Enzyme-catalyzed resolution of chlorinated esters.

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M.Sc. Biotechnology Dissertation.

Enzyme-Catalyzed Resolution of Chlorinated Esters.

1. A Study of the Stereospecific Hydrolysis of R,S-methyl-2-chloropropionate by *Candida rugosa* Lipase in an Aqueous/Organic Solvent Two-Phase System.

2. The Development of a Triple Linked-Enzyme Assay for D- or L-(α)-chloropropionic acid.

3.Isolation of Lipase Secreting Microorganisms from Ester Contaminated Soil.

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ABSTRACT.

It has been found that lipase from the yeast *Candida rugosa* (formerly *C. cylindracea*) will stereoselectively hydrolyze racemic mixtures of the partially water soluble methyl-2-chloropropionate. This will not occur in an aqueous reaction medium, so the substrate was made less water soluble by dissolving it in a water-immiscible organic solvent. The complete reaction mixture was a biphasic system comprising the water immiscible organic solvent plus reactant and an aqueous phase containing the enzyme and buffer component. The hydrolysis products were ideally present in the aqueous phase. The course of the reaction could be followed by the determination of the proportions of D- and L-(α)-chloropropionic acid using a triple-linked enzyme assay. The resolutions of the racemic ester were of a practical or pre-pilot scale.

Screening of the 5 soil samples was undertaken in an attempt to isolate some microorganisms that may possess some unusual or enantiospecific lipase or esterase enzymes. Individual microbes were isolated but not identified, several of which were shown to be secreting an exolipase capable of hydrolysing an olive oil emulsion.

CONTENTS.

1. INTRODUCTION.

1.1 Herbicides as Optically Active Compounds.

The production of optically active compounds for use in the manufacture of herbicides, insecticides, pharmaceuticals and metabolites is becoming increasingly important. The reasons for this are financial, ecological and more recently legal. Producing an isomer of a compound which is biologically active, and not a mixture where 50% is biologically inactive or worse, toxic, has major financial implications particularly in the agrochemical industry, where pesticide production may be in thousands of tonnes per year. The legal considerations are not yet so important to the manufacturers of agrochemicals, but will follow as today's sound ecological practice may become a legal requirement in the future. This is particularly likely to occur with the pharmaceuticals industry (Sih *et al.*, 1987). An example was thalidomide (Calton, 1987), administered originally as a racemic compound. The R-(+)-isomer relieved symptoms associated with pregnancy, the S-(-)-isomer was teratogenic. Thalidomide is currently on sale in countries other than the UK but is now manufactured with the S-(-)-isomer removed. Biologically active molecules are known to be highly dependent upon their interactions with particular enzymes or receptors within complex biological pathways. Enzymes and receptors typically recognize molecules specifically using their three dimensional configuration.

The compound of interest here was D,L-2-chloropropionic acid, which is used as a precursor compound or synthon in the manufacture of 2-(ρ-chlorophenoxy)propionic acid herbicides, such as Fusilade (fluazifop), mecoprop (Iso-Cornox) and dichlorprop (Cornox RK).

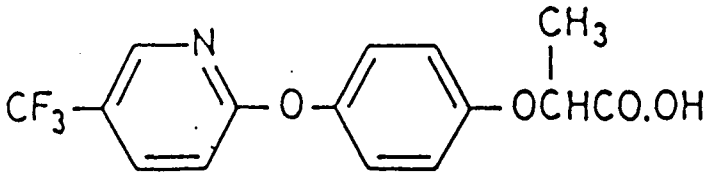

Figure 1. Fluazifop.

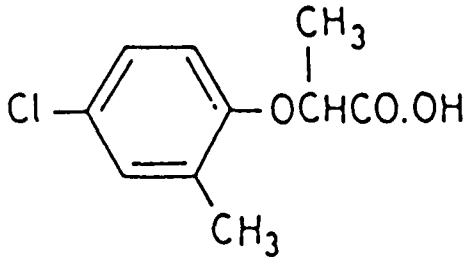

Figure 2. Mecoprop.

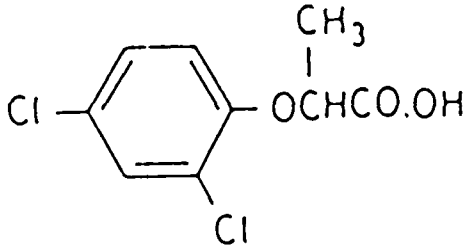

Figure 3. Dichlorprop.

Fusilade is produced as an enantiomerically pure compound, the R-(+)-isomer being the optically active component (Calton, 1987). The herbicidal activity of mecoprop and dichlorprop although sold as chiral pesticides, is due to the R-(+)-enantiomer (Worthing, 1984).

The 2-(ρ-chlorophenoxy)propionic acids and 2-(ρ-chlorophenoxy)acetic acids are synthetic auxins (Corbett, 1974) mimicking the action of the plant growth hormone indole-3-acetic acid (IAA). IAA is the narurally occurring auxin and occurs almost universally in plant tissues.

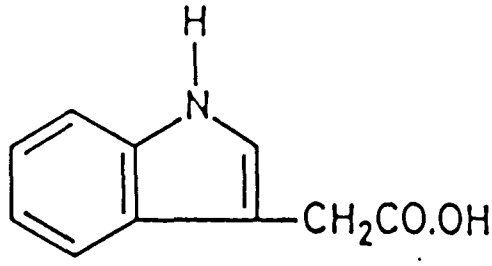

Figure 4. Indole-3-acetic acid.

Other closely related compounds have been found to be present in plants, and some cause the same responses as IAA. Many remained unidentified until very recently, others such as phenyl acetic acid are often more abundant in plants than IAA, but are far less active. It is these compounds which the ρ-phenoxyalkanoic acids have been synthesized to mimic, they cause many of the physiological responses common to IAA and are considered as auxins, but as they are not synthesized by the plants they cannot be called hormones. Examples of the ρ-chlorophenoxy acetic acids include 2,4-D, 2,4,5-T, and MCPA.

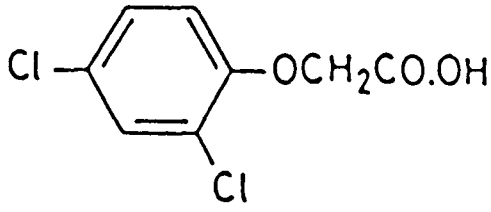

Figure 5. 2,4-D.

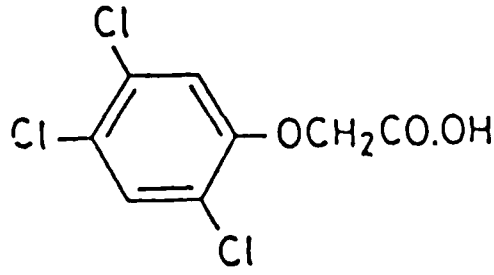

Figure 6. 2,4,5-T.

These auxins have been applied as herbicides since the 1940's and were popular due

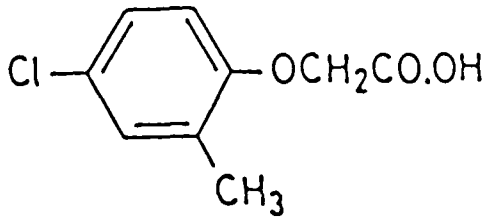

Figure 7. MCPA.

to their toxicity, relatively low cost, and their selectivity in affecting dicots much more than monocots.

One way to produce optically active compounds is to employ an enzyme that will act stereospecifically on one enantiomer. It is commoner to find enzymes that react stereoselectively on one isomer in preference to the other. Such reactions are known as enzymatic kinetic resolutions and they are currently being researched and developed with the hope that they may become commercially viable. Method which is commercially employed is the chemical approach of fractional recrystallization or cocrystallization which typically involves reacting or complexing a racemic mixture with a chiral auxiliary, there are several naturally occuring suitable compounds. The diastereometric pair formed can be separated on differences in their physical properties eg. solubility where one complex will crystallize before the other. Once separated the desired optically active isomers can be recovered along with the chiral auxiliary. The drawback with this method is that several cycles of the process are required to obtain high enantiomeric excesses and is therefore a labour intensive process. This process is used in the manufacture of the analgesic Naproxen and the commercial synthesis of L-lysine (Maugh II, 1983). It is more economic to employ a chemico-enzymatic approach using enzymes to prepare small optically active molecules known as isomerc synthons or chirons, which are then put together by more

traditional methods (Jones & Hinks, 1986; Ohno *et al.*, 1986; Calton, 1987; Bianchi *et al.*, 1988), than treatment of the finished product. Presently the high cost of producing these isomeric synthons for herbicides is preventing their use. It is also thought that L-2-haloalkanoic acids such as L-(α)-chloropropionic acid may have potential for their use in the manufacture of optically active pharmaceuticals.

There have been three published attempts (Cambou & Klibanov, 1984b; Kirchner *et al.*, 1985; Dahod & Siuta-Mangano, 1987) at producing optically active L-2-chloropropionic acid or its esters and one resolving ρ-chlorophenoxypropionic acid (Cambou & Klibanov, 1984a), using a lipase to catalyze a stereoselective hydrolysis, esterification or transesterification reaction.

1.2. Lipases as Suitable Hydrolytic Catalysts.

Considerable interest has been focused by academic and industrial researchers on the potential of enzymes in synthetic organic chemistry. The enzymes known broadly as the lipases and esterases have attracted much of this attention as many have been shown to be stereoselective in their activities and remain active in the presence of organic solvents (Kawamoto *et al.*, 1987).

The reactions that they have been reported to catalyze include transesterification (Cambou & Klibanov, 1984c; Kirchner *et al.*, 1985; Deetz & Rozzell, 1988), aminolysis (Margolin & Klibanov, 1987), acyl exchange (Klibanov, 1986), thiotransesterification, oximolysis (Zaks & Klibanov, 1985) and hydrolysis (Iriuchijima & Kojima, 1982; Iriuchijima *et al.*, 1982; Lavayre *et al.*, 1982; Cambou & Klibanov, 1984a).

All except hydrolysis require unusual catalytic conditions, as in excess water the

hydrolytic reaction suppresses all others (Martinek *et al.*, 1981; Carrea, 1984; Kirchner *et al.*, 1985). Lipases possess several characteristics that make them attractive as potential catalysts in commercial processes:

1. They have a broad substrate specificity and they are able to hydrolyze insoluble esters better than the other hydrolases (Lavayre *et al.*, 1982; Ladner & Whitesides, 1984).
2. There are a wide range of lipases available.
3. Nearly all exhibit some degree of enantiospecificity (Gil *et al.*, 1987; Ramos Tombo *et al.*, 1987).
4. Many are fairly inexpensive.
5. They do not require enzyme cofactors (Zaks & Klibanov, 1985).
6. Important for a commercial process they should be available in large enough quantities eg. 10's to 100's of kilogrammes.

For an enzymatic resolution the enzyme must show a preference for one enantiomer over the other. Any stereoselectivity is generally unpredictable and has to be investigated for each enzyme substrate combination.

Lipases do not require common cofactors or coenzymes for their activity, which are expensive non-reusable and are insoluble in organic solvents (Semeriva & Desnuelle, 1979). Although animal lipases do not require cofactors, a colipase has been isolated (Borgström *et al.*, 1979). This small (Mw 10000) protein has been found to enhance the activity of pancreatic lipase in it's action at the substrate interface (Junge *et al.*, 1983).

The attraction of lipases as catalytic agents extends to the application of immobilization techniques and the possibility of their reuse (Fukui & Tanaka, 1982; Yokozeki *et al.*,

1982; Marlot *et al.*, 1985). Immobilization also tends to improve the enzyme's stability. The lipase may be immobilized to a porous support which is soaked in an aqueous pH buffered solution and this forms the aqueous phase of the biphasic system, the immobilized enzyme being stirred in the organic solvent phase , with no other water being present. The adsorption of lipase from *Pseudomonas fragi* 22.39B to a magnetic fluid has also been accomplished, enabling the recovery of the enzyme using a strong magnetic field, without the loss of activity (Takahashi *et al.*, 1987; Mihama *et al.*, 1988). The advantages of immobilized enzymes over free enzymes when they are used as catalysts are clear:

1. Substrates and products are easily separated from the enzyme.
2. Continuous procedures can be used.
3. Immobilized enzymes generally posess greater stability.
4. Higher catalyst densities can be achieved than could in free solution.

1.3. Organic Solvents in Enzyme Catalysis.

A problem regularly encountered with useful substrates of lipases eg. insoluble esters and triglycerides,is total insolubility or only partial solubility in aqueous milieu. Therefore being able to dissolve the water-insoluble substrate in an organic solvent and then use this as a reaction medium would be extremely useful. This has become possible with the realisation that enzymes are not restricted solely to catalysis in aqueous solutions (Lugaro *et al.*, 1973; Cremonesi *et al.*, 1975; Klibanov *et al.*, 1977), many being highly active in the presence of hydrophobic water-immiscible solvents, even with only sufficient water to form a monolayer coating the enzyme's surface (Butler, 1979; Klibanov, 1986;

Zaks & Klibanov, 1988a). For much preparative chemistry it is advantageous that enzymic reactions can be carried out in the presence of organic solvents, or an aqueous / organic solvent biphasic system, rather than in purely aqueous milieu for several reasons:

1. Many organic substrates are poorly if not completely insoluble in water.
2. Water is often a party to unwanted side reactions eg. hydrolysis in esterification reactions.
3. The thermodynamic equilibrium of many reactions can be reversed in organic solvents where the water content has been reduced to the absolute minimum.
4. Recovery of a product or unreacted substrate from an organic solvent is much easier than from an aqueous phase.
5. Some enzymes have been found to be stabilized in the presence of an organic solvent (Zaks & Klibanov, 1988a).
6. If for no other reason the solvents would actually inhibit or prevent microbial contamination, which leads to the loss of enzyme from unwanted proteases (Klibanov, 1986).

Attempting to increase the solubility of a water insoluble substrate by the addition of a water-miscible solvent such as ethanol, acetone, methanol, acetonitrile or dioxane does not solve the problem for several reasons (Antonini *et al.*, 1981) :

1. Although low concentrations do not affect enzyme stability or activity, perhaps even enhancing these, higher solvent concentrations progressively give rise to inhibition (competitive or due to 'extractive' phenomena), decreased specificity and unfolding.
2. Attempts to overcome these hindrances by using immobilized enzymes fail if the concentration of miscible organic solvent is high.

3. Use of a co-solvent does not reduce the effects of enzyme inhibition exerted by high substrate or product concentrations.

A biphasic system with an equivalent or greater proportions of aqueous phase was the model to be studied in this project. The simple idea of such biphasic systems (Lilly, 1982; Brink & Tramper, 1985; Lilly *et al.*, 1987) is to add a water-immiscible organic solvent to the aqueous phase. The enzyme will be present only in the aqueous phase, either because it is immobilized there or if not proteins being highly soluble in water are unlikely to solubilize in the hydrophobic phase. This type of system makes stabilizing the enzyme against the denaturing effects of solvents unnecessary (Klibanov *et al.*, 1977; Antonini *et al.*, 1981).

The advantages in the use of such two-phase aqueous water-immiscible organic solvent arrangements are especially evident when enzyme-catalyzed reactions are carried out with substrates that are poorly soluble in water and when water is a reagent directly involved in the reaction. Attempts to carry out enzymatic reactions with poorly soluble substrates in homogeneous mixtures of water and miscible organic solvent often fail to give satisfactory results. This is due, in particular, to the instability of the enzymes in such systems (Butler, 1979). On stirring or shaking the substrate is transferred from the organic to the aqueous phase (Antonini et al., 1981; Cambou & Klibanov, 1984c), the enzyme-catalyzed transformation occurs and the products are free to return to the organic phase. Hence the effects of substrate or product inhibition on the enzyme are reduced to an absolute minimum.

The concentration of solvent in water is low and not dependent on the ratio of the two phases, even if the volume of the organic phase is much greater than the aqueous phase,

and therefore the inhibitory and denaturing effects are much less than those induced by comparable concentrations of water-miscible solvents.

A fundamental requirement for a suitable organic solvent (or mixture of solvents) is a high capacity to solubilize the reagents and products. Clearly the characteristics of the solvent influence the partition coefficients of the substrates and products between the organic and the aqueous phases. When high substrate or product concentations give rise to enzyme inhibition, solvents with high partition coefficients should be used; the opposite is the case when substrates with high Km values are to be transformed (Antonini *et al.*, 1981; Carrea, 1984).

The choice of organic solvent is also highly dependent on the type of enzyme (Butler, 1979). However generally solvents of lower polarity have a smaller effect on the enzyme's stability. The stability of enzymes can be improved by the addition of serum albumin, co-enzymes (Cremonesi *et al.*, 1977), and by avoiding the complete transformation of substrates when they have a stabilizing effect (Buckland *et al.*, 1975; Antonini *et al.*, 1981). Though co-enzymes are known to be inactive in the presence of organic solvents.

The potential inhibitory effect of solvents also must be taken into account. No general rule seems to exist and solvents have to be tested individually (Cremonesi *et al.*, 1977; Antonini *et al.*, 1981). Solvents that partially destabilize and inhibit the enzyme but solubilize large amounts of substrate are more productive than those not affecting enzyme activity but solubilizing only small concentrations of substrate (Butler, 1979; Laane *et al.*, 1987). Other factors affecting the choice of organic solvent particularly when considering a practical or commercial scale process include:

1. Solvent capacity for reactant / product.
2. Reactant / Product partition coefficients.
3. Denaturing or inhibitory effects.
4. Toxicity.
5. Flammability.

1.4. The Quantitative Analysis of Kinetic Resolutions.

In a normal hydrolytic reaction water is one of the substrates as well as the reaction milieu, and its high relative concentration (55.5 M) drives the reaction toward completion at equilibrium (Kirchner *et al.*, 1985; Chen *et al.*, 1987). Although the reverse condensation reaction is achievable, the excess of water in the hydrolysis makes the reaction virtually irreversible. It is possible by replacing the water in such biphasic aqueous/organic solvent reaction medium to lower the water activity and reverse the thermodynamic equilibrium in the direction of the synthetic or condensation reaction (Martinek *et al.*, 1981; Zaks & Klibanov, 1986; Cassells & Halling, 1988). Such systems may contain only sufficient water to form a monolayer surrounding the enzyme. Another way to reduce any reversal of the hydrolytic reaction is to make the substrate more soluble in the organic phase and the product more soluble in the aqueous phase (Antonini *et al.*, 1981), the opposite favours synthetic esterification reactions.

Expressions have been formulated (Chen *et al.*, 1982) to relate the three key parameters of the biochemical kinetic resolution : the enantiomeric excess (ee) or optical purity of the product or remaining substrate, the enantiomeric ratio (E), and the extent of conversion of the racemic substrate (c). Where the enantiomeric ratio (E) is a quantitative

measure of the discrimination between the two competing enantiomers by the enzyme or enzymes.

Assuming the hydrolysis is a simple three-step kinetic mechanism; where A and B are the fast and slow reacting enantiomers respectively, both competing for the same active site on the enzyme, and the reaction is irreversible or negligibly so.

$$\mathrm{A + enz} \underset{k_2}{\overset{k_{1A}}{\rightleftharpoons}} \mathrm{Enz.A} \xrightarrow{k_3} \mathrm{Enz.P} \xrightarrow{k_5} \mathrm{enz + P} \qquad 1.$$

$$\mathrm{B + enz} \underset{k_{2'}}{\overset{k_{1B}}{\rightleftharpoons}} \mathrm{Enz.B} \xrightarrow{k_{3'}} \mathrm{Enz.Q} \xrightarrow{k_{5'}} \mathrm{enz + Q}$$

It is possible to use both the enantiomeric excess of the remaining substrate fraction (ee (S)) and the product (ee (P)). The knowledge of any two of c, ee (S) or ee (P), allows the definition of the third. Here it is the relative rates of the two reactions (v_A and v_B) that determines the enantioselectivity (E). The enzyme binds with each chiral enantiomer to form two diastereometric complexes Enz.A and Enz.B.

The ratio of the two partial reaction rates (v_A and v_B) may be shown by steady state kinetics to be,

$$\frac{v_A}{v_B} = \frac{V_A}{V_B}\frac{K_B}{K_A}\frac{A}{B} \qquad 2.$$

Where V_A,K_A and V_B, K_B denote maximal velocities and Michaelis constants of the fast- and slow-reacting enantiomers respectively. Integration of equation (2) affords the

homocompetitive equation (3),

$$\frac{ln\,(A/A_o)}{ln\,(B/B_o)} = \frac{V_A/K_A}{V_B/K_B} = E \qquad 3.$$

Which shows that E is the ratio of the specificity constants V/K. E is a kinetic parameter whose value will vary with different catalysts, however it is sensitive to environmental changes such as pH, temperature and the organic solvent.

The relationship between the extent of conversion (c) and the enantiomeric excess of the recovered substrate, ee (S) or product fraction, ee (P) can be related to obtain a value for E. For the remaining substrate fraction,

$$\frac{ln\,((1-c)\,(1-ee\,(S)))}{ln\,((1-c)\,(1+ee\,(S)))} = \frac{V_A/K_A}{V_B/K_B} = E \qquad 4.$$

and for the recovered product fraction,

$$\frac{ln\,(1-c\,(1+ee\,(P))}{ln\,(1-c\,(1-ee\,(P))} = \frac{V_A/K_A}{V_B/K_B} = E \qquad 5.$$

Where

$$c = \frac{(A+B)}{(A_o+B_o)} \quad , \quad ee\,(S) = \frac{(B-A)}{(A+B)} \;\; and \;\; ee\,(P) = \frac{(P-Q)}{(P+Q)} \quad .$$

These two equations (4) and (5) are available for the analysis of reactions where there is either a selective removal of one of the substrate enantiomers (4) or (5) which relates the extent of conversion to the optimization of enantiomeric excess of the products.

These equations have been applied to various biochemical kinetic resolutions (Sih *et al.*, 1987; Sonnet, 1988; Sonnet & Antonian, 1988) and have recently been modified for their use with reverse reactions where the enzymes may have their thermodynamic equilibrium shifted in order to favour the reverse reaction (Chen *et al.*, 1987). In such systems the rate of reaction in both directions has to be taken into account.

1.5. The Stereospecific Resolution of 2-chloropropionate Esters.

It has been reported (Cambou & Klibanov, 1984a) that *C. rugosa* lipase expressed total enantiospecificity in the hydrolysis of R,S-octyl-2- chloropropionate to yield a high enantiomeric excess of S-(-)-octyl-2- chloropropionate and D-(α)-chloropropionic acid. But under the same conditions, the lipase showed no enantiospecificity in the hydrolysis of R,S-methyl-2-chloropropionate .

They observed that the methyl ester was soluble in water at the concentrations used, whereas the octyl ester was insoluble actually forming an emulsion. They hypothesised that the enantiospecificity of the lipase was most likely due to the presence of the substrate / aqueous interface which it requires to exhibit its enantiospecific activity. Lipase from *C. rugosa* was also found to be a highly stereospecific catalyst in the resolution of chiral acids and alcohols via asymmetric esterification (Cambou & Klibanov, 1984a, 1984b ; Kirchner *et al.*, 1985).

However it was the stereoselective hydrolysis of R,S-methyl-2-chloropropionate by *C. rugosa* which was the simplest system reported (Dahod & Siuta-Mangano, 1987). Many of the enzymic resolutions of enantiomeric compounds reported have only been accomplished in laboratories on gram or milligram scales. This process was being described as a practical scale resolution, with potential for it's application to a large-scale preparative process (Dahod, US and European patents). From the earlier work (Cambou & Klibanov, 1984a) it was known that *C. rugosa* lipase would not stereoselectively hydrolyze R,S-methyl-2-chloropropionate in a purely aqueous system so the problem was approached:

1. By using a biphasic system, and dissolving the substrate in the organic phase, employing a buffered aqueous phase which would maintain the partitioning of the substrate in the organic solvent.

2. They also chose their solvent very thoughtfully experimenting only with chlorinated hydrocarbons :- perchloroethylene, chloroform, carbon tetrachloride, trichloroethylene , deciding eventually to use carbon tetrachloride in their practical scale experiments.

3. Also by carrying out the bioreactions at 4°C and 22°C, finding the lower temperature gave a better resolution presumably because it further reduced the solubilty of the ester in the aqueous phase.

4. The enzyme was found to be stabilized by the carbon tetrachloride in the presence of the substrate . The lower temperature of 4°C would also increase its stability although reducing its activity.

All of these assist in maintaining the partitioning of the ester substrate in the organic phase. The aqueous phase was essential removing the reaction products and using it as a 'dump-phase' the 2-chloropropionic acid and methanol produced going into aqueous solution. To prevent the acid from repartitioning back into the organic phase, the aqueous phase was carefully buffered. They employed several types of buffering agent to prevent the acid from repartitioning back into the organic phase and to maintain the ester in the organic phase. Buffers included powdered calcium carbonate and various strengths of sodium phosphate buffer. The aqueous buffer had a secondary effect in that it helped maintain near optimal lipase activity with the pH in the region of 4.0 to 8.5. This biphasic system is slightly different to many previously reported , in that the organic phase only

constituted 35% to 50% of the total reaction volume, the surplus of water favouring the ester hydrolysis and reducing any reverse reactions (eg. esterifications) to a minimum.

1.6. An Assay for L- and D-(α)-chloropropionic Acid Isomer Proportions.

Another attraction of following this work was the dehalogenated product of 2-chloropropionic acid is lactic acid. D,L-(α)-chloropropionic acid plus a hydroxyl in the presence of a 2-haloacid dehalogenase gives D,L-2-hydroxy acid plus a halide ion.

So it should be possible to accurately assay for the 2-chloropropionic acid production and follow the course of the hydrolysis reaction. Another possible method of measuring the relative proportions of L- and D-(α)-chloropropionic acid is to compare a known standard against the samples in a polarimeter , a device that allows the measurement of the degree of refraction of polarized light due to the chemical in question. However a polarimeter may not be particularly accurate only giving a result $\pm$5%. So it was to be attempted to devise an enzyme assay which theoretically should have a greater sensitivity and accuracy to about 1% in a 10 mM sample. The assay was intended to be more of a qualitative than a quantitative assay, to enable the relative proportions of the isomers to be determined. The assay was a triple-linked enzyme assay using D,L-2-haloacid dehalogenase [EC 3.8.1.2], either D-lactate dehydrogenase [EC 1.1.1.28] or L-lactate dehydrogenase [EC 1.1.1.27], and glutamic pyruvic transaminase [EC 2.6.1.2]. Dehalogenation of 2-chloropropionic acid in the presence of water gives either L- or D-lactic acid (Motosugi *et al.*, 1981), this lactate can then assayed for using the lactate dehydrogenase/β NAD complex followed by glutamic pyruvic transaminase in the presence of L-glutamate (Gawehn, 1984; Noll, 1984). The conversion of lactate to pyruvate

by lactate dehydrogenase requires β NAD as a cofactor. This is reduced to NADH and can be detected using a UV spectrophotometer at 339/340 nm.

The critical reaction is that GPT in the presence of L-glutamate and pyruvate has an equilibrium well in favour of L-alanine and 2-oxoglutarate formation. This removal of pyruvate prevents the reversal of the lactate dehydrogenase enzymes which have an equilibrium in favour of lactate formation. By removing the pyruvate any lactate present will be transformed to pyruvate, thus pulling the series of reactions in the direction of L-alanine and 2-oxoglutarate. The reactions are stoichiometric, and one molecule of 2-chloropropionic acid will give one molecule of lactate which can be oxidized to give a molecule of pyruvate. Pyruvate plus one molecule of L-glutamate gives one molecule each of L-alanine and 2-oxoglutarate. The conversion of lactate to pyruvate requires the reduction of one molecule of β NAD to NADH. It was therefore possible to calculate the exact amount of D- or L-(α)-chloropropionic acid required to produce a spectrophotometer full scale deflection of 1.000.

1.7. The Screening of Soil Samples for Novel Lipases.

The screening of the soil samples for microorganisms able to grow on some unusual esters was an exercise to see if some more specific or novel lipases could be isolated, because most microbial (and animal) lipases have been found to have broad substrate specificities. Demand from industrial and academic researchers is stimulating the search for sources of novel and more specific enzymes (Sztajer *et al.*, 1988). With the considerable interest in lipases and hydrolytic enzymes for enantioselective catalyses and for their application in synthetic or interesterification reactions, where a surplus of triglyc-

eride may be converted in to one with more desirable properties eg. palm oil into cocoa butter, such characteristics will be of great value to some researchers (Nakatsuka, 1987; Sonnett & Antonian, 1988).

1.8. AIMS.

The aims of this research project were threefold:

1. The preliminary screening of soil samples for microorganisms with either the ability to utilize some enantiomeric substrates as carbon sources or secreting some unusual lipase or esterase enzymes capable of hydrolyzing the esters.
2. Repetition of some work by two workers (Dahod & Siuta-Mangano, 1987), on the stereoselective hydrolysis of R,S-methyl-2-chloropropionate in a biphasic aqueous / water immiscible organic solvent system. Employing mainly carbon tetrachloride as the organic solvent and concentrating on the effects of various buffers on the partitioning of the substrate and products. Also the application of some quantitative analyses on the results of the bioreactions.
3. Development of an assay to detect the relative proportions of 2-chloropropionic acid isomers and it's application the above resolution experiments.

3. MATERIALS AND METHODS.

2.1. Chemicals. S-(-)-methyl-2-chloropropionate, R-(+)-methyl-2-chloropropionate, D-(α)-chloropropionic acid, L-(α)-chloropropionic acid, D-(-)-lactic acid, L-(+)-lactic acid ethyl ester, ρ-nitrophenylpalmitate (ρ-NPP), and β nicotinamide-adenine dinucleotide (grade III from yeast) were all purchased from the Sigma Chemical Co.

R,S-methyl-2-chloropropionate, L-(-)-lactic acid ethyl ester and R-(-)-lactic acid isobutyl ester were all purchased from the Aldrich Chemical Co.

L-(+)-isobutyl-2-chloropropionate was supplied by ICI Fine Chemicals and Colours, Huddersfield. All other chemicals were of analytical reagent grade.

2.2. Enzymes. *Candida rugosa* lipase Type VII, EC 3.1.1.3 (500 U mg^{-1} protein), D,L-2-haloacid dehalogenase from a *Pseudomonas* species, EC 3.8.1.2 (19 U mg^{-1} protein), L-lactate dehydrogenase Type II from rabbit muscle, EC 1.1.1.27 (940 U mg^{-1} protein), D-lactate dehydrogenase from *Lactobacillus leichmannii*, EC 1.1.1.28 (340 U mg^{-1} protein), (also D- or L-lactate: NAD oxidoreductase) and glutamic pyruvic transaminase from porcine heart EC 2.6.1.2 (120 U mg^{-1} protein), (also ALT; alanine aminotransferase, L-alanine:2-oxoglutarate aminotransferase) were all obtained from the Sigma Chemical Co.

2.3. The D- and L-(α)-chloropropionic Acid Assay.

Glycylglycine / Glutamate Buffer.

(Gly.gly 0.6 mol l^{-1} : L-glutamate 0.1 mol l^{-1} , pH 10.5).

Dissolve 47.5 g gly.gly free base and 8.8 g L-glutamate free acid in approximately 500 ml water, adjust the pH to 10.5 with 10 M NaOH, then make up the volume to 600 ml with water. Store at 4°C.

β Nicotinamide-adenine dinucleotide (β NAD^+). Dissolve 265mg in 4 ml water (100 mM). Store frozen until required.

Prepare six 2-chloropropionic acid standards using 10mM solutions of D- and L-(α)-chloropropionic acid using the ratios:

Table 1. The 2-chloropropionic Acid Standards.

D-(α)-CPA (ml)	L-(α)-CPA (ml)	D-(α)-CPA (mM)	L-(α)-CPA (mM)
20	0	10	0
16	4	8	2
12	8	6	4
8	12	4	6
4	16	2	8
0	20	0	10

'CPA 'refers to 2-chloropropionic acid and standards prepared in this manner were stored at 4°C for upto 8 weeks.

These standards were used to construct the calibration curves for each assay.

D,L-2-haloacid dehalogenase (D,L-2-HAD). This was obtained in 50 U quantities, and stored frozen in a lyophilized state until required. Dissolved in 4.0 ml gly.gly buffer and stored at 4°C. The L- and D-lactate dehydrogenases and glutamic pyruvic transaminase were supplied as ammonium sulphate suspensions and were stored as recommended.

All the reagents were stored on ice whilst being used.

The assay was developed to detect NADH evolution, at 340nm.

UV Spectrophotometer.

Wavelength	340 nm
Cuvette	1.0 cm light path
Incubation	28°C to 30°C for $\geq$ 45 mins
Final volume	1.0 ml
Sample volume	20 μl
Blank	water

All reagents were aliquoted using gilson micropipettes, except the 2-chloropropionic acid standards and samples to be assayed where a capillary micropipetter was employed to make this repetitive task more consistent. All samples were diluted so the final 2-chloropropionic acid concentration was approximately $\leq$ 10 mM.

Table 2. Volumes of Reagents for Assay.

Reagents	Blank	Control	Control	Standards and Samples	Standards and Samples
	(μl)	D-(α)-CPA (μl)	L-(α)-CPA (μl)	D-(α)-CPA (μl)	L-(α)-CPA (μl)
Water	1000	500	540	480	520
Buffer		330	330	330	330
β NAD		60	60	60	60
GPT		40	40	40	40
D-/ L-LDH		D- 50	L- 10	D- 50	L- 10
Sample				20	20
D,L-2-HAD		20	20	20	20

'CPA 'refers to 2-chloropropionic acid. All volumes are in μ l.

The assay employed 2 blanks, 1 control, 6 calibrations and 25 or 30 samples for analysis. The reagents plus calibration or sample were mixed and allowed to stand for about five minutes the initial absorbances ($A1$) were then read on the spectrophotometer. Allowing the samples to stand before reading $A1$ will ensure that any lactate present will be converted to L-alanine and 2-oxoglutarate and not interfere with any subsequent increase in absorbance from the addition of the D,L-2-HAD. The spectrophotometer was zeroed using the water blanks, then the absorbance of the control (- D,L-2-HAD, - substrate) was read against a water blank, the remaining cuvettes were read against this control. Following the addition of the D,L-2-HAD to the control, any change in absorbance in this control can be used to subtract any absorbance due to the D,L-2-HAD from the rest of the cuvettes. Following the reading of the initial absorbances ($A1$) the

cuvettes were incubated at 28°C for 45 to 60 minutes, or until the NADH evolution was completed, which in some assays took slightly longer. This was attributed to variations in the batches of D,L-2-HAD. After the period of incubation the final absorbances ($A2$) could be read, using the procedure already described. All the absorbances $A1$ and $A2$ were recorded. Changes in absorbance (ΔA) were calculated using:

$$C = (control + HAD) - (control - HAD),$$

then

$$\Delta A = (A2 - A1) - C.$$

This corrected for any absorbance due to the D,L-2-HAD, variations due to the different concentrations of 2-chloropropionic acid and variations in the disposable cuvettes used.

From the assay a calibration curve was drawn for each assay using the ΔA for each of the six standards. The concentrations (mM) of the samples could be read directly from the calibration curve, and then multiplied by the dilution factor.

2.4. The HPLC Analysis of the Total 2-chloropropionic Acid Content.

The analysis of samples was performed by reverse phase chromatography using isocratic elution.

HPLC Conditions. Hplc equipment, Waters M501 pump linked to an 840 data handling computer. Column; Spherisorb 35 ODS 2(250 × 4.9 mm), Eluent; Millipore H_2O (18 MΩ cm) / Rathburns MeOH / Aristar H_3PO_4 (79.5 : 20 : 0.5 w/v), Run time; 15 minutes, Sample injection; 20 μl, Detection; Waters M490 detector at 210 nm, Flow rate; 1.0 μl minute^{-1}, Temperature; Ambient. Standards preparation. A series of standards, 1 - 3 g l^{-1} CPA and 195 - 760 ppm w/v 2-2-dichloropropionate were prepared using Aldrich 2-chloropropionic acid and Fluka 2,2-dichloropropionic acid, sodium salt (95%). The standards were not mixed because the CPA standard contained 2,2-dichloropropionate as an impurity. The separation of 2-chloropropionic acid (2 g l^{-1}) and 2,2-dichloroproionate (730 ppm) standards can easily be detected.

2.5. The Modified ρ-Nitrophenylpalmitate (ρ-NPP) Lipase Activity Assay.

Modified from the original (Winkler & Stuckman, 1979) by the substitution of Mops buffer for Sörensen phosphate buffer, as the Mops was considered to be more suitable in a biological application and the sodium taurocholate replaced sodium deoxycholate as this stabilized the ρ-NPP reagent more effectively. 30 mg of ρ-NPP flakes were dissolved in 10 ml of propan-2-ol, taking 15 to 30 minutes of vigorous shaking. Then 268 mg of sodium taurocholate and 100 mg of gum arabic were mixed with 90 ml of 0.055 M Mops buffer, pH 8.0, and the dissolved ρ-NPP was added to this. This reagent was measured out into 3 ml aliquots and frozen as soon as possible. Freezing this reagent appears to help stabilize the ρ-NPP preventing any unwanted yellow colour developing prematurely. The assay was carried out by adding 100 μl of sample to 2.9 ml of ρ-NPP reagent preheated to 25°C, this was incubated, mixing gently, for 10 minutes, then the absorbance was measured at 410 nm against a ρ-NPP blank. It was important to obtain a maximum absorbance reading no greater than 0.3 - 0.4. Timing of the incubation period was critical as all values obtained were comparative, and if left longer would increase. Reduction of the incubation period or addition of smaller samples may be necessary to obtain absorbances in the 0.3 to 0.4 range. The assay was designed to detect 10 lipase units in 2.5 ml of reagent.(12 U in 3.0 ml)

2.6. The Kinetic Resolutions of R,S-Methyl-2-chloropropionate.

The practical scale resolutions were carried out in 1 l or 500 ml stoppered bottles on magnetic stirrers in a cold room (4° – 6°C). Racemic R,S-methyl-2-chloropropionate was dissolved in the organic solvent; carbon tetrachloride, chloroform or di-isopropyl ether, and left to cool in the cold-room. The buffer solution (aqueous phase) was measured into the reaction vessel and left stirring to cool. The lipase was either dissolved for several hours in 0.5 M Tris buffer, pH 8.5 in the cold room or it was added directly to the reaction mixture as a powder. The addition of the lipase was used to signify the start of the reaction (zero hour). Two molarities of R,S-methyl-2-chloropropionate were investigated, 3.7 M (or 3.67 M) and 1.0 M, and the reactions were run for between 20 to 52 hours. The variations in the bioreactions were the solvents and the buffer or acidity regulating component of the aqueous phase. Stirring of the reaction mixtures was at an unknown speed but was sufficient to produce a fine emulsion of the organic phase (discontinuous) in the aqueous phase (continuous). Extra stirring was considered unnecessary. Samples (7- 10 ml) of the stirred reaction mixtures were removed at the required times, and allowed to settle out. No allowance was made to cover the removal of product and substrate with these samples as the concentrations would have remained the same. Sometimes it was necessary to centrifuge the samples to remove the calcium carbonate in suspension. The aqueous and organic phases were separated and stored frozen until required. The removal of the stirring was sufficient to halt the enzymatic hydrolytic reaction. Samples of the organic phase were taken from the end of a bioreaction run, except with runs 8,9 and 10, where samples of the organic phase were taken along with those of the aqueous phase to be assayed for 2-chloropropionic acid content. The

pH of the reaction mixture was measured in the presence of carbon tetrachloride using a Mettler organic solvent resistant pH probe.

2.7. Screening of the Soil Samples.

Five soil samples were obtained from around a 2-chloropropionate ester off-loading pipe and pump, from immediately beneath the pipe end, a drain gutter, and from below a drain-tap in the ester pipe. All five samples smelt heavily of ester compounds. The soil samples were cultured initially on 50 ml nutrient broth, either without any additional ester, or with 5 mM or 25 mM ester concentration at 28°C for at least 72 hours. The esters used in the selection were L-(+)-ethyl lactate, L-(-)-ethyl lactate, S-(-)-methyl-2-chloroproionate, R-(+)-methyl-2-chloropropionate, L-(+)-isobutyl-2-chloropropionate and R-(+)-isobutyl lactate. The intention was to allow organisms to grow utilizing the ester as a carbon source, or tolerating it's presence , the two different ester concentrations were supposed to help select between these conditions. Following this initial culture flasks containing 50 ml of the minimal seed 2 or MV broths were inoculated with 5 ml of the nutrient broth culture, and ester at a 50 mM concentration, to increase the selection pressure. This was repeated using the seed 2 cultures to inoculate the next set of seed 2 broths, but with the lower concentration of 20 mM. In the first set of minimal media cultures it was difficult to distinguish between a small amount of growth in the culture and turbidity remaining from the previous inoculant. The second set of minimal media cultures helped to clarify this problem. Flasks showing high turbidities from this set of cultures were selected for plating out onto their respective agars (seed 2 or MV). The esters were not mixed with the molten agar, but were spread onto

the agar surface and allowed to dry. Typically 100 μl of an ester was applied in this fashion. Initial dilutions of the cultures were used to help obtain individual colonies, but subsequently loop-streaking was employed.

After several subculturings of colonies from the plates and further streaking onto plates plus ester to maintain the selection pressure, colonies of individual organisms were obtained, about 25 in all. These organisms were then plated onto olive oil emulsion plates in an attempt to distinguish whether they were secreting an extracellular enzyme ,or simply growing in the presence of the ester. Production of an exolipase was characterized by the development of zones of clearance around the colonies as the olive oil was hydrolized by the secreted lipase or esterase.

2.8. Broth and Agar Recipes.

Nutrient Broth.

13 g l^{-1}	Oxoid nutient broth powder

Seed 2 Broth.

20 ml	phosphate buffer
5 ml	ammonium sulphate
1 ml	trace elements
0.5 ml	magnesium sulphate
0.1 ml	ferric chloride

adjust pH to 7.2 and make upto 1 l with water.

MV (yeast minimal) Broth.

5 g	ammonium sulphate
1.7 g	yeast nitrogen

For seed 2 and MV (yeast minimal) Agar - add 1.5% purified agar to the broth recipes.

Olive Oil Emulsion Agar (BYPO / BYPT Agar).

	10 g l^{-1}	Oxoid peptone
	5	sodium chloride
	3	yeast extract
	5	LAB Lemco
	15	purified agar
add as substrate	10 g l^{-1}	olive oil (BYPO)
or	10	tributyrin (BYPT)

The substrate is autoclaved separately as an emulsion of 10 g substrate , 1 g polyvinyl

alcohol (PVA Type II, of low molecular weight, which is water soluble) in 50 g distilled water. The emulsion is produced using a Sorvall-Omni mixer for 15 minutes.

Sterilization : 20 minutes autoclaving at 120°C.

3. RESULTS.

3.1. Results of the Soil Sample Screening.

This was a preliminary screen for lipase secreting organisms. From the five soil samples via 300 nutrient, seed 2 and MV broth shake-flask cultures, 24 agar plates carrying individual microorganisms were obtained by streaking colonies onto the relevant agars plus ester. These microorganisms were streaked onto BYPO agar (plus a *Pseudomonas gladioli* control, known to be a lipase secretor) and of the 24 only 7 plates showed zones of clearance, where the olive-oil emulsion had been hydrolysed. (See plates.) All of the microbes showing zones of clearance were organisms screened on the lactic acid ethyl esters. None of those screened on the R-(+)- or S-(-)-methyl-2-chloropropionate or on the R-(+)-isobutyl-2-chloroprpopionate showed any signs of olive-oil hydrolysis. Neither did some of the microbes screened with the lactic acid ethyl esters and lactic acid isobutyl ester.

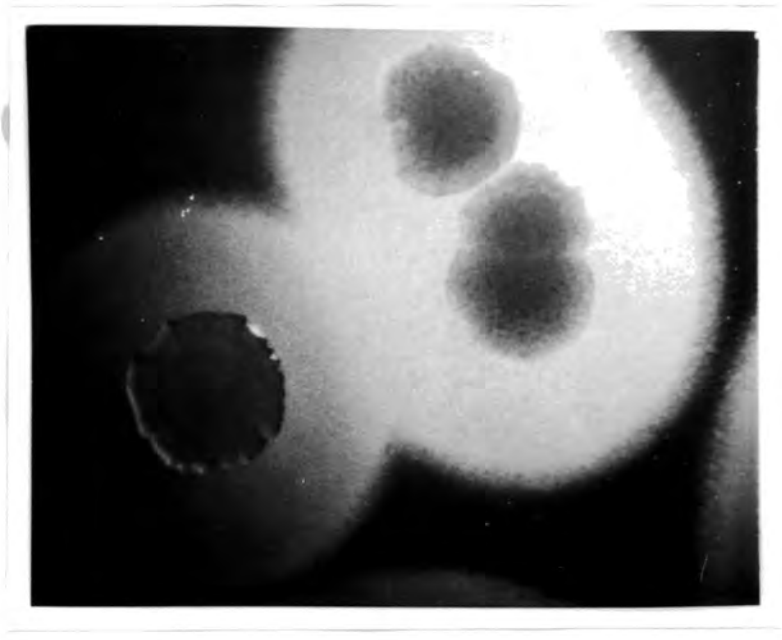

Plate 1.

Plate showing a microorganism grown on BYPO agar in the presence of L-(+)-ethyl lactate. Showing areas of olive oil emulsion hydrolysis.

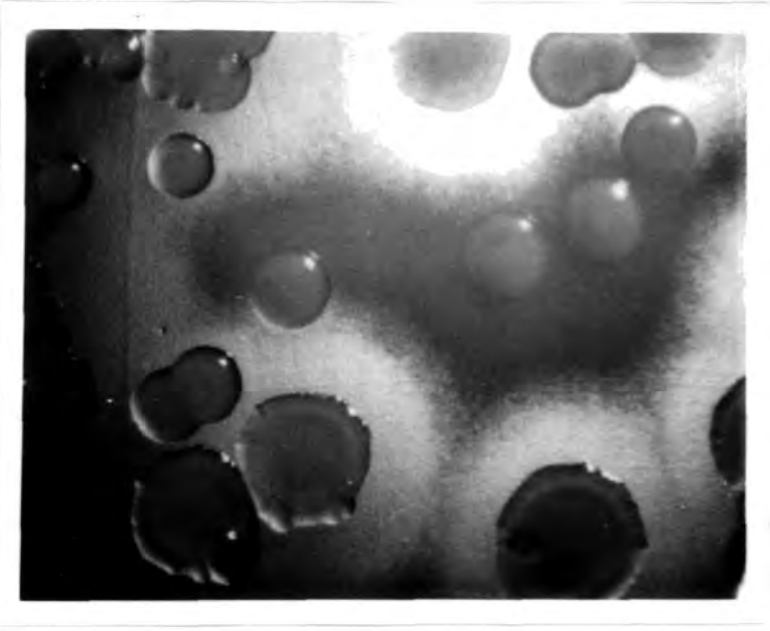

Plate 2.

Plate showing microorganisms grown on BYPO agar in the presence of L-(+)-ethyl lactate. Showing two colony types one secreting a lipase the other able to grow in the presence of the ester but not secreting any exolipase.

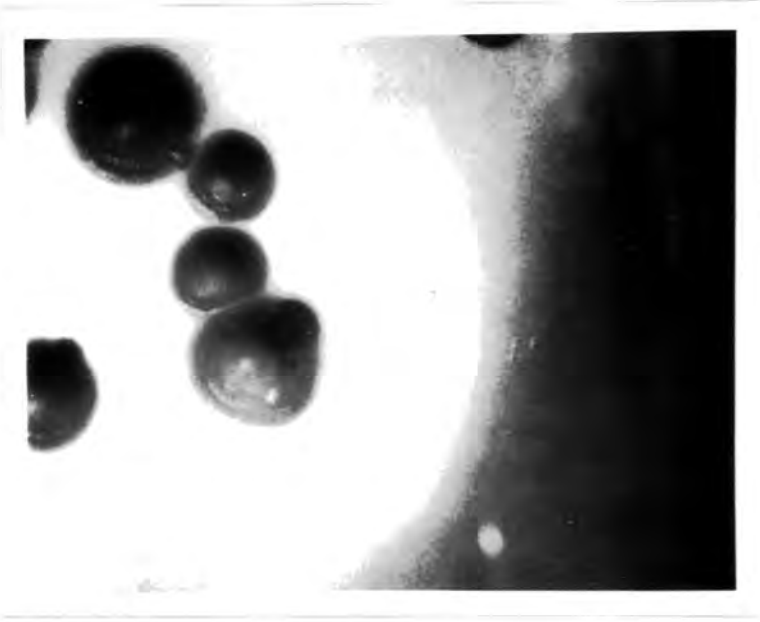

Plate 3.

Plate showing a microbe grown on BYPO agar in the presence of L-(+)-ethyl lactate. Showing a more diffuse boundary to the area of olive oil emulsion hydrolysis than plate 1, perhaps due to two species of hydrolytic enzyme being secreted.

3.2 The Results of the 2-chloropropionic Acid Assay Development.

The development of the assay required the optimization of several parameters, in order to adapt the lactate assay methodologies to one suitable for the L- and D-(α)-chloropropionic acid enantiomer determinations. Buffer pH ,and β NAD and enzyme concentrations were all investigated. Three analytical methods were compared; one for L-(+)-lactate (Noll, 1984), one for D-(-)-lactate (Gawehn, 1984) and a dual assay which could be used for either (Boehringer Mannheim, 1986).

It was necessary to calculate the sample volume and concentration required for the D- and L-(α)-chloropropionic acid standards to produce a spectrophotometer full scale deflection of 1.000. Using the general equation for concentration,

$$c = \frac{V \times M_r}{v \times \varepsilon \times d \times 1000} \times \Delta A$$

Where,

V =	final volume (1.0 ml).
M_r =	molecular weight of substance to be assayed (2-chloropropionic acid, 108.5).
d =	light path (1.0 cm).
ε =	the absorption coefficient of NADH at 340 nm (6.31 $l.mol^{-1}.cm^{-1}$).
ΔA =	the change in absorbance (1.000).
v =	sample volume (ml).
c =	the concentration of the sample (g l^{-1}).

It was calculated that 18.7 μl of a 10 mM L- or D-(α)-chloropropionic acid standard would give a full scale deflection of 1.000. It was possible to use a 20 μl sample and construct calibration curves for the enantiomer concentrations, eg. a 0 to 10 mM gradient, reading the concentration of a sample directly from the calibration curve. The L-(+)-lactate assay differed from the other protocols in that it employed a lower pH buffer and the enzyme and β NAD concentrations were lower. The gly.gly : L-glutamate buffer gave a faster reaction completion time than the pH adjusted L-glutamate buffer. An assay was taking 60 to 90 minutes to complete. The β NAD concentration in the reagent was increased from 50 mM to 100 mM and reduced the time taken for an assay, doubling the volume of β NAD solution added further increased the rate of reaction, so an endpoint was being reached in 20 to 30 minutes on a 2-chloropropionic acid standard. Increasing the amount of GPT initially improved the reaction rate but additional LDH had no effect. Reducing the amount of D,L-2-HAD to 0.25 Units per assay was possible. Though this increased the time to about 35 minutes. The enzyme ratio for the assay of D,L-2-HAD : D- or L-LDH : GPT was now ; 0.25 : 85 or 90 : 36 , in units of enzyme. The pH optimum of D,L-2-HAD was reported to be pH 10.5 (Motosugi *et al.*, 1981) and the pH of the lactate assays varied from pH 8.9 to 10.0, so a range of gly.gly buffers were prepared from pH 8.0 to 11.0 in 0.5 steps to investigate their effect on the assay's performance. With a 2-chloropropionic acid standard the optimum pH was 9.5, but with a bioreaction sample diluted to give approximately 10 mM 2-chloropropionic acid, pH 10.5 was found to be optimal.

The assays were carried out at ambient, 26° - 27°C in the spectrophotometer. This was acceptable as the cuvettes following their initial absorbance readings ($A1$) were

incubated at 28°C for > 45 minutes. Though the time for an assay had to be extended for some, due to variations in the activity of batches of D,L-2-HAD, such that some assays were taking 75 minutes to reach completion. Low concentrations of 2-chloropropionic acid eg. 2 mM, were only taking 20 minutes or less to complete. The higher concentrations of 10 to 12 mM were taking the longest. Calibration curves were drawn from the absorbances obtained for the standard mixes of known 2-chloropropionic acid concentration. Any differences between the L- and D-(α)-chloropropionic acid calibrations are negligible, and regression analysis applied to the calibration gave correlation coefficents of 0.999, and origin intercepts were frequently obtained. Plotting the lines by freehand was possible in most cases on the 6 calibration points.

Although it was not intended to be an L- or D-(α)-chloropropionic acid quantitative measure, it was a useful check to compare the concentrations of 2-chloropropionic acid enantiomers obtained from the assay with the results from the Hplc analyses of the total 2-chloropropionic acid contents of the bioreaction samples. Comparisons showed the enzyme assay to be $\pm$ 10 g l^{-1}, after taking any dilutions into account, so at low 2-chloropropionic acid concentrations there was potential for error. The errors arose from the calibration curves. Some samples had to be diluted 50 or 100 times, any errors only becoming exaggerated as the dilutions were corrected for. Due to this potential for large errors it was not completely reliable as a quantitative assay, but it was useful in determining the relative proportions of the 2-chloropropionic acid isomers. Enabling the calculation of the ee (P) and then the (ee (S)) of the remaining substrate.

3.3. The R,S-methyl-2-chloropropionate Kinetic Resolution Experiments.

The report (Dahod & Siuta-Mangano, 1987), on which this work was based, was rather uninformative particularly regarding the use of solid calcium carbonate as the aqueous phase buffer and why the reaction was only taken to 60% conversion.
It was not made obvious that the calcium carbonate was present in an amount calculated to counteract the developed acidity from the 2-chloropropionic acid. Once the calcium carbonate was depleted the aqueous phase cleared of the particulate suspension. It was known that 0.5 g calcium carbonate in powder form would not completely dissolve in 200 ml water at 4.0°C, giving a solution with a pH of 8.5 to 8.8 , the optimal for the activity of the *C. rugosa* lipase.

Runs 1, 2 and 3 showed that the 2-chloropropionic acid was partitioning back into the chlorinated organic solvents, carbon tetrachloride and chloroform but not the di-isopropyl ether, however this reaction gave the lowest yield of 12%. Compared with 1 and 2 which were able to continue to 16% and 19.5% respectively. It was critical to ensure the insolubilization of the partially water soluble R,S-methyl-2-chloropropionate, by using salts and buffers or by any other means during the enzymatic hydrolysis. Solubilization of the substrate would reduce the potential degree of conversion. With reaction 5 came the realization that insufficient calcium carbonate had been used, here a specific concentration of sodium bicarbonate was used as a buffer giving a conversion of 76%. Reaction 4 used calcium carbonate in a 50 mM salt solution but only improving the yield of acid to 32.5%.

Table 3. The Bioreaction Conditions.

Reaction	Time	Solvent	Aqueous phase buffer	Substrate /enzyme ratio	M-2-CPA concn. in organic phase	Aqueous /organic ratio	Aqueous phase volume	Expected 2-CPA concn.
No.	(Hours)			(w/w)	(M)	(v/v)	(ml)	($g\ l^{-1}$)
1.	48	Chloroform	0.5 g calcium carbonate	450	3.70	2.0	200	120.4
2.	48	Carbon tetrachloride	0.5 g calcium carbonate	450	3.70	2.0	200	120.4
3.	48	Di-isopropyl ether	0.5 g calcium carbonate	450	3.70	2.0	200	120.4
4.	48	Carbon tetrachloride	0.5 g calcium carbonate in 50 mM NaCl	450	3.67	2.0	100	119.5
5.	52	Carbon tetrachloride	8.86 g sodium bicarbonate	450	3.67	2.0	100	119.5
6.	20	Carbon tetrachloride	600 mM sodium phosphate buffer, pH 8.2	122.6	1.0	1.0	50	65.1
7.	20	Carbon tetrachloride	600 mM sodium phosphate buffer, pH 8.2	122.6	1.0	2.0	100	32.6
8.	48	Carbon tetrachloride	1.0 g calcium carbonate in 50 mM NaCl	450	3.67	2.0	200	119.5
9.	30	Carbon tetrachloride	600 mM sodium phosphate buffer, pH 8.2	450	1.0	2.0	200	32.6
10.	30	Carbon tetrachloride	11.1 g calcium carbonate	450	3.67	2.0	200	119.5

Table 4. The Bioreaction Results.

Reaction	Aqueous /organic phase ratio	Aqueous phase at end of run	2-CPA concn. in aq. phase	2-CPA concn. in org. phase	Measured actual conversion	Measured 2-CPA in aqueous phase	Calculated M-2-CPA in organic phase	Calculated M-2-CPA in organic phase
No.	(v/v)	(pH)	(g l^{-1})	(g l^{-1})		ee (P)%	ee (S)%	% remaining
1.	2.0		25.6	12.0	15.8	50.6		84.2
2.	2.0		33.3	12.5	19.8	47.6		80.2
3.	2.0		25.3	none detected	12.7	41.0		87.3
4.	2.0		42.1	44.9	32.3	49.6		67.7
5.	2.0		98.3	106.6	75.8	15.2		24.2
6.	1.0	5.2	61.2	0.3	56.7	27.2	45.8	43.4
7.	2.0	3.5	45.1	<0.3	83.1	8.8	100	16.9
8.	2.0	2.4	35.4	14.3	21.3	58.4		41.6
9.	2.0	5.6	46.2	none detected	85.2	11.1	91.0	14.8
10.	2.0	3.4	133.9	none detected	66.9	34.4	90.0	33.1

"CPA "and "M-2-CPA "refer to 2-chloropropionic acid and methyl-2-chloropropionate respectively. For the calculation of ee (P) and ee (S) see section 1.4.

The 2-chloropropionic acid yields were increasing with the retention of the ester insolubilized in the organic phase improved, but there was still repartitioning of the acid into the organic solvent phase. Reactions 6 and 7 employed a 600 mM sodium phosphate buffer at pH 8.2 . This appeared to prevent the repartitioning of the acid into the solvent and to help obtain 2-chloropropionic acid yields in line with those expected. In these reactions the double organic volume aqueous phase system gave the better result giving a 100% ee (S) of the R-isomer.

Figure 8. The inability of 0.5 g in 100 ml of calcium carbonate to buffer the aqueous phase and the predicted dramatic fall in pH bringing the reaction to a premature finish at only 21.4% conversion.

Figures 9 and 10. The efficient buffering of the 600 mM sodium phosphate buffer at pH 8.2, allowing the reaction to go to completion.

Figures 11 and 12. Using the correct amount (11.1 g) of calcium carbonate powder to effectively buffer 60% of the acid produced from the hydrolysis. It exhibited a crude or undamped form of buffering.

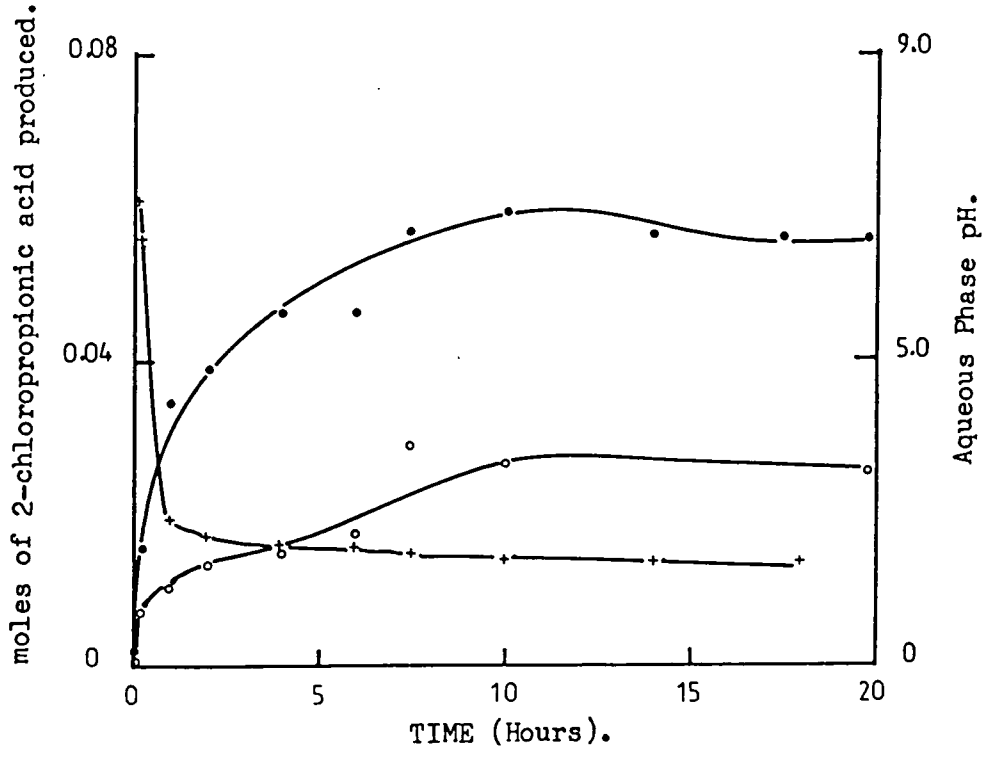

Figure 8.

Bioreaction number 8. The effect of the fall in pH on the repartitioning of product into the organic phase. (•) 2-chloropropionic acid in the aqueous phase,(o) 2-chloropropionic acid repartitioned to the organic phase,(+) the aqueous phase pH. The buffer was 0.5 g calcium carbonate in 50 mM NaCl.

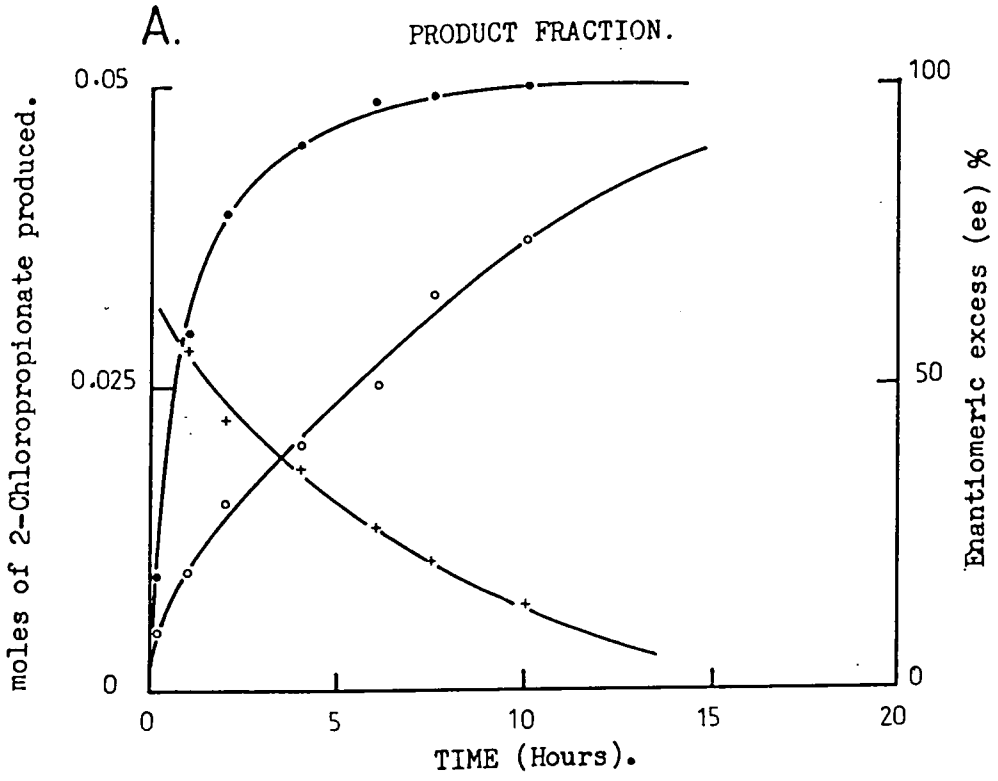

Figure 9.

Bioreaction number 9. A, the product fraction. The production of the two 2-chloropropionic acid isomers and the enantiomeric excess. (•) D-(α)-chloropropionic acid,(o) L-(α)-chloropropionic acid, (+) enantiomeric excess, (ee (P)%).

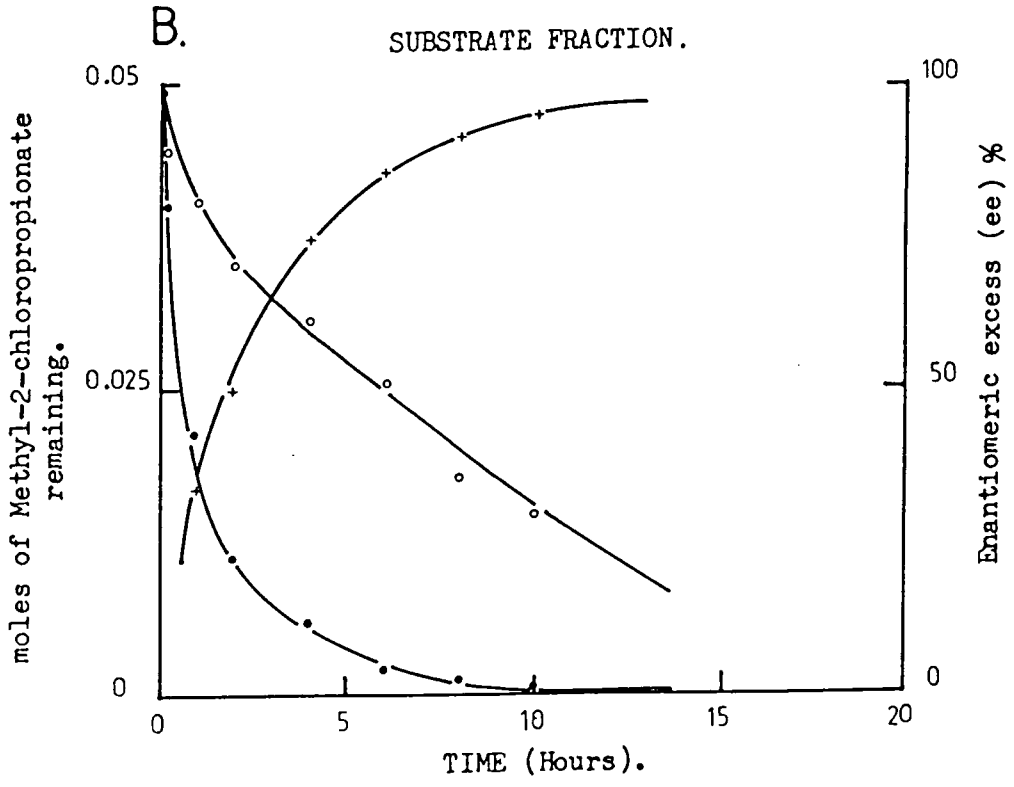

Figure 10.

Biorection number 9. B, the substrate fraction. Showing the more rapid removal of one isomer of the ester preferentially over the other. (•) S-(-)-methyl-2-chloropropionate, (o) R-(+)-methyl-2-chloropropionate, (+) enantiomeric excess, (ee (S)%).

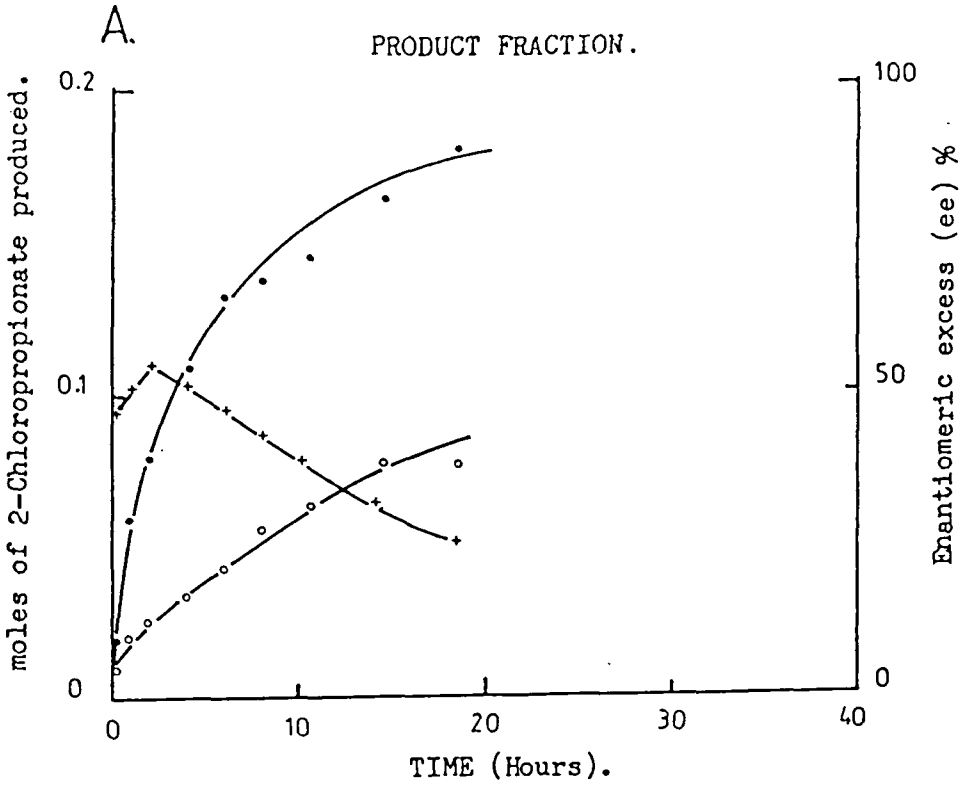

Figure 11.

Bioreaction number 10. A, the product fraction. The production of the 2-chloropropionic acid isomers and the enantiomeric excess. (•) D-(α)-chloropropionic acid, (o) L-(α)-chloropropionic acid, (+) enantiomeric excess, (ee (P)%).

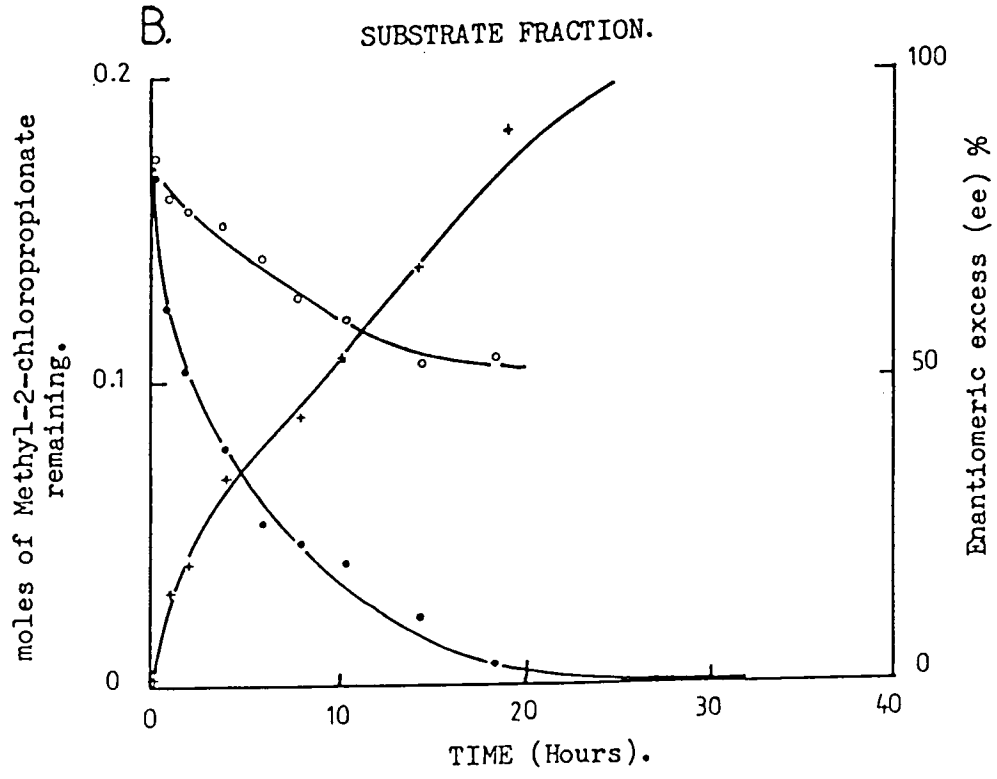

Figure 12.

Bioreaction number 10. B, the substrate fraction. Showing the preferential hydrolysis of one ester isomer over the other and the enantiomeric excess.

(•) S-(-)-methyl-2-chloropropionate , (o) R-(+)-methyl-2- chloropropionate, (+) enantiomeric excess, (ee (S)%).

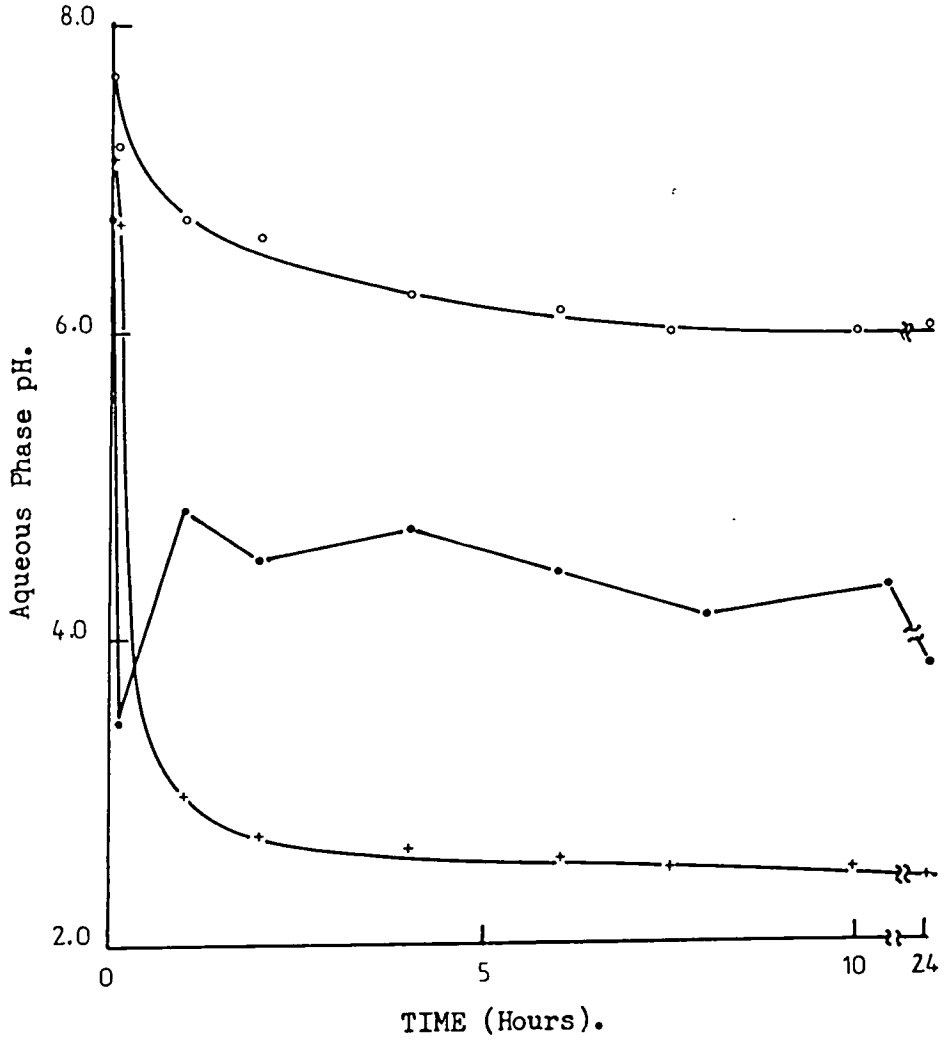

Figure 13.

Comparison of the pH buffering in bioreactions 8, 9 and 10. Bioreaction 8, (+) 1.0 g calcium carbonate in 200 ml water, bioreaction 9, (o) 600 mM sodium phosphate buffer, pH 8.2 and biorection 10, (•) 11.1 g calcium carbonate in 200 ml water.

Reactions 8,9 and 10 were to study the effects of the pH buffering of the aqueous phase, relating this to the repartitioning of the 2-chloropropionic acid and to the degree of substrate conversion. From reaction 10 and the plots of ester remaining and acid produced it can be seen that reaction 7 could have been terminated earlier to give a higher yield of R-(+)-methyl-2-chloropropionate yet retaining the high enantiomeric excess. Following reaction 10 it can be seen that this would have been at about 67% - 70% conversion and the ee (S) would have been > 95%. Reaction 6 had an aqueous/organic ratio of 1:1 which was reported to have yielded R-isomer ester with an ee (S) of 94%, however not having been able to repeat this reaction it can perhaps be concluded that either the reaction was performed incorrectly or that the analysis was suspect.

3.4. The Site of Lipase Activity.

The ρ-NPP assay was used to detect the presence of enzyme subsequent to the termination of a bioreaction, to show that :

a. Lipase activity was detectable even 48 hours after the end of a reaction run.

b. A mixed sample (emulsion) when allowed to settle out exhibited no lipase activity in the aqueous phase until a sample was withdrawn from immediately adjacent to the aqueous / organic interface, where the lipase activity was found to be concentrated.

This correlated with the generally recognized site of lipase activity on triglycerides where the enzyme acts on the substrate's surface. By dissolving the substrate in a water-immiscible organic solvent and reducing any possible solubilization into the aqueous phase, mixing of the two phases would present the enzyme with an aqueous / organic

emulsion of considerable surface area.

Although it should have been possible to quanitatively assay the lipase activity, it was found to be variable due presumably to the presence of organic solvent, and the concentrating effect of the enzyme at the interface of the two phases. Attempts to obtain lipase activity from the separated phases met with little success.

3.5. Results of the Quantitative Analyses of the Kinetic Resolutions.

Using the equations (4) and (5) (see introduction) to obtain values for the enantiomeric ratio, E. Only bioreactions 6, 7, 9 and 10 could be analysed in this way, as they had no measurable 2-chloropropionic acid in their organic phases. The proportions of 2-chloropropionic acid isomers in the organic phase could not be assayed, and although it would be safe to assume they had the same proportions this was not included in any calculations. The 2-chloropropionate repartitioning to the organic phase makes it impossible to calculate the enantiomeric excess of the remaining substrate.

The enantiomeric ratios can be calculated from the values of L- and D-(α)-chloropropionic acid production obtained with the assay.

An E of 5 to 10 has been described as modest (Chen *et al.*, 1982), such E values require a reaction to proceed to 80% completion to achieve the enantiomeric excesses of substrate. For an E value of 3.0 the conversion (c) has to be as high as 0.95, this would reduce the yields of recoverable substrate.

The amounts of substrate remaining in reactions 9 and 10 can be calculated using the results from the enzyme assay and the ee (P) obtained. These values were plotted against

the percent conversion to give figures 14 and 15. These plots of substrate and product fraction can be used to predict the the optimal point for termination ofthe hydrolysis reaction. By comparing these to the computer generated curves for various values of E (Chen *et al.*, 1982) it can be estimated that reactions 9 and 10 had enantiomeric ratios in the range ≤ 3 to ≤ 5. This corresponds to the values for E obtained by calculation. Also bioreaction 10 could have been terminated earlier according to figure 14 and bioreaction 9 may never have reached 100% ee for the substrate fraction until the substrate yield would have been only a few percent.

The peculiar increase in the enantiomeric excess of the product fraction in figure 15 can be related to the pH course of this reaction, see figure 13 and table 5.

Table 5. The Enantiomeric Ratios.

Reaction	Time	Conversion	Fraction	Enantiomeric ratio	Aqueous phase
No.	(Hours)	(c)		(E)	(pH)
6	20	0.627	substrate	2.62	3.5
			product	2.63	
7	20	0.935		3.02	5.2
				2.94	
9 a	4	0.648		4.30	6.2
				4.31	
b	8	0.812		3.79	6.0
				3.79	
c	12	0.910		2.74	5.9
				2.74	
10 a	5 min	0.074		1.85	3.4
				1.85	
b	4	0.382		4.62	4.7
				4.43	
c	8	0.510		4.36	4.1
				3.83	
d	14	0.658		4.07	4.2
				3.93	
e	18	0.698		6.16	3.9
				6.12	

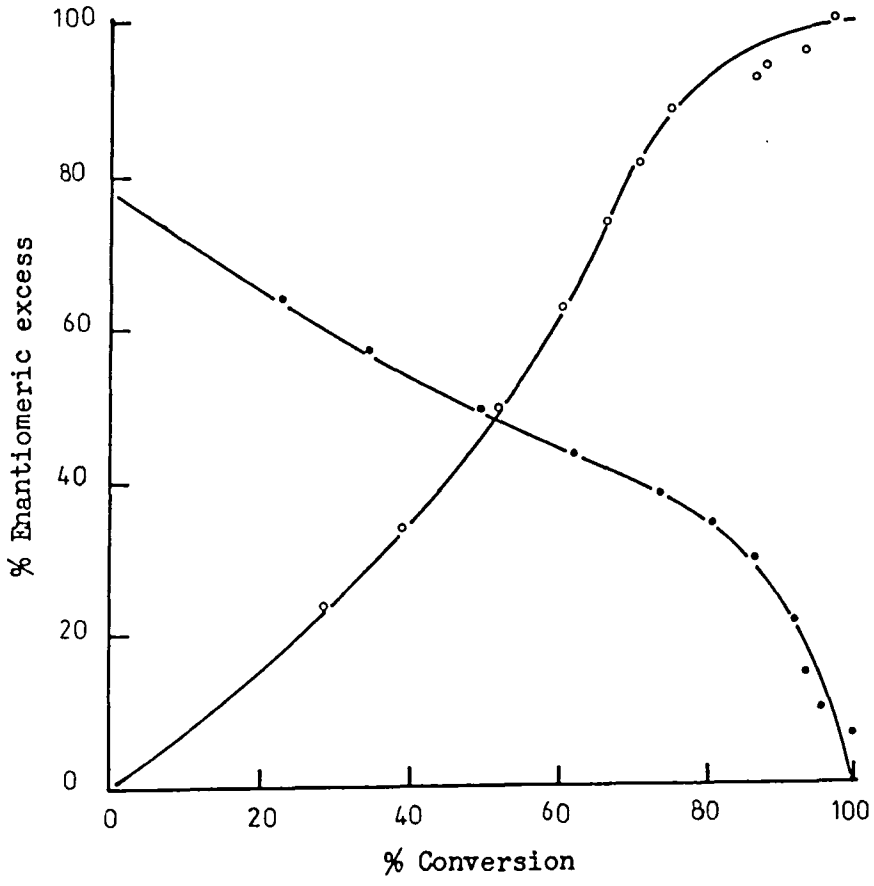

Figure 14.

Plot of enantiomeric excess (ee) as function of the percent conversion for (o) the substrate remaining (ee (S)) and (•) the acid product (ee (P)) for bioreaction 9.

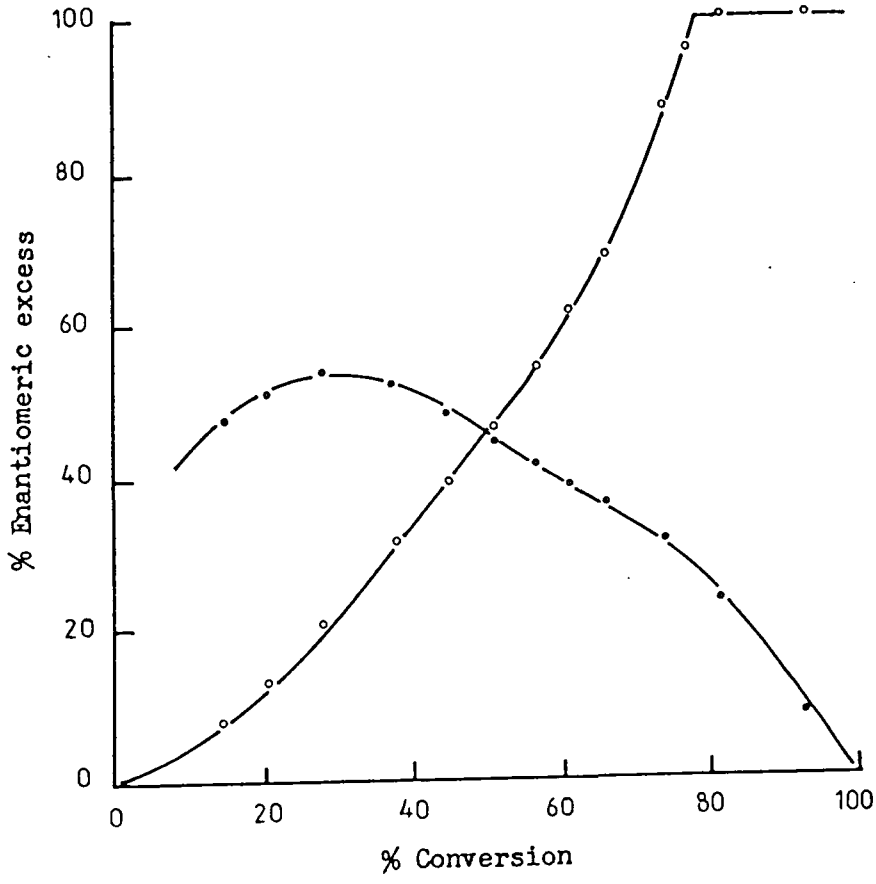

Figure 15.

Plot of enantiomeric excess (ee) as a function of the percent conversion for (o) the substrate remaining (ee (S)) and (•) the acid product (ee (P)) for bioreaction 10.

4. DISCUSSION.

4.1. Screening and Isolation of Novel Exolipase Secretors.

Recent published reports on the production of lipases by various microorganisms have dealt with the the optimization of enzyme secretion of known lipase producers (Nahas, 1988; Suzuki *et al.*, 1988). A factor in the search for new lipase enzymes is their characterization once they have been isolated. A microorganism that is utilizing both isomers of a compound may be in possession of one non-specific enzyme or a pair of stereospecific enzymes. Although lipase secreting microbes are easily isolated from the soil and raw milk (Sztajer *et al.*, 1988) there are few environments where an enantiospecific lipase would be an advantage. The site at ICI Huddersfield is unusual in that is has dealt with enantiomers of 2-chloropropionate esters for 5 years, though taking samples from this site is no guarantee of finding some novel enzymes it ~~will~~ should be one of the few from which some are to be found.

As this screening was only a preliminary procedure to find some lipase secretors, the characterization of the actual enzynes was not carried out, this aspect has been passed on to Dr John Colby at Sunderland Polytechnic. One idea was the synthesis of *p*-NPP - 2-chloropropionate ester derivatives which could be incorporated into the culture medium, together with a carbon source such as olive oil. Hydrolysis of these compounds would yield a colour change giving a fairly clear indication of lipase activity, as long as the substrate or products were also colourless. Other compounds which have been found to enhance lipase activity could also be incorporated into the medium (Winkler & Stuckmann, 1979; Schulte *et al.*, 1982; Jäger & Winkler, 1983; Wingender & Winkler,

1984; McKellar *et al.*, 1988).

The characterization of lipase activities on the interesterification of triglycerides has been attempted using *O*-alkylglycerol esters (Sonnet & Antonian, 1988) or by the lipase's ability to incorporate stearic acid in the conversion of olive oil to a cacao butter-like fat (Yokozeki *et al.*, 1982). However the selection and characterization of novel lipases is almost unreported.

4.2. The Kinetic Resolution of R,S-Methyl-2-chloropropionate by *Candida rugosa* lipase.

Lipase from *C. rugosa* was found to be a suitable catalyst in the practical scale preparation of the R-(+)- enantiomer of methyl-2-chloropropionate . This resolution was achieved contrary to the opinion of some researchers (Cambou & Klibanov, 1984a; 1984b). Using a biphasic aqueous / water immiscible solvent mixture was essential in the success of this enzyme-catalyzed resolution. The resolution of R,S-methyl-2-chloropropionate by *C. rugosa* lipase in a purely aqueous system has been shown to be totally non-stereospecific (Cambou & Klibanov, 1984a; Dahod & Siuta-Mangano, 1987).

The solvent and aqueous phase were equally important. Mixing of the phases was sufficient to form an emulsion of solvent in the aqueous phase. These are the discontinuous and continuous phases respectively. Agitation by the magnetic stirrer was used to mix the phases, ensuring no clear layer of aqueous phase was left at the top of the mixture when using carbon tetrachloride, or at the bottom with di-isopropyl ether. This was at an unknown speed achieved through trial and error. The effect of stirring on the activity and stability of enzymes in biphasic systems has been studied (Buckland *et al.*,

1975). With this system stirring has to be vigorous enough to maintain the calcium carbonate or sodium bicarbonate in suspension to prevent pockets of reduced pH from forming. Sodium phosphate buffer crystallized at 4°C and required considerable agitation to maintain the crystals in suspension. Dahod (1987) stated that "the hydrolysis process was conducted under sufficient agitation to maintain the reactants sufficiently in contact that the reaction can occur ". Dahod and Siuta-Mangano (1987) observed neither any increase in reaction rates, nor any reduction in enzyme stability with additional stirring.

The ratio of aqueous phase volume to organic phase volume was no less than 1:1. This was because Dahod and Siuta-Mangano (1987) found that ratios from 1:3 to about 5:1 were the easiest to work with and gave the best results. Though at high solvent to low aqueous volume ratios reversal of the emulsion could occur giving a suspension of aqueous phase droplets in the organic solvent. The solvent here becomes the continuous phase. This effect has not been investigated and in this system is probably disadvantageous. Emulsification of the organic phase is assisted by the *C. rugosa* lipase which has been observed to act as an effective emulsifying agent (Dahod & Siuta-Mangano, 1987). The reaction rate will benefit from having a greater substrate surface area for the enzyme to react with. This may also prevent a reversed emulsion from developing when the aqueous phase volume is low relative to the organic phase volume.

Choice of solvent is critical to the success of a biphasic system, but in many reports appears to be determined by it's availability on the bench (Laane *et al.*, 1987). Perhaps the most important consideration is the solubility of the reactants and the products in the organic solvent. The organic solvent will also affect enzyme activity and may have an influence on the recovery of any remaining substrate or products. Until recently

the choice of solvent was also considered to be 'hit and miss 'as the solvents had to be experimented with individually. This has been shown to be partially unnecessary as a solvent's physical properties, particularly it's hydrophobicity, can be used to predict it's behaviour on some enzymes in biphasic systems (Brink & Tramper, 1985; Laane *et al.*, 1985, 1987; Reslow *et al.*, 1987). Carbon tetrachloride, chloroform and di-isopropyl ether do not appear to inhibit the *C. rugosa* lipase relative to each other. Carbon tetrachloride and chloroform were both used by Dahod and Siuta-Mangano (1987) and di-isopropyl ether has been shown by other workers not to greatly inhibit yeast lipase activity (Zaks & Klibanov, 1985; Reslow *et al.*, 1987). Dahod and Siuta-Mangano (1987) showed that carbon tetrachloride promoted the kinetic resolution by this lipase in the presence of substrate. Ratios of carbon tetrachloride to substrate of $\geq$ 0.5 (v/v) showed no increase in the reaction rate. In the absence of substrate carbon tetrachloride irreversibly denatures the enzyme. Terminating the reaction before the substrate is completely hydrolyzed is important in maximizing the amount of recoverable enzyme and substrate. The plotting of data in the form of figures 14 and 15 is therefore useful not only in optimizing the yield of substrate.

At concentrations of methanol < 1 M carbon tetrachloride reduces the inhibitory effect methanol has on the reaction rate. However at concentrations $\geq$ 1 M methanol this advantage is lost and the rates of hydrolysis in the presence or absence of carbon tetrachloride are equal. Methanol was not the only alcohol found to inhibit lipase-catalyzed hydrolysis (Dahod & Siuta-Mangano, 1987). Methanol at 1 M concentration was shown to partially stabilize the enzyme in the presence of carbon tetrachloride. This is of no practical value as the activity is inhibited by the methanol. Methanol inhibition appears

to severely affect the reaction when the aqueous phase volume is relatively small, as in biorection 6, and can be seen by comparing bioreactions 6 and 7, where the only difference is the aqueous / organic phase ratio.

The optimal aqueous to organic phase ratio is to some extent determined by the concentration of methanol produced from the hydrolysis reaction. The aqueous phase is therefore necessary in diluting the methanol to reduce it's potential inhibitory effects. The aqueous phase is also the recipient of the 2-chloropropionic acid, which in it's unprotonated form is insoluble in carbon tetrachloride and chloroform. The aqueous phase has to be pH buffered to maintain the solubility of the acid and the activity of the enzyme. A pH of 4 to 8 will serve this purpose. There is no difference between the pH rate profiles for the lipase in the presence or absence of carbon tetrachloride. An insolubilizing salt is also required to reduce the solubility of the R,S-methyl-2-chloropropionate in the aqueous phase. This is soluble to a concentration of 130 mM in sodium tetraborate buffer containing 200 mM sodium chloride and 3 mM calcium chloride (Cambou & Klibanov, 1984a). This would reduce the stereospecificity of the lipase in the hydrolysis. Insolubilization of the ester is achieved by dissolving it in an organic solvent, salting it out from the aqueous phase and by lowering the reaction temperature. The simplest aqueous phase component is where the insolubilizing salt also acts as the buffer. The effect of using too little insolubilizing salt or buffer is shown in bioreactions 1 to 4 (table 4.) and 8 (figure 8.). This also shows the result of the pH being allowed to fall below 2.5 and the repartitioning of the acid. The presence of 2-chloropropionic acid in the organic phase does not affect the hydrolysis, but it will be more difficult to separate product from any remaining substrate in a commercial process. The enzyme assay cannot be applied to acid isomers in the

organic phase as the solvent affects both the enzymes and the absorbance. Therefore the proportions of substrate enantiomers remaining cannot be calculated.

Controlling the pH of the aqueous phase is required not only to retain optimal enzyme activity eg. above pH 4.0, but also to prevent the repartitioning of the 2-chloropropionic acid eg. above pH 2.5. If for no other reason this is essential for the assaying of the 2-chloropropionic acid isomers. Effective buffering can be seen clearly in bioreaction 9, figures 9 and 10 with the pH course of the reaction shown in figure 13. Compare this with bioreaction 10, figures 11 and 12 and it's pH course in figure 13. The sodium phosphate buffer appears to be a superior buffering system and the calcium carbonate appears rather erratic. However the final results for these bioreactions are comparable in that the enantiomeric excesses of substrate remaining are the same. But the lower pH with bioreaction 10 the reduced enzymic hydrolysis resulted in twice the amount substrate remaining with the same enantiomeric excess and figure 15 shows that the reaction could have been terminated earlier to achieve an even higher yield of resolved substrate. If figures 14 and 15 are compared, it would appear that hydrolysis at the elevated pH reduces the stereospecificty of the lipase by affecting it's enantiomeric ratio. Highest stereoselectivity occuring here at about pH 4.3. This observation is borne out by the enantiomeric ratios for these two reactions (see table 5.). The higher the enantiomeric ratio, the greater the enzyme's preference for one enantiomer over the other. The overall higher enantiomeric raios calculated for bioreaction 10 show this form of buffering, despite it's initial fluctuation, to be more useful than the sodium phosphate. Only one other report was found in the literature relating the pH to enantiomeric ratio (Sonnet & Antonian, 1988). They obtained an optimal pH to maximize the enantiomeric ratio in

the stereoselective hydrolysis of acetonide esters of glycerol by *Aspergillus niger*. The *A. niger* and porcine pancreatic lipase were found to be more stereoselective than *C. rugosa* or *Mucor miehei* lipases. However these hydrolyses were carried out in aqueous solutions and are therefore not relevant to biphasic systems.

The temperature at which the biorections were conducted was intended to reduce the solubility of the partially water soluble substrate to retain it in the organic solvent phase. Such low temperatures will greatly reduce the reaction rates, but will allow for more accurate control of the pH or termination of the reaction. Calcium carbonate during the initial stages of the hydrolysis cannot dissolve as quickly as the acidity is developing. Any great increase in the rate of reaction would probably find the calcium carbonate unable to buffer the hydrolysis. In such a situation a lower pH sodium phosphate buffer might be very useful. Lower reaction temperatures will also reduce any unwanted side-reactions to an absolute minimum, particularly non-enzymatic hydrolysis and the non-enzymatic dehalogenation of 2-chloropropionic acid. The former occurs non-stereoselectively and the latter, if allowed to occur to any extent, will interfere with the enzyme assay. Preventing non-enzymatic dehalogenation of the 2-chloropropionic acid will maximize the amount of recoverable acid, which can be reesterified and racemized for reuse. It has been observed that *C. rugosa* lipase is unstable at higher temperatures such that 30 minutes incubation at 37°C almost completely denatures the enzyme. Hence using lower temperatures will help stabilize the enzyme against heat denaturation. Many organic solvents are flammable or at best volatile. Carbon tetrachloride will easily evaporate at warmer temperatures but at 4°C the vapour pressure is lowered and evaporation is negligible over the period of these bioreactions. Carbon tetrachloride is non-flammable but carcinogenic,

di-isopropyl ether and chloroform are flammable but non-carcinogenic. At 4°C their combustibility will be lessened. Temperature is an important consideration when choosing the solvent as some may solidify or become too viscous at low temperatures.

The *C. rugosa* lipase was found to be associated with the aqueous / organic interface. This observation was also made by Cambou and Klibanov (1984a) in their resolution of water insoluble racemic octyl-2-chloropropionate which was stereospecifically hydrolyzed by this enzyme. The partially water soluble methyl ester was non-stereospecifcally hydrolyzed. This was attributed to the preference of lipase enzymes for acting on a substrate surface and not on dissolved reactants (Desnuelle, 1972). This observation correlates with the termination of the hydrolyis rection which can be effectively achieved by the removal of the stirring. The reduction in available surface area, from fine emulsion to the two phases, reduces the hydrolysis to negligible rates.

4.3. Possible Areas for Future Investigation.

The work discussed here was limited not only by the time available. The next bioreactions could be planned now bioreactions 6,7,9 and 10 have shown the main requirements for a successful hydrolysis:

1. A larger volume aqueous phase than organic phase
2. and for an effective, but not necessarily efficient buffer component. The next bioreactions would probably have been repetitions of 9 and 10 using chloroform and di-isopropyl ether as the organic solvents. Calcium chloride and sodium bicarbonate buffers were mentioned in the patents by Dahod (1987; 1988), though using the sodium phosphate buffer at a lower ph eg. pH 6.2 would be interesting to investi-

gate it's effect on the enantiomeric ratios. Replacement of portions of the aqueous phase with fresh solution at set intervals to increase the reaction rate by reducing the methanol inhibition is another possibility for investigation, mentioned by Dahod (1987) in the patent. There do not appear to be any other lipases mentioned in the literature that are either as stereoselective as the *C. rugosa* lipase in this type of biphasic system, or as inexpensive in it's crude form. Porcine pancreatic lipase is known to be enantioselective and active in aqueous organic systems but is considerably more expensive (Zaks & Klibanov, 1985). Chymotrypsin has also been used to hydrolyze substrates in the presence of organic solvents (Zaks & Klibanov, 1988), but not in an aqueous / organic biphasic system. Stereospecific or asymmetric hydrolyses have been carried out in the absence of organic solvent (Iriuchijima *et al.*, 1981; 1982a; 1982b; Ohno *et al.*, 1987) but usually on water insoluble substrates in aqueous reaction medium. Although *C. rugosa* lipase is suitable as a stereospecific catalyst and is fairly inexpensive when compared to other lipases, it would be a major cost in any commercial process. Immobilization of lipases has been carried out and shown in some reports to be active and stable in some organic solvents (Fukui & Tanaka, 1982; Yokozeki *et al.*, 1982; Koshiro *et al.*, 1985; Marlot *et al.*, 1985; Osanai, 1986; Nakatsuka, 1987; Kang & Rhee, 1988). Immobilization will allow the reuse of the lipase and for the development of a continuous or batch process where the aqueous phase is replaced only once or twice. Dahod (1988) developed a continuous system relying on the higher density of the carbon tetrachloride and the enzyme's affinity for the aqueous / solvent interface to reduce the loss of enzyme. Removal of the aqueous phase was to be up a wide tube where the loss of agitation would allow the carbon

tetrachloride to sink taking the enzyme with it. The aqueous phase could then be removed continuously from the top of this tube.

C. rugosa lipase was successfully entrapped in polyurethane and used to stereoselectively esterify D,L-menthol in isooctane (Koshiro, *et al.*, 1985). The adsorption of the enzyme onto cellite-like particles has been considered to be a possibility as it is unnaffected by organic solvents, unlike many support materials. However more enzyme may be required as the contact of adsorbed enzyme with the aqueous / organic interface may be reduced.

The modification of the enzyme by attaching short chain polyethylene glycol molecules (Takahashi *et al.*, 1984; Inada *et al.*, 1986; Kodera *et al.*, 1986) to make the enzyme soluble in organic solvents is unnecessary. The solubility of the lipase in a solvent is probably best discouraged in this system, as it operates effectively dissolved in the aqueous phase and acting at the interfacial surface. Also it has been observed that a comparison of PEG-modified and unmodified enzyme shows no advantage in the laborious and complex modification procedure (Zaks & Klibanov, 1985).

Other possibilities for further investigation:

1. The hydrolysis of other 2-chloropropionate esters eg. isobutyl-2-chloropropionate which is less water soluble than the methyl ester.
2. Conducting the reactions at higher temperatures eg. 10°C, 16°C, or 22°C.
3. Varying the environmental conditions of enzyme-catalyzed hydrolysis in biphasic systems and the effect on the enantiomeric ratio has not been investigated in any great detail.

In conclusion it can be said that the buffering or insolubilization salt was the most

important component in these bioreactions. It's importance was understated by Dahod and Siuta-Mangano (1987). The effect of pH on the enantiospecificity indicates that the process could be pH optimized enabling higher enantiomeric excesses and yields of resolved substrate to be obtained. This requires further experimentation with the buffers as these also appear to affect the enantiomeric ratio.

SUMMARY.

1. 7 unidentified microorganisms were isolated and found to be secreting an exolipase capable of hydrolysing an olive oil emulsion.
2. A further 17 microorganisms isolated were capable of growth in the presence lactate and 2-chloropropionate esters, but were not found to be secreting an exolipase.
3. A triple linked-enzyme assay was developed to accurately measure the proportions of 2-chloropropionic acid isomers in aqueous solutions.
4. The practical or pre-pilot scale enzyme-catalyzed resolution of R,S-methyl-2-chloropropionate, a useful chiron for the synthesis of optically active herbicides, was attempted.
5. The enzyme employed as the hydrolytic catalyst was a lipase from the yeast *Candida rugosa* (formerly *C. cylindracea*).
6. R-(+)-methyl-2-chloropropionate was successfully prepared with an enantiomeric excess of 90% to 91% at yields of 14% to 33% of original substrate.
7. The enzymic hydrolysis was conducted in an aqueous / organic biphasic system.
8. The pH buffering of the aqueous phase was crucial to the stereospecificity of the reaction in retaining the substrate in the organic phase and the 2-chloropropionic acid in aqueous solution.
9. Calcium carbonate was the most effective buffer used.
10. The ee (P) and ee (S) from the last two bioreactions were used in the quatitative analysis of the resolutions and the values for E obtained showed the enantiomeric ratio of the lipase being affected by the pH of the reaction mixture.

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