is ;

(4527820 ÷ 14397367) x 100%= <u>31.4% of 1%w/w sucrose peak</u>.

113

therefore, in the test sample there was a concentration of ~0.3g of sucrose per 100ml and since the test sample was 0.5g in 100ml, then there was 60% sucrose in the soluble fraction. Since the amount of insoluble material in the crude PPL is negligible then the overall sucrose content is approximately 60%. This is a substantial proportion of the mass of the enzyme which could be removed. However even though it is known that buffer salts are involved in the catalytic activity it is not known if sucrose has any role in the catalytic activity of the enzyme. Two methods were chosen to remove the sucrose. One involving anhydrous pyridine and the other involving Visking-tube dialysis in phosphate buffer.

5.9.5 Removal of Sucrose from Crude PPL by Extraction with Anhydrous Pyridine

A sample of crude PPL under an argon blanket was extracted with anhydrous pyridine to remove the sucrose. The resultant insoluble fraction was then allowed to dry in a vacuum desiccator prior to being tested for lipase activity and compared to untreated PPL.

Table 39. Enzyme activity after extraction and vacuum desiccation.

Reaction No.	Enzyme Type	Lipase Activity (units/mg protein)	Average 0.05M NaOH Titre (cm^3) (BromoThymol Blue Indicator)
94	Freeze-dried PPL + buffer	390	2.4
95	Vacuum Desiccated PPL	370	2.3
99	Pyridine washed	700	4.4
100	Pyridine washed	500	3.1

Even though the pyridine washed preparations showed increased activity compared to freeze-dried and desiccated PPL they surprisingly produced very low yields of product when used as catalyst in polytransesterification reactions. The sucrose-free system required further study, but there was insufficient time available to pursue this.

114

5.9.6 Removal of Sucrose from Crude PPL by Dialysis in Phosphate Buffer

Dialysis of the sucrose from a buffered solution of crude PPL into external phosphate buffer was run in parallel with the purification method using pyridine. HPLC analysis of the external buffer solutions showed that all the sucrose had been removed after the first submersion of the Visking tube in the buffer. The HPLC trace showed that the external phosphate buffer gave a peak at a retention time of 11.49 minutes and sucrose at 11.00 minutes.

The dialysed enzymes were then freeze-dried and tested for lipase activity.

Table 40. Enzyme activity after freeze-drying the dialysed enzyme.

Reaction No.	Enzyme Type	Lipase Activity (units/mg protein)	Average 0.05M NaOH Titre (cm^3) (BromoThymol Blue Indicator)
94	Freeze Dried PPL + buffer	390	2.4
95	Vacuum Desiccated PPL	370	2.3
101	Buffer Dialysed (3 hours)	480	3.0
102	Buffer Dialysed (20 hours)	720	4.5
103	Buffer Dialysed (9 days)	240	1.5

Even though the treated preparations (102) and (101) showed increased lipase activity compared to vacuum desiccated and freeze-dried PPL, they failed to produce any product when employed as the catalyst in polytransesterification reactions. This was disappointing since polymer had been obtained previously using the insoluble material from crude PPL in reactions (97 and 98). Presumably, this catalytically active insoluble fraction would still have been a component of the residual material which remained after dialysis of the crude PPL.

5.10 Conclusions

Modification of the enzyme showed some interesting results.

We found that similar polymer products were obtained from polytransesterification reactions when either desiccated or freeze-dried PPL enzyme was used. This is beneficial since if such an enzyme system is to become employed commercially it would need to be able to cope with less than the totally anhydrous reaction conditions.

Immobilisation of enzyme on a suitable support is an effective method for protecting the enzyme from denaturisation. However, we found that immobilisation of the enzyme proved to be detrimental to the attainment of high molecular weight material. Perhaps a better choice of support was needed, to mimic the conditions experienced by native crude PPL enzyme powder.

Purification of the crude enzyme by removing the sucrose was not found to be detrimental to the aqueous enzyme activity. However, use of this purified material in an organic polytransesterification reaction showed that further work is necessary for improved performance.

Separation of the enzyme into soluble and insoluble fractions indicated that it is not simply the lipases which give rise to reaction, but proteases also. This was not surprising since it was well known that the inexpensive crude PPL contains a complex mixture of enzymes which include α-amylase and protease activity. It is conceivable that one of the minor components of PPL is responsible for the catalytic activity. However it seemed that none of these was trypsin (with an activity of 9,000 units per mg of protein) or chymotrypsin which has a low activity (40-60 units per mg of protein); both were individually tested and failed to produce polymer product. However, looking at **Table 36** it can be seen that the protease activity of the insoluble fraction (10,100 arb. units litre^{-1} hour^{-1}) is similar to the usual activity for trypsin. It was interesting that polymer product was obtained using the insoluble material. This would result in an overall reduction in the mass of enzyme required for reaction, by approximately 90%.

116

CHAPTER 6
CONCLUSIONS

6.0 CONCLUSIONS

Contrary to previous observations,[130] it was found that polymers were produced in the PPL-catalysed polytransesterification of divinyl adipate and 1,4-butanediol under a wide variety of conditions.

Polymers with Mn values ~9,000 were achieved on occasion but regardless of the fact that a wide variety of conditions were explored, it proved impossible to obtain higher molecular weights (Mn).

It had been suggested previously that the principal factors limiting the molecular weight were reversibility and hydrolysis due to the release of water from the enzyme promoted by the alcohol formed in the polytransesterification. Our observations suggest that reversibility might play some role in that higher molecular weights (Mn) were obtained from the non-reversible divinyl system than from the bis(2,2,2-trichloroethyl) system.

It was also found that higher molecular weight polymers were obtained in the presence of molecular sieve. The molecular sieve might promote the reaction in a number of ways. It could limit the back hydrolysis by absorption of ancillary water and also adsorb alcohol. It was found that molecular sieve promoted both the bis(2,2,2-trichloroethyl) and the divinyl reaction. In the divinyl case part of its effect was probably due to the adsorption of acetaldehyde.

It was observed that the divinyl system showed some inconsistency even though duplicate reactions in diethyl ether run in tandem gave very similar molecular weights. Relatively high molecular weights (Mn) were achieved early on in the investigation but these could not be reproduced subsequently. Tabulation of the results showed a seasonal variation and the highest molecular weights were achieved in the summer months. This suggested a temperature effect; however, it was found that raising the temperature of the diethyl ether system did not give higher molecular weights. At the time this seemed somewhat surprising considering the seasonal variation effect. It is now thought that concentration factors were responsible for the lower molecular weights obtained, since the higher temperature reactions in diethyl ether were run using a condenser, reducing the loss of the reaction solvent. This is in contrast to the reaction which produced polymer with the highest

molecular weight, where some of the solvent evaporated from the stoppered flask, effectively increasing the substrate concentrations.

On the basis of all our observations we believe that the major factor limiting molecular weight is the precipitation of the polymer on the enzyme surface preventing further penetration of the monomers to the enzyme. Support for this hypothesis comes from studies in the higher boiling ether solvents at higher temperature in which the polymer is insoluble. It was found that the *tert*-butyl methyl ether system at higher temperature gave consistently high molecular weights (Mn ~9,000).

Attempts to improve the efficiency of the enzyme by immobilisation were unsuccessful. In general, immobilised systems gave lower molecular weights.

Separation of the enzyme into soluble and insoluble fractions gave interesting results. The soluble fraction which should have contained mainly lipases was inactive, whereas the insoluble fraction which contained the proteases gave product. The readily available proteases, trypsin and pancreatin, were tested but gave no product.

Since the source of activity in the complex PPL system is not known, it may be that activity is dependent on the presence of a specific enzyme in the complex mixture. This could explain the observation that Kobayashi was unable to obtain polymeric products in the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol. It may be that the source of the PPL is a major factor and if the PPL does not contain the specific enzyme it will be inactive.

CHAPTER 7
EXPERIMENTAL

.

7.0 EXPERIMENTAL

7.0.1 Introduction

In this, the experimental section, the reader will find details of the materials used, the suppliers, their pre-reaction treatment and storage, experimental techniques and full details of all the actual experiments carried out. The experiments which were successful in producing polymer product have been given a cross-reference number (**bold** and in brackets) to be used in conjunction with the results presented in chapters 2 to 5.

7.0.2 Materials

Table 41. Materials used.

COMPOUND	R.M.M.	SUPPLIER
Acacia (gum arabic)	-	BDH
Acetone (SLR)	58.08	FISONS
Acetonitrile	41.05	FISONS
Adipic acid	146.14	BDH
Adipoyl chloride, 98%	183.03	ALDRICH
Argon	39.95	BOC
Azocoll	-	SIGMA
bis (2,2,2-trichloroethyl) adipate	408.82	SYNTHESISED
bis (2,2,2-trichloroethyl) glutarate	394.82	SYNTHESISED
Bromomethyl blue indicator	-	ALDRICH
1,4-Butanediol, 99%	90.12	ALDRICH
1-*tert*-Butoxy-2-methoxyethane, 99%	132.20	ALDRICH
tert-Butyl methyl ether, 98%	88.15	ALDRICH
Calcium hydride	42.10	ALDRICH
Celite 545	-	BDH
Chloroform	119.38	FISONS
colipase	-	GENZYME
Dialysis tubing (Visking tubing)	-	-
Dichloromethane	84.93	FISONS
1,3-Dicyclohexylcarbodiimide, 99%	206.33	ALDRICH
Diethyl ether	74.12	FISONS

Diisopropyl ether, 99%	102.18	ALDRICH
1,2-Dimethoxybenzene	138.17	ALDRICH
1,3-Dimethoxybenzene	138.17	BDH
4-Dimethylaminopyridine, 99%	122.17	ALDRICH
2,4-Dinitrophenyl hydrazine	198.14	BDH
Diolpate (PPA 350)	-	KEMIRA POLYMERS
Divinyl adipate (+ HQ inhibitor)	198.0	FLUOROCHEM (TCI)
Ethanol (SLR)	46.07	FISONS
Ethylene glycol diethyl ether	118.18	ALDRICH
Glutaric acid, 99%	132.12	ALDRICH
Glutaryl dichloride, 97%	169.01	ALDRICH
Heptane	100.21	ALDRICH
Hexane	86.18	FISONS
Hydrochloric acid (0.05M)	36.46	ALDRICH
Isosorbide dimethyl ether (DMI)	174.20	ICI SURFACTANTS
Lipozyme IM	-	NOVO NORDISK
Magnesium sulphate, 97+%	120.37	ALDRICH
Mercury (II) acetate, 98+%	318.68	BDH
2-Methoxymethylether, 99%		ALDRICH
Molecular sieves 3Å and 4Å	-	BDH
Olive oil	-	SIGMA
Pancreatin, Type II	-	ALDRICH
Phenolphthalein indicator	-	ALDRICH
Phosphate buffer, pH7.2	-	ALDRICH
Phosphorous pentoxide, 97%	283.89	ALDRICH
Porcine pancreatic lipase (PPL), (Type II, 110-220 units/mg using olive oil)	-	SIGMA
Potassium hydroxide pellets	56.11	ALDRICH
1,3-Propanediol (purified)	76.10	BDH
Pyridine, 99.9%	79.10	ALDRICH
Silica gel (TLC Grade, without binder)	-	ALDRICH
Sodium chloride	58.44	BDH
Sodium hydroxide (0.05M)	40.00	ALDRICH
Sodium hydroxide pellets	40.00	FISONS
Sodium metabisulphite, 97%	190.10	BDH
Sodium wire	22.99	BDH
Sucrose 99+%	342.30	BDH
Tetrahydrofuran (HPLC, 99.9%)	72.11	FISONS

D-Trehalose dihydrate, 99%	378.33	SIGMA
2,2,2-Trichloroethanol, 99+%	149.4	ALDRICH
Trypsin	-	SIGMA

7.0.3 Drying and Storage of Reagents

The porcine pancreatic lipase enzyme (PPL) (approximately 25% protein, activity of 110-220 units per mg protein using olive oil, Sigma L3126) used during this project was kept in an open container over phosphorous pentoxide inside a desiccator. The sealed desiccator was refrigerated at night, and then placed under vacuum during the day at ambient temperature, daily, for up to 3 weeks prior to use. This regime was maintained throughout the project with the desiccator and enzyme being allowed to reach ambient temperature before use.

Molecular sieve 3Å & 4Å 1/16in pellets were oven dried at 300°C for at least 4 days before use.

Diethyl ether was dried over KOH pellets for 24hrs then dried over sodium wire and distilled immediately prior to use. Dichloromethane was distilled over CaH_2 prior to use.[140]

All reagents were weighed out on a Sartorius Balance 'Basic' model with four decimal place display.

Unless otherwise stated, all other reagents were used as purchased and stored according to the manufacturers' guidelines.

7.1 Experimental Methods

7.1.1 Freeze-Drying

Freeze-drying was carried out by the Pharmaceutical Sciences Dept., Aston University. The technique used a Edwards freeze-drying carousel, liquid nitrogen and an Edwards High Vacuum pump.

7.1.2 Gel Permeation Chromatography (GPC)

Gel permeation chromatography was performed by RAPRA Technology Ltd., Shawbury, Shrewsbury, Shropshire, SY4 4NR, UK. The analysis was carried out usually in THF at ambient temperature using a Knauer 64 HPLC Pump. The column used in the system was a styrene and divinylbenzene heavily crosslinked PL-gel; dimensions 300x7.5mm; 10μm diameter mixed bed. A Knauer differential refractometer was used as a detector. The polymer samples were calibrated against a

polystyrene standard and the samples were run in duplicate. A polyester "Diolpate" reference was also analysed with each batch of GPC samples to detect variation in the GPC technique. The molecular mass averages were computated using Viscotek software.

7.1.3 Infrared Spectroscopy

All IR spectra were recorded on either Perkin Elmer 1710 or Perkin Elmer Paragon 1000 Fourier Transform Infrared Spectrometers. Solid samples were prepared as a KBr disc and liquids as thin films between sodium chloride plates.

7.1.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

All NMR analysis was carried out on a Brüker AC300 spectrometer in solution (deuterated chloroform). ^{13}C spectra were recorded as PENDANT (Polarisation Enhancement Nurtured During Attached Nucleus Testing).

The Mn values of the polymers obtained in the time study reactions for bis(2,2,2-trichloroethyl) alkanedioates and divinyl adipate, were determined by ^{1}H NMR end group analysis using the method of Wallace and Morrow.[101] The area of the methylene proton absorption (δ 4.8 ppm) for the unreacted trichloroethyl group plus the area of the methylene proton absorption (δ 3.7 ppm) for terminal CH_2OH groups are added together and this area is divided into the area of absorption of the esterified methylenes of the butanediol moiety (δ 4.1); the quotient gives a DP (degree of polymerisation) for the polymer.

7.1.5 HPLC Analysis

All HPLC test samples were filtered through a 0.2μm syringe filter and placed into a vial on an automatic carousel. The injector was pumped with degassed HPLC water for 20s at a flow rate of 0.5 cm^3 min^{-1} and then the sample was drawn up over 10s and injected for 10s by a Bio-Rad pump. The runtime was 15 minutes and the pressure was maintained at 50 psi. The separating column was an Aminex HPX-87C column (30 cm) kept at 85°C and the components were detected by a Bio-Rad refractive-index detector. Retention times of the sucrose and buffer salts were 11.01 minutes and 11.49 minutes respectively.

7.1.6 Melting Point Determinations

Melting points were determined in capillary tubes with a Gallencamp Melting Point Apparatus, Model No. ME-370, and are uncorrected.

7.1.7 Thin Layer Chromatography (TLC)

This technique was used to determine product purity. Metal backed plates of silica were used and the separated components were visualised by heating after using a 50% H_2SO_4 in methanol spray mix. The results obtained using TLC allowed the best solvent system to be determined for the separation of mixtures by 'Dry Flash Column Chromatography'.

7.1.8 Dry Flash Chromatography

A cylindrical porosity no.4 sinter funnel was filled with a slurry of TLC grade silica and mobile phase and then suction was applied. The silica was compacted using the screw motion of an inverted glass stopper to eliminate cracks and voids to give a column in which the solvent front moved in a horizontal line. The column was eluted by adding predetermined volumes of the mobile phase and allowed to run dry before the addition of the following fraction.[140]

The mobile phase was chosen by TLC as the one in which the product was soluble and gave an R_f value of 0.5 by TLC analysis. For the majority of separations, the solvent system was any suitable mix of hexane, ether, ethyl acetate and methanol.

The separation was followed by TLC analysis and the required fractions were gathered together and the solvent removed under reduced pressure to yield the purer product.

7.1.9 Karl Fischer Moisture Analysis

A Mitsubushi Moisture Meter Model CA-20 was used for moisture determinations of the substrates and solvents.

Karl Fischer titrimetry involves an electrolytic solution containing iodide ions, sulphur dioxide, a base and an alcohol which is electrolysed in a titration cell to generate free iodine. The free iodine reacts with water in the test sample to produce hydrogen iodide according to the following equation;

Water-iodide reaction

$$H_2O + I_2 + SO_2 \rightarrow 2HI + SO_3$$

Generation of iodine

$$2I^- - 2e^- \rightarrow I_2$$

The quantity of electric charge required to electrolyse the H_2O is then recorded as a measure of the amount of water present in a sample of known mass.

7.1.10 Protease Activity Assay

Protease activity tests[141] involved insoluble Azocoll (a cowhide protein to which a bright red dye is attached). When proteases act on the cowhide to break it down, the dye is released. The rate at which the dye is released can be used as a measure of the catalytic activity of the protease. The assay is non-specific. One arbitrary unit is defined as that amount of enzyme that causes an increase in A_{580} of 0.1 under the conditions of the assay.

The assay involved a control which was a fraction of the supernatant liquid containing the enzyme, which was made inactive by boiling it for 15 minutes. A second fraction was made up from supernatant (0.5cm^3), 0.1M phosphate buffer (pH 7.2) (1.5cm^3) and Azocoll (10mg). The fraction was incubated for 30 minutes and mixed occasionally. Distilled water was added (3.0cm^3). The fraction was filtered and the absorbance was measured against the inactive control. The activity of the enzyme was then assessed. (50mg/cm^3 in pH 7 buffer 0.2g of enzyme in 4cm^3 of buffer.)

7.1.11 Lipase Olive Oil Activity Assay

Lipase activity was assayed by the 'Olive Oil Emulsion' method according to Mustranta et al. [80] The olive oil emulsion was prepared by homogenising olive oil ($30cm^3$) with emulsification reagent ($70cm^3$) for 3 minutes. (The emulsification reagent (1.0L) contained NaCl (17.9g), KH_2PO_4 (0.41g), glycerol ($540cm^3$), gum arabic (10.0g) and distilled water.)

The enzyme solution ($1.0cm^3$ (15mg of enzyme cm^{-3} of 0.2M buffer)) was added to the olive oil emulsion ($5cm^3$) and 0.2M phosphate buffer pH 7.2 ($4cm^3$). The mixture was incubated for 10min at 37°C. The reaction was terminated by the addition of $10cm^3$ of an acetone-ethanol mixture (1:1). The liberated fatty acids were titrated with 0.05M sodium hydroxide solution (standardised using 0.05M HCl solution). The activity of crude PPL is defined on the basis that one unit of enzyme will hydrolyse 1μmol equivalent of fatty acid from a triglyceride in one hour at the indicated pH and temperature.

7.1.12 Protein Content Determinations

The Bio-rad assay is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue shifts from 465nm to 595nm when binding to a protein occurs. Beer's law may be applied for accurate quantification of protein by selecting an appropriate ratio of dye volume to sample concentration. Over a broader range of protein concentrations, the dye binding method gives an accurate, but not entirely linear response. Generally no sample preparation was required. The procedure involved adding standard solutions ($0.1cm^3$) and test samples ($0.1cm^3$) into test tubes. Buffer ($0.1cm^3$) was added to a blank test tube. Diluted dye reagent ($5.0cm^3$) was added to each of the sample tubes. The solutions were mixed and after 5 minutes the samples were measured using a spectrophotometer set at OD_{595} with the control blank. From a graphical plot of OD_{595} versus concentration of standards, the test protein contents were read from the graph.

Biuret Method

The assay detailed above was found to be inaccurate and so the Biuret method was used.[142] This method is unaffected by the amino acid

composition of the protein. $CuSO_4.5H_2O$ (1.5g) and sodium potassium tartrate (6.0g) were dissolved in distilled water (500cm^3). 10%(w/v) NaOH (300cm^3) was then added and made upto 1.0L with water. In order to keep the solution indefinitely, KI (1.0g) was added. To a known concentration of enzyme in solution was added 2.5cm^3 of the reagent. The mixture was allowed to stand for 25 minutes before a reading was taken at A_{540}.

7.2 Synthesis and Analysis of Reagents

7.2.1 Bis(2,2,2-trichloroethyl) alkanedioates
Synthesis of the bis(2,2,2-trichloroethyl) alkanedioates was carried out as described by Wallace and Morrow[101] with modifications outlined below.

A nitrogen-filled 500cm^3 three-necked round bottomed flask was charged with diacid (glutaric acid (9.11g, 69mmol) or adipic acid (10.08g, 69mmol)), 2,2,2-trichloroethanol (15cm^3, 140mmol), dry dichloromethane (175cm^3) freshly distilled from CaH_2. The flask was then cooled in an ice-bath. To the resulting stirred suspension was added 4-(dimethylamino)pyridine (DMAP) (0.2g, 1.64mmol) followed by slow addition of N,N'-dicyclohexyl-carbodiimide (DCC) (28.9g, 140mmol) dissolved in freshly distilled dichloromethane (75cm^3). The mixture was then stirred at ambient temperature under a nitrogen blanket for at least 12 hrs. A voluminous precipitate of N,N'-dicyclohexylurea (DHU) was formed. The precipitate was filtered and the residue was washed successively with distilled dichloromethane (2x100cm^3). The filtrate was washed with 5% aqueous HCl (12x100cm^3), saturated aqueous sodium bicarbonate solution (8x100cm^3) and saturated brine solution (4x100cm^3). The organic layer was dried over magnesium sulphate, filtered and the solvent was removed on the rotary evaporator. The bis(2,2,2-trichloroethyl) alkanedioate structures were confirmed by IR, ^{1}H and ^{13}C NMR analyses and characterised by TLC and purified using 'Dry Flash Chromatography' where necessary.

Bis(2,2,2-trichloroethyl) adipate

$Cl_3CCH_2OC(O)(CH_2)_4C(O)OCH_2CCl_3$

^1H NMR (δ; $CDCl_3$): 4.66 (s, 4H; Cl_3CCH_2),

2.43 (t, 4H; J=5.69Hz; $(O)C(CH_2CH_2CH_2CH_2C(O))$,

1.89 (t, 4H; J=3.13 Hz; $(O)CCH_2CH_2CH_2CH_2$)

^{13}C NMR (δ; $CDCl_3$): 171.0 (C(O)), 94.0 (Cl_3CCH_2), 73.8(Cl_3CCH_2O),

33.0 ($C(O)(CH_2CH_2CH_2CH_2)$,

23.0 ($C(O)(CH_2CH_2CH_2CH_2)$.

Bis(2,2,2-trichloroethyl) glutarate

$Cl_3CCH_2O(O)C(CH_2)_3C(O)OCH_2CCl_3$

^1H NMR (δ; $CDCl_3$): 4.70 (s, 4H; Cl_3CCH_2),

2.53 (t, 4H; J = 7.26 Hz; $(O)C(CH_2CH_2CH_2C(O)$,

2.00 (t, 2H; J = 7.43 Hz; $CH_2CH_2CH_2C(O)$).

^{13}C NMR (δ; $CDCl_3$): 171.0 (C(O)), 94.7 (Cl_3CCH_2O), 73.8(Cl_3CCH_2O),

32.6 ($C(O)(CH_2CH_2CH_2)$,

19.6 ($C(O)(CH_2CH_2CH_2)$.

The bis(2,2,2-trichloroethyl) adipate (crude yield 90%) was recrystallised from cyclohexane, m.p. 44-45°C (lit.,[101] m.p. 44.5-46°C), and the bis(2,2,2-trichloroethyl) glutarate (crude yield 90%) was recrystallised from cyclohexane m.p. 41-42°C (lit.,[101] m.p. 41-42°C).

The diols, 1,4-butanediol and 1,3-propanediol were purified by distillation under reduced pressure, discarding the first 30cm³.

Divinyl adipate (with hydroquinone inhibitor) was used as supplied.

7.3 PPL-CATALYSED POLYTRANSESTERIFICATION OF BIS (2,2,2-TRICHLOROETHYL) ADIPATE WITH 1,4-BUTANEDIOL IN DIETHYL ETHER

7.3.1 Without Molecular Sieves

A 100cm^3 round bottomed flask was charged with typical reactants (except for molecular sieve), 1,4-butanediol (0.90g, 10mmol) and bis(2,2,2-trichloroethyl) adipate, (4.08g, 10mmol). Freshly distilled diethyl ether (over sodium wire) (50cm^3) was then added to the flask. Finally, a weighed amount of the vacuum desiccated PPL (2.5g) was added to the flask promptly and the contents swirled.

A series of these reactions were carried out (**reactions 1-6**). The flasks were stoppered, lagged and the contents stirred at ambient temperature for fixed lengths of time before they were terminated by filtration and the products recovered.

The contents of the reaction flask were filtered on Whatman no.1 filter paper and the enzyme residue was washed with dichloromethane (freshly distilled from CaH$_2$) (50cm^3), or HPLC grade THF. The clear filtrate was added to a round bottomed flask and the solvent removed by rotary evaporation and gentle heating.

Freshly distilled diethyl ether (50cm^3) was then added to the remaining oil. Any white precipitate formed over 24 hours was filtered on Whatman no.1 filter paper and washed with freshly distilled diethyl ether (50cm^3). (If no precipitate formed with diethyl ether then hexane was used to precipitate the oligomers.) The white product was dried in a vacuum desiccator and then characterised by ^{13}C and ^{1}H NMR (see **Figures 7 & 8**).

poly(1,4-butylene adipate) Cl_3CCH_2-$[OC(O)(CH_2)_4C(O)O(CH_2)_4O]_n$-H

^{1}H NMR (δ; $CDCl_3$):   1.65 (m, 8H; $(CH_2CH_2)_2$),

2.28 (t, 4H; J =5.93 Hz; $(CH_2)_2C(O)$),

3.62 (t, 4H; J =5.77 Hz; terminal $(CH_2)_2OH$),

4.05 (t, 4H; J =2.97 Hz; $(CH_2CH_2)_2$-O))

^{13}C NMR (δ; $CDCl_3$):   173.26 ($C(O)$), 64.13 (O-$(CH_2CH_2CH_2CH_2)$),

33.78 ($C(O)(CH_2CH_2CH_2CH_2)$),

25.21 (O-$(CH_2CH_2CH_2CH_2)$),

24.29 ($C(O)(CH_2CH_2CH_2CH_2)$)

.
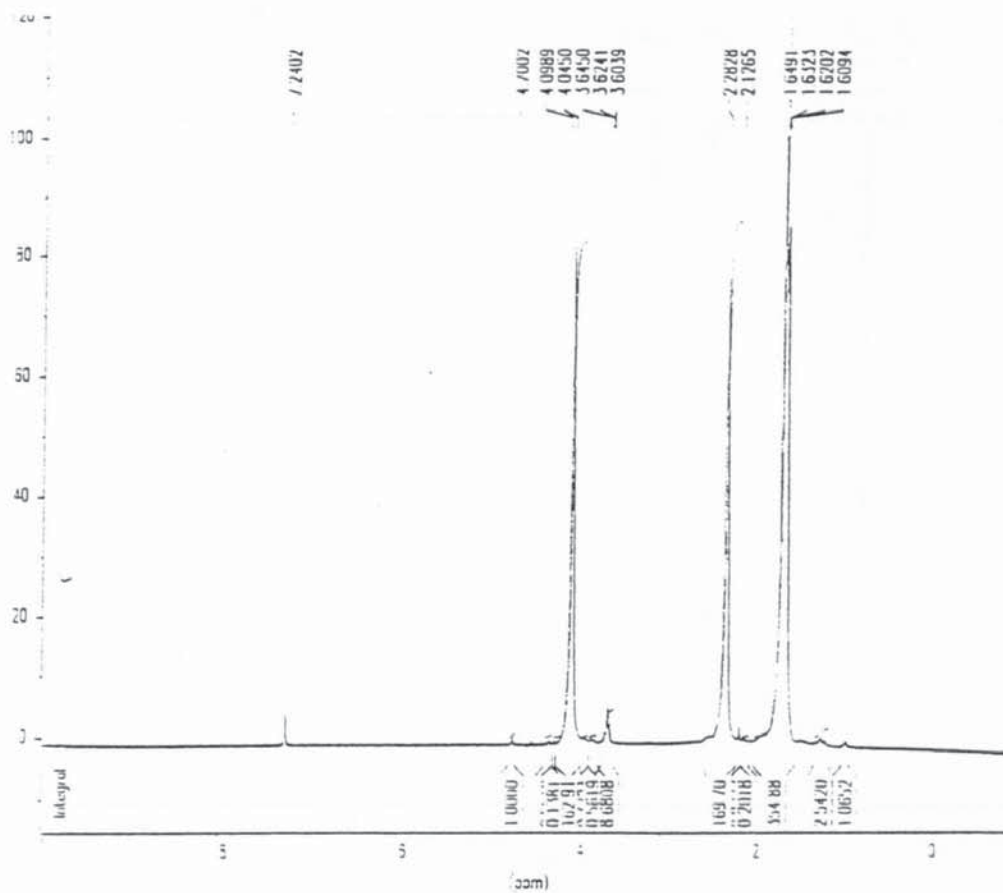
Figure 7. A typical ^{1}H NMR for the polymer obtained from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) adipate with 1,4-butanediol in anhydrous diethyl ether without molecular sieves.
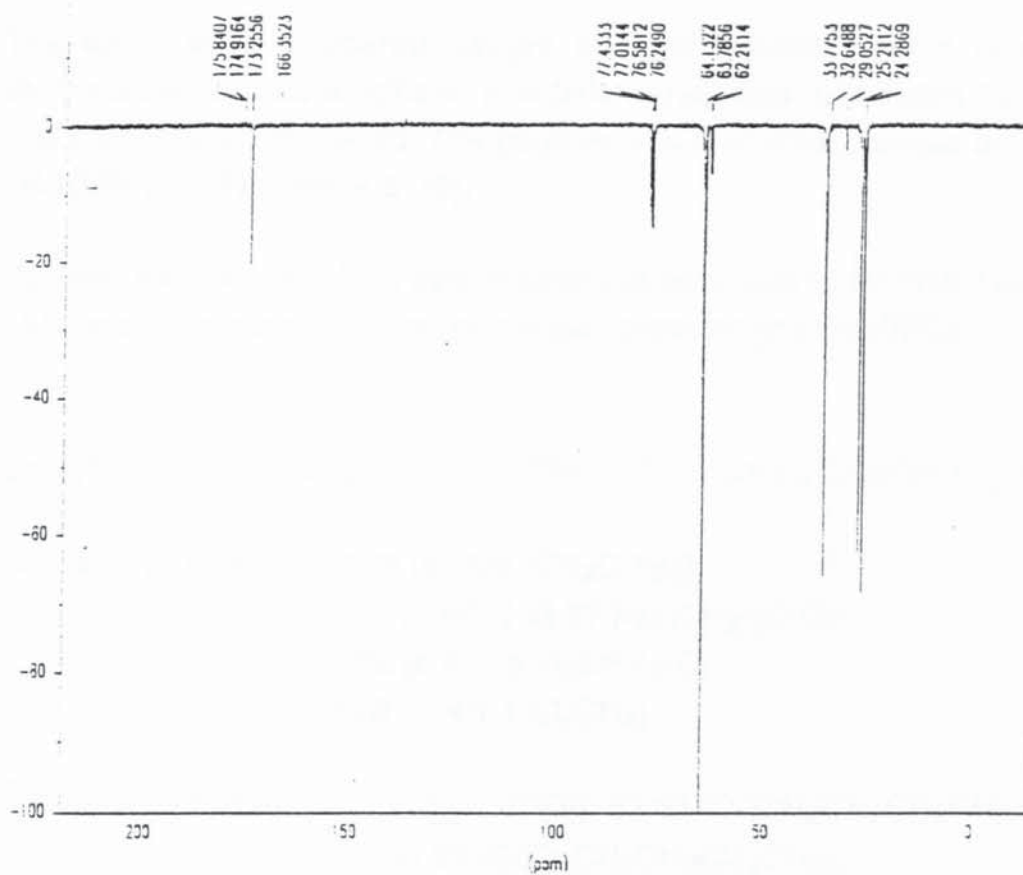
Figure 8. A typical ^{13}C NMR for the polymer obtained from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) adipate with 1,4-butanediol in anhydrous diethyl ether without molecular sieves.

7.3.2 With Molecular Sieves

A series of typical reactions (as described above) were carried out in the presence of dried 4Å molecular sieves (3.0g) **(reactions 7-11)**. The sieves were removed from the oven (260°C) and allowed to cool in a desiccator over phosphorous pentoxide. They were immediately weighed and then added to each of the flasks, prior to the addition of PPL.

The flasks were stoppered, lagged and the contents stirred at ambient temperature for fixed lengths of time before they were terminated by filtration and the products recovered. The polymer was then characterised by ^{13}C and ^{1}H NMR (see **Figures 9 & 10**).

Polymer samples from both sets of reactions were sent to RAPRA Technology Ltd. for characterisation by gel permeation chromatography (GPC).

poly(1,4-butylene adipate) Cl_3CCH_2-$[OC(O)(CH_2)_4C(O)O(CH_2)_4O]_n$-H

^{1}H NMR (δ; CDCl$_3$):   1.63 (m, 8H; $(CH_2CH_2)_2$),

                       2.26 (t, 4H; J=5.87 Hz; $(CH_2)_2C(O)$),

                       4.02 (s, 4H; $(CH_2CH_2)_2$-O)),

                       4.68 (s, 4H, Cl_3CCH_2).

^{13}C NMR (δ; CDCl$_3$):   173.08 $(C(O))$, 63.63 $(O$-$(CH_2CH_2CH_2CH_2)$,

                         33.63 $(C(O)(CH_2CH_2CH_2CH_2)$,

                         25.07 $(O$-$(CH_2CH_2CH_2CH_2)$,

                         24.14 $(C(O)(CH_2CH_2CH_2CH_2)$
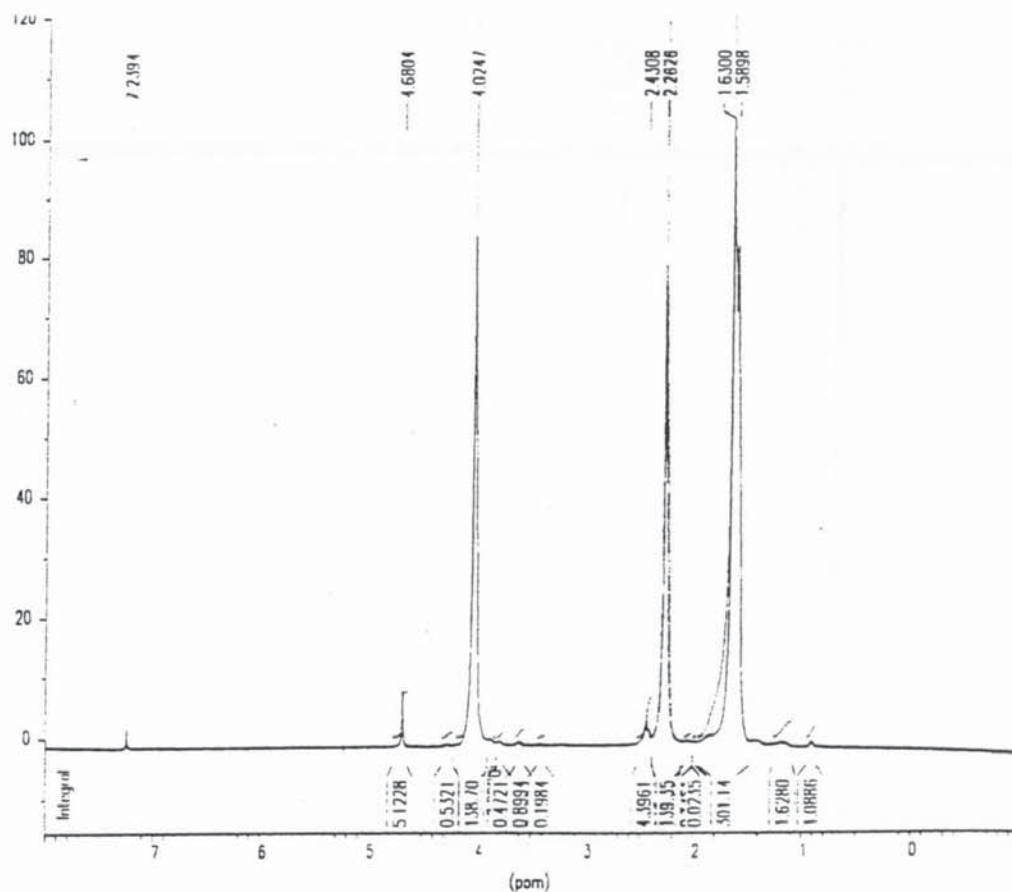
Figure 9. A typical ¹H NMR for the polymer obtained from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) adipate with 1,4-butanediol in anhydrous diethyl ether with molecular sieves.
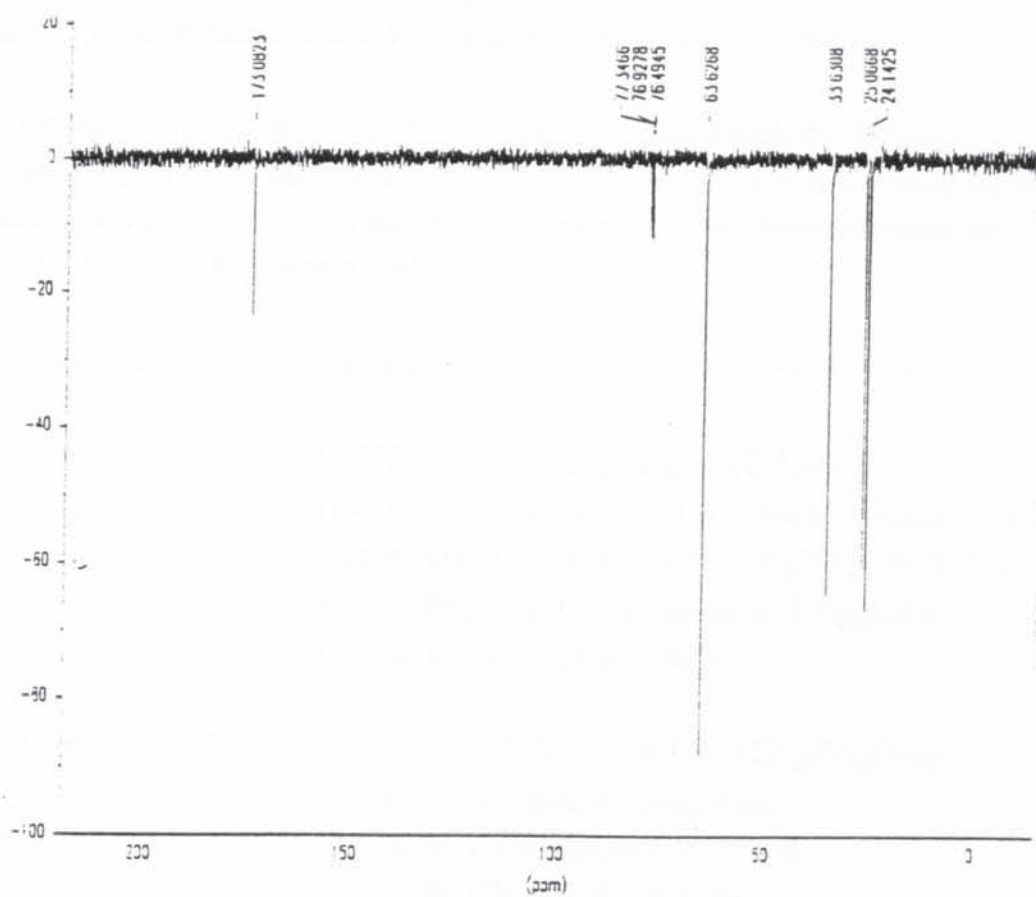
Figure 10. A typical ^{13}C NMR for the polymer obtained from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) adipate with 1,4-butanediol in anhydrous diethyl ether with molecular sieves.

7.4 PPL-CATALYSED POLYTRANSESTERIFICATION OF BIS (2,2,2-TRICHLOROETHYL) GLUTARATE WITH 1,4-BUTANEDIOL IN DIETHYL ETHER

7.4.1 Without Molecular Sieves

A series of typical reactions were carried out (except for molecular sieves), with bis(2,2,2-trichloroethyl) glutarate (3.94g,10mmol) **(reactions 12-15)** being used in place of the bis(2,2,2-trichloroethyl) adipate (4.08g, 10mmol).

The flasks were stoppered, lagged and the contents stirred at ambient temperature for fixed lengths of time before they were terminated by filtration and the products recovered. The polymer was then characterised by ^{13}C and ^{1}H NMR (see **Figures 11 & 12**).

poly(1,4-butylene glutarate) $Cl_3CCH_2-[OC(O)(CH_2)_3C(O)O(CH_2)_4O]_n-H$

^{1}H NMR (δ; CDCl$_3$):   1.67 (m, 4H; -O-CH$_2$CH$_2$CH$_2$CH$_2$-OH),

1.94 (quintet, 2H; J = 7.43 & 7.26Hz; CH$_2$CH$_2$CH$_2$C(O)),

2.34 (t, 4H; J =7.25 Hz; (O)C(CH$_2$CH$_2$CH$_2$C(O)),

3.64 (t, 4H; J =6.11 Hz; terminal (CH$_2$)$_2$OH),

4.08 (s, 4H; CH$_2$CH$_2$-O-C(O))

^{13}C NMR (δ; CDCl$_3$):   172.85 (C(O)), 63.83 (O-(CH$_2$CH$_2$CH$_2$CH$_2$),

33.14 (C(O)(CH$_2$CH$_2$CH$_2$)),

25.20 (O-(CH$_2$CH$_2$CH$_2$CH$_2$)),

19.98 (C(O)(CH$_2$CH$_2$CH$_2$))

Figure 11. A typical ^1H NMR for the polymer obtained from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) glutarate with 1,4-butanediol in anhydrous diethyl ether without molecular sieves.
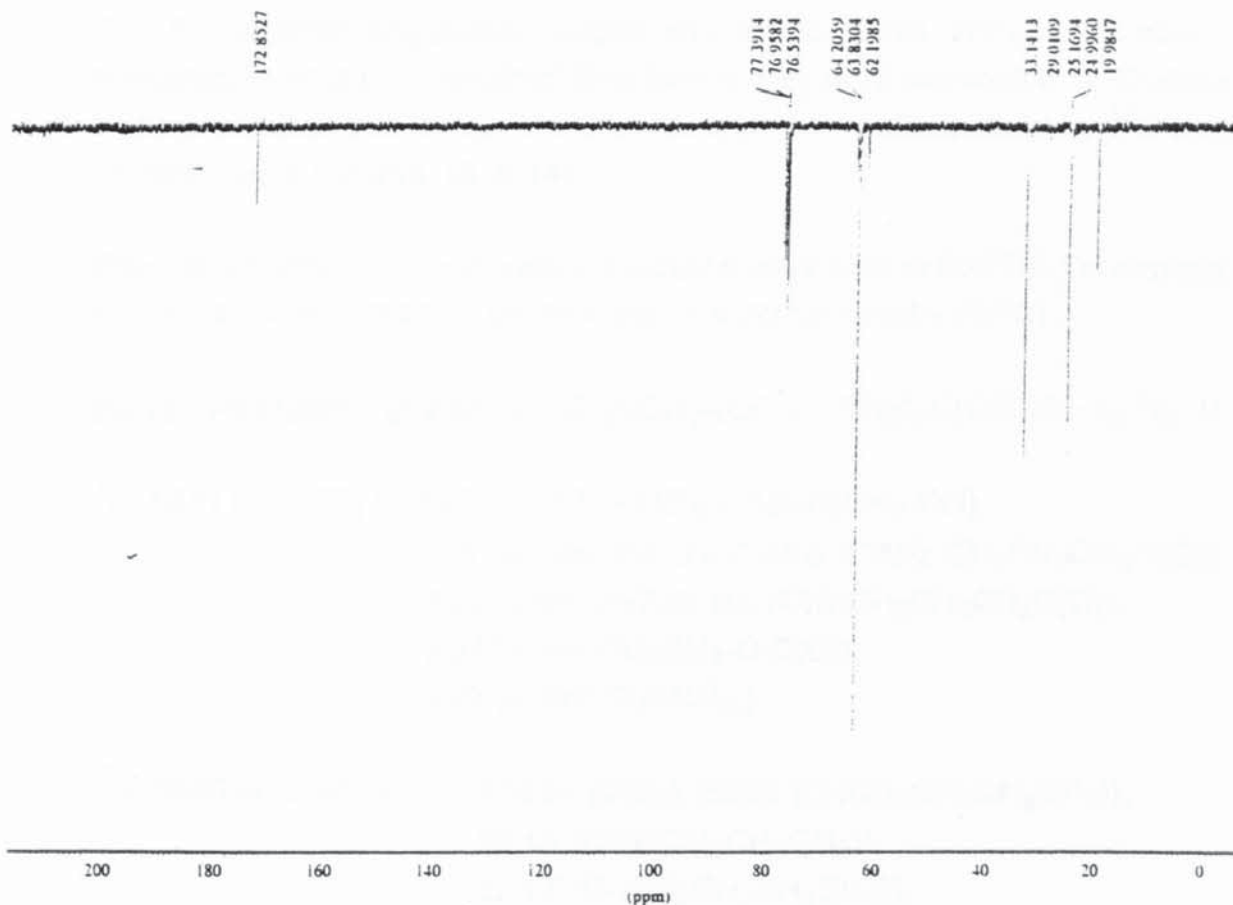
Figure 12. A typical ^{13}C NMR for the polymer obtained from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) glutarate with 1,4-butanediol in anhydrous diethyl ether without molecular sieves.

7.4.2 With Molecular Sieves

A series of typical reactions (as described above) were carried out in the presence of dried 4Å molecular sieves (3.0g) (**reactions 16-20**). The sieves were removed from the oven (260°C) and allowed to cool in a desiccator over phosphorous pentoxide. They were immediately weighed and then added to each of the flasks, prior to the addition of PPL.

The flasks were stoppered, lagged and the contents stirred at ambient temperature for a fixed length of time before they were terminated by filtration and the product recovered. The polymer was then characterised by ^{13}C and ^{1}H NMR (see **Figures 13 & 14**).

Polymer samples from both sets of reactions were sent to RAPRA Technology Ltd. for characterisation by gel permeation chromatography (GPC).

poly(1,4-butylene glutarate) $Cl_3CCH_2-[OC(O)(CH_2)_3C(O)O(CH_2)_4O]_n-H$

^{1}H NMR (δ; $CDCl_3$): 1.67 (m, 4H; $-O-CH_2CH_2CH_2CH_2-OH$),

1.91 (quintet, 2H; $J = 7.43$ & $7.25Hz$ $CH_2CH_2CH_2C(O)$),

2.32 (t, 4H; $J = 7.26$ Hz; $(O)C(CH_2CH_2CH_2C(O))$,

4.07 (s, 4H; $CH_2CH_2-O-C(O)$),

4.72 (s, 4H; Cl_3CCH_2)

^{13}C NMR (δ; $CDCl_3$): 172.84 ($C(O)$), 63.83 ($O-(CH_2CH_2CH_2CH_2)$),

33.14 ($C(O)(CH_2CH_2CH_2)$),

25.17 ($O-(CH_2CH_2CH_2CH_2)$),

20.00 ($C(O)(CH_2CH_2CH_2)$)
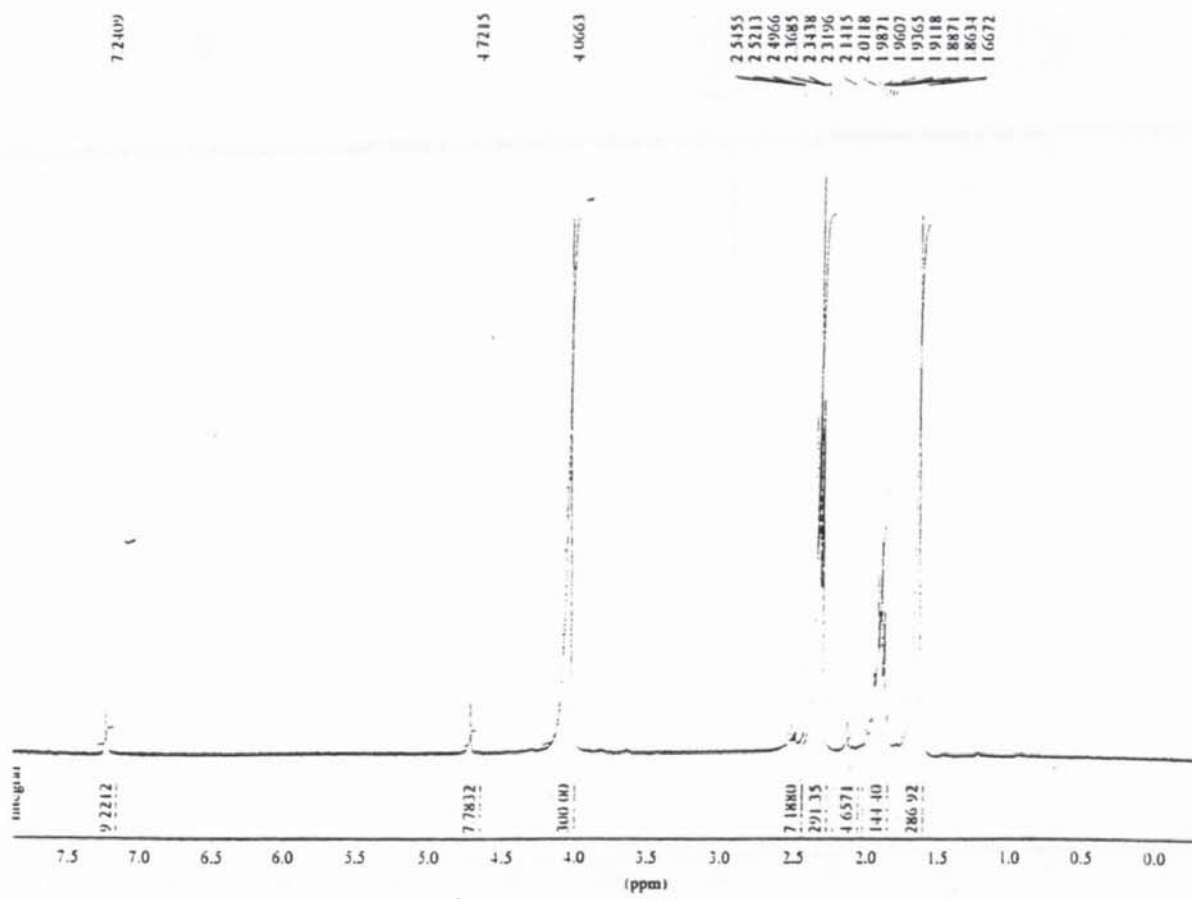
Figure 13. A typical ^{1}H NMR for the polymer obtained from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) glutarate with 1,4-butanediol in anhydrous diethyl ether with molecular sieves.
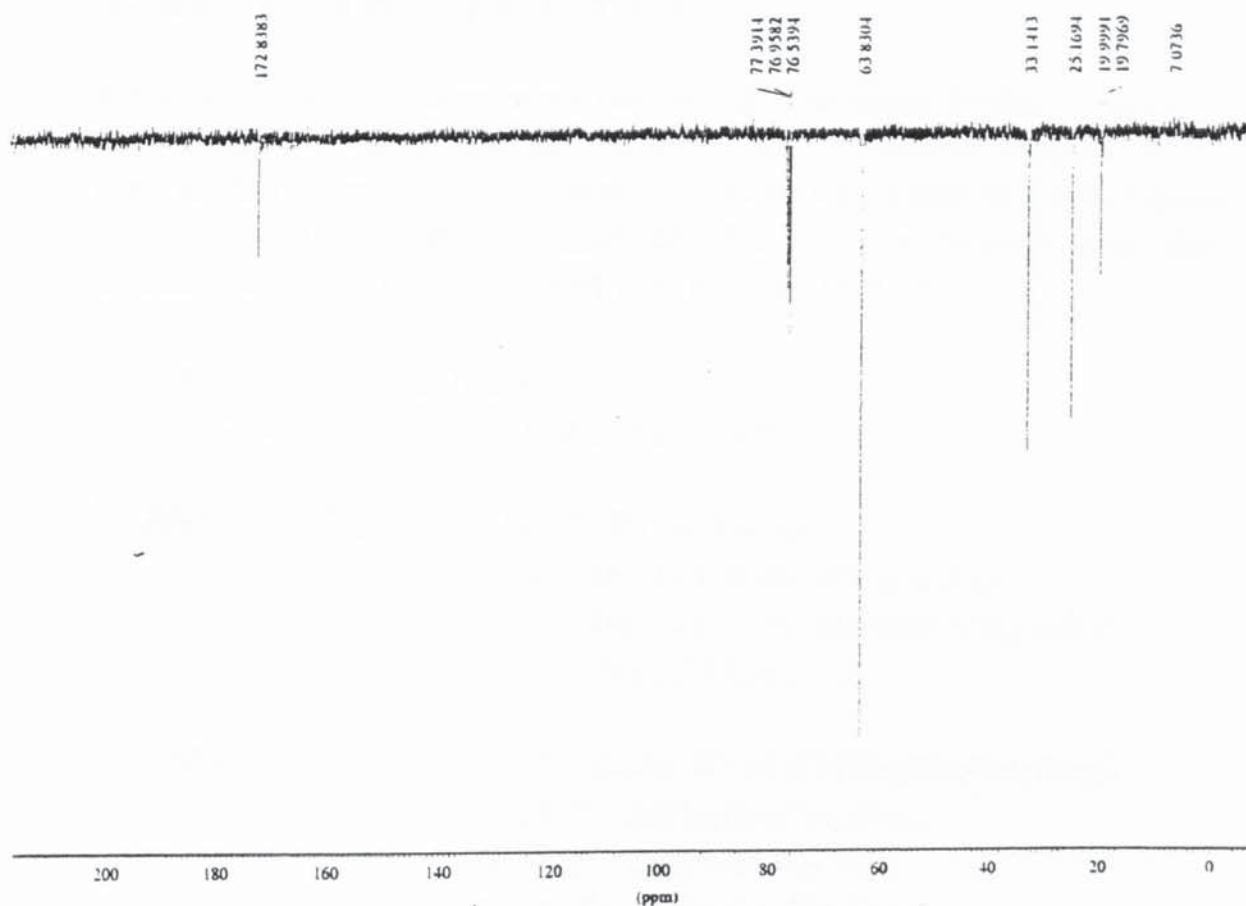
Figure 14. A typical ^{13}C NMR for the polymer obtained from the PPL-catalysed polytransesterification of bis(2,2,2-trichloroethyl) glutarate with 1,4-butanediol in anhydrous diethyl ether with molecular sieves.

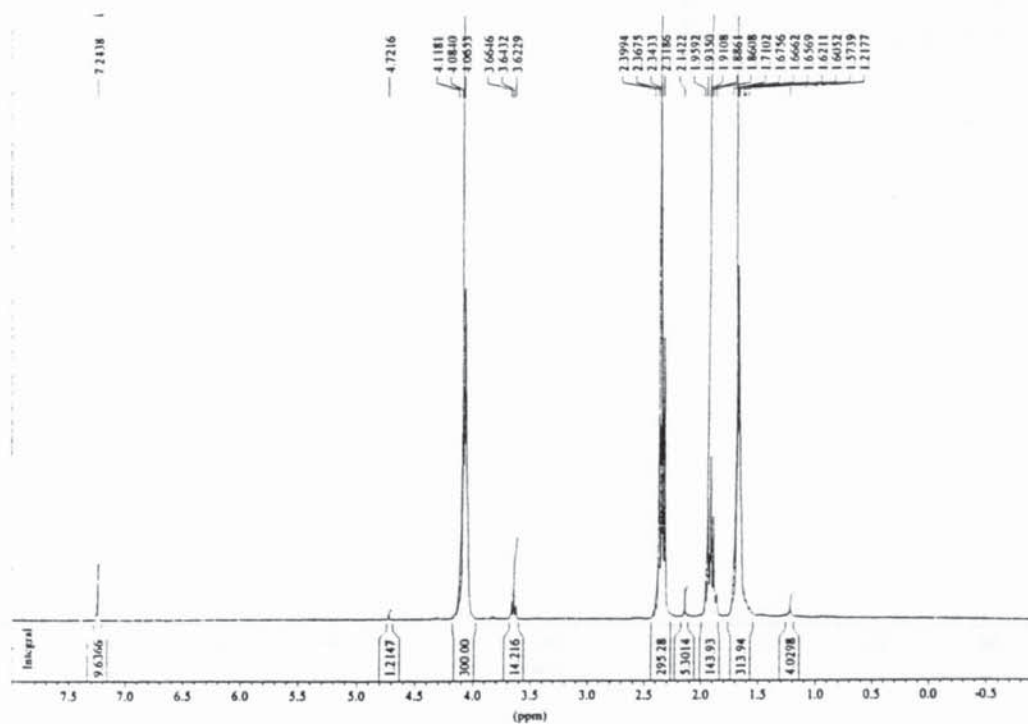## 7.5 PPL-CATALYSED POLYTRANSESTERIFICATION OF DIVINYL ADIPATE WITH 1,4-BUTANEDIOL IN DIETHYL ETHER

7.5.0.1 Without Molecular Sieves

A 100cm^3 round bottomed flask was charged with typical reactants (except for molecular sieves); 1,4-butanediol (0.90g, 10 mmol) and divinyl adipate (1.98g, 10 mmol). Freshly distilled diethyl ether (over sodium wire) (50cm^3) was then added to the flask. Finally, a weighed amount of the vacuum desiccated PPL (2.5g) was added promptly, and the contents swirled.

A series of these reactions were carried out **(reactions 21-26)**. The flasks were stoppered, lagged and the contents stirred at ambient temperature for fixed lengths of time before they were terminated by filtration and the products recovered as described in **Section 7.3.1**. The polymer was then characterised by ^{13}C and ^{1}H NMR (see **Figures 15 & 16**).

poly(1,4-butylene adipate)

CH$_2$=CH-[OC(O)(CH$_2$)$_4$C(O)O(CH$_2$CH$_2$)$_2$O]$_n$-H
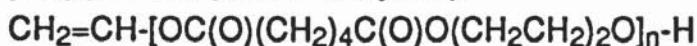
^{1}H NMR (δ; CDCl$_3$):   1.60 (m, 8H; (CH$_2$CH$_2$)$_2$),
2.24 (t, 4H; J=5.94 Hz; (CH$_2$)$_2$C(O)),
3.57 (t, 4H; J=6.11 Hz; terminal (CH$_2$)$_2$OH)),
4.01 (s, 4H; (CH$_2$CH$_2$)$_2$-O))

^{13}C NMR (δ; CDCl$_3$):   173.13 (C(O)), 63.66 (O-(CH$_2$CH$_2$CH$_2$CH$_2$)),
33.64 (C(O)(CH$_2$CH$_2$CH$_2$CH$_2$)),
25.08 (O-(CH$_2$CH$_2$CH$_2$CH$_2$)),
24.16 (C(O)(CH$_2$CH$_2$CH$_2$CH$_2$))
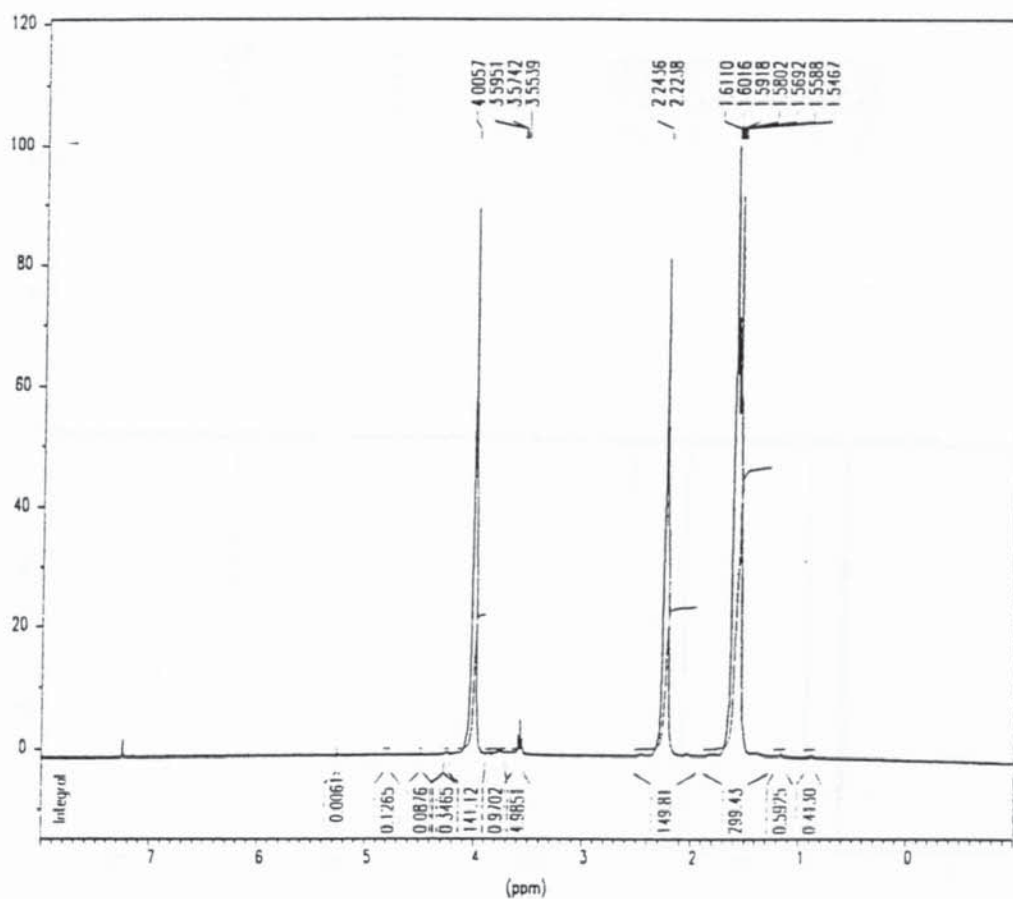
Figure 15. A typical ^{1}H NMR for the polymer obtained from the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether without molecular sieves.
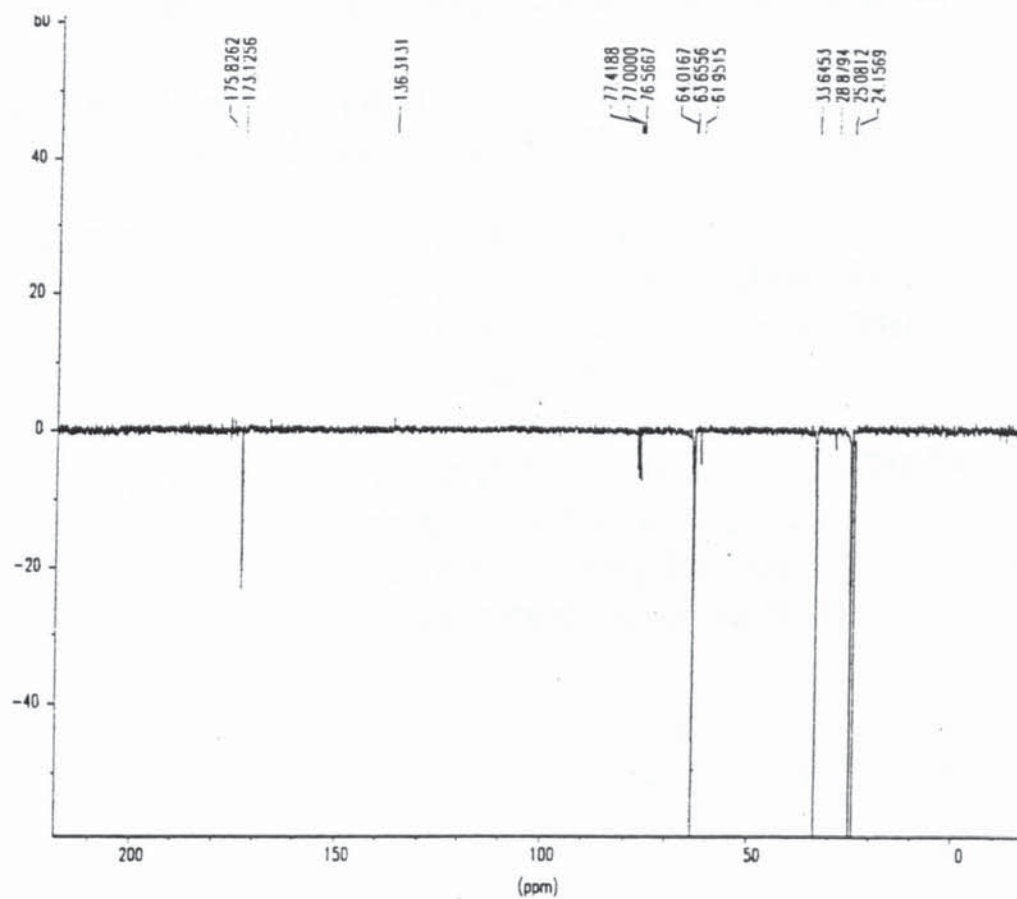
Figure 16. A typical ^{13}C NMR for the polymer obtained from the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether without molecular sieves.

146

7.5.0.2 With Molecular Sieves

A series of typical reactions (as described above) were carried out in the presence of dried 4Å molecular sieves (3.0g) **(reactions 27-31)**. The sieves were removed from the oven (260°C) and allowed to cool in a desiccator over phosphorous pentoxide. They were immediately weighed and then added to each of the flasks, prior to the addition of PPL. The flasks were stoppered, lagged and the contents stirred at ambient temperature for a fixed length of time before they were terminated by filtration and the product recovered. The polymer was then characterised by ^{13}C and ^{1}H NMR (see **Figures 17 & 18**).

poly(1,4-butylene adipate)
$CH_2=CH-[OC(O)(CH_2)_4C(O)O(CH_2CH_2)_2O]_n-H$
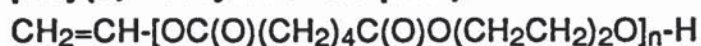
^{1}H NMR (δ; $CDCl_3$):   1.60 (m, 8H; $(CH_2CH_2)_2$),
2.27 (t, 4H; $J=5.94$ Hz; $(CH_2)_2C(O)$),
3.63 (t, 4H; $J=6.27$ Hz; terminal $(CH_2)_2OH$)),
4.05(s, 4H; $(CH_2CH_2)_2$-O))

^{13}C NMR (δ; $CDCl_3$):   173.20 (C(O)), 63.73 (O-$(CH_2CH_2CH_2CH_2)$),
33.73 (C(O)$(CH_2CH_2CH_2CH_2)$),
25.17 (O-$(CH_2CH_2CH_2CH_2)$),
24.24 (C(O)$(CH_2CH_2CH_2CH_2)$)
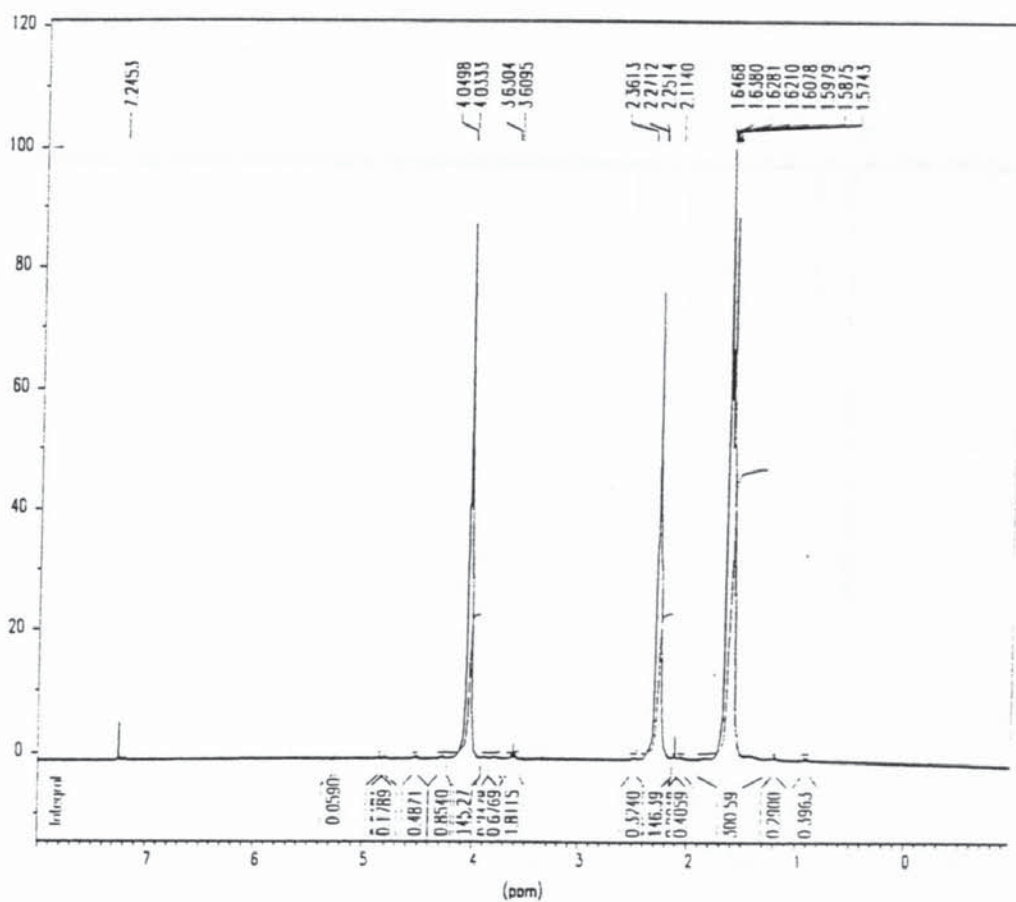
Figure 17. A typical ¹H NMR for the polymer obtained from the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether with molecular sieves.
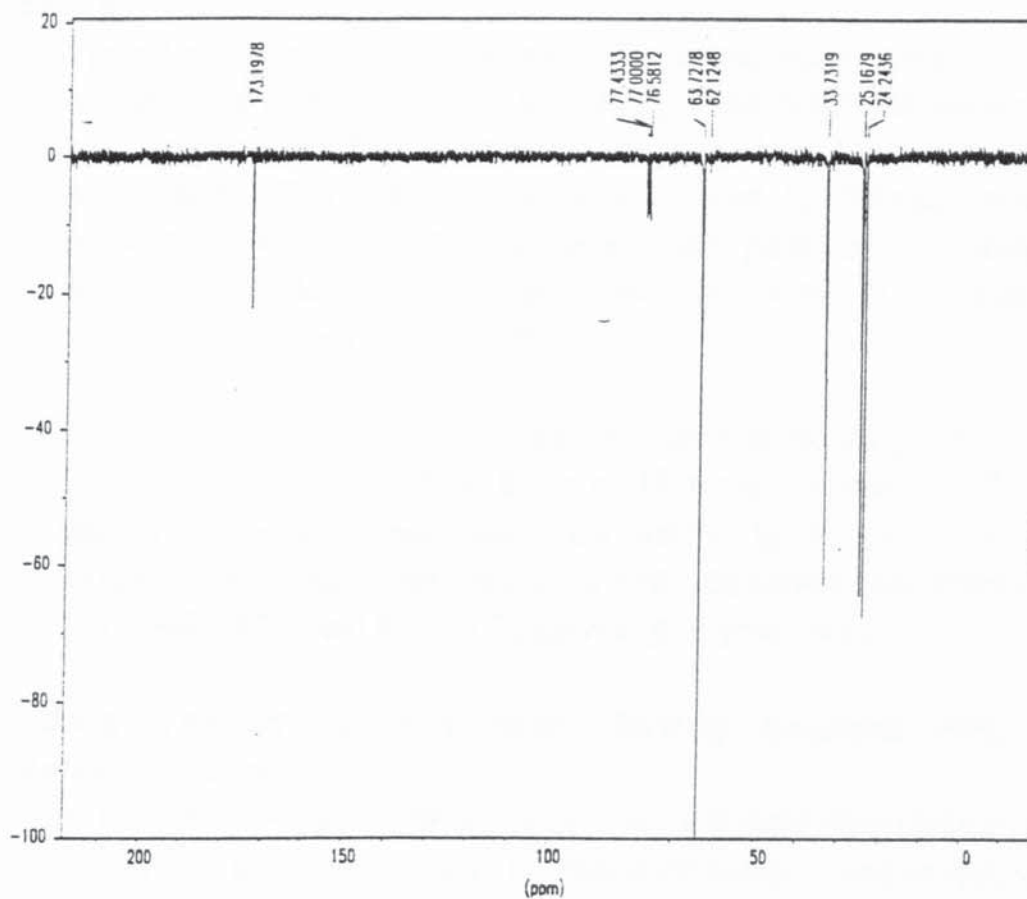
Figure 18. A typical ^{13}C NMR for the polymer obtained from the PPL-catalysed polytransesterification of divinyl adipate with 1,4-butanediol in anhydrous diethyl ether with molecular sieves.

### 7.5.0.3 The Reproducibility of PPL-Catalysed Reactions with Molecular Sieve

A 100cm^3 flask was charged with typical substrates and molecular sieves (3g) and was stirred for 3 days at ambient temperature to yield solids ((30); yield 40%), ((61); yield 64%), ((52); yield 41%), ((48); yield 15%), ((33); yield 51%), ((62); yield 32%), ((32); yield 45%), ((95); yield 28%), ((47); yield 42%).

7.5.0.4 The Effect of Varying the Mass of Pelleted Molecular Sieve

Typical substrates (except for molecular sieves) were charged to each of five flasks containing 0.5, 1.0, 3, 6, 9g of 4Å molecular sieve 1/16th inch pellets respectively, and were then stirred for 3 days at ambient temperature. The reactions were terminated by filtration and the products were recovered. (9g sieves (34); yield not recorded); (6g sieves (35); yield 51%); (3g sieves (36); yield 45%); (1.0g sieves; no yield); (0.5g sieves (37); yield 45%).

Typical substrates (except for molecular sieve) were charged to each of four flasks containing 1, 3, 6, 9g of 4Å molecular sieve 1/16th inch pellets respectively, and were each stirred for 4 days at ambient temperature. (9g sieves (38); yield 1%); (6g sieves (39); yield 25%); (3g sieves (40); yield 6%); (1.0g sieves (41); yield 24%).

7.5.0.5 The Effect of Intimate Mixing Between PPL and Molecular Sieve

A 150cm^3 two-vessel flask with a porous sinter connection was charged with typical substrates (except for molecular sieve) in one vessel, whilst molecular sieve (6g) was placed in the other vessel. The vessel containing the enzyme was stirred for 3 days at ambient temperature. ((42); yield 18%)

7.5.0.6 The Effect of Varying the Molecular Sieve Porosity

Two flasks each containing typical substrates, but 3Å molecular sieve pellets (3g) and 4Å molecular sieve pellets (3g) respectively, were stirred for 3 days at ambient temperature. The two reactions were terminated by filtration and the products were recovered. (3Å molecular sieves (43); yield 40%; 4Å molecular sieves (29); yield 35%).

7.5.0.7 The Effect of Changing the Physical Form of the Molecular Sieve

Two flasks each containing typical substrates but with 1g and 3g of macerated 4Å molecular sieve respectively, were stirred for 3 days at ambient temperature. (The 4Å molecular sieves were macerated using a pestle and mortar and then treated under the same conditions as the molecular sieve pellets before use in the reactions.) (3g sieves yielded no product, 1g of sieves (44); yield 14%).

A flask containing typical substrates but with 3g of ground up 4Å molecular sieve powder was stirred for 17 hours at ambient temperature, but yielded no product.

7.5.0.8 The Effect of Varying the Type of Drying Agent

Two flasks were each filled with typical substrates, but without the 4Å molecular sieves (3g). One was charged with oven dried magnesium sulphate (10.8g) plus 4Å molecular sieves (6.2g) and the other was charged with oven dried NaCl (7.5g). Both flasks were stirred for 3 days at ambient temperature and then terminated by filtration. The products were recovered and yielded solids ((45); yield 25%) and ((46); yield 33%).

A flask was charged with typical substrates and oven dried sodium metabisulphite (3.7g), and was stirred for 3 days at ambient temperature, and yielded no product.

7.5.1 The Effect of Varying Reaction Temperature

At Ambient

Two flasks charged with typical substrates were stirred at ambient temperature for 3 days. The reactions yielded solid products ((47); yield 48%) and ((48); yield 16%).

At 27°C

Typical substrates (in the same reflux setup as above) were stirred at 27°C for 3 days in a water bath. The reaction yielded solid product ((49); yield 65%).

At Reflux

A three-necked 50cm^3 flask was fitted with a thermometer and an argon blanket which was applied to the reaction through the top of the water cooled condenser. The same substrates as detailed in the typical reaction were added, heated and stirred in a water bath until the contents were at reflux. Two reactions of this type were carried out; one for 8 hours with no molecular sieve which yielded no product, and the other for 48 hours with oven dried 4Å molecular sieves (3g) ((50); yield 50%).

7.5.2 The Effect of Using Denatured PPL

A sample of crude PPL was denatured by heating at 65°C for 14 days and then it was stored under dry conditions for a period of 14 days. A 100cm^3 flask containing typical substrates was charged with the denatured PPL (2.5g) in place of the usual PPL, and was stirred for 2 days at ambient temperature. It yielded no solid product.

7.5.2.1 Control reactions

A 100cm^3 flask was charged with typical substrates but no molecular sieves or PPL, and was subjected to UV radiation from a UV lamp (wavelength range 100-350nm) and then stirred for 120 days at ambient temperature. A very small amount of white material was produced.

A 100cm^3 flask was charged with typical substrates (except for PPL) and stirred for 3 days at ambient temperature yielding no solid product.

7.5.3 The Effect of Increasing Substrate Concentration

A 100cm^3 flask was charged with typical substrates but with only 25cm^3 diethyl ether and stirred for 3 days at ambient temperature which yielded a solid product ((51); yield 38%).

A 100cm^3 flask was charged with typical substrates and was stirred for 3 days at ambient temperature to yield a solid ((52); yield 41%).

A 100cm^3 flask was charged with typical substrates but with divinyl adipate (9.9g, 50mmol) and 1,4-butanediol (4.5g, 50mmol) and were stirred for 3 days at ambient temperature which yielded no solid product (53).

A 100cm^3 flask was charged with 1,4-butanediol (1.8g, 20mmol), divinyl adipate (3.96g, 20mmol), 2.5g of PPL, 4Å molecular sieves (3g) and diethyl ether (50cm^3). It was stirred for six days at ambient temperature. The reaction yielded no solid product (54).

A 100cm^3 flask was charged with typical substrates (except for molecular sieves) and was stirred for three days at ambient temperature, at which point more 1,4-butanediol (0.9g, 10mmol) and divinyl adipate (1.98g, 10 mmol) were added to the flask and stirring was continued for a further 3 days at ambient temperature. A solid product was recovered ((55); yield 64%).

The above reaction was repeated in the presence of molecular sieves yielding a solid ((56); yield 15%).

7.5.3.1 PPL-Catalysed Polymer Modification in Diethyl Ether
Separate reactions were carried out with these two polymer types, reacting each with the corresponding substrate; only 1,4-butanediol was added to a reaction with ester-terminated polymer (57) (0.5g) and likewise only divinyl adipate was added into another reaction with hydroxy-terminated polymer (59) (0.5g). The reactions were stirred for 48hrs at ambient temperature after which the polymer was recovered in the usual way. The polymer (58) recovered (0.86g, 61%) from the original ester-terminated polymer (57), and the polymer (60) recovered (yield 0.72g, 29%) from the original hydroxy-terminated polymer (59) were analysed by GPC.

7.5.4 The Effect of Varying the Degree of Agitation

A 100cm^3 flask was carefully charged with typical substrates diol, adipate, solvent, molecular sieves (without the usual swirling) followed

by addition of the enzyme, and was stirred at ambient temperature for 3 days. The product recovered was a solid ((61); yield 64%).

A basic lobed propeller powered by an overhead mechanical stirrer in a permanent direction was air sealed to a 3 necked 50cm^3 round bottomed flask containing typical substrates which were stirred vigorously at ambient temperature for 3 days yielding a solid ((62); yield 32%).

7.6 THE EFFECT OF CHANGING REACTION SOLVENT ON THE PPL-CATALYSED POLYTRANSESTERIFICATION OF DIVINYL ADIPATE WITH 1,4-BUTANEDIOL

7.6.1 The Effect of Using Alternative Ether Solvents

7.6.1.1 Diisopropyl Ether
A 100cm^3 flask was charged with typical substrates and diisopropyl ether (distilled and dried over sodium wire) in place of diethyl ether, and was stirred for 3 days at ambient temperature. The product recovered was a solid ((63); yield 6.5%).

7.6.1.2 *tert*-Butyl Methyl Ether
Two 100cm^3 flasks were charged with typical substrates and *tert*-butyl methyl ether (distilled and dried over sodium wire) in place of diethyl ether. Both were stirred at ambient temperature; one for 4 days and the other for 3 days. The products recovered were solids (3 days (64); yield 51%; 4 days (65); yield 26%).

7.6.1.3 Ethylene Glycol Diethyl Ether
Two 100cm^3 flasks were each charged with typical substrates and ethylene glycol diethyl ether (distilled over CaH$_2$ (50cm^3)) in place of diethyl ether, and were stirred at ambient temperature for 3 days. No product was recovered from either reaction.

7.6.1.4 1-*tert*-Butoxy-2-methoxyethane
Two 100cm^3 flasks were each charged with typical substrates and 1-*tert*-butoxy-2-methoxyethane (distilled from CaH$_2$) (50cm^3) in place of diethyl ether. There was a solubility of the 1,4-butanediol in this solvent

of 50%. One was stirred at ambient temperature for 3 days and the other for 5 days without molecular sieves. No products were recovered from the reactions.

7.6.2 The Effect of Using High Boiling Ethers at an Elevated Temperature

The following ether solvents were tested at an elevated temperature in a 3 necked 100cm^3 flask and standard reflux set-up along with the typical substrates. The flask was submerged in a silicone oil filled jacketed beaker connected to a thermostatically controlled bath. The bath temperature was set to 43°C and was calibrated to ±1.0°C. The jacketed beaker was mounted on a magnetic stirrer and the reaction contents stirred for a set period of time.

7.6.2.1 Diisopropyl ether (bp 68-69°C)

A 100cm^3 flask was charged with typical substrates and diisopropyl ether (distilled and dried over sodium wire (50cm^3)) in place of diethyl ether and was stirred for 3 days at 43°C in the silicone oil filled jacketed beaker. The product recovered was a solid ((66); yield 6.5%).

7.6.2.2 *tert*-Butyl Methyl Ether (bp 55-56°C)

Two reactions with typical substrates and *tert*-butyl methyl ether (distilled and dried over sodium wire (50cm^3)) in place of diethyl ether, were stirred in the silicone oil bath at 43°C; one for 3 days with an argon purge and the other for 3 days without purge. The products recovered were solids (3 days with purge (67); yield 56%); (3 days (no purge) (68); yield 38%).

The Effect of Varying Substrate Concentration

A three necked 50cm^3 flask was fitted with a thermometer, and an argon blanket which was applied to the reaction through the top of the water cooled reflux condenser. The flask was charged with typical substrates but 4Å molecular sieves (6g) and *tert*-butyl methyl ether (50cm^3) (distilled from sodium wire) in place of diethyl ether. The reaction was heated in the silicone oil bath set at 43°C. A reaction with the above substrates was stirred for 24 hours ((69); yield 52%).

Three additional reactions were carried out as described above but with varying reagent concentrations (5mmol of divinyl adipate and 1,4-butanediol 24 hours (70); yield 22%) (3mmol of divinyl adipate and 1,4-butanediol 72 hours (71); yield 9%) (1mmol of divinyl adipate and 1,4-butanediol 24hours, yield very small).

7.6.3 The Effects of Using Hydrocarbon Solvents

7.6.3.1 Hexane

A 100cm^3 flask was charged with typical substrates but with 4Å molecular sieves (6g) and hexane SLR (50cm^3)) in place of diethyl ether and was stirred in a water bath at 35°C for 3 days. No solid product was recovered.

7.6.3.2 Heptane

A 100cm^3 flask was charged with typical substrates but HPLC heptane (50cm^3) in place of diethyl ether, and was stirred at ambient temperature for one day when additional PPL was added (2.5g). After 3 days the reaction yielded a semi-solid.

7.6.4 The Effects of Using Polymer Solubilising Solvents

7.6.4.1 THF

A typical reaction involved 1,4-butanediol (0.90g, 10 mmol) and divinyl adipate (1.98g, 10 mmol) which were weighed directly into a 100cm^3 round bottomed flask. HPLC grade THF (50cm^3) was then added to the flask. Finally, a weighed amount of the vacuum desiccated PPL (2.5g) was added promptly and the contents swirled. A series of these reactions were carried out (reactions 72-76). The flasks were stoppered, lagged and the contents stirred at ambient temperature for fixed lengths of time before they were terminated by filtration and the products recovered.

The above typical reaction was carried out but in the presence of the molecular sieves. (Hot dried 4Å molecular sieves (3g) were removed from the oven and allowed to cool over phosphorous pentoxide in a desiccator before they were added to the reaction flask.) Finally, a

weighed amount of the vacuum desiccated PPL (2.5g) was added promptly and the contents swirled. A series of these reactions were carried out (**reactions 77-80**). The flasks were stoppered, lagged and the contents stirred at ambient temperature for fixed lengths of time before they were terminated by filtration and the products recovered.

7.6.4.2 Dimethyl Isosorbide (DMI)

Typical substrates and untreated dimethyl isosorbide (DMI), in place of diethyl ether, were stirred at ambient temperature for 3 days. The reaction yielded no apparent product.

Typical substrates and DMI (dried over $MgSO_4$) ($50cm^3$) in place of diethyl ether were stirred at ambient temperature for 3 days. The reaction yielded no apparent product.

7.6.4.3 Dichloromethane

A $100cm^3$ flask was charged with typical substrates but HPLC dichloromethane ($50cm^3$) in place of diethyl ether, and was stirred at ambient temperature for 3 days to yield no solid product.

7.6.4.4 THF and Diethyl Ether Mixtures

Typical substrates and mixed distilled diethyl ether : THF HPLC grade solvent systems (total volume of $50cm^3$) were stirred at ambient temperature. Chosen ratios (diethyl ether : tetrahydrofuran) were:- 100% THF (4 hours, 0g and 3g of 4Å molecular sieves) yielding brown solids (8% and 5%); 50:50 (72 hours (**81**); 3g of 4Å molecular sieves) yielding a solid (22%) and 90:10 (72 hours (**82**); 3g of 4Å molecular sieves) yielding a solid (40%).

A $100cm^3$ flask was charged with 1,3-propanediol (0.76g, 10mmol), divinyl adipate (1.98g, 10mmol), distilled diethyl ether ($25cm^3$), tetrahydrofuran HPLC ($25cm^3$) (solvent ratio 50:50), PPL (2.5g) and 4Å molecular sieves (6g) and was stirred at ambient temperature for 2 days. Only a clear liquid was obtained which solidified into a gel on storage.

157

7.6.4.5 Chloroform

Typical substrates and distilled chloroform (50cm^3) in place of diethyl ether were stirred at ambient temperature for 3 days. No solid product was recovered.

7.6.4.6 Acetonitrile

A 100cm^3 flask was charged with typical substrates but HPLC acetonitrile (50cm^3) in place of diethyl ether, and was stirred at ambient temperature for 3 days to yield solid product ((83); yield 9%).

7.7 ENZYME MODIFICATION

7.7.1 The Effect of Enzyme Immobilisation on the Lipozyme IM-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol In Anhydrous Diethyl Ether

A 100cm^3 flask was charged with typical substrates (except molecular sieve), and Lipozyme IM™ (0.5g) in place of PPL, which were stirred for 4 hours at ambient temperature with an argon purge. The product recovered was a solid ((84); yield 75%).

A 100cm^3 flask was charged with typical substrates (except molecular sieve), and Lipozyme IM™ (0.5g) in place of PPL, which were stirred for 24 hours at ambient temperature. The product recovered was a solid ((85); yield 64%).

A 100cm^3 flask was charged with typical substrates (except molecular sieve), and Lipozyme IM™ (0.5g) in place of PPL, which were stirred for 48 hours at ambient temperature with argon purge. The product recovered was a solid ((86); yield 63%) which had a melting point of 40-80°C.

A 100cm^3 flask was charged with typical substrates (except molecular sieve), and Lipozyme IM™ (0.5g) in place of PPL, which were stirred for 96 hours at ambient temperature. The product recovered was a solid ((87); yield 70%) which had a melting point range of 20-68°C.

A 100cm³ flask was charged with typical substrates (except molecular sieve), and Lipozyme IM™ (0.5g) in place of PPL, which were stirred for 120 hours at ambient temperature. The product recovered was a solid ((88); yield 75%).

7.7.2 The Effect of Increasing Substrate Concentration

A 100cm³ flask was charged with 20mmol of the typical substrates (except molecular sieve), and Lipozyme IM™ (0.5g) in place of PPL which were stirred for 72 hours at ambient temperature. The product recovered was a solid ((89): yield 79%).

7.7.3 The Effect of Using Polymer Solubilising Solvent

7.7.3.1 THF
A 100cm³ flask was charged with typical substrates but HPLC grade THF (50cm³) in place of diethyl ether and Lipozyme IM™ (0.5g) in place of PPL, which were stirred for 6 hours at ambient temperature again yielding no product.

7.7.3.2 THF and Diethyl Ether Mixtures
A 100cm³ flask was charged with typical substrates, but HPLC grade THF (45cm³), distilled diethyl ether (5cm³) and Lipozyme IM™ (0.5g) which were stirred for 6 hours at ambient temperature. The product recovered was a solid ((90); yield 39%).

7.7.4 The Effect of Using Hydrocarbon Solvents

7.7.4.1 Toluene
A three-necked 50cm³ flask was charged with typical substrates but no molecular sieves, distilled toluene (50cm³) in place of diethyl ether, Lipozyme IM™ (0.5g) in place of PPL. The flask was stirred for 7 days at ambient temperature yielding a very small amount of solid (8%).

7.7.4.2 Hexane

A three-necked 50cm^3 flask was charged with approximate amounts of typical substrates but no molecular sieves, distilled hexane (50cm^3) in place of diethyl ether and Lipozyme IM™ (0.5g) in place of PPL, and were stirred for a few days at ambient temperature yielding a semi-solid (69%).

7.7.5 The Effect of Employing High Boiling Ethers

7.7.5.1 Ethylene glycol diethylether

A 100cm^3 flask was charged with typical substrates but distilled Ethylene glycol diethylether (dried over MgSO$_4$ and sodium wire) in place of diethyl ether and Lipozyme IM™ (0.5g) in place of PPL which were stirred for 5 days at ambient temperature. The product recovered was a solid ((91); yield 22%).

7.7.5.2 Diisopropyl ether

A three-necked 50cm^3 flask was charged with typical substrates but diisopropyl ether (distilled and dried over sodium wire) in place of diethyl ether and Lipozyme IM™ (0.5g) in place of PPL which were stirred for 2 days at ambient temperature. The product recovered was a solid mp 42-46°C ((92); yield 63%).

7.7.5.3 Diglyme

A 100cm^3 flask was charged with typical substrates, but with 0.5g of Lipozyme™ in place of PPL and sodium wire dried diglyme (50cm^3) in place of diethyl ether. The flask was stirred at ambient temperature for 24 hours (with 4Å molecular sieves (6g)) and 96 hours (with 4Å molecular sieves (3g)). Product recovery resulted in liquids in both cases.

7.7.6 The Effect of Immobilising PPL on Silica on the Lipase-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol

A 100cm^3 flask was charged with typical substrates but no molecular sieves and PPL/silica mix (1.5g) ($\equiv$0.25g of PPL) in place of PPL which

were stirred for 3 days at ambient temperature and yielded no solid product.

A 100cm^3 flask was charged with typical substrates. Oven dried silica (2.5g) was also added and the reaction was stirred for 3 days at ambient temperature. The reaction yielded solid product ((93); yield 28%). A control reaction with the enzyme absent was carried out alongside and it yielded no solid product.

7.7.7 The Effect of Water Content on the PPL-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol

PPL (3.0g) was suspended in buffer (20cm^3) and then freeze-dried to yield 3.67g of preparation. The dry material was assayed for lipase activity before being used in an organic polytransesterification reaction.

A 100cm^3 flask was charged with typical substrates but with 1,3-propanediol (0.76g, 10mmol) and freeze-dried PPL with buffer (3.67g) in place of desiccated PPL. They were stirred at ambient temperature for 3 days. The reaction yielded solid ((94); yield 42%). This reaction was run alongside a control which involved typical reactants and vacuum desiccated PPL. The reaction yielded a solid ((95); yield 28%).

Analysis of the water absorbancy of the desiccated PPL was determined by weight gain. An open vial containing a pre-weighed amount of desiccated PPL (0.9495g) was exposed to the atmosphere for 3 hours and then re-weighed.

7.7.8 Determination of the Substrate Water Content by Karl Fischer Titration.

Karl Fischer water content determinations were made on 1,4-butanediol, divinyl adipate, diethyl ether (distilled from sodium wire) and PPL. Molecular sieve (3Å) dried ethanol was the medium used to absorb water from PPL which was subsequently analysed to determine the water content in the PPL.

7.7.9 PPL-Catalysed Polymer Degradation In Aqueous Media

A sample of poly(1,4-butylene adipate) from a previous enzyme-catalysed reaction (69) was placed in situ with PPL (0.5g) in phosphate buffer (pH 7.2) (20cm^3) for a period of 2 months at ambient temperature with periodic shaking. The polymer was filtered and the contents washed with water (96).

7.7.10 Separation of Crude PPL into Soluble and Insoluble Fractions

Crude PPL (7.5g) was suspended in 500cm^3 of distilled water to give a concentration of 15mg/cm^3. The suspension was centrifuged and the supernatant filtered through Whatman no.1 filter paper. The insoluble fraction was repeatedly washed with distilled water to remove any soluble material. The insoluble fraction was re-suspended in distilled water. Both the supernatant and the re-suspended mixtures were freeze-dried. Lipase activity tests of the two fractions and crude PPL were carried out. The protein content of the two fractions was also determined.

7.7.10.1 Use of Soluble and Insoluble PPL Fractions in Organic Polytransesterification Reactions

A 100cm^3 flask was charged with typical substrates but no molecular sieves or PPL. Freeze-dried insoluble material (0.25g) was added to the flask which was stirred at ambient temperature for 48 hours yielding

a solid ((97); yield 47%). The reaction was repeated to yield a solid ((98); yield 49%).

A 100cm^3 flask was charged with typical substrates but no molecular sieves or PPL. Freeze-dried soluble material (0.25g) was added to the flask which was stirred at ambient temperature for 48 hours. The reaction yielded no solids. The same reaction was repeated, but again it yielded no solid material.

A 100cm^3 flask was charged with typical substrates but no molecular sieves or PPL. Freeze-dried soluble material (2.5g) was added to the flask which was stirred at ambient temperature for 48 hours. The reaction yielded no solid.

7.7.10.2 Use of Soluble PPL Fraction in Organic Polytransesterification Reactions in the Presence of Silica

A 100cm^3 flask was charged with typical substrates and silica gel (1.0g) but no molecular sieves or PPL. Freeze-dried soluble material (0.25g) was added to the flask which was stirred at ambient temperature for 48 hours. The reaction yielded no solid.

Soluble fraction (2.5g) was mixed with TLC grade silica (12.5g) and buffer added to make a paste which was spread on a watch glass and dried in a vacuum desiccator over phosphorous pentoxide for 7 days. The silica supported preparation (1.5g ≡0.25g soluble fraction) was added to a 100cm^3 flask along with typical substrates but no PPL or molecular sieves, and was stirred at ambient temperature for 3 days, but yielded no solid product.

7.7.11 The Effects of Using Proteolytic Enzymes on the Enzyme-Catalysed Polytransesterification of Divinyl Adipate with 1,4-Butanediol in Anhydrous Diethyl Ether

7.7.11.1 Trypsin

A 100cm^3 flask was charged with typical substrates but no molecular sieves and trypsin (2.5g) in place of PPL which were stirred for 48 hours at ambient temperature and yielded no solid product. This reaction

163

was repeated again but with HPLC grade THF (50cm^3) in place of diethyl ether, but no solid product was obtained.

7.7.11.2 Silica Supported Trypsin

Trypsin enzyme (2.5g) was added to TLC grade silica (12.5g) and mixed thoroughly. Sufficient phosphate buffer (pH 7.2) was added to make a paste which was then spread onto a watchglass and placed into a desiccator over phosphorous pentoxide and under vacuum for 7 days. This same procedure was also carried out for pancreatin and for PPL.

A 100cm^3 flask was charged with typical substrates but no molecular sieves and trypsin/silica mix (1.5g) ($\equiv$0.25g of trypsin) in place of PPL which were stirred for 3 days at ambient temperature and yielded no solid product.

7.7.11.3 Pancreatin

A 100cm^3 flask was charged with typical substrates but no molecular sieves and pancreatin (2.5g) in place of PPL which were stirred for 48 hours at ambient temperature and yielded no solid product.

7.7.11.4 Silica Supported Pancreatin

A 100cm^3 flask was charged with typical substrates but no molecular sieves and pancreatin/silica mix (1.5g$\equiv$0.25g of pancreatin) (see silica supported trypsin) in place of PPL which were stirred for 3 days at ambient temperature and yielded no solid product.

7.7.12 Extraction of Sucrose from Crude PPL

7.7.12.1 Using Anhydrous Pyridine

Crude PPL (3.0g) and anhydrous pyridine (50cm^3) were placed into a round bottomed flask along with a magnetic flea and stirred for 24hrs at 15°C (99). The contents were filtered under vacuum and the residue was washed under an argon blanket with additional anhydrous pyridine (3x10cm^3 portions). The residue was suction dried for 10 minutes and then placed in an open container inside a desiccator and refrigerated overnight. The desiccator was removed and then placed under

constant vacuum until the contents reached ambient temperature. The procedure of refrigeration followed by the application of a vacuum until the contents reached ambient temperature was repeated daily for 7 days. A sample of the sucrose-free enzyme was tested for lipase activity. The remainder was employed in an organic polytransesterification reaction along with typical substrates. The reaction was stirred at ambient temperature for 48 hours, but yielded no solid.

All the above procedure was repeated with another batch of crude PPL. The resultant pyridine extracted crude PPL (100) was later employed in a polytransesterification reaction, but failed to yield product.

7.7.12.2 Removal of Sucrose from Crude PPL by Dialysis in Phosphate Buffer

An enzyme solution (crude PPL (2.5g) in 20cm^3 0.2M pH 7.2 Buffer) was placed inside a knotted length of Visking tubing which had been allowed to soak in buffer for 10 minutes. The tubing was filled, tied, submerged into a 600cm^3 beaker containing buffer, covered and then placed in the fridge at 5°C. After about 2hrs the external buffer was sampled and analysed by HPLC against a 1% sucrose standard and a buffer blank. The external buffer was replaced by fresh buffer. This was repeated until the HPLC showed the absence of sugar in the samples. Three dialyses were carried out - one for 3 hours (101), another for 20 hours (102) and another for 9 days (103).

Once the HPLC analysis indicated the absence of sucrose in the external buffer for each of the dialyses, the Visking tube contents were emptied into separate containers and then freeze-dried.

All the resulting freeze-dried enzymes were tested for lipase activity by the 'Lipase Olive Oil Assay' using Bromothymol Blue indicator (transition interval pH 6.0 (yellow) to pH 7.6 (blue)). The set of three freeze-dried buffer dialysed PPL materials (0.39g (101), 0.47g (102) and 0.28g (103)) were then each employed in an organic polytransesterification reaction and were added separately to each of three 100cm^3 flasks charged with typical substrates (except for PPL).

The reactions were stirred at ambient temperature for 72 hours. The reactions failed to yield polymer.

REFERENCES

167

1. T. Palmer, "Understanding Enzymes", 3rd ed., Ellis Horwood, London, (1991).

2. M. Dixon and E. C. Webb, "Enzymes", 3rd ed., Longman Group Limited, London, (1979).

3. H. S. Mosher and J. D. Morrison, "Current status of asymmetric synthesis", *Science*, **221(4615)**, 1013-1019, (1983).

4. W. A. Konig, W. Francke and I. Benecke, "Gas chromatographic enantiomer separation of chiral alcohols", *J. Chromatogr.*, **239**, 227-231, (1982).

5. K. Faber, "Biotransformations in Organic Chemistry", 2nd ed., Springer Verlag Berlin Heidelberg, (1995).

6. A. M. P. Koskinen and A. M. Klibanov, "Enzymatic Reactions in Organic Media", 1st ed., Chapman and Hall, London, (1996).

7. H. L. Brockman, "Triglyceride Lipase from Porcine Pancreas-Triacylglycerol Hydrolase", *Methods in Enzymology*, Academic Press, **71**, 619-627, (1981).

8. P. Desnuelle and P. D. Boyer, "The Enzymes", **7**, 3rd ed., Academic Press, New York, (1972).

9. T. Godfrey and J. Reichelt, "Industrial Enzymology - The Application of Enzymes in Industry", Nature Press, London, (1983).

10. H. Brockerhoff and R. G. Jensen, "Lipolitic Enzymes", Academic Press, New York, (1974).

11. C. Chapus and M. Sémériva, "Mechanism of Pancreatic Lipase Action. 2. Catalytic Properties of Modified Lipases", *Biochemistry*, **15**, 4988-4991, (1976).

12. L. Sarda and P. Desnuelle, "Action de la lipase Pancréatique sur les esters en émulsion", *Biochim. Biophys. Acta*, **30**, 513-521, (1958).

13. W. A. Pieterson, J. C. Vidal, J. J. Volwerk and G. H. D. Haas, "Zymogen-Catalysed Hydrolysis of Monomeric Substrates and The Presence of a Recognition Site for Lipid-Water Interfaces in Phospholipase A$_2$", *Biochemistry*, **13**, 1455-1459, (1974).

14. R. Verger, M. C. E. Mieras and G. H. De Haas, "Action of Phospholipase A at Interfaces", *J. Biol. Chem.*, **248**, 4023-4034, (1973).

15. A. G. Marangoni, "Enzyme Kinetics of Lipolysis Revisited: The Role of Lipase Interfacial Binding", *Biochem. Biophys. Res. Commun.*, **200(3)**, 1321-1328, (1994).

16. Z. S. Derewenda and A. M. Sharp, "News from the interface - the molecular structures of triglyceride lipases", *Trends Biol. Sci.*, **18(1)**, 20-25, (1993).

17. "Enzymes for Biochemistry, Analytical Chemistry, Molecular Biology, Organic Synthesis", Fluka BioChemica, 2nd ed., (1994).

18. Swiss Protein Database, "lipase, pancreatic",P00591, (1997).

19. J. D. De Caro , M. Boudouard, J. J. Bonicel, A. A. Guidoni, P. Desnuelle and M. Rovery, "Porcine pancreatic lipase - completion of the primary structure", *Biochim. Biophys. Acta*, **671**,129-138, (1981).

20. J. D. Bianchetta, J. Bidaud, A. A. Guidoni, J. J. Bonicel and M. Rovery, "Porcine Pancreatic Lipase - sequence of the first 234 amino acids of the peptide chain", *Eur. J. Biochem.* , **97**, 395-405, (1979).

21. A. Guidoni, J. Bonicel, J. Bianchetta and M. Rovery, "Porcine pancreatic lipase. Sequence between the 235th and 307th amino acids", *Biochimie* , **61**, 841-845, (1979).

22. S. H. Pine, "Organic Chemistry", McGraw-Hill International Editions, (5th ed.), 846-7, (1987).

23. A. M. Klibanov, "Enzymes that work in organic solvents", *Chemtech.*, **16**, 354-359, (1986).

24. F. G. Mann and B. C. Saunders, "Practical Organic Chemistry", 4th ed., Longman Group Ltd., London, (1960).

25. P. M. Bailie, S. E. McNerlan, E. Robinson and W. R. Murphy, "The Immobilisation of Lipases for the Hydrolysis of Fats and Oils", *Trans IChemE Part C*, **73**, 71-76, (1995).

26. W. -R. Sheih, D. -M. Gou and C. -S. Chen, "Computer aided substrate design for biocatalysis - an enzymatic access to optically active propranolol", *J. Chem. Soc., Chem. Commun.*, (9), 651-653, (1991).

27. F. Boreix, F. Monot and J. -P. Vandecasteele, "Strategies for enzymatic esterification in organic solvents: Comparison of microaqueous, biphasic and micellar systems", *Enzyme Microb. Technol.*, **14**, 791-797, (1992).

28. L. Cantarella, F. Alfani and M. Cantarella, "Stability and activity of immobilised hydrolytic enzymes in two-liquid-phase systems: acid phosphatase, β-glucosidase and β-fructofuranosidase entrapped in poly(2-hydroxyethylmethacrylate) matrices", *Enzyme Microb. Technol.*, **15**, 861-867, (1993).

29. B. Cambou and A. M. Klibanov, "Production of optically-active esters and alcohols using esterase-catalysed stereospecific transesterification in organic media", *J. Am. Chem. Soc.*, **106**(9), 2687-2692, (1984).

30. A. M. Klibanov, G. P. Samokhin, K. Martinek and I. V. Berezin, "A new approach to preparative enzymatic synthesis", *Biotechnol. Bioeng.*, **19**, 1351-1361, (1977).

31. A. M. Klibanov, "Asymmetric transformations catalysed by enzymes in organic solvents", *Acc. Chem. Res.*, **23**, 114-120, (1990).

32. A. M. Klibanov, "Enzymatic catalysis in anhydrous organic solvents", *Trends Biol. Sci.*, **14**, 141-144, (1989).

33. J. S. Dordick, "Enzymatic catalysis in monophasic organic solvents", *Enzyme Microb. Technol.*, **11**, 194-211, (1989).

34. E. Santaniello, P. Ferraboschi, P. Grisenti and A. Manzocchi, "The biocatalytic approach to the preparation of enantiomerically pure chiral building blocks", *Chem. Rev.*, **92**, 1071-1140, (1992).

35. A. Zaks and A. M. Klibanov, "Enzymatic Catalysis in Non-Aqueous Media", *J. Biol. Chem.*, **263**, 3194-3201, (1988).

36. A. Zaks and A. M. Klibanov, "The Effect of Water on Enzyme Action in Organic Media", *J. Biol. Chem.*, **263**, 8017-8021, (1988).

37. A. J. Russell and A. Zaks, "Enzymes in Organic Solvents: Properties and Applications.", *J. Biotech.*, **8**, 259-270, (1988).

38. R. F. Kazandjian, J. S. Dordick and A. M. Klibanov, "Enzymatic Analyses in Organic Solvents", *Biotechnol. Bioeng.*, **28**, 417-421, (1986).

39. A. Zaks and A. M. Klibanov, "Enzyme Catalysed processes in organic solvents", *Proc. Natl. Acad. Sci. USA*, **82**, 3192-3196, (1985).

40. G. Langrand, M. Secchi, G. Buono, J. Baratti and C. Triantaphylides, "Lipase-Catalysed Ester Formation in Organic Solvents. An Easy Preparative Resolution of Substituted Cyclohexanols", *Tetrahedron Lett.*, **26(15)**, 1857-1860, (1985).

41. B. Cambou and A. M. Klibanov, "Comparison of Different Strategies for the Lipase-Catalysed Preparative Resolution of Racemic Acids and Alcohols: Asymmetric Hydrolysis, Esterification, and Transesterification", *Biotechnol. Bioeng.*, **26**, 1449-1454, (1984).

42. M. Degueil-Castaing, B. D. Jeso, S. Drouillard and B. Maillard, "Enzymatic Reactions in Organic Synthesis: 2-ester Interchange of vinyl esters", *Tetrahedron Lett.*, **28(9)**, 953-954, (1987).

43. J. B. Jones, "Enzymes in Organic Synthesis", *Tetrahedron*, **42(13)**, 3351-3403, (1986).

44. J. S. Wallace, B. W. Baldwin and C. J. Morrow, "Separation of Remote Diol and Triol Stereoisomers by Enzyme Catalysed Esterification in Organic Media or Hydrolysis in Aqueous Media", *J. Org. Chem.*, **57**, 5231-5239, (1992).

45. J. S. Deetz and J. D. Rozzell, "Enzyme-Catalysed Reactions in Non-Aqueous Media", *Trends Biotechnol.*, **6**, 15-19, (1988).

46. A. Gubelman, "Bioprocess production of flavour, fragrance and colour ingredients", Elsevier, New York, (1994).

47. G. A. Hills, A. R. Macrae and R. R. Poulina, "Ester Preparation", (EP 0 383 405 A1/1990).

48. J. H. Atherton, "Methods for Study of Reaction Mechanisms in Liquid/Liquid and Liquid Solid Reaction Systems and their Relevance to the Development of Fine Chemical Processes", *Trans IChemE Part A*, **71**, 111-118, (1993).

49. P. L. Dubin, P. A. Fitzpatrick, A. M. Klibanov and A. L. Margolin, "Chemoenzymatic Synthesis of Optically Active (Meth)acrylic Polymers", *J. Am. Chem. Soc.*, **113(12)**, 4693-4694, (1991).

50. C. Laane, S. Boeren, K. Vos and C. Veeger, "Rules for Optimisation of Biocatalysis in Organic Solvents", *Biotechnol. Bioeng.*, **30**, 81-87, (1987).

51. P. Aldercreutz, "Activation of Enzymes in organic media at low water activity by polyols and saccharides", *Biochim. Biophys. Acta*, **1163**, 144-148, (1993).

52. K. Dabulis and A. M. Klibanov, "Dramatic enhancement of enzymatic activity in organic solvents by lyoprotectants", *Biotechnol. Bioeng.*, **41**, 566-571, (1993).

53. N. Khalaf, C. P. Govardhan, J. J. Lalonde, R. A. Persichetti, Y. -F. Wang and A. L. Margolin, "Cross-linked enzyme crystals as highly active catalysts in organic solvents", *J. Am. Chem. Soc.*, **118**, 5494-5495, (1996).

54. M. Otamiri, P. Aldercreutz and B. Mattiasson, "A differential scanning calorimetric study of chymotrypsin in the presence of added polymers", *Biotechnol. Bioeng.*, **44**, 73-78, (1994).

55. L. A. S. Gorman and J. S. Dordick, "Organic Solvents Strip Water Off Enzymes", *Biotechnol. Bioeng.*, **39**, 392-397, (1992).

56. A. Zaks and A. M. Klibanov, "Enzymatic Catalysis in Organic Media at 100°C", *Science*, **224**, 1249-1251, (1984).

57. J. Otera, "Transesterification", *Chem. Rev.*, **93**, 1449-1470, (1993).

58. F. Binns, S. M. Roberts, A. Taylor and C. F. Williams, "Enzymic Polymerisation of an Unactivated Diol/Acid System, *J. Chem. Soc., Perkin Trans. 1*, 899-904, (1993).

59. D. O'Hagan and N. A. Zaidi, "Polymerisation of 10-hydroxydecanoic acid with the lipase from *Candida cylindracea*", *J. Chem. Soc., Perkin Trans.1*, 2389-2390, (1993).

60. J. A. K. Howard, D. O'Hagan, N. A. Pitchford and N. A. Zaidi, "The solid state structure of the 34-membered macrocyclic diolide of 16-hydroxyhexadecanoic acid, formed by porcine pancreatic lipase mediated cyclisation in hexane", *J. Chem. Res. (M)*, 2601-2606, (1996).

61. D. O'Hagan and N. A. Zaidi, "Enzyme-Catalysed condensation of 11-hydroxyundecanoic acid with lipase from *Candida cylindracea*", *Polymer*, **35(16)**, 3576-3578, (1994).

62. P. A. Fitzpatrick and A. M. Klibanov, "How can the solvent affect enzyme enantioselectivity?", *J. Am. Chem. Soc.*, **113**, 3166-3171, (1991).

63.   Y. L. Khmelnitsky, A. V. Levashov, N. L. Klyachko and K. Martinek, "Engineering biocatalytic systems in organic media with low water content", *Enzyme Microb. Technol.*, **10**, 710-724, (1988).

64.   C. R. Westcott and A. M. Klibanov, "The solvent dependence of enzyme specificity", *Biochim. Biophys. Acta*, **1206**, 1-9, (1994).

65.   C. Laane, J. Tramper and M. D. Lilly (eds.), "Biocatalysis in organic media", Elsevier, Amsterdam, (1987).

66.   D. S. Clark, J. S. Dordick, Y. L. Khmelnitsky and S. H. Welch, "Salts dramatically enhance activity of enzymes suspended in organic solvents", *J. Am. Chem. Soc.*, **116**, 2647-2648, (1994).

67.   M. Harnisch, H. J. Möckel and G. Schulze, "Relationship between log P_{OW} shake flask values and capacity factors derived from reversed-phase high-performance liquid chromatography for *n*-alkylbenzenes and some OECD reference substances", *J. Chromatogr.*, **282**, Dec, 315-332, (1983).

68.   S. Fukui and A. Tanaka, "Enzymatic reactions in organic solvents", *Endeavour, New Series*, **9**(1), 10-17, (1985).

69.   A. M. Klibanov, "Immobilised Enzymes and Cells as Practical Catalysts", *Science*, **219**, 722-727, (1983).

70.   B. K. Samra, "The Use of Enzyme Catalysts in Organic Media for the Synthesis of Biodegradable Polyesters", Ph.D. Thesis, The University of Aston in Birmingham, (1996).

71.   A. W. P. Jarvie, B. K. Samra and A. J. Wiggett, "The Effect of a Molecular Sieve on the Enzyme-Catalysed Polytransesterification of Alkanedioates with Butane-1,4-diol in Organic Media", *J. Chem. Res (S)*, 129, (1996) and *J. Chem. Res (M)*, 0701-0715, (1996).

72.   A. J. Wiggett, "Enzyme-catalysed esterification in organic solvents using *Candida cylindracea* lipase", Final Year Project, (1994).

174

73. Z. -L. Wang, Y.-Y. Linko and J. Seppälä, "Lipase-catalysed polytransesterification in an organic solvent", *Biotechnology Techniques*, **9(5)**, 349-354, (1995).

74. T. Garcia, M. Martinez and J. Aracil, "Enzymatic synthesis of an analogue of jojoba oil: Optimization by statistical analysis", *Enzyme Microb. Technol.*, **15**, 607-611, (1993).

75. A. K Chaudhary, E. J. Beckman and A. J. Russell, "Rational Control of Polymer Molecular Weight and Dispersity during Enzyme-Catalysed Polyester Synthesis in Supercritical Fluids", *J. Am. Chem. Soc.*, **117**, 3728-3733, (1995).

76. M. Indlekofer, M. Funke, W. Claassen and M. Reuss, "Continuous enantioselective transesterification in organic solvents. Use of suspended lipase preparations in a microfiltration membrane reactor", *Biotechnol. Prog.*, **11**, 436-442, (1995).

77. S. K. Dalvie and R. E. Baltus, "Distribution of Immobilised Enzymes on Porous Membranes", *Biotechnol. Bioeng.*, **40**, 1173-1180, (1992).

78. F. Wagner, F. Kleppe, W. Lokotsch, A. Ziemann and S. Lang, "Synthesis of uncommon wax esters with immobilised lipases", *Enzyme Engineering XI, New York Academy of Sciences*, **672**, 484-491, (1992).

79. G. Winter and A. R. Fersht, "Engineering Enzymes", *Trends Biotechnol.*, **2(5)**, 115-119, (1984).

80. A. Mustranta, P. Forssell and K. Poutanen, "Applications of immobilized lipases to transesterification and esterification reactions in nonaqueous systems.", *Enzyme Microb. Technol.*, **15**, 133-139, (1993).

81. G. Kirchner, M. P. Scollar and A. M. Klibanov, "Resolution of Racemic Mixtures via Lipase Catalysis in Organic Solvents", *J. Am. Chem. Soc.*, **107**, 7072-7076, (1985).

82. G. Lagrand, J. Baratti, G. Buono and C. Triantaphylides, "Lipase Catalysed Reactions and Strategy for Alcohol Resolution", *Tetrahedron Lett.*, **27(1)**, 29-32, (1986).

83. E. Santaniello, P. Ferraboschi and P. Grisenti, "Lipase-catalysed transesterification in organic solvents: Applications to the preparation of enantiomerically pure compounds", *Enzyme Microb. Technology*, **15**, 367-379, (1993).

84. C. -H. Wong and G. M. Whitesides, "Enzymes in Synthetic Organic Chemistry", Tetrahedron Organic Chemistry Series, **12**, Elsevier Science Ltd., London, (1994).

85. J. S. Wallace, K. B. Reda, M. E. Williams and C. J. Morrow, "Resolution of a chiral ester by lipase-catalysed transesterifications with poly(ethylene glycol) in organic solvent", *J. Org. Chem.*, **55**, 3544-3546, (1990).

86. J. B. West, J. Scholten, N. J. Stolowich, J. L. Hogg, A. I. Scott and C. -H. Wong, "Modification of proteases to esterases for peptide synthesis - methylchymotrypsin", *J. Am. Chem. Soc.*, **110(11)**, 3709-3710, (1988).

87. H. M. Sweers and C. -H. Wong, "Enzyme-catalysed regioselective deacylation of protected sugars in carbohydrate synthesis", *J. Am. Chem. Soc.*, **108**, 6421-6422, (1986).

88. Y. -F. Wang and C. -H. Wong, "Lipase-catalysed irreversible transesterification for preparative synthesis of chiral glycerol derivatives", *J. Org. Chem.*, **53**, 3127-3129, (1988).

89. Y. -F. Wang, J. J. Lalonde, M. Momongan, D. E. Bergbreiter and C. -H. Wong, "Lipase-catalysed irreversible transesterifications using enol esters as acylating reagents: Preparative enantio- and regioselective syntheses of alcohols, glycerol derivatives, sugars and organometallics", *J. Am. Chem. Soc.*, **110**, 7200-7205, (1988).

90. K. Laumen, D. Breitgoff and M. P. Schneider, "Enzymic preparation of enantiomerically pure secondary alcohols. Ester synthesis by irreversible acyl transfer using a highly selective ester hydrolase from *Pseudomonas sp.*; an attractive alternative to ester hydrolysis", *J. Chem. Soc., Chem. Commun.*, 1459-1461, (1988).

91. H. Frykman, N. Ohrner,T. Norin and K. Hult, "S-ethyl thiooctanoate as acyl donor in lipase catalysed resolution of secondary alcohols", *Tetrahedron Lett.*, **34**, 1367-1370, (1993).

92. A. Ghogare and G. S. Kumar, "Oxime esters as novel irreversible acyl transfer agents for lipase catalysis in organic media", *J. Chem. Soc., Chem. Commun.*, 1533-1535, (1989).

93. V. Gotor and R. Pulido, "An improved procedure for regioselective acylation of carbohydrates - novel enzymatic acylation of α-D-glucopyranose and methyl α-D-glucopyranoside", *J. Chem. Soc., Perkin Trans.1*, **(2)**, 491-492, (1991).

94. M. Pozo, R. Pulido and V. Gotor, "Vinyl carbonates as novel alkoxycarbonylation reagents in enzymatic synthesis of carbonates", *Tetrahedron*, **48(31)**, 6477-6484, (1992).

95. T. Nishida, T. Nihira and Y. Yamada, "Facile Synthesis of Racemic 2-Hexyl-3-Hydroxy-4-Pentanolide(NFX-2) and its Optical Resolution", *Tetrahedron*, **47**, 6623-6634, (1991).

96. H. J. Bestmann and U. C. Philipp, "Enzymatic Synthesis of Chiral C_4 Building Blocks from *meso* -Tartaric Acid", *Angew. Chem., Int. Ed. Engl.*, **30**, 86-88, (1991).

97. B. Berger and K. Faber, "Immunisation of Lipase Against Acetaldehyde Emerging in Acyl Transfer Reactions from Vinyl Adipate", *J. Chem. Soc., Chem. Commun.*, 1198-1200, (1991).

98. K. A. Babiak, J. S. Ng, J. H. Dygos, C. L. Weyker, Y. -F. Wang and C. -H. Wong, "Lipase-catalysed irreversible transesterification using enol esters - resolution of Prostaglandin synthons 4-hydroxy-2-alkyl-2-cyclopentenones and inversion of the 4S enantiomer to the 4R enantiomer", *J. Org. Chem.*, **55(10)**, 3377-3381, (1990).

99. N. Ogata and S. Okamoto, "Synthesis of Polyesters from Active Diesters", *J. Polym. Sci., Polym. Chem. Ed.*, **11**, 2537-2545, (1973).

100. J. M. G. Cowie, "Polymers, Chemistry and Physics of Modern Materials", 2nd ed., Blackie, Glasgow, (1991).

101. J. S. Wallace and C. J. Morrow, "Biocatalytic Synthesis of Polymers II. Preparation of [AA-BB]x Polyesters by Porcine Pancreatic Lipase Catalysed Transesterification in Anhydrous, Low Polarity Organic Solvents", *J. Polym.Sci.; Part A: Polym. Chem.*, 27, 3271-3284, (1989).

102. J. S. Wallace and C. J. Morrow, "Biocatalytic Synthesis of Polymers. Synthesis of an Optically Active Epoxy-Substituted Polyester by Lipase-Catalysed Polymerisation.", *J. Polym.Sci.; Part A: Polym. Chem.*, **27**, 2553-2567, (1989).

103. B. D. Martin, S. A. Ampofo, R. J. Linhardt and J. S. Dordick, "Biocatalytic synthesis of sugar-containing Poly(acrylate)-based hydrogels", *Macromolecules*, **25(26)**, 7081-7085, (1992).

104. S. Kobayashi, S. Shoda and H. Uyama, "Enzymatic polymerisation and oligomerisation", *Adv. Polym. Sci.*, **121**, 1-30, (1995).

105. P. F. Bruins, "Polyurethane Technology", John Wiley and Sons, New York, (1969).

106. D. Knani, A. L. Gutman and D. H. Kohn, "Enzymatic polyesterification in organic media. Enzyme-catalysed synthesis of linear polyesters. I. Condensation polymerisation of linear hydroxyesters. II. Ring-opening polymerisation of ε-caprolactone", *J. Polym. Sci., Part A: Polym. Chem.*, **31**, 1221-1232, (1993).

107. S. Kobayashi, "Enzymatic polymerisation using hydrolysis enzymes as catalyst for producing polysaccharides and polyesters", *Poly Preponite*, **35**, 772-773, (1994).

108. J. S. Dordick, "Enzymatic and chemoenzymatic approaches to polymer synthesis", *Trends Biotechnol.*, **10(8)**, 287-293, (1992).

109. J. S. Dordick, "Enzymatic and chemoenzymatic approaches to polymer synthesis and modification", *Annals New York Academy of Sciences*, **672**, 352-362, (1992).

110. Y. -Y. Linko, Z. -L. Wang and J. Seppälä, "Lipase-catalysed linear aliphatic polyester synthesis in organic solvent", *Enzyme Microb. Technol.*, **17**, 506-511, (1995).

111. S. Okumura, M. Iwai and Y. Tominaga, "Synthesis of Oligomer by *Aspergillus niger* Lipase", *Agric. Biol. Chem.*, **48**, 2805-2808, (1984).

112. A. Ajima, T. Yoshimoto, K. Takahashi, Y. Tamura, Y Saito and Y. Inada, "Polymerization of 10-Hydroxydecanoic Acid in Benzene with Polyethylene Glycol-Modified Lipase", *Biotechnol. Lett.*, **7(5)**, 303-306, (1985).

113. A. L. Margolin, J. -Y. Crenne and A. M. Klibanov, "Stereoselective Oligomerizations Catalysed by Lipases in Organic Solvents", *Tetrahedron Lett.*, **28**, 1607-1610, (1987).

114. E. M. Brazwell, D. Y. Filos and C. J. Morrow, "Biocatalytic Synthesis of Polymers. III. Formation of a High Molecular Weight Polyester through Limitation of Hydrolysis by Enzyme-Bound Water and through Equilibrium Control", *J. Polym.Sci.; Part A: Polym. Chem.*, **33**, 89-95, (1995).

115. C. J. Morrow, "Biocatalytic Synthesis of Polyesters Using Enzymes", *MRS Bulletin*, Biology and Materials Synthesis Part II, 43-47, (November 1992).

116. G. Mezoul, T. Lalot, M. Brigodiot and E. Maréchal, "Enzyme-catalysed synthesis of poly(1,6-hexanediyl maleate) and poly(1,6-hexanediyl fumarate) in organic medium", *Macromol. Rapid Commun.*, **16(8)**, 613-620, (1995).

117. S. Kamat, E. J. Beckman and A. J. Russell, "Role of diffusion in non-aqueous enzymology.1. Theory", *Enzyme Microb. Technol.*, **14**, 265-271, (1992).

118. J. S. Dordick, R. J. Linhart and D. G. Rethwisch, "Chemical and biochemical catalysis to make swellable polymers", *Chemtech, J. Am. Chem. Soc.*, 33-39, January (1994).

119. D. R. Patil, J. S. Dordick and D. G. Rethwisch, "Chemoenzymatic synthesis of novel sucrose-containing polymers", *Macromolecules*, **24**, 3462-3463, (1991).

120. L. A. Henderson, Y. Y. Svirkin and R. A. Gross, "Enzyme-catalysed polymerisations of ε-caprolactone: Effects of initiator on product structure, propagation kinetics and mechanism", *Macromolecules*, **29**, 7759-7766, (1996).

121. R. Nomura, A. Ueno and T. Endo, "Anionic Ring-Opening Polymerisation of Macrocyclic Esters", *Macromolecules*, **27**(2), 620-621, (1994).

122. H. Uyama, H. Kikuchi and S. Kobayashi, "Single-Step Acylation of Polyester Terminals by Enzymatic Ring-Opening Polymerisation of 12-Dodecanolide in the Presence of Acyclic Vinyl Esters", *Bull. Chem. Soc. Jpn.*, **70**, 1691-1695, (1997).

123. H. Uyama, K. Takeya, N. Hoshi and S. Kobayashi, "Lipase-Catalysed Ring-Opening Polymerisation of 12-Dodecanolide", *Macromolecules*, **28**, 7046-7050, (1995).

124. K. S. Bisht, L. A. Henderson and R. A. Gross, "Enzyme-Catalysed Ring-Opening Polymerisation of ω-Pentadecalactone", *Macromolecules*, **30**, 2705-2711, (1997).

125. K. S. Bisht, Y. Y. Svirkin, L. A. Henderson and R. A. Gross, "Lipase-catalysed ring-opening polymerisation of trimethylene carbonate", *Macromolecules*, **30**, 7735-7742, (1997).

126. D. R. Patil, D. G. Rethwisch and J. S. Dordick, "Enzymatic synthesis of a sucrose-containing linear polyester in nearly anhydrous media", *Biotechnol. Bioeng.*, **37**, 639-646, (1991).

127. H. Uyama, K. Takeya and S. Kobayashi, "Enzymatic ring-opening polymerisation of lactones to polyesters by lipase catalyst: unusually high reactivity of macrolides", *Bull. Chem. Soc. Jpn.*, **68**, 56-61, (1995).

128. V. D. Athawale and S. R. Gaonkar, "Enzymatic synthesis of polyesters by lipase-catalysed polytransesterification", *Biotechnol. Lett.*, **16(2)**, 149-154, (1994).

129. H. Uyama and S. Kobayashi, "Enzymatic ring opening polymerisation of lactones catalysed by lipase", *Chem. Lett.*, 1149-1150, (1993).

130. H. Uyama and S. Kobayashi, "Lipase-catalysed polymerisation of divinyl adipate with glycols to polyesters", *Chem. Lett.*, 1687-1690, (1994).

131. A. K. Chaudhary, E. J. Beckman and A. J. Russell, "Biocatalytic polyester synthesis: Analysis of the evolution of molecular weight and end group functionality", *Biotechnol. Bioeng.*, **55(1)**, 227-239, (1997).

132. R. T. MacDonald, S. K. Pulapura, Y. Y. Svirkin and R. A. Gross, "Enzyme-catalysed ε-caprolactone ring-opening polymerisation", *Macromolecules*, **28**, 73-78, (1995).

133. S. Kobayashi, H. Kikuchi and H. Uyama, "Lipase-catalysed ring-opening polymerisation of 1,3-dioxan-one," *Macromol. Rapid Commun.*, **18**, 575-579, (1997).

134. Y. -Y. Linko, Z. -L. Wang and J. Seppälä, "Lipase-catalysed synthesis of poly(1,4-butanediol succinate) in organic solvent", *Biocatalysis*, **8**, 269-282, (1994).

135. Novo Nordisk - Enzyme Process Division data sheet, "Lipozyme IM", (1992).

136. R. A. Clendinning, J. E. Potts and W. D. Niegisch, (Union Carbide Corp.), "Blends of biodegradable thermoplastic dialkanoyl polymer, a naturally occurring biodegradable product, a plastic additive and a filler", (USP 3 932 319/1976).

137. P. A. Tack - Personal communication.

138. P. Lambert - Personal communication.

139. N. Overton - Personal communication.

140. L. M. Harwood and C. J. Moody, "Experimental Organic Chemistry - Principles and Practice", 1st ed., Blackwell Scientific Publications Ltd., London, (1989).

141. R. Chavira, T. J. Burnett and J. H. Hageman, "Assaying Proteinases with Azocoll", *Anal. Biochem.*, **136(2)**, 446-450, (1984).

142. A. G. Gornall, C. J. Bardawill and M. M. David, "Determination of Serum Proteins by Means of the Biuret Reaction", *J. Biol. Chem.*, **177**, 751-766, (1949).